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FOREWORD

The III Asian Horticultural Congress (AHC2020), which was convened during December 15-17, 2020, Bangkok, Thailand, under the theme "Asian Horticulture for a Sustainable World", was jointly hosted by the Horticultural Science Society of Thailand, the International Society for Horticultural Science, the Department of Agriculture, the Department of Agricultural Extension and Kasetsart University with the support from National Research Council of Thailand, Agricultural Research Development Agency (Public Organization), Thailand Convention and Exhibition Bureau (TCEB). The congress provided a platform for researchers, professors, students, government agencies, associations, growers, entrepreneurs and other professionals having an interest in horticulture to share recent research and development findings and innovation in various fields of horticulture as well as creating technical and business cooperation network among Asian horticulturists and relevant parties.

AHC2020 could struggle through critical crisis of COVID-19 pandemic. It was the first time for the AHC that keynote and invited speakers, as well as registered participants from overseas could not travel from their countries to participate in the congress. The convener had to take due consideration about the best solution to further organize the congress in a suitable and practical format. Therefore, a hybrid congress was our way out. Virtual meeting space had to be used to facilitate all parties concerned, in particular foreign participants. Over a two-day scientific program, various technical issues on recent research and development in horticultural science including related innovation were presented and shared through the congress which comprised those from 5 keynote, 9 invited, 53 oral and 79 poster presentations.

It was proved that since the AHC2016 in Chengdu, the People's Republic of China, scientific studies in horticultural science were conducted on and on for novel findings and innovation. I truly commended the deliberations of all scientists who are involved in making horticulture more technically progressed and proposed fruitful recommendation for this congress. I would like to take this opportunity to express my sincere thanks to the International Society for Horticultural Science, the three editors namely Professor Dr. Jingtair Siripanich, Professor Dr. Ian Warrington, and Dr. Robert J. Nissen including Dr. Nipat Sukvibul, all the reviewers, the fellows from the Japanese, Korean, and Chinese Societies for Horticultural Science, the participating government organizations, the private sector, especially professional excursion sites, distinguished keynote and invited speakers, on-line and on-site participants for their valuable contribution in making this congress a success in specific challenges of COVID-19 pandemic.

My thanks are also given to Thailand Convention and Exhibition Bureau for generous support and provision of virtual meeting space, the Steering Organizing Committee, the Academic Sub-Committee, the Organizing Sub-Committee, the Secretariat and all those who worked hard behind the scene for their untiring efforts. I appreciate the full team of the Idext MICE Company who worked professionally in terms of on-line meeting arrangement which was rather new to us. Without their cooperation, it would not have been possible for me to carry out the task of convening this congress to its successful conclusion. I would like also to convey my thanks to VNU Asia Pacific for working with us and shared contribution at the outset of the AHC2020, but could not work with us later on due to certain circumstances.

The AHC2020 Scientific Committee selected the best oral and the best poster presentation for ISHS young minds award. The awardees of best oral presentation was Yu Kinoshita from Kyoto University, Japan and the best poster presentation was Katsuhisa Futagami from Kyushu University, Japan. The certificate of award was sent to them, accordingly via e-mail.

As the next AHC will be hosted by the Japanese Society for Horticultural Science in Tokyo in August 2023, which will coincide with the centennial celebration, I wish Japan the very best success. I do believe that cordial bond among horticultural community who share the same goal will be even fostered. See all of horticulturists at the next AHC2023.

A. Dalodom
Convener

PREFACE

The papers contained in this volume of *Acta Horticulturae* report the scientifically reviewed Proceedings of the III Asian Horticultural Congress – AHC2020. Keynote speakers and authors of selected contributed oral and poster presentations were given the opportunity to submit a manuscript for publication.

The manuscripts were reviewed by the Editors and members of the Editorial Board. Only those papers judged suitable for publication following the authors' consideration of reviewer suggestions appear in this volume of *Acta Horticulturae*.

The ISHS acknowledges and appreciates the contribution of all editors and reviewers. They have made a significant contribution to improving the quality of this publication.

The ISHS Board of Directors

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LIST OF AUTHORS

Novel orchid breeding to meet future market demands

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Abstract

New cultivars of many orchids have been actively developed and released to the market worldwide. Different countries or areas may have different requirement for the types of orchid products, such as double spikes and certain flower sizes of *Phalaenopsis* pot plants are set as market standard for the trade. We have been working with breeding *nobile*-type *Dendrobium* and novelty *Phalaenopsis* hybrids for easy flowering, novel color and long lasting here in our university. Miniature type in small pot size, e.g., 6-cm pot, of *nobile*-type *Dendrobium* cultivars with free flowering under optimal cool temperature to save energy cost is one of the breeding goals. For novelty *Phalaenopsis* breeding hybrids with double/multiple spikes, multi flowers of heavy substance are being pursued by introducing germplasms with desirable traits for the cross hybridization, such as *Phal. micholitzii* which has compact leaves, multiple spikes and heavy flower substance. We have being using the species or its progeny with several spikes and more flower counts to cross pollinate with commercial cultivars. Some of the intermediate hybrids derived from this breeding program will be used as examples for explaining novelty *Phalaenopsis* breeding works. Polyploidy cultivars of *Phalaenopsis* are more popular than other ploidy in the flower market. Here we use sporad analysis of pollen mother cells to look for unreduced gametes so that the percentage of polyploid progeny can be increased after choosing suitable parents for cross hybridization. Recently developed alliance intergeneric hybrids derived from genera *Holcoglossum* and *Rhynchostylis* may have the chance to be integrated into *Phalaenopsis* cultivars to breed for novel traits. Molecular tools such as genomic and transcriptomic data mining may also help develop efficient breeding. Alternative splicing of a gene *PhAGL6b* leading to change of lip into petal like morphology will be discussed for future potential application of big lip breeding.

Keywords: *Phalaenopsis*, *nobile*-type *Dendrobium*, cross hybridization, intergeneric hybrids, horticultural traits, polyploid, molecular analysis

INTRODUCTION

Potted orchid plants are widely traded in global markets. To meet year-round production and market demands, the flowering ability of orchids is required. The easiest way of flowering is by manipulation of temperature for several orchid species, including *Cymbidium*, *nobile*-type *Dendrobium*, *Phalaenopsis*, and some other minor orchids. Breeding of *nobile*-type *Dendrobium* has been mainly developed by private sectors, such as Yamamoto *Dendrobium*, with the release of numerous hybrids in the past. However, many *nobile*-type *Dendrobium* hybrids require rather lower temperature, such as 13°C for *D. nobile*, for flowering induction (Rotor, 1952). Yen et al. (2008) revealed that cooling of *Dendrobium nobile* hybrids at 10-21°C will induce either delayed or fasten flowering of Sea Mary 'Snow King', with 13 or 15°C conditions being recommended for production cost saving. *Phalaenopsis* nowadays is the leading flower crops for potted plant and cut flower production worldwide. In Taiwan, both private sectors and public research institutes, including universities, are actively working on all aspects of orchid production, with our university focused more on *nobile*-type *Dendrobium* and *Phalaenopsis* breeding. Variety right of authorized by foreign country for 281 *Phalaenopsis* hybrids has been applied and 177 cases approved by 2018. A total of 1,207

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application of *Phalaenopsis* variety right has been filed between 1991 to 2018, November (Council of Agriculture, 2019), indicating the very active breeding work of this orchid in Taiwan constantly.

BREEDING *NOBILE*-TYPE *DENDROBIUM*

There are about eight companies/nurseries working on the breeding or production of *nobile*-type *Dendrobium* orchids in the world, among them Yamamoto *Dendrobium* probably is the leading company for the breeding. The vegetative and reproductive phase of the *nobile*-type *Dendrobium* is quite different from *Phalaenopsis* orchids, including fertilizer and flowering requirements. In order to year-round production and marketing of the flowering plants, several key points or horticultural traits need to be satisfied to fit the breeding program:

1) The finished pot plant should be vertical, flowering at the lower nodes throughout the whole pseudobulb;
2) The flowering plant should bear with green leaves, various flower color choices, good flower shape and display;
3) The production time from flask, young plants up to flowering finished plants should be as short as possible for higher turn-over rate;
4) Flowering of *nobile*-type *Dendrobium* should be controllable either by photoperiod or temperature;
5) The desk appreciation period should be at least six weeks long;
6) The cultivars are tolerant to environmental shifts and pest and disease resistant/tolerant.

To meet the above criteria, we began the breeding in the low land of southern Taiwan by choosing suitable species and commercial hybrids years ago and selected many novel lines that can be grown in 6-cm pot and flower in the temperature-controlled cooling room used for *Phalaenopsis* spiking condition (day/night ~26/18°C). In brief, we have selected novel hybrids with low chilling requirement for flower induction and also with above mentioned criteria (Figure 1).

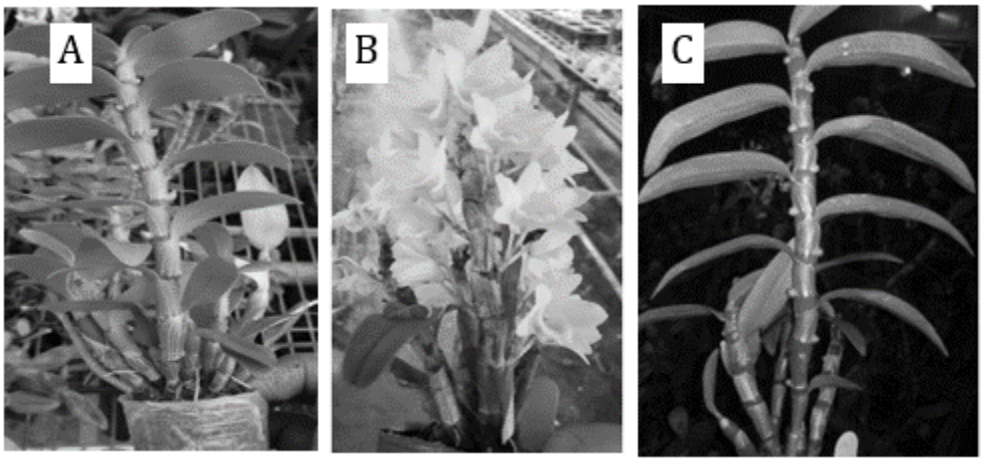

Figure 1. Selected miniature *nobile*-type *Dendrobium* with desirable flowering traits. Line selection 16035 (A, B), ideal reproductive pseudobulb with most nodes, especially lower positions bearing floret primordia (C).

BREEDING NOVEL *PHALAENOPSIS* HYBRIDS

Breeding hybrids with multiple spikes

Commercial cultivars of *Phalaenopsis* require at least double spikes and certain plant height for 9 cm pot or above size, despite some markets like Japan focusing only on single spike of good spray arrangement. In order to create double or multiple spikes, several species,

such as *P. micholitzii* and *P. tetraspis* (Figure 2), carrying this trait can be adopted in the breeding program for novel hybrids development. The species *P. micholitzii* bears compact leaves, multiple but very short spikes and one or two flowers per spike (Figure 2A). The species *P. tetraspis* bears multiple long spikes but long slender leaves (Figure 2B-D). It has several flowers per spike and the color or spot patterns vary from plant to plant, and their fragrance is apparent. Currently the so called violet or indigo colored hybrids in the progenies of *P. tetraspis* and *P. violacea* f. *coerulea* Norton indigo (Figure 2E) are hotly pursued in some hobby markets. In both species, the older spikes do not wilt and dry out but still can produce flowers when encounter the suitable climate conditions. In the near future both commercial and hobby markets may rely on these two species to breed for many innovative *Phalaenopsis* hybrids, and probably intergeneric hybrids with other alliance genera such as *Holcoglossum*, *Rhynchostylis* and *Vanda*. Currently we have been using an intergeneric hybrid *Holcostylis* M S Sunlight (*Holcoglossum flavescens* × *Rhynchostylis gigantea*) (Figure 2F) to cross with some *Phalaenopsis* germplasms or *Rhynchostylis* species in order to develop novelty hybrids having flowering forcing ability, despite a long way is expected to reach the goal.

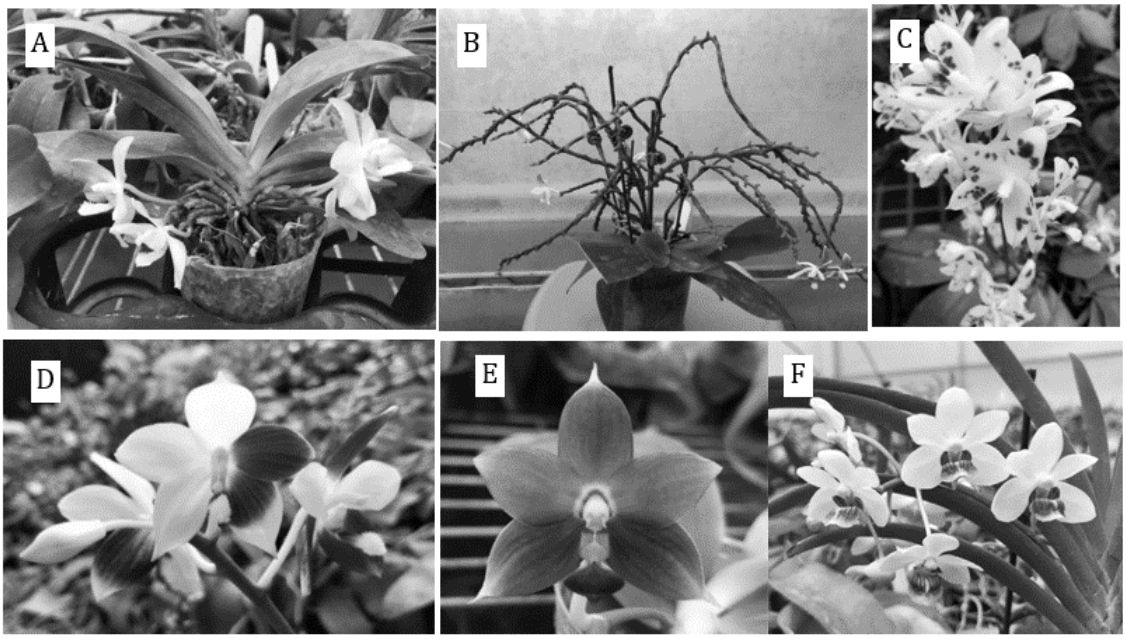

Figure 2. Potential *Phalaenopsis* and alliance genera for breeding novel hybrids with multiple spikes. The species *Phalaenopsis micholitzii* (A) with short, lanceolate and compact leaves as well as multiple and short spikes. The species *Phalaenopsis tetraspis* (B) is with multiple but long spikes, in addition to long leaves and interesting spot patterns (C, D). The indigo colored *P. violacea* f. *coerulea* Norton (E), with strong fragrance is popular among amateur breeders, *Holcostylis* M S Sunlight (F), a hybrid between *Holcoglossum flavescens* × *Rhynchostylis gigantea*, with fragrant flowers, can produce spike in respond to low temperature .

We and other breeders have developed several *Phalaenopsis* hybrids bearing multiple spikes using above mentioned species or interspecific hybrids. Several examples of such novel hybrids are as follows. The hybrid *P.* Nobby's Green Finger available in local market was used to cross with the multiple spikes species *P. micholitzii* or interspecific hybrid *P.* Tzu Chiang Tetralitz (*P. micholitzii* × *P. tetraspis*), which may have as many as five spikes (Figure 3A). The new cross was registered as *P.* Pingtung Green Michol (*P.* Nobby's Green Finger × *P. micholitzii*) which has compact leaves, short and multiple spikes, and in general white and waxy flowers with good shape or even flava yellow colored spots (Figure 3B, D). These sib plants may have the potential to further be used to cross hybridize with commercial cultivars to create novel

colors and multiple spikes. The second hybrid is from a local breeder, which was registered as *P*. HRB Tetralitz (*P*. Nobby's Green Finger × P. Tzu Chiang Tetralitz) (Figure 3E, F). Both hybrids *P*. Pingtung Green Michol and *P*. HRB Tetralitz possess about 56-81% *P. micholitzii* in their genetic background, thus parental traits, such as multiple spikes, could be reiterated in their progeny. Further breeding using these early generation interspecific hybrids with commercial cultivars may produce desirable novel cultivars carrying desirable horticultural traits.

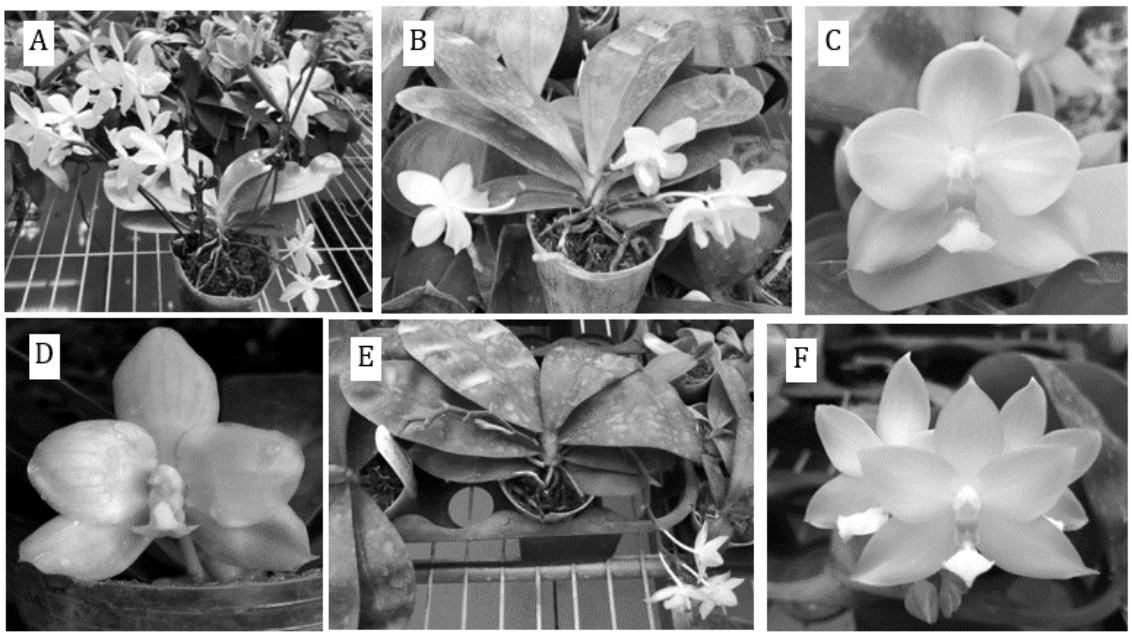

Figure 3. Novel *Phalaenopsis* hybrids generated by cross hybridizing *P. micholitzii* and *P. tetraspis* (*P*. Tzu Chiang Tetralitz) (A), *P*. Nobby's Green Finger × *P. micholitzii* (B-D), and *P*. Nobby's Green Finger × *P*. Tzu Chiang Tetralitz (*P*. HRB Tetralitz) (E-F).

Breeding polyploid *Phalaenopsis* hybrids

Two approaches can be adopted to obtain polyploids or hybrids with doubled chromosome sets. The first is to look for unreduced gametes in desirable pollen parents by analyzing pollen mother cells with sporad types (Bolaños-Villegas et al., 2008). We can also apply mitotic inhibitors on young flower buds when reaching certain sizes before pollen meiotic stage (Hsu et al., 2010; Bolaños-Villegas et al., 2017). We have used lactophenol-acidafuchsin (Lim et al., 2001) to stain pollen mother cells of some *Phalaenopsis* (then *Doritaenopsis*) hybrids and checked the frequency of sporads with different types, such as monads, dyads or *2n* gametes (Bolaños-Villegas et al., 2008). The results indicated small percentage of monad, dyad and triad were present in most hybrids examined, and depending on the genetic background, the higher percentage of tetrads, with regular meiosis, the higher percentage of fertility as indicated by viable seed amounts. We then asked if mitotic inhibitors could be applied to young flower buds to induced unreduced gametes. Two large standard flower cultivars, one with large white *P*. Sogo Yukidian 'V3' and another with large purple *P.* Tai Lin Redangel 'V31', were selected for the experiment. Lower dosage of colchicine (0.05 and 0.10%) and herbicide-type inhibitor trifluralin (0.09 and 0.13%) were mixed separately in lanolin paste and then smeared on the bud surface. The result indicated colchicine could induce increased number of three types of unreduced gametes (Hsu et al., 2010). The pollinia derived from the inhibitor treated flower buds can then be used to cross pollinate with a selected female parent so higher percentage of polyploid progeny can be expected.

A second approach to obtain polyploid is through tissue culture. In general, the young plants of *Phalaenopsis* cultivars are produced by flower stalk node culture and subsequent shoot multiplication or by inducing protocorm-like bodies (PLBs) and further proliferation in

suitable induction media using optimized growth regulator combinations, including auxin and cytokinin and perhaps coconut water (Wu and Chen, 2008; Huang et al., 2014). We have collected a miniature cultivar of commercial origin *P*. Liu's Twilight Rainbow 'Sogo F2006' derived from flower stalk node culture and shoot multiplication. Among the population several tetraploid plants were obtained and larger flower size and thicker petals and roots. A cross between the diploid and tetraploid clones produced progenies with some multiple spikes of commercial value (Figure 4).

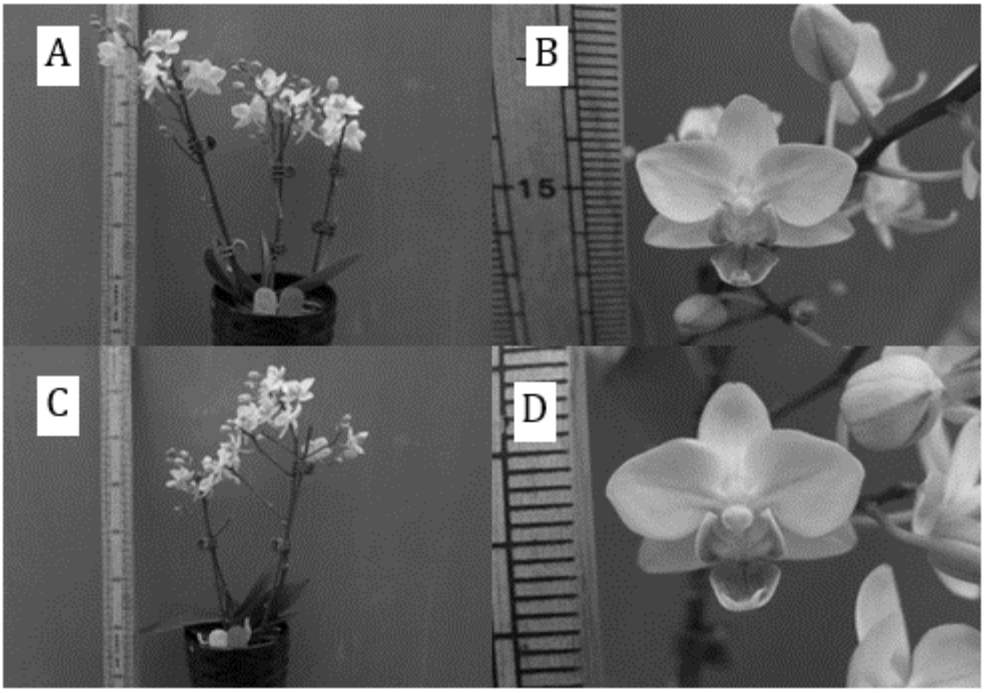

Figure 4. Appearance of triple spikes (A, B) and double spikes (C, D) progenies from sibling of diploid and tetraploid *Phal*. Liu's Twilight Rainbow 'Sogo F2006'.

We crossed the diploid 'Sogo F2006' with the tetraploid and obtained over 100 seedlings. Individuals with double and triple spikes were selected during flowering season (Figure 5). The progenies obtained probably were mostly triploid and the flowering seedlings with ideal plant architecture were chosen for further cross hybridizing to generate novel hybrids with multiple spikes and probably other desirable traits when suitable parents are chosen.

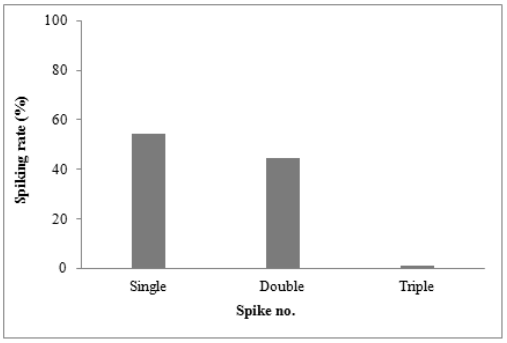

Figure 5. Spiking rate and spike number in a sib cross between diploid and tetraploid *P*. Liu's Twilight Rainbow 'Sogo F2006'.

***Phalaenopsis* lip as a target for novelty flower shape breeding**

A commercial hybrid *P*. World Class 'Big Foot', which has a mutation in the labelum (lip) with petal like morphology, was introduced to the market decade ago. The mutation probably occurred in one of its parent *P*. Kathy Sagaert (Wyche Poole, pers. commun.). *P*. World Class 'Big Foot' was then actively crossed with many commercial cultivars and produced numerous hybrids with novel lip shape and diverse flower colors. We have investigated the novel genes which may be involved in the lip development by analyzing transcriptome and genome library (Huang et al., 2015, 2016). After detailed study by bioinformatics, RT-PCR, real-time PCR and DNA sequencing, the alternative splicing of one candidate gene *PhAGL6b* was found to correlate with the formation of the big lip morphology (Figure 6) (Huang et al., 2015). When the activity of L (lip) complex proteins in the so called Perianth Code was reduced by virus induced gene silencing (VIGS) in *Phalaenopsis* orchid, the lip shape became petal like (Hsu et al., 2015).

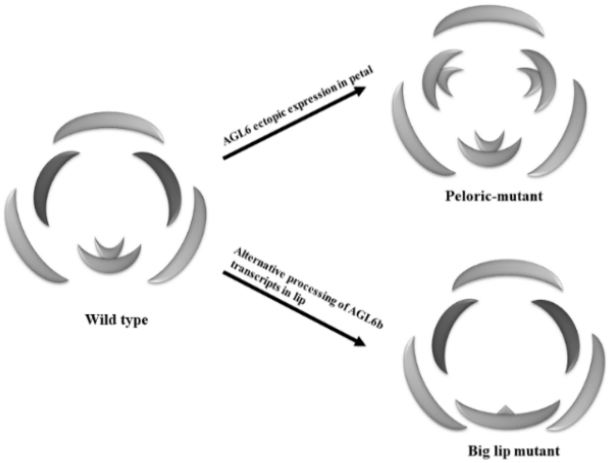

Figure 6. Relationship of *PhAGL6b* expression pattern in the regulation of lip formation in *Phalaenopsis* orchid (modified after Huang et al., 2015).

When the big lip mutant was cross hybridized to the wild-type cultivars as well as reciprocal cross, the ratio of the big lip with the normal lip in the progeny was from 1:1 to 1:2 (unpublished observation). This segregation does not fit the Mendel recessive gene model, and since the alternative splicing of the *PhAGL6b* gene led to the big lip formation, there may be some genes or unknown factors acting together to control the lip development. Therefore, VIGS or the advanced technology of gene editing (Ahmar et al., 2020) may be further used for precise trait modification of *Phalaenopsis* hybrids to fulfil future market demands. One challenge to the genetic modification of *Phalaenopsis* orchid is the use of *Agrobacterium*-mediated transformation (Belarmino and Mii, 2000; Chan et al., 2005), in addition to the difficulty in callus induction and the regeneration through protocorm-like body formation (Huang et al., 2014; Lee et al., 2013). This means an efficient plant regeneration system is required when gene editing technique is to be applied for precise breeding of *Phalaenopsis* and other orchids.

CONCLUSIONS

Breeding of *nobile*-type *Dendrobium* and *Phalaenopsis* orchids was achieved with numerous novel lines with desirable horticultural traits in our university. The breeding is a long-term work because of the market demands or changes of consumer preference from time to time. Besides, many new commercial orchid cultivars are produced and released to the global market, as a breeder one should attempt every possibility to obtain available advanced cultivars. Intra-specific or inter-specific hybrids may also have the opportunity to combine superior traits from both parents and contribute novel genetic advantages for cross

hybridization. Molecular tools such as transcriptome and bioinformatics, gene editing and other potential new technologies can be adopted to the breeding program for high efficiency and precision gene modification to create unusual innovative hybrids to meet the future market trends.

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Characterization of a gene co-expression network associated with *MGST*, the pollen modifier gene of gametophytic self-incompatibility in sweet cherry (*Prunus avium* L.)

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Abstract

Most sweet cherry (*Prunus avium* L.) cultivars exhibit S-RNase-based gametophytic self-incompatibility (SI), a major obstacle for fruit production. Clarifying the molecular mechanism behind the SI reaction will contribute to future improvements in sweet cherry productivity. Although the *S* haplotype combination of pollen and seed parent determines SI specificity in *Prunus*, as in other S-RNase-based SI plants, accumulated experimental results imply the presence of a *Prunus*-specific SI mechanism. The sweet cherry cultivar 'Cristobalina', with a functional *S* locus, shows pollen-part self-compatibility (SC) owing to the downregulated expression of an SI modifier in pollen. This modifier has been identified as *M*-locus-encoded glutathione S-transferase-like (*MGST*), but it has not been well characterized. In this study, we investigated the transcriptomic profiles of pollen with relatively higher and lower *MGST* expression levels, *M*-pollen and *m*-pollen, respectively. 'Cristobalina' (*Mm*) F_1 populations segregating for SC (*Mm*) and SI (*MM*), 'Cristobalina' selfed progeny of the *mm* genotype (SC), and diverse SI sweet cherry cultivars (*MM*) were used. Illumina mRNA-seq reads obtained from a pollen grain or germinated pollen tube of each sample were mapped to the sweet cherry reference-coding sequence. A differential expression analysis among the genotypic combinations yielded 985 and 853 differentially expressed genes in the pollen-grain and germinated pollen-tube stages, respectively; however, only *MGST* was detected in all the pollen-grain comparisons. This supported our hypothesis that *MGST* functions in the S-RNase activation of pollen. However, a differentially expressed gene clustering analysis and a weighted gene co-expression network analysis revealed significant correlations between MGST's expression and the expression levels of functional gene sets, such as those involved in lipid metabolism and stress responses. Thus, it is also possible that *MGST* may function in some pollen development- or viability-related processes. Further analyses are necessary to elucidate the molecular function of *MGST* in the *Prunus*-specific SI reaction.

Keywords: glutathione S-transferase, S-RNase, differential gene expression, weighted gene co-expression network analysis, transcriptome analysis

INTRODUCTION

Sweet cherry (*Prunus avium* L.) is an important fruit crop worldwide, with more than 2.5 million t produced in 2018 (Food and Agricultural Organization, 2018). The presence of self-incompatibility (SI) in sweet cherry leads to the requirement of additional labor to assist in pollination and subsequent fruit set during fruit production. The SI system prevents fertilization by self-pollen and pollen from closely related individuals. The low fertilization rates of SI crops are disadvantageous for their fruit production, although high outcrossing rates are advantageous for the generation of genetic diversity within wild plant species.

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Clarifying the SI mechanism may lead to the development of artificial SI control measures. In the *Prunus* SI system, pollen tube growth is arrested in the middle of the style when the haploid pollen bears the same *S* haplotype, defined by a combination of pollen and pistil *S* alleles, as one of the two *S* haplotypes in diploid pistils at the *S* locus (Crane and Lawrence, 1929). Self/non-self is determined by the genes encoded by the *S* locus, consisting of the pollen-part determinant, which is the F-box protein, and the pistil-part determinant, which is the S-RNase (Ushijima et al., 1998; Tao et al., 1999; Entani et al., 2003; Ushijima et al., 2003; Yamane et al., 2003). S-RNase-based gametophytic SI occurs not only in *Prunus* (*Rosaceae*) but also in the *Malinae* (*Rosaceae*), *Solanaceae* and *Plantagenaceae* (Anderson et al., 1986; Sassa et al., 1992). However, accumulated data indicate that the underling molecular mechanism in *Prunus* SI is different from that of other species, although these species commonly utilize S-RNase and F-box proteins in self/non-self recognition (Hauck et al., 2006; Ushijima et al., 2004; Morimoto et al., 2015; Matsumoto and Tao, 2016).

In addition to the self/non-self determinants, the characterization of SI-modifier factors, which are located outside of the *S* locus, is indispensable to fully understand the molecular mechanism of SI. Modifiers should play important roles at certain steps in the SI reaction cascade, and thus, they have been studied to elucidate molecular bases behind the SI reaction. One possible modifier was determined from the sweet cherry 'Cristobalina' (S^3S^6) found in eastern Spain. Although its S-RNases and S haplotype-specific F-box proteins seemed to be functional, 'Cristobalina' exhibits pollen-part self-compatibility (SC) (Wünsch and Hormaza, 2004; Wünsch et al., 2010). A bulked segregant analysis of 'Cristobalina' progeny identified a molecular marker linked to SC, and it was mapped to the sweet cherry linkage group 3 (Cachi and Wünsch, 2011). Subsequently, whole-genome re-sequencing and subsequence (35-mer) cataloging identified the causal gene for SC in 'Cristobalina', named *M* locus-encoded glutathione S-transferase-like, *MGST*, which was annotated as a GST kappa-like protein (Ono et al., 2018). This finding was corroborated by the identification of an orthologous *MGST* gene in apricot (*P. armeniaca*), which was named *P. armeniaca M*-locus DsbA-like oxidoreductase, *ParMDO*, in accordance with its structural similarity to the oxidoreductase containing disulfide bond A-like (DsbA-like) domain, that acts as an SI modifier (Muñoz-Sanz et al., 2017). However, the molecular functions of *MGST* and *ParMDO* remain to be clarified. Unfortunately, the functions of their homologs in dicots are not well characterized; therefore, they are of limited use in making comparative functional inferences.

'Cristobalina' is heterozygous for *MGST*, producing both mutated SC pollen with downregulated *MGST* expression (*m*-pollen) and wild-type SI pollen with normal *MGST* expression (*M*-pollen) (Ono et al., 2018). In this study, we characterized the transcriptomic profiles of *M*-pollen and *m*-pollen at two different developmental stages, the mature pollen grain (PG) and the germinated pollen tube (PT), in an artificial medium. Based on the transcriptomic analysis, a presumptive molecular function of *MGST* is discussed.

MATERIALS AND METHODS

Plant materials and pollen sample preparation

Samples were divided into three groups, the PG of 'Cristobalina' progeny, the PG of *MM* cultivars, and the PT. There were 39 PG samples of 'Cristobalina' progeny derived from the following crosses: 'Brooks' × 'Cristobalina' (BC), 'Lambert' × 'Cristobalina' (LC), 'Rainier' × 'Cristobalina' (RC), 'Cristobalina' × 'Cristobalina' (CC), and three yearly replicates of 'Cristobalina' (*Mm*). BC, LC, and RC contained *MM* and *Mm* individuals, while for CC, only *mm* individuals were used. Because pollen is haploid, pollen samples of *Mm* individuals have a mixture of *M*-pollen with relatively higher *MGST* expression levels and *m*-pollen with relatively lower expression levels. There were 18 PG samples from the *MM* cultivars, including 'Ambrunes', 'Takasago', 'Van', 'SonMiro', two replicates of 'Benishuho', two replicates of 'Napoleon', three replicates of 'Rainier', five replicates of 'Satonishiki', and two replicates of 'Stella'. In addition, we used 11 PT samples from four CC (*mm*) progeny and seven (*MM*) cultivars: 'Benishuho', 'JI2434', 'Naporeon', 'Rainier', 'Satonishiki', 'Takasago', and 'Seneca'. Medium containing 12.5% polyethylene glycol 6000, 5% sucrose, 300 ppm casein, 0.025 M

MES, 1 mM $CaCl_2$, 1 mM KCl, 0.8 mM $MgSO_4$ and 1.6 mM H_3BO_3 was utilized for pollen cultivation to obtain PT samples. Approximately 20 mg of pollen was added to 1 mL of the medium, which was then rotated at room temperature for 4-5 h. Afterwards, PTs were collected by centrifugation.

In this study, we collected PGs from RC and CC progeny and PTs from all the individuals to prepare mRNA libraries. Illumina mRNA sequence data of other PG samples were downloaded from the Short Read Archives database (BioProject ID PRJDB6734) (Ono et al., 2018). Pollen of CC was collected from orchards of Centro de Investigación y Tecnología Agroalimentaria de Aragón in Zaragoza (Spain). That of 'Seneca' was collected from the experimental orchard of Yamagata University (Japan), and those of other cultivars and RC were collected from the experimental orchard of Kyoto University (Japan).

mRNA library preparation and quantification of mRNA expression levels

Sequencing libraries were prepared as described by Ono et al. (2018) using PureLink® Plant RNA Reagent (Thermo Fisher) for RNA extraction, Dynabeads™ mRNA Purification Kit (Thermo Fisher), Superscript III reverse transcriptase (Life Technologies), DNA polymerase I (New England BioLabs; NEB) and RNaseH (NEB) for second strand synthesis, NEBNext dsDNA Fragmentase (NEB), AMPureXP (Beckman Coulter Life Sciences) for removing fragments less than 250 bp, and PrimeStar Max DNA polymerase (TAKARA) for enrichment. End repair, A-tailing, and adaptor ligation steps were substituted using a KAPA HyperPlus Kit (Kapa Biosystem). The Illumina sequencing libraries were then sequenced using Illumina HiSeq4000 (150-bp paired-end) or 2500 (100-bp paired-end). Reads with low quality base calling (Phred score<20) and with "N" bases were trimmed, and reads shorter than 35 bp were removed using python scripts (https://github.com/Comai-Lab/allprep). Sweet cherry reference-coding sequence (Shirasawa et al., 2017), as well as *MGST*- and Pav_sc0000661.1_g340.1-2-coding sequences (Ono et al., 2018) were mapped with the clean RNA-seq reads using Burrows-Wheeler Aligner version 0.7.15 (Li and Durbin, 2009) with default parameters. The normalized expression values per gene were recorded as reads per kilobase of transcript per million mapped reads (RPKM). Pearson's correlation coefficient analysis was applied to detect correlations between the gene and *MGST* expression levels.

Identification of differentially expressed genes (DEGs)

The read count data were analyzed using the DESeq2 software package (Love et al., 2014) to identify DEGs between self-incompatible pollen (*M*) and self-compatible mutated pollen (*m*). First, for PG-progeny group samples, genes with an average RPKM value lower than one across whole libraries were filtered out. Then, DEGs for the "*MM* vs. *mm*", "*Mm* vs. *mm*" and "*MM* vs. *Mm*" comparisons were detected independently using the threshold of a false discovery rate (FDR) lower than 0.01. All the DEGs detected were then hierarchically clustered with the average-linkage method based on one minus Pearson's correlation value using Morpheus (2018). For the PT data, the "*MM* vs. *mm*" comparison was used owing to sample limitations. The results were clustered by the method used for the PGs.

Discovering SI associated gene co-expression network

The Weighted Gene Correlation Network Analysis (WGCNA) R package (Langfelder and Horvath, 2008) was used to characterize the co-expression network. The gene co-expression network in the *MGST*-containing module was first visualized using the VisANT program (Hu et al., 2004). The soft-thresholding power was determined by the lowest number that the scale-free topology model fitted at greater than 0.85. The correlation values (weights) between two genes were calculated by raising Pearson's correlation coefficient to a certain soft-thresholding power. Intramodular connectivity was calculated based on the correlation with the expression of the module's eigengene. Genes that had the top 5% values for intramodular connectivity among *MGST*-containing modules were selected as the hub genes, which may have central roles in controlling the overall gene expression in the module.

Functional analysis

The cherry-coding sequences were annotated using the National Center for Biotechnology Information protein non-redundant database and Blast2GO software (Conesa et al., 2005) ($E<10^{-3}$), with default parameters. Then, an enrichment analysis was conducted for DEGs or genes in the *MGST*-containing module (Fisher's exact test; FDR<0.1).

RESULTS

Differential expression and gene ontology (GO) enrichment analyses between *M*- and *m*-pollen at the PG and PT stages

Among the PGs of the 'Cristobalina' progeny group, 985 DEGs were detected in the three comparisons (*MM* vs. *mm*, *Mm* vs. *mm* and *MM* vs. *Mm*), including 410 and 575 *M* up- and downregulated genes, respectively. Interestingly, *MGST* was the only gene detected in all three comparisons. This suggests the possibility that *MGST* itself does not have a large effect on the transcriptome profile at the PG stage. An enrichment analysis (Fisher's exact test) of clusters defined by the hierarchical clustering analysis using the linkage method based on Pearson's correlation values was performed on the 383 genes in the *M* upregulated cluster (1), and 366 genes in the *M* downregulated cluster (4), of the DEGs (Figure 1A). Several GO terms, including lipid metabolism, were enriched in each cluster (Table 1).

Table 1. Representative enriched GO terms of *M* up- and downregulated clusters and *MGST*-containing modules, with false discovery rates in parentheses.

M-upregulated cluster of DEGs for PG
lipid metabolic process (0.035); oxylipin metabolic process (0.100)
M-downregulated cluster of DEGs for PG
microtubule-based movement (0.020); positive regulation of MAPK cascade (0.069); developmental vegetative growth (0.080); mitotic cell cycle (0.080); mitochondrial respiratory chain complex III (0.080)
M-upregulated cluster of DEGs for PT
lipid metabolic process (0.003); sphingolipid biosynthetic process (0.036); regulation of exocytosis (0.038); C-4 methylsterol oxidase activity (0.048); arachidonic acid secretion (0.048)
M-downregulated cluster of DEGs for PT
carbohydrate metabolic process (3.45E-4); response to oxygen-containing compound (0.030)
MGST-including module for PG (WGCNA)
chromatin remodeling (0.005); transcription by RNA polymerase II (0.006); translational initiation (0.018); regulation of response to biotic stimulus (0.049)
MGST-including module for PT (WGCNA)
protein disulfide isomerase activity (0.461); regulation of hydrogen peroxide metabolic process (0.821); positive regulation of translation (0.821); regulation of lipid kinase activity (0.821)

At the PT stage, 853 DEGs, including 309 *M* up- and 544 *M* downregulated genes, were detected. *MGST* was included in the DEGs and had the 342nd lowest FDR among all the genes. Among the DEGs, 182 were detected in both the PG and PT stages. An enrichment analysis of the hierarchical clusters was performed for the clusters containing the 300 *M* upregulated genes (Cluster 2) and the 336 *M* downregulated genes (Cluster 4) of the DEGs (Figure 1B). Several GO terms were enriched in each cluster (Table 1).

WGCNA of *MGST*-related genes in the PG and PT stages

Expression data of mapped genes with RPKM values >1 in the PG progeny group (13,619 of 41,234) were analyzed using a soft-thresholding power of 7. The WGCNA function cutreeDynamic clustered them into 31 modules with a tree-height cut-off of 0.15. One of the 31 modules, which contained 553 genes, included *MGST*. The expression levels of the genes in this module were positively correlated with MGST's expression, but some showed negative correlations. Several GO terms were enriched in this module (Table 1). However, *MGST* showed a relatively low intramodular connectivity, which indicates that *MGST* was not a

central hub gene in this module.

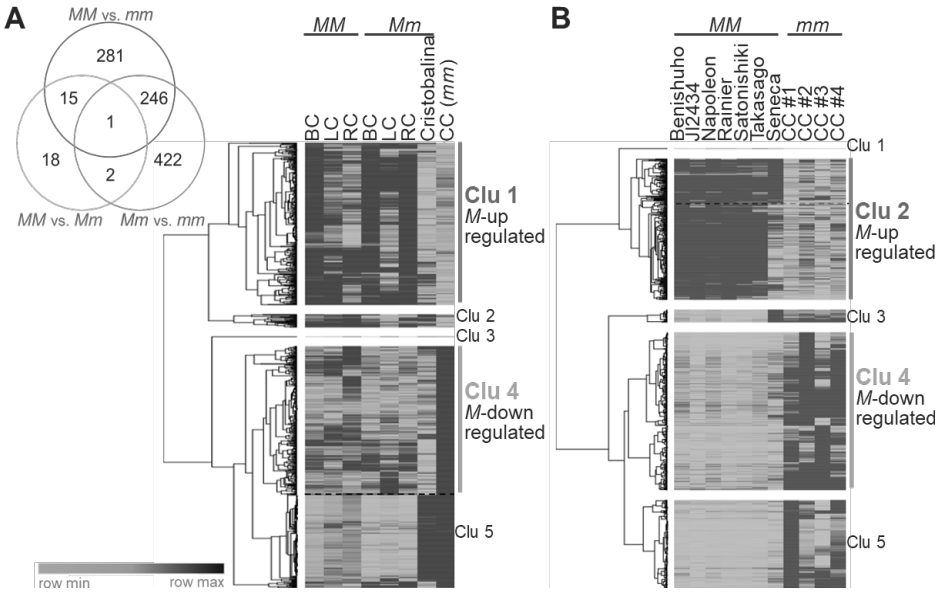

Figure 1. Differential expression between *M*- and *m*-pollen at the PG and PT stages. (A) Venn diagram of DEGs in the PG stage; (A, B) hierarchical clustering of *M* up- and downregulated genes in the PG (A) and PT (B) stages. Average RPKM values of yearly replicates of cultivars or individual replicates of lineage are represented.

The genes in the PG cultivar group with RPKM values >1 (8,351 of 41,234) were also analyzed using a soft-thresholding power of 7 and they clustered into nine modules. The module that included *MGST* contained 613 genes. The expression levels of genes in this module were both positively and negatively correlated with MGST's expression. As in the PG progeny group, *MGST* showed a relatively low intramodular connectivity.

Expression data of mapped genes with RPKM values >1 in the PT stage (11,257 of 32,369) were analyzed using a soft-thresholding power of 18 and they clustered into 26 modules. The module that included *MGST* contained 245 genes. Several GO terms were enriched in this *MGST*-containing module (Table 1) when $p<0.05$ was set as the enrichment threshold rather than FDR<0.1 owing to an insufficient gene number. Similar to the PG results, *MGST* did not have features of a central hub gene in this module.

DISCUSSION

In this study, we analyzed the transcriptomic profiles of two pollen stages to investigate the molecular function of *MGST* in the *Prunus* SI reaction. In the 'Cristobalina' progeny group, *MGST* was the only DEG found across all three comparisons (*MM* vs. *mm*, *Mm* vs. *mm*, and *MM* vs. *Mm*). In addition, the WGCNA revealed that *MGST* was not a candidate hub gene. These results suggest that the *MGST* expression level is not a direct transcriptomic regulator of a specific pathway, at least not in the PG and PT stages. This result supported our hypothesis that MGST only functions in the S-RNase activation in pollen after direct interaction with S-RNase in pollen (Matsumoto and Tao, 2019). However, it may also be possible that MGST may become functional after the pollen and stigma interaction. Qin et al. (2009) suggested that pollen may be activated and many genes newly transcribed after the interaction with the stigma as indicated by genes being differentially expressed between PT germinated in an artificial medium and PT pollinated on a stigma.

However, some GO terms were significantly enriched in DEGs between *M*- and *m*-pollen, and in *MGST*-containing WGCNA modules (Table 1), which suggested that *MGST* is functional during the PT and PG stages. Because GOs related to sugar metabolism were enriched in *m*-pollen with lower *MGST* expression levels, while GOs related to lipid metabolism were

enriched in *M*-pollen, *MGST* might be involved in switching from primary to secondary metabolism. Fatty acids, such as oxylipins, are sources of many secondary metabolites. Thus, *m*-pollen with a relatively active primary metabolism might grow faster in the style to accomplish fertilization before being rejected by the SI reaction. Other *m*-enriched GO terms such as "mitochondrial respiratory chain complex II" and "microtube-based movement", support this hypothesis. A comparison of pollen tube growth between *M*- and *m*-pollen should be conducted in the future. Because MGST is a possible member of the thioredoxin-like superfamily, MGST might function as an oxidoreductase to regulate the antioxidative environment. This hypothesis is supported by the enrichment of GOs related to stress responses, such as "oxylipin metabolic process", "regulation of hydrogen peroxide metabolic process" and "regulation of response to biotic stimulus" (Table 1). Because S-RNase is toxic to pollen, active defense processes in pollen might be involved in the SI response.

In this work, we proposed experimentally supported hypotheses for the molecular function of *MGST* in the *Prunus* SI reaction. The data serve as a basis for further experiments designed to clarify MGST's function.

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Allelic distribution at SNP loci within bud dormancy-related QTLs in Japanese apricot (*Prunus mume*) collections

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Abstract

Bud dormancy broadly affects factors related to tree growth and development, including blooming date and leafing date (LD), which are important phenological traits associated with fruit production. We previously identified significant quantitative trait loci (QTLs) for the leaf bud chilling requirement, heat requirement, and LD on *Prunus* consensus linkage group 4 (LG4) in Japanese apricot (*Prunus mume*). However, the significance of QTLs in LG4 for the regulation of dormancy and bud break in diverse *P. mume* germplasm collections remains undetermined. Accordingly, we first searched for single nucleotide polymorphisms (SNPs) that are in the LG4 QTL interval and associated with LD variations in a segregating F_1 population. We genotyped these SNP loci in 19 *P. mume* cultivars with a Kompetitive allele-specific PCR genotyping assay. Additionally, we recorded the LD of these collections in the 2016-2017 and 2017-2018 seasons. Our allelic frequency distribution analysis suggested that specific alleles at selected SNP loci that tended to be distributed in late-LD cultivars were not present in early-LD cultivar. However, the early-LD-specific alleles and genotypes of some SNP loci were detected in late-LD cultivars. These results imply that the identified LG4 QTLs for LD is not a single causal determinant of the early-LD phenotype but is one of the loci associated with the LD variation in *P. mume* germplasm.

Keywords: Japanese apricot, chilling requirement, heat requirement, bud break, Kompetitive allele-specific PCR (KASP)

INTRODUCTION

Bud dormancy, which evolved as a crucial trait for overcoming the extreme winter climate, is important for perennial crops because it determines the timing of vegetative bud break and blooming (Beauvieux et al., 2018; Rohde and Bhalerao, 2007; Yamane, 2014). Dormancy establishment is regulated by environmental cues, including the chilling temperature and a short photoperiod (Horvath et al., 2003). Dormancy is released by a genetically determined specific chilling period (i.e., chilling requirement fulfillment) and a subsequent warming period (i.e., heat requirement fulfillment), culminating in bud break and normal blooming. Although dormancy is regulated by environmental cues, genetic variations influencing the dormancy progression rate and dormancy depth have been reported for *Rosaceae* fruit tree species (Yamane, 2014), and thus dormancy is controlled by genetic as well as environmental factors.

To decipher the genetic regulation of dormancy, quantitative trait loci (QTLs) have been analyzed to identify the genetic factors underlying dormancy-associated quantitative traits in *Rosaceae* species, including Japanese apricot (*Prunus mume*) (Kitamura et al., 2018), apricot (*Prunus armeniaca*) (Olukolu et al., 2009), sweet cherry (*Prunus avium*) (Castède et al., 2014, 2015), peach (*Prunus persica*) (Blaker and Chaparro, 2012; Fan et al., 2010; Romeu et al., 2014), European pear (*Pyrus communis*) (Gabay et al., 2018), apple (*Malus domestica* Borkh.) (Miotto et al., 2019; Van Dyk et al., 2010), and strawberry (*Fragaria vesca* L.) (Samad et al.,

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2017). In peach, the fine-mapping of QTLs and a genome wide association study suggested that low-chill-specific polymorphisms are located in the linkage group 1 (LG1) region containing *DORMANCY-ASSOCIATED MADS-box* genes, which encode well-known dormancy regulators (Li et al., 2019; Zhebentyayeva et al., 2014). Additionally, these dormancy-associated QTLs in peach had been used to develop a marker-assisted selection platform via a pipeline of high-resolution melting and principal component analyses (Chou et al., 2020).

Prunus mume is ideal for analyzing bud dormancy because this fruit species has a small sequenced genome that is genetically diverse (Zhang et al., 2018). We recently applied a genotyping-by-sequencing method to analyze QTLs for bud chilling requirement (CR), heat requirement (HR), leafing date (LD), and blooming date, and identified significant QTLs for CR, HR of leaf buds and LD on the terminal region of *Prunus* consensus LG4 (equivalent to the Pm3 chromosome) (Kitamura et al., 2018). However, the importance of the LG4 QTLs for the regulation of dormancy and CR in diverse *P. mume* germplasm collections remains unclear. In this study, we conducted a Kompetitive allele-specific PCR (KASP) genotyping assay to investigate an association between genotypes with the identified LG4 QTL regions and the LD phenotype in diverse germplasm collections.

MATERIALS AND METHODS

SNP selection

The sequence data of individuals in an F_1 population derived from a cross between late LD 'Nanko' and early LD 'SC' (i.e., the NKSC F_1 population) used in this study were generated in an earlier investigation by Kitamura et al. (2018). After the single nucleotide polymorphism (SNP) calling and initial screening steps, only SNPs that were heterozygous in either or both parents were selected as described by Kitamura et al. (2018). The SNP loci missing in more than 10% of individuals and those exhibiting a significant segregation distortion based on a χ^2 test ($p<0.05$) were excluded. A genetic map was constructed with the default settings of the maximum likelihood mapping method in JoinMap 4.1 (van Ooijen, 2006). The QTLs were identified according to an LOD score ($p<0.05$) determined by a permutation test with 1,000 replicates in MapQTL 6 (van Ooijen, 2009). The confidence interval of each QTL was determined as 1.0- and 1.5-LOD intervals from the QTL peak. The SNP markers in the LG4 QTL region were phased based on the segregation pattern, so that the recombinant haplotypes could delimit the QTL region. The SNPs in this region were further selected based on the correlation between genotype and the phenotype data of bud dormancy-associated traits (Kitamura et al., 2018).

Plant materials and leafing date phenotyping

Adult trees (>15 years old) of 19 *P. mume* cultivars grown at the Experimental Farm of the Graduate School of Agriculture, Kyoto University (35.032N; 135.785E) were used in this study. The LD phenotype data for the 2015-2016 and 2016-2017 seasons (LD2016 and LD2017) were recorded. The LD was defined as the date when bud break (visible bud scale loosening) was observed in the middle portions of long branches. The Pearson correlation coefficient was calculated with the Python pandas package.

Plant DNA extraction

Frozen leaf tissue was ground to a fine powder, after which 1 mL pre-heated extraction buffer containing 1% (v/v) 2-mercaptoethanol (65°C) was added and the sample was gently mixed before being incubated at 65°C for 30 min. The sample was centrifuged at 5,000×g for 5 min, and the supernatant was transferred to a tube with an equal volume of chloroform: isoamyl alcohol (24:1 (v/v)). The solution was slowly shaken and then centrifuged at 5,000×g for 10 min. The upper phase was transferred to a new tube, to which 0.6 µL 100 mg mL^{-1} RNase A was added before an incubation at 37°C for 15 min. The upper phase was transferred to a new tube and nucleic acids were precipitated with 5 M NaCl (0.5 volume) and chilled (-20°C) 95% ethanol (3 volumes) at -20°C for 30 min. The solution was centrifuged at 5,000×g for 10 min and the pellet was washed with 600 µL 70% ethanol and then centrifuged at 5,000×g for

5 min. The pellet was air-dried and re-suspended in 30 µL sterilized water.

Kompetitive allele-specific PCR genotyping

A 10-µL KASP reaction solution was prepared comprising 5 µL 2× KASP master mix, 50 ng genomic DNA, and 0.14 µM KASP assay mix. The KASP genotyping analysis was completed with the Light Cycler® 480 system (F. Hoffmann-La Roche, Basel, Switzerland). The PCR thermal cycling conditions were set according to the instruction manual.

RESULTS AND DISCUSSION

Haplotype analysis of the NKSC F_1 population

The SNP candidates in the LG4 QTL region that were heterozygous in high-chill individuals and homozygous in low-chill individuals in the NKSC F_1 population (Kitamura et al., 2018) were selected based on a haplotype analysis (data not shown). These SNP loci were considered to be putative dormancy-associated SNP loci. An example of the genotypes of selected dormancy-associated SNP loci in the NKSC F_1 population is presented in Figure 1.

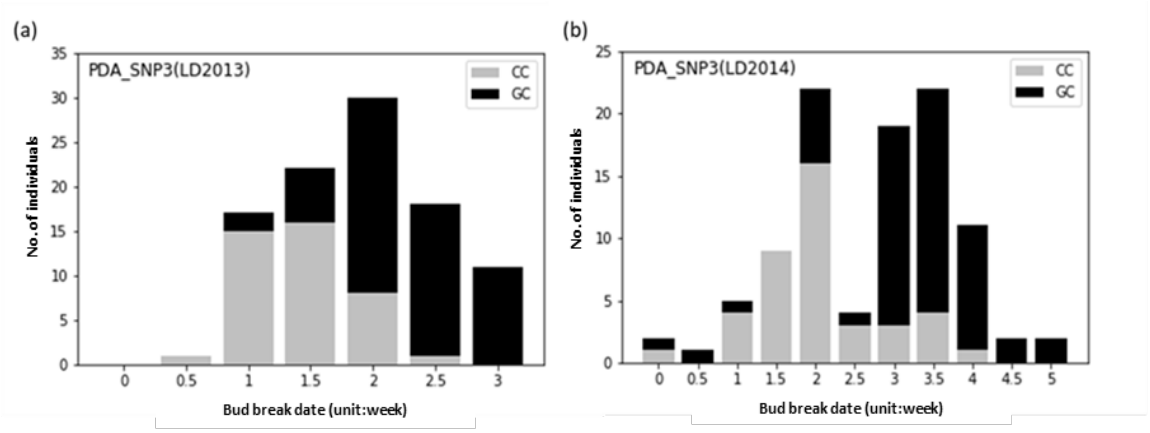

Figure 1. NKSC F_1 genotype composition of PDA_SNP3 and the leafing date (LD) frequency distribution in 2013 (a) and 2014 (b).

KASP genotyping and leafing date phenotyping of 19 cultivars

The LD phenotyping results suggested that the 19 cultivars included one early-LD cultivar ('Ellching'). The rank correlation coefficient of LD between years was high (i.e., 0.695 between 2016_LD and 2017_LD), implying that genetic factors contributed to the LD variations in these collections. The KASP assay genotyping results for the 19 *P. mume* cultivars are provided in Figure 2. Different genotypes were clearly discriminated (Figure 2). The SNP genotypes of all cultivars are listed in Table 1. The genotype distributions of SNPs relative to the LD scores are presented in Figure 3. In PDA_SNP1, allele 'C' was not detected in the early-LD cultivar 'Ellching', but alternative allele 'T' was distributed in both late- and early-LD individuals of the19 cultivars. In PDA_SNP3, allele 'G' was undetectable in 'Ellching', but alternative allele 'C' was detected in both late- and early-LD individuals (Table 1; Figure 3). Several late-LD individuals had the same genotype as 'Ellching' at these two loci, and individuals with the latest LD were homozygous in PDA_SNP1. These observations imply that allele 'C' of PDA_SNP1 and allele 'G' of PDA_SNP3 may be associated with a late LD. In contrast, for PDA_SNP2, the main genotype of high-chill collections was the same as that of 'Ellching' and the genotype pattern of this locus was not correlated with LD (Table 1; Figure 3). Regarding PDA_SNP4, most late-LD individuals had an 'AA' genotype, whereas 'Ellching' had a 'GG' genotype. The allelic distributions of PDA_SNP1, 3 and 4 were consistent with those in the early- and late-LD individuals of the segregating NKSC population. Specifically, alleles 'C' of SNP1, 'G' of SNP3 and 'A' of SNP4 were preferentially distributed in the late-LD F_1 individuals (Figures 1 and 3).

Table 1. Results of the KASP genotyping of four putative dormancy-associated SNP loci in 19 *Prunus mume* cultivars.

Cultivar	PDA_SNP1	PDA_SNP2	PDA_SNP3	PDA_SNP4	LD2016[a]	LD2017[b]
Rinshu	CC	AA	GC	AA	85	35
1KO-26	TT	AA	GC	AA	74	23
Kensaki	TT	AA	GC	AA	84	28
Shirokaga	CC	AA	GC	AA	90	38
Koshukoume	TC	AA	GC	AA	81	23
Ryukyokoume	CC	AA	GC	AA	66	23
1C1-10	TC	AA	GC	AA	66	14
Aojiku	TC	AA	GC	AA	78	23
Oshuku	TT	AA	GC	AA	74	28
Kagajizo	TC	AA	CC	AA	74	28
Nanko	TC	AG	GC	AG	70	23
Kotsubnu-nanko	CC	AA	GG	AA	74	28
Koshinoume	TT	AA	GG	AA	76	23
Ellching	TT	AA	CC	GG	19	3
Gyokuei	CC	AA	GC	AA	90	38
Kairyo-uchida	CC	AA	GG	AA	81	23
Hayazakinanko	TC	AG	GC	AG	NA[c]	23
Benisashi	TT	AA	CC	AA	78	23
Hachiro	TC	AA	GG	AA	81	28

[a]LD2016 was calculated from January 1, 2016.
[b]LD2017 was calculated from March 1, 2017.
[c]Missing value is represented by the symbol NA.

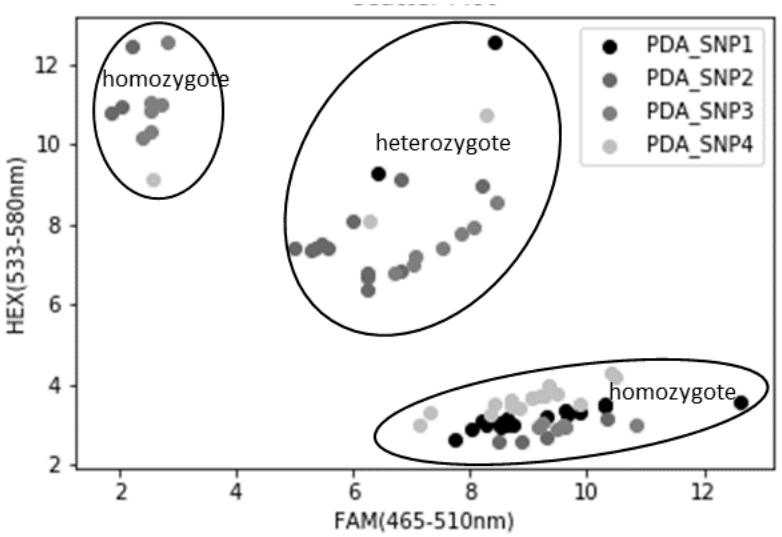

Figure 2. Discrimination of the putative dormancy-associated alleles in KASP systems based on 19 *Prunus mume* cultivars.

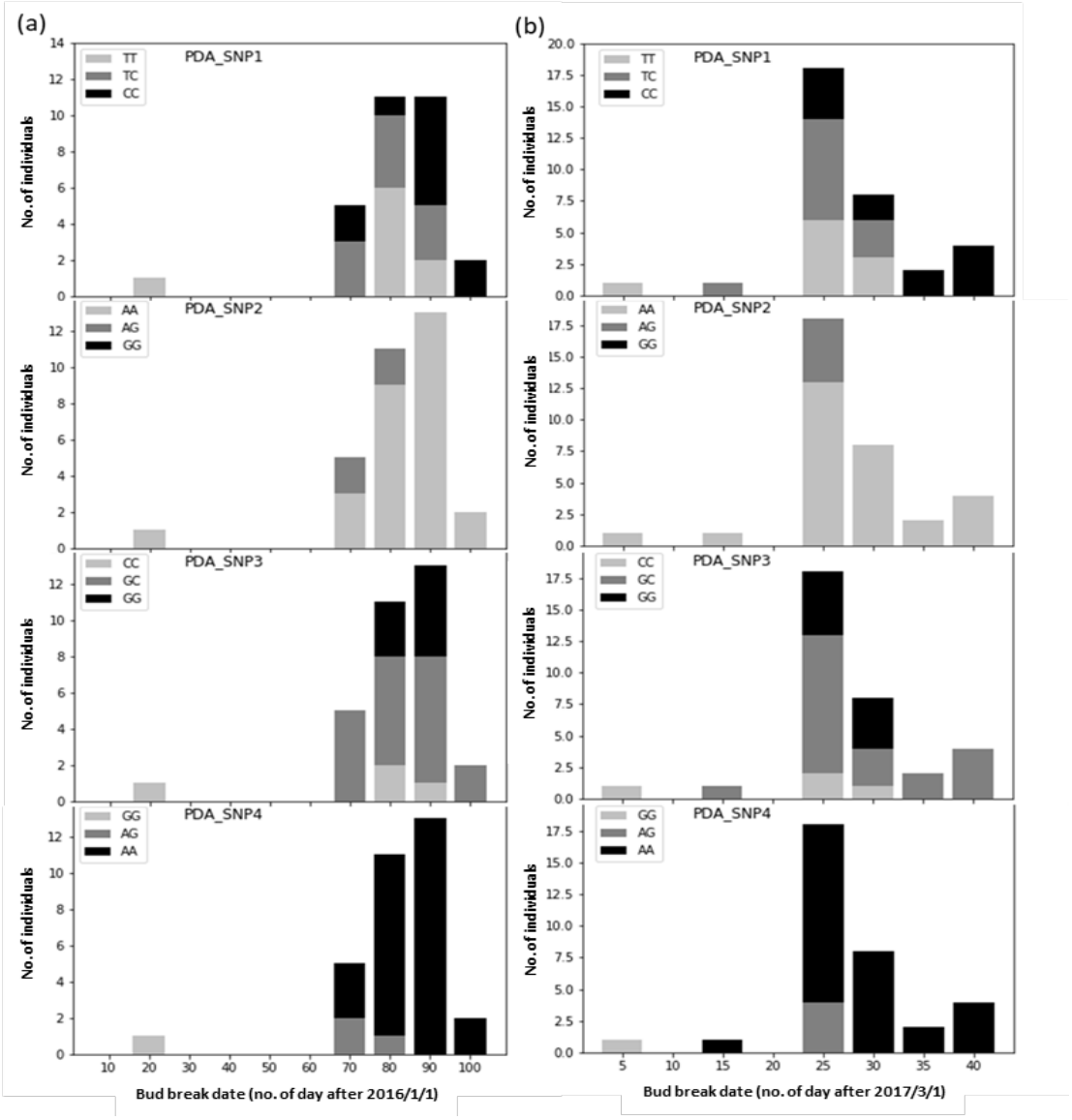

Figure 3. Genotype composition of putative dormancy-associated SNPs and the leafing date frequency distribution in 2016 (a) and 2017 (b) for 19 *Prunus mume* cultivars.

CONCLUSIONS

In this study, we established a KASP genotyping platform for *P. mume*. After identifying SNPs highly correlated with LD in the NKSC F_1 cultivars, we analyzed five putative dormancy-associated SNP candidates in 19 *P. mume* collections that were present in late-LD-related genetic variations. Three SNP loci present mostly in late-LD individuals in the NKSC F_1 population were also detected in the late-LD collections. We will validate our results using larger *P. mume* germplasm collections. In addition, we will identify and evaluate additional putative dormancy-associated SNP loci to elucidate the universal dormancy-associated genomic characteristics.

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Evaluation of the effects of pollination on fruit size and quality of highbush blueberry

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Abstract

Pollination is an important factor affecting fruit development in highbush blueberry (*Vaccinium corymbosum* L.). Generally, cross-pollination produces many mature seeds in fruits, resulting in a relatively high fruit set, large fruits, and early maturation. However, the effects of cross-pollination on fruit quality vary depending on the seed parent and remain unclear. The aim of this study was to re-evaluate the pollination effects on fruit size and quality by comparing the fruits derived from a controlled pollination with single-genotype pollen and those resulting from an open pollination. Additionally, the effects of mature seed number on fruit size and quality were also evaluated by a correlation analysis. Fruit size and quality for 'O'Neal', 'Blue Muffin', 'Sharpblue', 'Biloxi', and 'Sunshine Blue' plants that had been controlled pollinated with 'O'Neal' pollen were compared with the corresponding data for open-pollinated samples. The mature seed number and percentage of mature seeds were higher for controlled pollination than for open pollination, with the exception of the controlled self-pollination of 'O'Neal' plants. Additionally, larger fruits were produced by controlled pollination than by open pollination for 'Sharpblue' and 'Biloxi'. In contrast, the soluble solids content as well as the fructose and glucose concentrations did not differ between controlled cross-pollination and open pollination. Additionally, the effects of controlled cross-pollination on the acidity and anthocyanin concentration varied in a seed parent- and treatment-dependent manner. A significant correlation was detected between mature seed number and fruit weight; however, fruit quality parameters were not clearly correlated with mature seed number for most cultivars. Our re-evaluation of the relationship between mature seed number and fruit quality implies that controlled cross-pollination may not significantly improve fruit quality even though it significantly increases the number of mature seeds.

Keywords: *Vaccinium corymbosum* L., cross combination, mature seed, pollen viability, southern highbush blueberry

INTRODUCTION

Cultural practices promoting cross-pollination, such as mixed planting and pollinator release, are recommended for highbush blueberry (*Vaccinium corymbosum* L.) production. In an earlier study by Coville (1921), self-pollination resulted in a lower fruit set, smaller fruits, and delayed maturation. Because fruit set and fruit size affect yield, cross-pollination may be applied to increase productivity. Additionally, the maturation time affects growers' income. Specifically, early-season fruits can be sold at a higher price, suggesting cross-pollination may be useful for increasing profits.

Fruit size and maturation are influenced by the number of mature seeds. For example, Eaton (1967) reported that highbush blueberry fruit size is positively correlated with the number of mature seeds. Moreover, controlled cross-pollination involving an appropriately timed application of a sufficient number of pollen grains to the stigma reportedly enhances fruit set (Filmer and Marucci, 1963) and fruit size and promotes maturation with an increase in the number of mature seeds (Brewer and Dobson, 1961). These results suggest that a specific cross-compatible pollen or cross combination between pollen and a seed parent may

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increase mature seed production, which may affect fruit size and quality. This is supported by the results of a previous study by Kobashi et al. (2002) in which controlled cross-pollination of highbush blueberries increased seed number and promoted sugar accumulation. Gupton (1997) revealed that pollination with pollen from late-ripening cultivars tends to delay maturation, whereas pollination with pollen from early-ripening cultivars tended to accelerate maturation. However, Ehlenfeldt (2003) reported that although the maturation time differs significantly among cross combinations, the diversity depends on the combination and not on the specific pollen source. Additionally, pollen viability has been suggested to differ across cultivars (Lang and Parrie, 1992), which may result in pollen source-dependent variability in fruit quality (Vander Kloet, 1983). These reports imply that a controlled cross-pollination with a specific pollen source or combination may improve blueberry fruit quality; however, the effects of controlled cross-pollination on fruit quality remain unclear.

The aim of this study was to understand the effects of pollination on fruit size and quality. Considering that a specific pollen or pollen-pistil combination may affect fruit quality in various ways, an evaluation of the relationship between mature seed number and fruit quality should be based on fruits resulting from a controlled pollination with single-genotype pollen. We thus used pollen from 'O'Neal' to analyze the effects of controlled cross-pollination and/or the mature seed number on fruit size and quality.

MATERIALS AND METHODS

Plant materials and pollination procedure

This study was completed with 2- to 6-year-old pot- or field-grown plants of the following highbush blueberry cultivars: 'O'Neal', 'Blue Muffin', 'Sharpblue', 'Biloxi' and 'Sunshine Blue'. All plants were grown at the Kyoto Experimental Farm of Kyoto University. Pollen grains were collected from 'O'Neal' flowers. Briefly, flowers immediately before or after opening were collected, after which anthers were removed and dried for approximately 24 h with silica gel. Pollen grains were detached from anthers using a G-560 vortex mixer (Scientific Industries, Inc., NY, USA) and then stored at 4°C for the subsequent controlled pollination experiments. In 2019, non-emasculated and open flowers were control pollinated at an appropriate time by gently touching the stigma with a glass rod containing 'O'Neal' pollen grains three or four times per cluster to ensure all flowers were pollinated. Pollinated flowers were covered with paper bags from the pre-blooming stage to two weeks after blooming to avoid unexpected pollination. Approximately 40 fruits were collected at the appropriate harvesting period for each pollination combination.

Measurement of fruit size and fruit quality parameters

Fruit weight, size, and quality were measured. Briefly, the fruit longitudinal and transverse diameters were measured with a AD-5764A-150 digital caliper (A&D Co., Tokyo, Japan) and the fruit weights were determined with an XPE105 electronic balance (Mettler, Toledo, OH, USA). Additionally, total and mature seed numbers were recorded. Mature seeds were large and dark brown, whereas aborted seeds were smaller and lighter brown (Figure 1). The soluble solids content (SSC) and acidity were measured with a PAL-BX/ACID F5 portable refractometer/acidimeter (ATAGO Co., Tokyo, Japan). Fructose (Fru), glucose (Glu), and sucrose (Suc) concentrations were determined with an HPLC system (Shimadzu Co., Kyoto, Japan) and the total anthocyanin content was measured with a UV1800 spectrophotometer (Shimadzu).

Statistical analysis

Significant differences in the size and quality parameters between fruits derived from the open-pollinated and control cross-pollinated flowers were assessed with a t-test ($p<0.05$) if the data were normally distributed according to the Kolmogorov-Smirnov test. If the data were not normally distributed, the non-parametric Mann-Whitney U test was applied ($p<0.05$) to test the significance of any differences. Pearson's correlation analysis ($p<0.05$) was used to

investigate the relationship between mature seed number and fruit quality. To analyze the correlation between seed numbers and the SSC and acidity, each fruit was analyzed individually. To evaluate the correlation between seed numbers and Fru, Glu, and anthocyanin concentrations, the fruits were divided into groups based on mature seed number (n=3 for each group) and subjected to statistical analysis. In this case, the mean value in each group was used as the mature seed number.

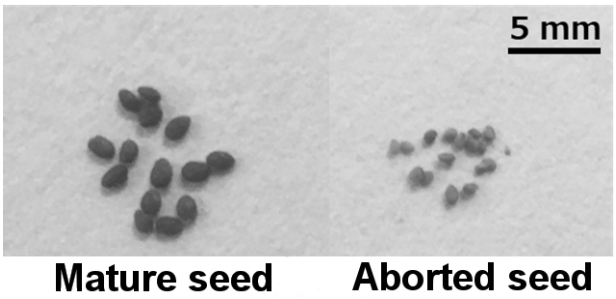

Figure 1. Seed classification. Large and brown seeds were designated as mature seeds and small and light brown seeds were designated as aborted seeds.

RESULTS AND DISCUSSION

Comparison of fruit size and quality between controlled and open pollinations

The mature seed number and percentage of mature seeds were both greater for the controlled cross-pollination than for the open pollination for all cultivars, except for self-pollinated 'O'Neal'. However, the controlled cross-pollination did not increase total seed number for most cultivars (Figure 2a-c). These observations imply that the fertilization frequency was similar, but that seed fertility varied between the controlled pollination and open pollination. This indicates that a controlled cross-pollination and/or the 'O'Neal' pollen may promote seed development after fertilization better than an open pollination. How the cross-pollination with 'O'Neal' pollen enhances seed development and increases the mature seed percentage in several seed parents will need elucidation in future investigations. Fruit weight, as well as longitudinal and transverse diameters, were also significantly greater for the controlled cross-pollination than for the open pollination of 'Sharpblue' and 'Biloxi' (Figure 2d-f). For 'Blue Muffin', there were no differences between the effects of the controlled cross-pollination and open pollination regarding fruit weight and size. Unlike cross-pollination, the self-pollination of 'O'Neal' plants decreased total seed number, mature seed number, mature seed percentage, and fruit weight and size compared with the effects of open pollination (Figure 2), possibly because of self-sterility, which is common in highbush blueberry (Ehlenfeldt, 2001).

The SSC as well as Fru and Glu concentrations did not significantly differ across treatments (Figure 2g-i). Additionally, Suc was scarcely detected in all cultivars (data not shown). Acidity was significantly higher in the 'Biloxi' fruits resulting from the cross-pollination than in the fruits derived from open pollination (Figure 2j). Cross-pollination resulted in lower anthocyanin contents than the open pollination of 'Sharpblue' and 'Biloxi', whereas the opposite pattern was observed for 'Sunshine Blue' (Figure 2k).

Relationship between the mature seed number and fruit size and quality

Regardless of the type of pollination, mature seed number and fruit weight were positively correlated with 'O'Neal', 'Blue Muffin', and 'Biloxi'. However, with 'Sharpblue' and 'Sunshine Blue', this positive correlation was observed only following cross-pollination (Figure 3). The correlation coefficients calculated for the controlled cross-pollination ranged from 0.32 (cross-pollinated 'Sunshine Blue') to 0.77 (self-pollinated 'O'Neal').

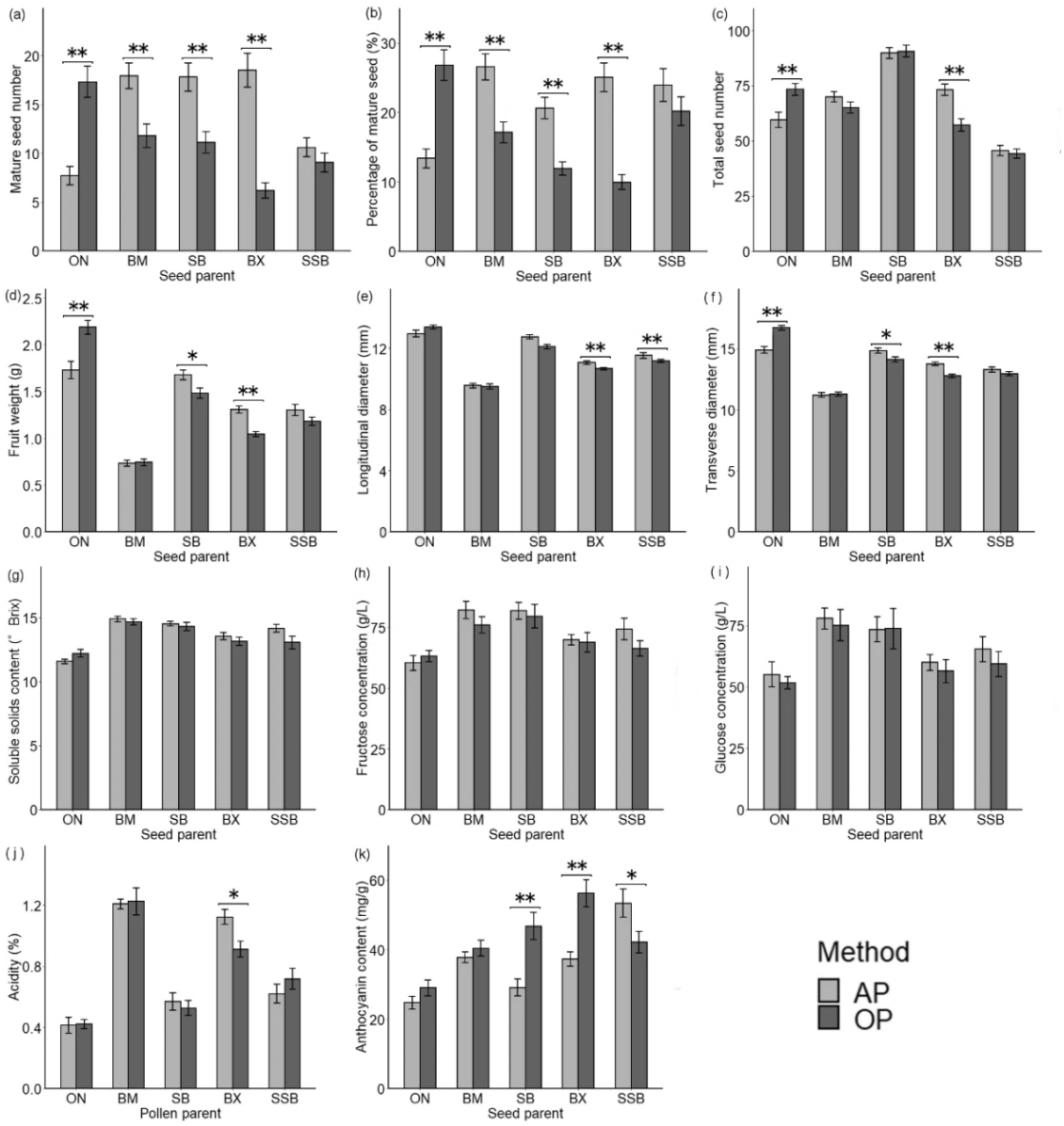

Figure 2. Differences in the quality of fruits resulting from the controlled (or artificial) pollination with 'O' Neal' pollen (AP) and open pollination (OP). ON = 'O'Neal', BM = 'Blue Muffin', SB = 'Sharpblue', BX = 'Biloxi' and SSB = 'Sunshine Blue'. Fruit weight (a), longitudinal diameter (b), transverse diameter (c), total seed number (d), mature seed number (e), mature seed percentage (f), soluble solids content (g), fructose concentration (h), glucose concentration (i), acidity (j), and anthocyanin concentration (k). Data are presented as the mean ± standard error. * and ** indicate significant differences at $p<0.05$ and 0.01, respectively.

Significant correlations between mature seed number and Fru and Glu concentrations were observed for some seed parents and treatments (Table 1). For example, mature seed number was positively correlated with Fru concentration of open-pollinated 'Blue Muffin' as well as with the Glu concentration of open-pollinated 'O'Neal' and cross-pollinated 'Sunshine Blue'. A significant correlation was not detected between mature seed number and SSC. Kobashi et al. (2002) reported that controlled cross-pollination of highbush blueberries increases the number of seeds and promotes sugar accumulation. They hypothesized that the increased number of mature seeds in the fruits produced by cross-pollination may increase

abscisic acid contents, thereby enhancing the invertase activity to induce sugar accumulation. Consistent with this hypothesis, we revealed a positive correlation between mature seed number and Fru/Glu concentrations in several seed parents. However, our results also indicated that the SSC as well as Fru and Glu concentrations did not differ between controlled pollination and open pollination even though the fruits resulting from the artificial pollination contained a higher number of mature seeds (Figure 2g-i). Therefore, the positive effect of the number of mature seeds on sugar accumulation may be minor and often masked by other factors.

	Seed parent				
Method	ON	BM	SB	BX	SSB
AP	0.77	0.65	0.62	0.33	0.32
OP	0.66	0.68	0.25	0.66	0.21

<0.4	>0.4, <0.7	>0.7
R-Value		

Figure 3. Relationship between mature seed number and fruit weight resulting from controlled or artificial pollination (AP) and open pollination (OP). ON = 'O'Neal', BM = 'Blue Muffin', SB = 'Sharpblue', BX = 'Biloxi' and SSB = 'Sunshine Blue'. Pearson's correlation coefficients are presented. The shaded values indicate significant correlations at $p<0.05$.

Table 1. Relationship between mature seed number and fruit quality following the controlled or artificial pollination (AP) and open pollination (OP). Pearson's correlation coefficients are listed.

Seed parent	Method	Soluble solids cont. (°Brix)	Fructose conc. (g L^{-1})	Glucose conc. (g L^{-1})	Acidity (%)	Anthocyanin cont. (mg g^{-1})
ON	AP	-0.62	-0.28	-0.03	-0.22	-0.36
	OP	-0.23	0.26	0.67*	-0.50	-0.26
BM	AP	0.67	-0.11	-0.17	0.26	0.19
	OP	0.37	0.89*	0.63	-0.55	0.004
SB	AP	0.09	-0.0007	-0.06	0.69*	-0.40
	OP	0.16	-0.11	-0.16	-0.40	0.01
BX	AP	-0.20	-0.21	-0.06	0.47	-0.75*
	OP	0.18	0.38	0.48	-0.30	-0.36
SSB	AP	0.45	0.62	0.72*	-0.05	0.25
	OP	0.10	0.12	-0.03	0.72*	-0.10

ON = 'O'Neal', BM = 'Blue Muffin', SB = 'Sharpblue', BX = 'Biloxi' and SSB = 'Sunshine Blue'.
*indicates a significant correlation at $p<0.05$.

Acidity was positively correlated with mature seed number for the 'Sharpblue' fruits generated by cross-pollination and the 'Sunshine Blue' fruits resulting from open pollination. In contrast, the cross-pollination of 'Biloxi' resulted in a negative correlation between mature seed number and anthocyanin concentration. Additionally, considering that the effects of controlled cross-pollination on acidity and anthocyanin content differed in a seed parent- and treatment-dependent manner (Figure 2j, k), cross-pollination may have diverse effects on acidity and anthocyanin accumulation depending on the cross combination. Our results imply that inherent tree factors and environmental conditions, as well as pollination/fertilization affects, impact on fruit quality.

CONCLUSIONS

This study showed that controlled pollination with 'O'Neal' pollen can increase mature seed number and fruit size in several highbush blueberry cultivars. However, the differences

in pollination methods did not affect different measures of fruit quality consistently. Since fruit quality is influenced by genetic and non-genetic factors, such as tree internal and environmental conditions, the factors other than pollination may also considerably affect fruit quality.

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Expression analysis of bulb development-related genes in onion cultivars

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Abstract

Onion (*Allium cepa* L.) is one of the most widely produced and consumed vegetable crops worldwide. The bulb size of onion is an important factor in its productivity. It is known that bulb development is induced and continues under long day length and is inhibited under short day length. These responses have also been characterized at the genetic level, with studies indicating that expression of *AcFT1*, which encodes an FT-like protein for bulb development, is upregulated under long day length and downregulated under short day length. Conversely, a further *AcFT* gene, *AcFT4*, which promotes leaf initiation and suppresses bulb development, is upregulated under short day length, and downregulated under long day length. In this study, we conducted expression analysis of *AcFT1* and *AcFT4* using individual leaves of the cultivar 'Momiji No. 3' to evaluate the difference in day length response. Our expression analysis from 0 weeks after transplanting (WAT) to 8 WAT indicated that the expression of *AcFT1* and *AcFT4* in the preceding leaf was higher than that in newly initiated leaves from 4 to 8 WAT. These results indicate that the preceding leaves play an important role in the response of onions to day length for leaf initiation or bulb development. We also conducted an RNA-seq analysis to identify candidate genes for bulb development.

Keywords: *AcFT* gene, day length response, RNA-seq analysis

INTRODUCTION

Onion (*Allium cepa* L.) is one of the most important vegetables in the world, and its bulb size is an important factor in determining productivity. Bulb development is controlled by day length and temperature (Khokhar, 2017). We previously reported that temperature influences the final leaf number, which is closely related to the final bulb size (Ikeda et al., 2019). There have been many studies on bulb development, and it is known that bulb development is initiated by a critical day length, which differs among onion cultivars (Brewster, 2008). Therefore, to achieve optimal onion production, studies of the characteristics and environmental responses of various cultivars are necessary. Previous studies have demonstrated that *flowering locus T (FT)*-like genes play a key role in bulb development (Lee et al., 2013; Rashid et al., 2019). These studies indicated that the expression of *AcFT1*, which encodes an FT-like protein for bulb development, is upregulated under long day lengths and downregulated under short day lengths. Conversely, *AcFT4*, which promotes leaf initiation and suppresses bulb development, is upregulated under short day length and downregulated under long day length. We previously examined *AcFT1* and *AcFT4* expression in three onion cultivars grown in the field and found that these genes are expressed in accordance with the maturity of the cultivars (Ikeda et al., 2020). However, genetic studies of bulb development are still insufficient because onion has a large genome. A previous study revealed differences in gene expression at different positions on the leaf blades (Rashid et al., 2019), but the differences in gene expression between individual leaves are unknown.

In this study, we conducted expression analysis of *AcFT1* and *AcFT4* using individual leaves of the cultivar 'Momiji No. 3' to evaluate the difference in day length response in each leaf. We also conducted RNA-seq analysis for the early maturing cultivar 'Turbo' and the late-maturing cultivar 'Okhotsk 222' grown in growth chambers under short-day and long-day

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treatments to identify candidate genes for bulb development.

MATERIALS AND METHODS

Expression analysis of *AcFT* genes

Expression analysis of *AcFT1* and *AcFT4* was conducted every two weeks from April 18, 2017 (0 weeks after transplanting; 0 WAT) until June 13 (8 WAT) at the NARO Tohoku Agricultural Research Center in Morioka, Iwate, Japan (39.7°N; 141.1°E). The commercially grown onion cultivar 'Momiji No. 3' (Shippo Co., Japan) was used in this study. Seeds were sown on 16 February 2017 in plug trays, and seedlings were transplanted by hand on 18 April in triplicate plots and cultivated as described in Ikeda et al. (2019). Leaf blades from 10 plants were individually sampled from triplicate plots and counted as shown in Figure 1. Samples in each plot were composited, homogenized, and stored at -80°C. Total RNA was extracted from leaf blades using the RNeasy Plant Mini Kit (Qiagen, The Netherlands). Reverse transcription and quantitative real-time PCR (qPCR) were performed as described by Ikeda et al. (2020) and the day length response was evaluated as the expression levels of *AcFT1* and *AcFT4*. Plant growth surveys were conducted every week for five plants per plot, and these results are shown in Ikeda et al. (2020).

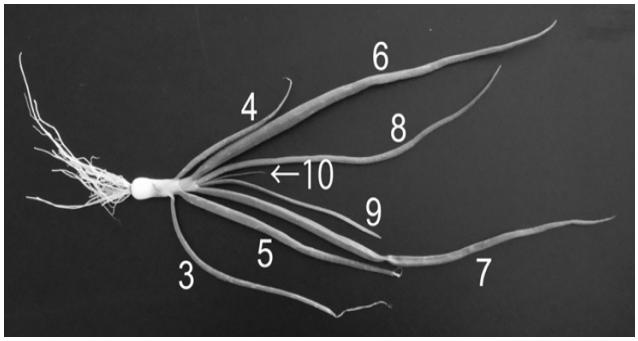

Figure 1. Method for counting the number of leaf blades. Leaf blade numbers were counted from emergence. In this figure, 1st and 2nd leaf blades were naturally dropped during growth, and these leaves were not sampled for expression analysis.

Transcriptome analysis

Seeds of 'Turbo' (Takii & Co., Japan) and 'Okhotsk 222' (Shippo Co.) were sown on October 21, 2016 in plug trays and grown in the greenhouse at the NARO Tohoku Agricultural Research Center. Seedlings were transplanted on December 21 in 18-cm plastic pots and grown in a greenhouse. On January10, 2017, seedlings were transferred to growth chambers at 20°C under a metal halide and high-pressure sodium lamp (8 h light/16 h dark) for a month. From February 10 to 20, short-day (SD, 8 h light/16 h dark) and long-day (LD, 16 h light/8 h dark) treatments were conducted in the growth chambers. After finishing the SD and LD treatments, the 8th initiated leaf blade which showed moderate growth was sampled from 15 plants of each cultivar and stored at -80°C. Total RNA was extracted as previously described. For RNA-seq analysis, the sequencing library was constructed using the NEBNext Ultra RNA Library Prep Kit (New England Biolabs, US) and sequenced on a HiSeq instrument (Illumina, US) by Novogene (China). The fragments per kilobase of transcript sequence per million base pairs sequenced (FPKM) method was used to calculate relative transcript levels of genes.

Statistical analysis

Statistical analysis for the expression analysis of *AcFT1* and *AcFT4* was performed using Excel (Microsoft, US) and Bell Curve for Excel (Version 3.20; Social Survey Research Information Co., Japan). The statistical significance of the gene expression was analyzed with the Tukey-Kramer test at the 5% level using Bell Curve for Excel (Version 3.20) software.

RESULTS AND DISCUSSION

AcFT1 and *AcFT4* expression in individual leaf blades

The *AcFT1* and *AcFT4* expressions in individual leaf blades of 'Turbo' and 'Okhotsk 222' were determined from 0 to 8 WAT in 2017 (Figure 2). In this experiment, the oldest leaf (e.g., 1st leaf at 0 WAT, 3rd leaf at 6 and 8 WAT) was used as a reference and gene expression among individual leaves was compared at each growth stage. The expression of *AcFT1* and *AcFT4* immediately following transplantation (0 WAT) was the highest in the 2nd leaf blades (i.e., the leaf blade initiated following the first leaf). At 2 WAT, while the expressions of *AcFT1* and *AcFT4* were high in the 1st to 2nd and 4th leaf blades, the lowest expression was observed in the 3rd leaf blade. *AcFT1* and *AcFT4* expression from 4 to 8 WAT were high in the oldest leaves and decreased in accordance with the order of leaf initiation. In other words, the expression of *AcFT1* and *AcFT4* in each preceding leaf was higher than that in more recently initiated leaves from 4 to 8 WAT. These results indicate that the older leaves may play an important role in the response to day length for leaf initiation or bulb development in onion. We used only 'Momiji No. 3' for this experiment; however, there are many cultivars suitable for spring-sown cultivation methods in northern Japan (Tsukazaki et al., 2020). Onions are also grown using autumn-sowing cultivation in most parts of Japan, including Morioka, Iwate Prefecture. Therefore, to obtain further details on the day length response of onion leaf blades, expression analysis of *AcFTs* with various cultivars or different cultivation methods is necessary.

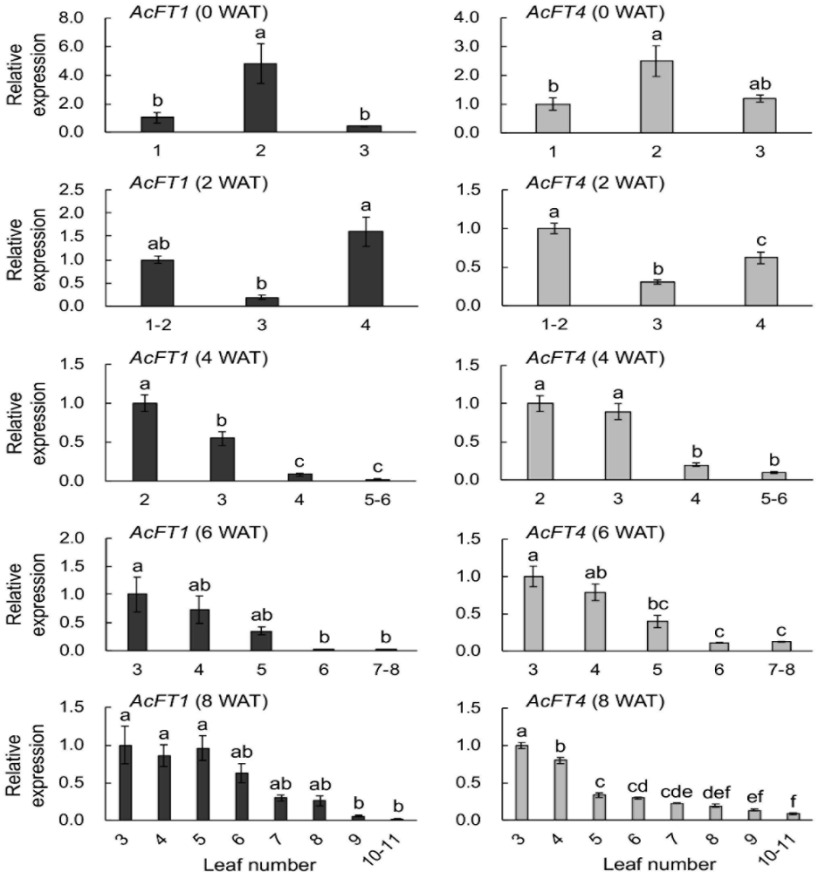

Figure 2. Relative levels of *AcFT* gene transcripts during plant growth of individual leaves of the cultivar 'Momiji No. 3'. *AcFT1* and *AcFT4* expression levels were determined by quantitative real-time PCR and normalized against *β-tubulin* expression. Values indicate the mean ± SE (n=3), and values with the same letter were not significantly different at $p<0.05$ (Tukey-Kramer test).

Transcriptome analysis

To identify genes that showed differential expression between SD- and LD-treated onion cultivars, we conducted RNA-seq analysis and identified 146,009 unigenes that were selected from de novo assembled transcriptomes. The levels of transcripts detected by these unigenes were subjected to differential expression analysis, and volcano plots of differentially expressed genes in the comparison between long-day and short-day treatments are shown in Figure 3. A total of 415 genes were identified that were differentially expressed between LD and SD treatments in the leaf blades of 'Turbo', and the expression of 193 genes was upregulated under SD conditions. Similarly, 264 differentially expressed genes were identified in 'Okhotsk 222', and 91 genes were upregulated under SD treatment. Among the differentially expressed genes, 109 unigenes were identified as being common to both cultivars. Interestingly, *AcFT4* and *GIGANTEA (GI)* were identified among these 109 genes. As we mentioned, *AcFT4* promotes leaf initiation and suppresses bulb development (Lee et al., 2013; Rashid et al., 2019). *GI* regulates circadian rhythms and flowering and acts earlier than *FT* genes in *Arabidopsis thaliana* (Mizoguchi et al., 2005). Therefore, it is possible that onion *GI* regulates *AcFTs* that are located upstream of this gene. However, further investigation will be necessary to confirm this hypothesis, and identification of genes that are located downstream of *AcFTs* may also lead to interesting results. Several transcriptome analyses, including our study, have been conducted (Zhang et al., 2016, 2018), and more detailed analysis (e.g., coexpression analysis) will enable us to obtain further details about the day length response and bulb formation of onion.

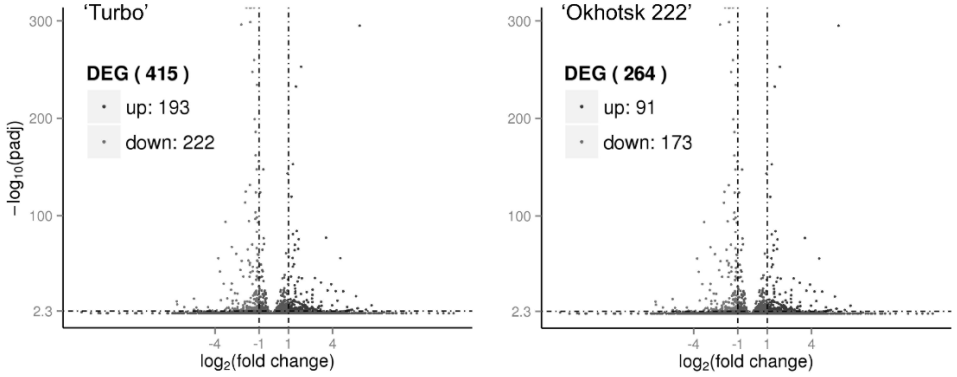

Figure 3. Volcano plots of differentially expressed genes (DEG) in the comparison between short- and long-day treated 'Turbo' and 'Okhotsk 222'. Up- and downregulated genes under SD conditions are shown in this Figure. The threshold was set as follows: [$\log_2$(fold change)]>1 and q-value<0.005.

CONCLUSIONS

Expression analysis of *AcFTs* using individual leaf blades was conducted for the cultivar 'Momiji No. 3' to evaluate the difference in day length response. Our expression analysis from 0 to 8 WAT indicated that the expression of *AcFT1* and *AcFT4* in each preceding leaf was higher than that in more recently initiated leaves from 4 to 8 WAT. In addition, older leaves therefore play an important role in the day length responses of onions. We also conducted RNA-seq analysis for two differently maturing cultivars, 'Turbo' and 'Okhotsk 222', to identify candidate genes for bulb development. In total, 109 unigenes were identified as differentially expressed genes in both cultivars and *AcFT4* and *GI*, which are known as key genes for day length responses, were detected.

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Transcriptome analysis of the effect of modified atmosphere packaging and low temperature on the mature green tomatoes during storage

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Abstract

Tomato is a major vegetable crop in the world. It is rich in potassium, vitamin A, vitamin B, vitamin C, lycopene and beta-carotene. Tomato is regularly consumed in the diet, having a defensive role for reducing the risk of some types of cancer and ischemic heart disease. It is reported that the huge amount of produce, including tomato, is discarded due to postharvest losses caused by quality deterioration. Low storage temperature and modified atmosphere packaging (MAP) have the potential to keep the quality of tomato after harvest. The objective of this investigation was to analyze the effect of low temperature (15°C) and MAP on the ripening and the transcription profile of 'KEK-1' tomatoes. Mature green tomatoes var. 'KEK-1' were stored at 25°C with MAP or without MAP (perforated film package) or stored at 15°C without MAP. The packaging materials used were low-density polyethylene (LDPE) film (40 µm). The O_2 concentration of 3 to 5% in the MA packaged tomatoes was maintained throughout the storage period. Microarray analysis was conducted using total RNA extracted from tomato samples, before storage and from the three different storage conditions for five days. The pericarp color value of the tomatoes and the ripening related genes expression from four treatments confirmed a clear difference between the ripening stages among samples. Comparison of the gene expression profile was conducted by using microarray data analysis software. The effects of storage and storage conditions applied in this study were clearly seen by a profile plot of genes. A principal component analysis using three components showed the clear difference among the four samples. The gene showing a distinctive expression pattern among samples was selected and genes showing a similar pattern were also selected. Four distinctive expression patterns were found and genes that have similar pattern were grouped. There is no overlap among the groups, suggesting these groupings reflect different biological status. The results showed that several ripening related genes (including ACC synthase and ACC oxidase, histidine decarboxylase, alcohol acyl transferase) and defense reaction related genes (including chitinase, pathogenesis related leaf protein 6, PR-1a1 protein) were up/downregulated by low temperature or MA packaging.

Keywords: tomato, ripening, MAP, low temperature, gene expression, microarray

INTRODUCTION

Tomato is one of the world's most popular, economically significant, and widely grown irrigated vegetable crops that contain a valuable specific nutritive in its fruit (Gallardo et al., 2006). It has been broadly reported that the ripening process in tomato is associated with the development of red color and onset of the rise in ethylene production. Considering tomato as the fleshy-fruit model in omics research, ripening behavior and the control mechanism of ripening in tomato fruit have been extensively studied (Giovannoni et al., 2017; Li et al., 2019). However, the effects of the postharvest practices on the ripening behavior and the quality attributes of tomato based on gene expression analysis including transcriptome is limited. The

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aims of this study were to investigate the possibility of mature green tomato distribution system using the Japanese tomato cultivar 'KEK-1' and to understand the effect of low temperature and modified atmosphere packaging (MAP) on the shelf life extension. To obtain the greater understanding of gene expression behavior during storage of tomato and find the important genes involving quality alteration, a microarray analysis of the representative samples was conducted.

MATERIALS AND METHODS

Plant materials

Greenhouse grown tomatoes 'KEK-1' (called as super tomato fruit containing 9 °Brix) were procured from the farmer's market (Chikusei City, Japan) and taken to the Institute (Tsukuba City, Japan). Samples were selected based on the maturity stage (mature green) and uniformity of shape and size. Tomatoes were stored at 15 or 25°C for 20 or 15 days, respectively. Low density polyethylene (LDPE) film (40 μm in thickness) with heat sealing was used for MAP to study the effects of gas modification on the ripening of tomato during storage at 25°C. The storage temperatures of 15°C was selected to study the effect of low temperature on the ripening of tomato. Storage test have been conducted by using three to four replicate samples for each treatment and analyses were conducted at regular intervals (Vanitha et al., 2019). About 0.6 g of pericarp sample was taken from top of the fruit (blossom end) and immediately frozen with liquid nitrogen and stored at -80°C until the analysis. Samples for microarray analysis are shown in Table 1.

Table 1. Samples for the microarray analysis.

Treatments	Storage		
	Period (day)	Temperature (°C)	Packaging
T1	0	-	-
T2	5	25	without MAP
T3	5	25	with MAP
T4	5	15	without MAP

RNA extraction

Frozen tomato sample was ground under liquid nitrogen with a pre-chilled mortar and pestle. Total RNA from each sample was extracted and purified using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) as described in Thammawong et al. (2012). After quantifying the RNA concentration using spectrophotometric analysis, RNA samples with sufficient yield (>approximately 30 mg L^{-1}) and ratio of absorbance at 260 nm and 280 nm (~2.0) were confirmed and used for the microarray analysis.

Microarray analysis

Ten μL of total RNA from each sample was sent on dry ice to the Hokkaido System Science (Hokkaido, Japan) who performed the microarray analysis. The 4×44 k format Tomato Gene Expression Microarrays (Agilent, *Solanum lycopersicum*) having 43,803 tomato probes manufactured using Agilent 60-mer SurePrint technology was used for the analysis. For further data analysis, the probe sequence and annotation information were taken from Agilent's website through microarray data analysis software (GeneSpring, Agilent Technology, USA).

RESULTS AND DISCUSSION

Outline of the microarray analysis results

Postharvest ripening of the tomatoes harvested at mature green stage of development is characterized by the burst of ethylene production and sharp rise in respiration rate.

Tomatoes stored under 25°C without MAP (T2) showed visible color change after five days of storage. On the other hand, there were no changes in appearance for the tomatoes stored at 25°C with MAP (T3) or stored at 15°C without MAP (T4). These observations were confirmed by the color values of the pericarp on the top of the fruit (blossom end). CIE a* values of the tomato for the microarray analysis were -12.3, 0.51, -12.06 and -11.93 for T1, T2, T3 and T4, respectively. These results show that the tomato of T2 was at breaker stage and others were at mature green stage of development (Ciptaningtyas et al., 2020).

Figure 1 shows the profile plot of all genes obtained by microarray analysis. *y*-axis in Figure 1 is the $\log_2$ value of each genes after normalization. Gene expression levels were differently shown depending on the treatments.

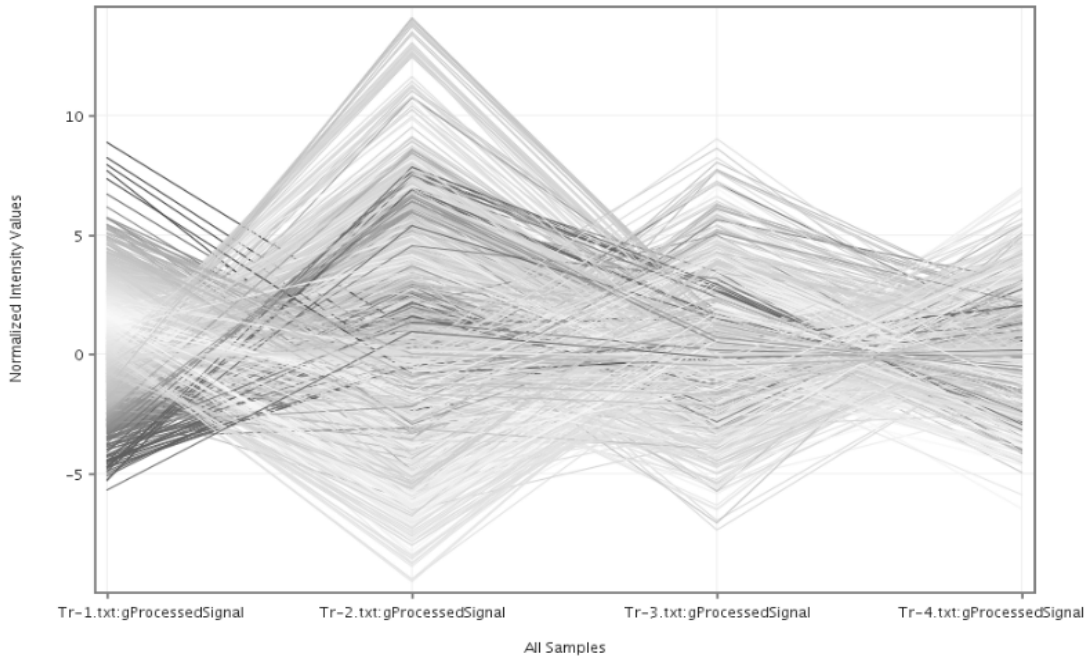

Figure 1. Profile plot of expression values of all genes in four samples tested.

Figure 2 shows the principal component analysis of current microarray data of four samples from T1, T2, T3 and T4. By using 3 principal components (component 1, 2 and 3), the difference among samples were clearly shown as in Figure 2. The ranges of *x*-axis (component 1), *y*-axis (component 2) and *z*-axis (component 3) were 346, 332 and 260, respectively. Table 2 shows the calculated distances between treatments obtained from PCA analysis (Figure 2).

Table 2. Distances between treatments in Figure 2.

Treatments	T1	T2	T3	T4
T1	-	371	305	310
T2	371	-	364	357
T3	305	364	-	335
T4	310	357	335	-

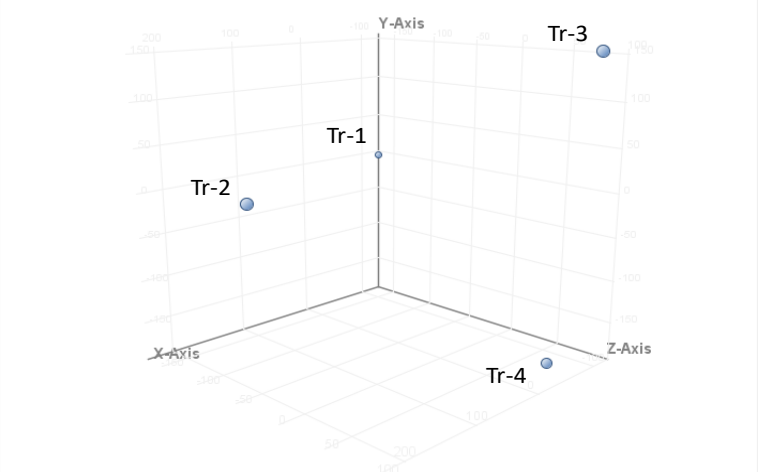

Figure 2. Principal component analysis result of all genes expression in four samples from respective treatments (T1, T2, T3 and T4).

Effects of ripening behavior and suppression of ripening on the gene expression

1. Up/downregulated genes.

Probes having a 2-fold change or more (upregulated) and a fold change of 0.5-fold or less (downregulated) were selected with the limitation filters (Control, gIsFeatNonUnif0L, gIsFeatPopn0L and gIsWellAboveBG) by using the data sheet reported by microarray analysis company. Table 3 shows the results of the detailed analysis (upregulated or downregulated) among treatments of the selected genes.

Table 3. Numbers of the genes up/downregulated between treatments.

Comparison	Upregulated				Downregulated			
	2-	10-	100-	1000-	-1/2	-1/10	-1/100	-1/1000
T2/T1	5643	626	104	19	5258	793	60	1
T3/T1	3437	186	17	0	3095	255	13	0
T4/T1	3827	193	7	0	3036	156	8	0
T3/T2	4706	581	86	5	4830	551	86	28
T4/T2	5238	725	47	0	4327	485	103	8
T4/T3	18053	12376	5454	1430	9783	4955	1092	102

It is notable that T4/T3 shows different characteristics from others, although both treatments (T3 and T4) resulted in a similar effect of suppressing the ripening process. This is probably due to the different mechanisms of suppressing the ripening process by modification of atmosphere or by lowering the temperature.

2. Expression of important ripening related genes.

The ripening stages of the fruits tested for microarray analysis can be confirmed further by the genes involved in the ripening process of tomato fruits. Figure 3 shows the relative expression level of three most important genes, *RIPENING-INHIBITOR* gene (*Le-MADS-RIN*) (Vrebalov et al., 2002; Li et al., 2019) and the 1-aminocyclepropane-1-carboxylate (ACC) synthase (ACS) encoding genes (*LeACS2*, *LeACS4*) (Giovannoni et al., 2017; Karlova et al., 2014; Li et al., 2019), obtained by the microarray analysis.

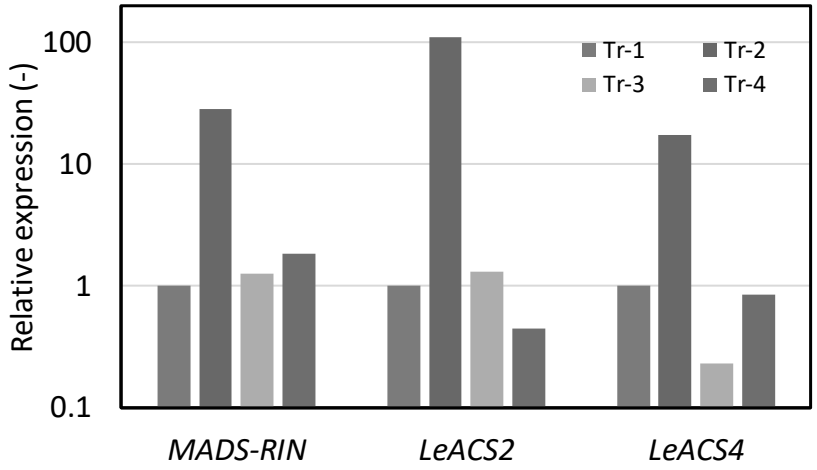

Figure 3. Relative expression levels of ripening related genes (the values for *MADS-RIN* and *LeACS2* are based on average of three probes and that for *LeACS4* are based on the single probe on the microarray).

The expression level of *MADS-RIN* increased to 28.5-fold after five days of storage at 25°C without MAP (T2) compared with the tomato just after harvesting (T1). Whereas the *MADS-RIN* expression level were 1.23 and 1.93 for the tomato stored at 25°C with MAP (T3) and the tomato stored at 15°C without MAP (T4), respectively.

LeACS2 encodes the ACS which is the rate limiting enzyme of ethylene production in tomato fruit during ripening. The relative expression level of *LeACS2* in the tomato stored at 25°C without MAP (T2) was 111-fold. Whereas the *LeACS2* expression level was 1.30 and 0.44 for the tomato stored at 25°C with MAP (T3) and the tomato stored at 15°C without MAP (T4), respectively. The downregulation of the *LeACS2* in the tomato stored at 15°C might be responsible for the reduced change in color and texture of the fruit, and the combination of low temperature and the MAP enhanced the storability of the tomato (Vanitha et al., 2019). Relative expressions of *LeACS4* were similar to that of *LeACS2,* except for the lesser upregulation than *LeACS2* at T2 (17.5-fold) and downregulation at T3 (0.23-fold).

The differences of color change and the gene expression level among stored tomatoes indicate that the storage under MAP (T3) (3-5% O_2 and 5% CO_2 shown by Vanitha et al., 2019) or low temperature (15°C) (T4) resulted the inhibition/delay of the onset of ripening process during five days of storage when compared with the storage at 25°C without MAP (T2). Little upregulation or downregulation of the most important genes involved in ripening process including *MADS-RIN* together with the genes involving ethylene production at T3 and T4 compared with T2 might be the cause of the inhibition of ripening process at these two conditions.

3. Ripening associated genes found by microarray analysis.

The different expression patterns were selected by using the analysis function "Find similar entity" of the GeneSpring. Some of these patterns are shown in Figures 4-7. The effects of onset of the ripening process on the gene expression of tomato fruit can be seen in Figure 4 (upregulated genes) and in Figure 5 (downregulated genes).

From Figure 4, it is clear that many genes were upregulated by storing at 25°C without MAP (T2) including histidine decarboxylase (*HDC*), 2-oxoglutarate/Fe(II)-dependent dioxygenase-like protein (*E8-6*), alcohol acyl transferase (*AAT*), endo-1,4-beta-glucanase precursor (*Cel2*), alcohol oxidase, expansin (*EXP1*), etc. Among these genes, *HDC* seems to play an important role in the ripening of tomato (Kim et al., 2019; Kumar et al., 2016; Picton et al., 1993) however, the effect of *HDC* on ripening is not clearly identified.

Figure 5 shows the genes that were downregulated during ripening process under 25°C

storage including pathogenesis-related leaf protein 6 precursor, pathogenesis-related leaf protein 6 (*PR1b1*), chitinase (*CHI9*), endochitinase 1 precursor - *Solanum tuberosum*. These are responsible for the defense system against microorganisms, implying the increased susceptibility of tomato fruit to the decay during storage.

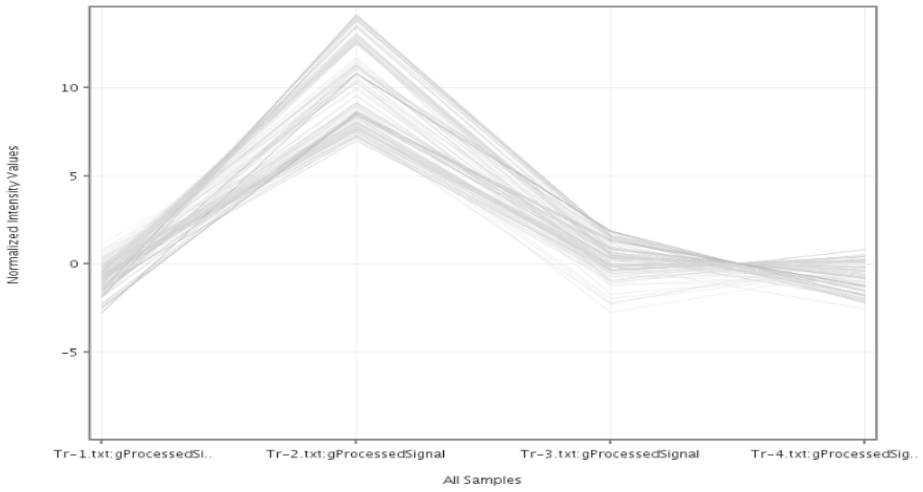

Figure 4. Upregulated genes under T2.

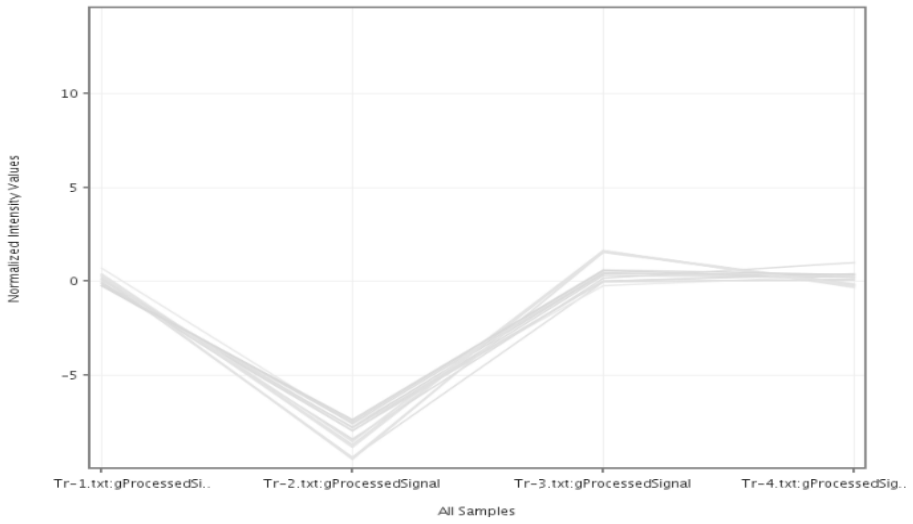

Figure 5. Downregulated genes under T2.

From Figure 6, pathogenesis-related protein 1A1 (PR-1a1) and PR5-like protein were detected in tomato of T3, suggesting the improved resistance of fruit against the microorganisms by using MAP. From Figure 7, wound-induced proteinase inhibitor 1 precursor, FtsH protease (ftsH6), polyphenol oxidase F, chloroplastic, TBG6 protein (TBG6), NADH-ubiquinone oxidoreductase chain 4, probable galacturonosyltransferase-like 1, tonoplast dicarboxylate transporter-like, wound-induced proteinase inhibitor 1 precursor, anthocyanidin 3-O-glucosyltransferase 5-like, glutamine synthetase cytosolic isozyme 1-1 (gts1), sulfate transporter 2.1-like, metallocarboxypeptidase inhibitor (mcpi), TAS14 peptide (AA 1-130) (TAS14), probable hexokinase-like 2 protein, etc., were found in tomato stored at 15°C, proposing the wide range of effects on the tomato by lowering the fruit temperature.

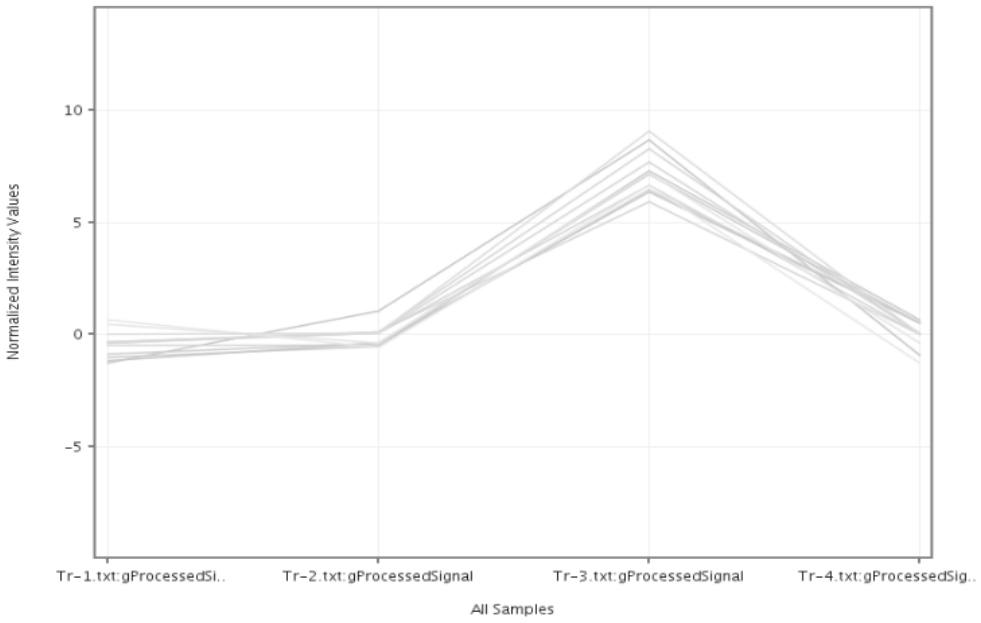

Figure 6. Upregulated genes under T3.

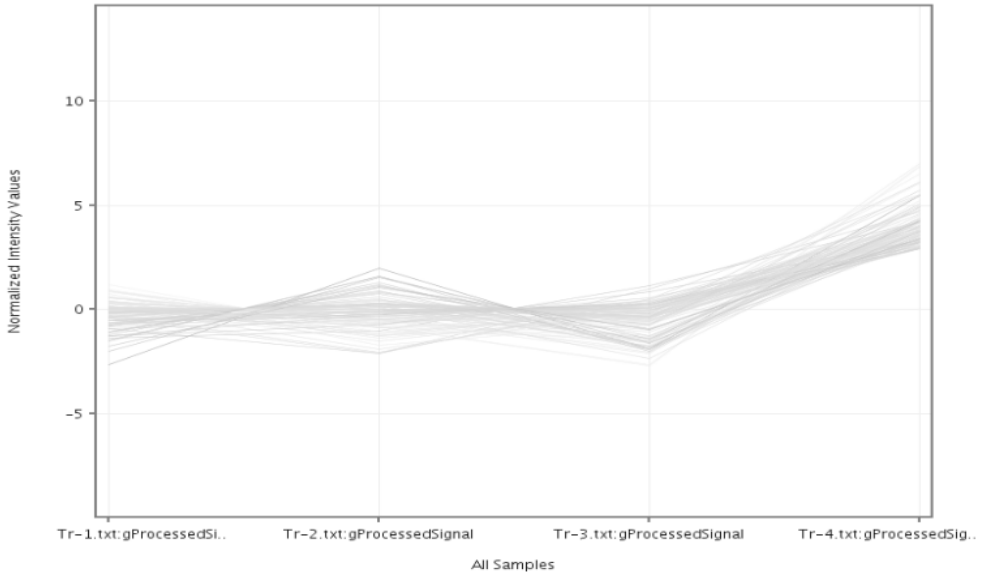

Figure 7. Upregulated genes under T4.

CONCLUSIONS

This study clearly showed the effect of MAP and low temperature on the delay/inhibition of the ripening of mature green tomato. Recent study on the functional evaluation of the genes involving the ripening process, resulted in the unveiling of the complicated control system in tomato fruit (Ito et al., 2020; Tomato Genome Consortium, 2012). Future study on the control mechanism of the ripening process of fresh produce including tomato will lead to an efficient supply chain, reducing food loss, and delivery of a high nutrient supply for human health.

ACKNOWLEDGEMENTS

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Investigation of different time points of anthesis for intrinsically isotopic deuterium labelling on the enrichment of deuterium-labelled indispensable amino acids in mung bean (*Vigna radiata* L. Wilczek)

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Abstract

Dietary protein quality has great importance for human health throughout the life cycle. Indispensable amino acid (IAA) digestibility of dietary protein is an integral component of protein quality evaluation, which can be properly measured by dual-stable isotope approach. This advanced technique requires intrinsically labelled protein sources (animals or plants). Nevertheless, the study of producing intrinsically labelled plant protein at different time points of anthesis is still scarce. Therefore, the present study examined the appropriate time points of anthesis to produce deuterium-labelled plant proteins. Mung bean (*Vigna radiata* (L.) Wilczek) was selected and watered with 20% deuterium oxide (^2H_2O) at different time points of anthesis (25, 50, 75 and 100%). The amount of ^2H_2O was technically calculated based on the amount of water lost by transpiration from the previous day, measured by the gravimetric method. Seed yield, the amount of ^2H_2O used, and deuterium-enriched IAA (^{2}H-IAA) analysis was investigated at the selected time points. Results revealed that the average amount of ^{2}H-IAA at the time of 25, 50, 75 and 100% anthesis were 0.20, 0.19, 0.23 and 0.25 atom percent excess, respectively. Remarkably, the treatment at anthesis time 75 and 100% produced the highest amount of ^{2}H-leucine, ^{2}H-phenylalanine, and ^{2}H-lysine; whereas the treatment at the anthesis stage of 50%, provided the lowest ^{2}H-valine and ^{2}H-isoleucine. Notably, positive relationship between the amount of ^2H_2O used and the average amount of ^{2}H-IAA was observed (R^2=0.828, p=0.09). The ^2H_2O watering based on the transpiration loss at the anthesis time of 75 and 100% appears to be a major factor for the efficient production of enriched ^{2}H-IAAs. This study provides a simple and efficient method for the production of highly enriched ^{2}H-IAAs in plant protein, which can be used to explore the protein digestibility in human-beings.

Keywords: intrinsic labelling, deuterated protein, protein quality, digestibility, anthesis, transpiration

INTRODUCTION

Dietary protein quality plays a crucial role in human health, especially during pregnancy, early childhood, and old age. The major sources of high-quality protein are derived from animals and plants (legumes). The latter (legumes), are sustainable crops; however, their digestibility is poor. Indispensable amino acid (IAA) digestibility of the protein in the diet is an integral component of protein quality evaluation. It is called a digestible indispensable amino acid score (DIAAS). Therefore, a minimally invasive dual-isotope approach was recently recommended by FAO/WHO Expert who provided consultation on Protein Quality Evaluation (FAO, 2013). Proteins are intrinsically labelled by different stable isotopes and compared with a reference protein for assessing IAA digestibility.

Stable isotopic labelling is a necessary technique for elucidating metabolic pathways.

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Since transamination after digestion and absorption blends the label through the amino acid pool, using ^{15}N is inappropriate for tracing the carbon skeleton of individual amino acids (FAO, 2013). The intrinsic isotopic labelling of the plants with $^{13}CO_2$ shows more effective outcomes in generating highly labelled compounds. However, the tracer is relatively expensive and requires a labelling chamber (Gleichenhagen et al., 2013; FAO, 2013). Deuterium oxide or $^{2}H_2O$ has been widely accepted for labelling the amino acid carbon skeleton in plants, as it is an inexpensive isotopic molecule, and can be absorbed by the root membrane transport system for incorporation into the hydrogen molecules of amino acids (Grusak, 1997).

The digestibility of IAA from chickpeas, mung beans, and yellow peas has been intensively studied in humans (Devi et al., 2018; Kashyap et al., 2019). Chickpeas and mung beans were planted and 25% $^{2}H_2O$ applied at the 50% flowering stage, followed by 2.5% $^{2}H_2O$ on the 3rd, 5th, 7th and 9th days. Furthermore, yellow peas were sown and irrigated with the 25% $^{2}H_2O$ at flowering stage followed by 2% $^{2}H_2O$ for the next 14 days (Kashyap et al., 2019). Nevertheless, the application of isotope should not only be performed at the onset of anthesis, but also during the latter half of growth to obtain higher incorporation of isotopes into the seed (Fox et al., 1991).

In general, amino acids from roots and leaves would be translocated to pods and secreted into vascular bundles of the seed coat, and then cotyledons would absorb them for the synthesis and accumulation of storage proteins. This process occurs mainly in seeds (Takuji et al., 2017). Moreover, the storage-protein accumulation period was different in three legumes; mung beans between 10 and 14 days, cowpeas between 7 and 14 days and soybeans between 20 and 30 days after flowering (Awolumate, 1983). Therefore, the different time points of anthesis might be a crucial variable for the efficient production of intrinsically labelled-IAA in plant proteins. Nonetheless, to the best of our knowledge, studies on the production of the intrinsically labelled plant protein at different time points of anthesis are still scarce.

In this investigation, we hypothesized that the accumulation of deuterium-enriched IAA (^{2}H-IAA) could depend on the administration of $^{2}H_2O$ at different time points of anthesis. Thus, the study's purpose was to examine the effect of $^{2}H_2O$ irrigation on the production of ^{2}H-IAA at disparate time points of anthesis (at 25, 50, 75 and 100%) in mung beans.

MATERIALS AND METHODS

Plant material

Mung bean (*Vigna radiata* L. Wilczek), 'ML5' cultivar was selected for this study. Seeds were obtained from the Faculty of Agriculture, Kasetsart University, Nakhon Pathom, Thailand.

Plant cultivation

Overall, mung beans were cultivated at Mahidol University, Kanchanaburi Campus, Thailand during the rainy season (July to September 2016). Mung bean plants were grown under 6×5×2.5 m^3 (length × width × height) greenhouse covered with white nylon net. Mung bean seeds were sown on mixed substrate (rice husk charcoal:coconut coir:soil in the ratio of 1:1:1) for 4-5 days. Subsequently, two healthy seedlings were transferred to a brown plastic pot (30 cm diameter) filled with a mixture of local soil and manure mixed with 5 g of N-P-K (15-15-15) fertilizer in the ratio 3:1. The holes of the pot were closed to prevent drainage.

Intrinsic labelling of mung bean protein at different time points of anthesis

The flow diagram of the study is illustrated in Figure 1. First, plants were grown in a greenhouse and watered daily to replace the amount of water lost by evapotranspiration. The daily rate of evapotranspiration was monitored by gravimetric method. A 10-kg load cell sensor was used to establish the weighing system with automatic real-time data logging. The pots were irrigated daily with tap water (EC 0.2-0.4 mS cm^{-1}, pH 6.8-7.5), except on the day of deuterium oxide ($^{2}H_2O$) administration.

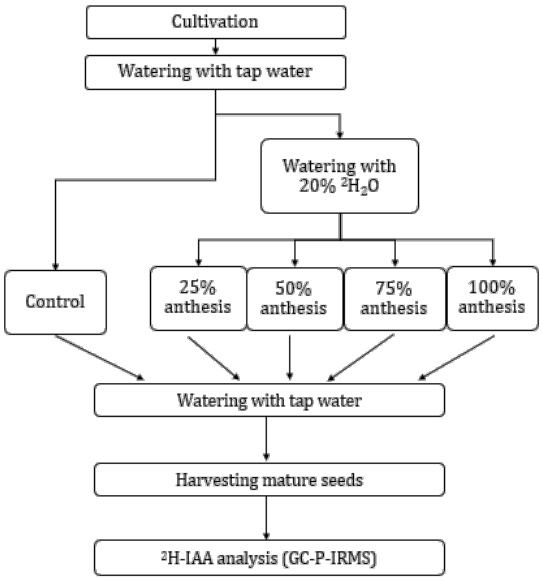

Figure 1. Flow chart diagram of the study.

To intrinsically label the plant protein, the 99.9 atom % ^2H_2O was diluted with tap water to 20% concentration, and watered as a single bolus at distinctive time points of anthesis, including 25, 50, 75 and 100% anthesis, or 3, 6, 8 and 10 days after flowering (four pots per group). The ^2H_2O water was pulsed into a 7 cm depth hole made in the pot's soil. This region of the soil includes the active root zone (Figure 2). The amount of 20% ^2H_2O provided for the plants replaced the water lost in the previous day, which was measured by weighing system. After watering, the hole was filled and the pot was subsequently closed by the transparent plastic sheet. After two days of the ^2H_2O application, all the pots were irrigated daily with tap water. Control group was watered with only tap water throughout the experiment. The seeds produced by all plants were harvested, dried, and stored at -20°C for further analysis.

Figure 2. The active mung bean root zone at 2-7 cm from the top of the soil.

Deuterium enrichment of indispensable amino acids analysis

All the samples of mung bean seeds were milled to fine powder using a ball mill (Retsch MM 301, Germany). Each seed powder sample was extracted in duplicate to obtain dried protein pellet. The dried protein pellet was digested following acid hydrolysis with 12 M hydrochloric acid and 0.02 M sodium thiosulphate at 150°C for 4 h, then 10 mM nor-valine was gradually added as an internal standard. The hydrolyzed proteins were further purified

using a strongly acidic cation exchange column and eluted with 4 M ammonium hydroxide for the formation of free amino acids. The amino acid fractions were then gathered and dried in a vacuum centrifuge. Amino acids were successfully derivatized to their *N*(O,S)-ethoxycarbonyl ethyl ester derivatives, and used for analysis.

The ^{2}H abundance of amino acids was analysed by gas chromatography-pyrolysis isotope ratio mass spectrometry (GC-P-IRMS) (Delta V Advantage, Thermo Fisher Scientific Inc., Bremen, Germany) at St John's Research Institute, Bangalore, India. The procedure of this analysis was described previously (Devi et al., 2018). The ^{2}H enrichment of IAA was represented as atom percent excess, which was calculated by subtracting the natural amount of ^{2}H in unlabelled (control) seeds.

Deuterated yellow pea was used as in-house quality control (QC) sample to validate the test runs. The results of QC samples were within mean ±2 SD for all amino acids. The amino acid standard mixture from Sigma-Aldrich were used for identifying individual amino acid peaks.

Statistical analysis

All data were reported as mean ± standard deviation (SD). Statistical differences between treatments were analysed using One-way ANOVA and Tukey's test, where $p<0.05$ was considered as statistically significant. Pearson's correlation was performed to evaluate the association between the amount of 20% $^{2}H_2O$ used and the average ^{2}H-IAA. Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) software version 17.0 (IBM Corp., Armonk, NY).

RESULTS AND DISCUSSION

The yield of intrinsically labelled mung bean seeds at different time points of anthesis

Weight of 1,000 seeds, seed yield plant^{-1} and the corresponding amount of 20% $^{2}H_2O$ used are presented in Table 1. The treatment at the stage of 25% anthesis significantly produced the lowest in number of seeds in each pot (91±19 seeds). Furthermore, the control plants generated the highest number (179±30 seeds). Likewise, seed yield plant^{-1} at the stage of 25% anthesis was lower than the control and other treatment groups (50, 75 and 100% anthesis). On the contrary, the treatment at the time of 25% anthesis resulted in the highest 1,000-seed weight.

Table 1. Yields of intrinsically labelled mung bean seeds at different time points of anthesis.

Treatments	Seeds pot^{-1} (no.)	1,000-seed weight (g)	Seed yield plant^{-1} (g)	The amount of 20% $^{2}H_2O$ pot^{-1} (mL)
Control	179±30a	75.60±4.99b	6.77±1.19a	0±0c
25% anthesis	91±19b	92.13±3.65a	4.16±0.84b	931±105a
50% anthesis	155±22a	78.89±5.82b	6.13±1.11ab	725±150b
75% anthesis	178±20a	74.63±6.23b	6.64±0.85a	1011±13a
100% anthesis	165±10a	81.59±2.83ab	6.74±0.48a	1042±23a

Data expressed as mean±SD (*n*=4). The values within the same column with different superscript letters showed significant differences between treatments at the $p\leq0.05$ by One-way ANOVA with Tukey test.

The results indicated that the weight of 1,000 mung bean seeds ranged between 74.63±6.23 and 92.13±3.65 g. This amount was higher than the previous report, which reported that the average weight of 1,000 seeds was 35.6 g (ranged 7.3 to 60.1 g) (Dahiya et al., 2015). Additionally, enhanced seed yield at the increased level of fertilizer can be attributed to a higher 1,000-seed weight, and affected the seed protein contents (Nadeem et al., 2004). Literately, seed size or 1000-seed weight is a necessary physical indicator of seed quality, that can vary due to the flow of nutrients into the seed from the mother plant (Ambika et al., 2014). Seed size of mung beans are positively associated with protein content, which contribute to higher seed weight (Trung and Yoshida, 1982).

Table 1 revealed the average of the amount of 20% ^2H_2O used in each treatment. Noticeably, there was no significant differences in the average of the amount of 20% ^2H_2O used per pot at the time of 25, 75 and 100% anthesis. However, the treatment at the time of 50% anthesis was watered with the lowest amount of 20% ^2H_2O (725±150 mL). Amount of 20% ^2H_2O used in this study was strongly related to the deuterium enriched IAA.

Deuterium enriched-IAA of intrinsically labelled mung bean seeds at different time points of anthesis

In the present investigation, mung beans were intrinsically labelled based on soil water conditions and the use of any specific instruments was not required. By watering plants with ^2H_2O, the deuterium atoms would be incorporated into amino acids by replacing the hydrogen atoms during the amino acid synthesis and metabolism. The deuterated amino acid molecules would be randomly generated depending on the number of hydrogen atoms in each amino acid.

Figure 3 shows the deuterium enrichment of IAAs including valine, leucine, isoleucine, serine/methionine, threonine, phenylalanine, and lysine. Noticeably, serine/methionine exhibited the lowest deuterium enrichment for all deuterated treatments. This ranged from 0.03 to 0.06 atom percent excess. Besides, the result also indicated that mung beans contain the lowest amount of methionine, and the highest amount was recorded for leucine. Therefore, these outcomes implied that the ^2H-IAAs was contributed by IAAs contents and not the total protein (mg g^{-1} protein). In short, the highest generation of ^2H-IAAs occurred for leucine, and the lowest was detected for methionine among IAAs.

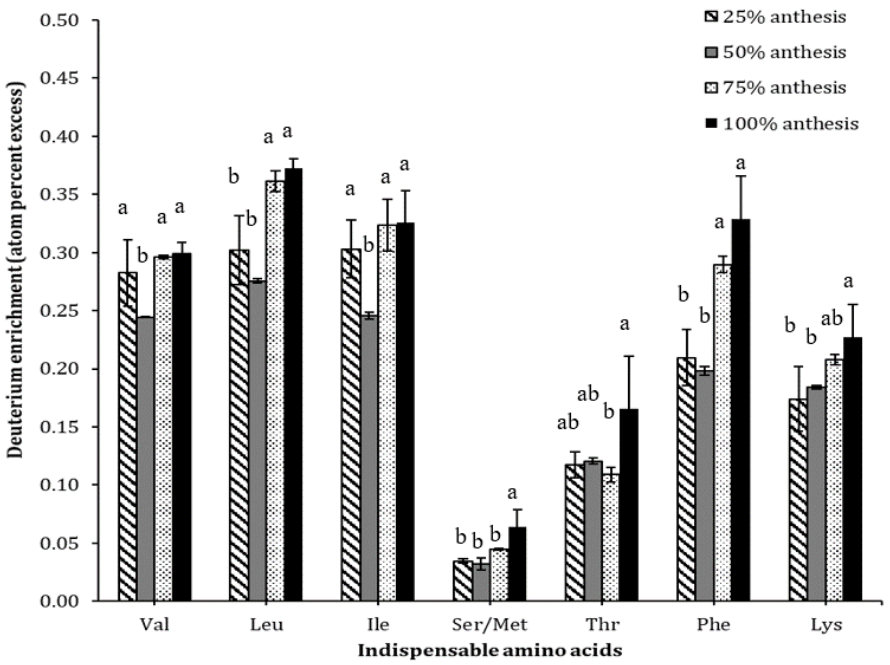

Figure 3. Average deuterium enrichment of indispensable amino acids in intrinsically labelled mung bean seeds (*n*=2). Data expressed as mean±SD. The values of the same amino acid with different superscript letters shows the significant differences between treatments at the p≤0.05, by One-way ANOVA with Tukey test. Val=Valine, Leu=Leucine, Ile=Isoleucine, Ser=Serine, Met=Methionine, Thr=Threonine, Phe=Phenylalanine and Lys=Lysine.

In addition, we found that there were no significant differences for ^2H-valine and ^2H-isoleucine at the time of 25, 75, and 100% anthesis. Treatments at the time of 75 and 100%

anthesis produced significantly higher amounts of ^{2}H-leucine and ^{2}H-phenylalanine compared to the stages of 25 and 50% anthesis. Remarkably, treatment at the time of 100% anthesis produced the highest amount of ^{2}H-serine/methionine, ^{2}H-threonine, and ^{2}H-lysine. The average amount of ^{2}H-IAAs at all time points was 0.20, 0.19, 0.23, and 0.25 atom percent excess, respectively. The treatments at the time of 75 and 100% anthesis (8 and 10 days after flowering) might be an appropriate period of mung bean protein labelling.

A previous study affirmed that the stable isotope of zinc would be assimilated and efficiently transported to plant's reproductive areas during anthesis, even up to the period of seed formation. For higher enrichment of isotope into the seed, the administration of isotope should be performed during the latter half of growth (Fox et al., 1991). The storage-protein accumulation of each legume seed had distinctive periods, these gradually declined until maturation. Awolumate (1983) showed that the period for storage-protein accumulation of mung beans was between 10 and 14 days after flowering. Hence, the proper time for intrinsic labelling of mung bean seed should be 0-2 days before protein synthesis, and then the accumulation of proteins takes place from 8 to 10 days after flowering.

Generally, the evapotranspiration appeared to be a reliable index for evaluating the absorption and translocation of water and nutrients into the plant's xylem vessels (Takuji et al., 2017). In similar circumstances, soil water capacity during anthesis is an important factor for assessing wheat grain quality (Zhao et al., 2009). Therefore, adjusted amounts of the $^{2}H_2O$ watering was based on the evapotranspiration from plants in each pot. This was done by replacing the amount of water lost in the previous day for all treatments. Figure 4 shows the association between the amount of 20% $^{2}H_2O$ used and the average ^{2}H-IAAs of mung bean seeds. There was a positive relationship between the amount of 20% $^{2}H_2O$ used and the average amount of ^{2}H-IAAs generated (R^2=0.828; p=0.09). These results indicated that evapotranspiration might influence the nutrients and its rate of uptake. Subsequently, the deuterated IAAs would be enhanced and incorporated into proteins, when a high rate of water loss occurred.

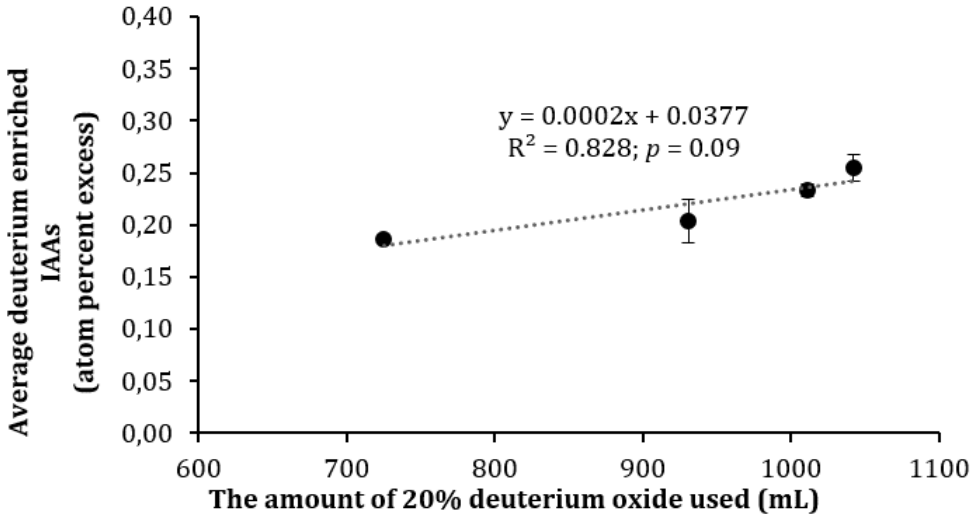

Figure 4. The association between the average amount of 20% deuterium oxide used and the average deuterium enriched indispensable amino acids of intrinsically labelled mung bean seeds.

CONCLUSIONS

For efficiently enriching ^{2}H-IAAs in mung bean seeds, the appropriate period for $^{2}H_2O$ watering was at the time of 75 and 100% anthesis or 0-2 days before the period of protein synthesis and accumulation. Furthermore, these outcomes also affirmed that

evapotranspiration is necessary for obtaining high degree of enrichment. However, several limitations can be acknowledged in the study. The current study represents the coelution of serine and methionine. Further studies are needed to analyse other amino acids, such as tryptophan and histidine. Therefore, to obtain a higher and efficient intrinsic labelling, additional environmental factors should be considered. For instance, relative humidity, temperature, and the moisture level during production.

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Breeding of new *Asparagus officinalis* purple cultivars 'RG murasakishikibu First' and 'RG murasakishikibu Luce'

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Abstract

Japan is home to several purple *Asparagus officinalis* cultivars, including 'Pacific Purple' and 'Purple Passion.' These cultivars have low yield, slow emergence, and a dark spear color. Therefore, two new cultivars, 'RG murasakishikibu First' and 'RG murasakishikibu Luce' were developed. These two new cultivars are hybrid asparagus cultivars bred by the single cross of different male and female plants selected from the tetraploid cultivar, 'Purple Passion.' The harvest start date of 'RG murasakishikibu First' was four to seven days earlier than 'Pacific Purple' and two to six days later than 'UC157'. The spear weight, number, and yield of 'RG murasakishikibu First' equaled 'Pacific Purple' and its yield was equivalent to 'UC157'. Its spear color had a chroma c* value greater than or equal to 'Pacific Purple.' The plant height, number of stems, stem diameter, growth index, and height up to the first branch were the same as those of 'Pacific Purple.' 'RG murasakishikibu First' plant height was greater, stem diameter was thicker, growth index was higher, and height up to the first branch was greater than 'UC157'. The harvest start date of 'RG murasakishikibu Luce' was two to four days earlier than 'Pacific Purple' and three to nine days later than 'UC157'. The spear weight was slightly heavier than that of 'Pacific Purple,' with the same number of stems and higher yield (equivalent to 'UC157'). The spear color had higher chromatic components, a* and chroma c* values, than 'Pacific Purple'. Plant height, number of stems, stem diameter, growth index, and height up to the first branch were equivalent to those of 'Pacific Purple.' Plant height was greater, stem diameter was thicker, growth index was higher, and height up to the first branch was higher than 'UC157'.

Keywords: development, spear, yield, emergence, color

INTRODUCTION

Asparagus officinalis (asparagus) is a popular vegetable in Japan. Hokkaido is the largest production area and produces mainly green asparagus. In recent years, demand for purple asparagus has been increasing due to diversification of consumer preferences and heightened health consciousness. Purple asparagus is reported to be richer in rutin content, total polyphenols, total soluble solids, and ascorbic acid content than green asparagus (Kohmura and Watanabe, 2005; Maeda et al., 2005). In Hokkaido, asparagus is sold as a gift. By selling green and purple asparagus as a set, when selling asparagus as a gift, the commercial value is improved.

Purple asparagus is a tetraploid, and its cultivars include 'Violetto D' Albenga' (Falavigna and Fantino, 1985), 'Purple Passion' (Benson et al., 1996), and 'Pacific Purple' (Falloon and Andersen, 1999). In Hokkaido, 'Purple Passion' and 'Pacific Purple' are cultivated. However, these cultivars are slower to emerge in spring, have lower yields, and have uneven spear and shoot growth compared to green asparagus (Nii et al., 2011; Takamura et al., 2015). Further, the dark color of the spears is a disadvantage as it is not highly favored by consumers. By increasing the planting density by 2 to 3 times, the yield of the 'Purple Passion' is equivalent to that of UC157, but the cost of seeds increases (Motoki et al., 2011). For this reason, purple asparagus cultivars that have better yield, clearer coloring of spears and that emerge faster in

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the spring are desired. In Fukushima Prefecture, 'Harumurasaki F' has been developed, which has higher yield, and higher uniformity of spear weight, and emerges earlier than 'Purple Passion' (Nii et al., 2011). However, this cultivar has the disadvantages of a lighter spear weight and darker spear color than 'Purple Passion' (Nii et al., 2011). Therefore, Rakuno Gakuen University has been working on breeding purple asparagus since 2011 and has bred the tetraploid 'RG murasakishikibu First' and 'RG murasakishikibu Luce' cultivars.

In this report, the breeding process for these two cultivars and the results of performance tests conducted from 2017 to 2019 are presented.

MATERIALS AND METHODS

Breeding process

Cross breeding was used to develop new asparagus cultivars with early emergence in spring, high yield, and vivid purple spears. From 2010 to 2012, four female and four male plants with these characteristics were selected from 'Purple Passion' production sites in Hokkaido and Fukushima Prefecture. Using the eight plants selected in 2013 as mother plants, 12 combinations were artificially crossed, and among them, seven lines with a fruit set rate of 60% or more were selected.

From 2014 to 2015, a combining ability test was performed in an open-field culture site and a semi-forcing culture site, and two lines RG1305 and RG1306, which emerged early in spring, had a high yield, and vivid coloring of spears, were selected. As a result of conducting a performance test in a semi-forcing culture site from 2015 to 2019, the characteristics of RG1305 and RG1306 were determined to be stable and practical. RG1305 was named 'RG murasakishikibu First' and RG1306 was named 'RG murasakishikibu Luce'.

Performance test

The performance test was conducted at a semi-forcing culture site at Rakuno Gakuen University from 2015 to 2019. The test used 'RG murasakishikibu First', 'RG murasakishikibu Luce', the standard colored cultivar 'Pacific Purple', and the green asparagus cultivar 'UC157'. Seeds of each cultivar were sown in a cell tray on January 28, 2015, potted into a 9-cm diameter poly-pot on February 23, and planted in the field on May 21. In the test, 60 plants per cultivar were planted in three different places in the test site. In the test site, 100 t compost, 250 kg ha^{-1} nitrogen, 200 kg ha^{-1} phosphoric acid, 230 kg ha^{-1} potassium and 1,000 kg ha^{-1} mica lime were applied. From the second year after planting, nitrogen 100 kg ha^{-1} was applied simultaneously with irrigation.

Production studies began in 2017, three years after planting. The harvesting period was 52 days from March 29 to May 19 in 2017, 55 days from April 1 to May 25 in 2018, and 54 days from March 31 to May 23 in 2019. The harvest start date was the date when 30% of the test plants commenced harvest. Spears with a length ≥28 cm were harvested and trimmed to a length of 25 cm. Thereafter, the weight and the number of spears which were not bent, flattened, or opened at the tip were measured. In addition, the distribution of the weight of 100 spears harvested in April 2018 was determined. The color of the spears was measured three times on a sample using a color difference meter (MINORUTA CM-700d) to determine lightness L* and the chromatic components a* and b*. The chroma c* value was calculated using the formula: $\sqrt{(a^*)^2+(b^*)^2}$. One month after the completion of the 2018 harvest, the plant height, stem diameter, number of stems, and height up to the first branch were investigated, and a growth index was calculated from the product of plant height, stem diameter, and number of stems.

RESULTS

At the start date of harvest, there was an interaction between the cultivar and the year. The harvest start date of 'RG murasakishikibu First' was 4-5 days earlier than 'Pacific Purple' and 2-6 days later than 'UC157'. The harvest date of 'RG murasakishikibu Luce' was 2-4 days earlier than 'Pacific Purple' and 3-9 days later than 'UC157' (Table 1).

Table 1. The harvest start date of 'RG murasakishikibu First', 'RG murasakishikibu Lice', 'Pacific Purple' and 'UC157'.

Cultivar	2017	2018	2019
RG murasakishikibu First	April 10±1.0[a]	March 30±0.3	April 10±0.9
RG murasakishikibu Luce	April 11±0.9	April 2±1.5	April 13±0.3
Pacific Purple	April 14±1.3	April 4±1.5	April 17±1.5
UC157	April 4±1.2	March 28±0.9	April 4±0.3

[a]Harvest start date is the date when spears of 30% of the test plants were ready for harvest (mean ± SD) (*n*=3).

The marketable yield of 'RG murasakishikibu First' was comparable to 'Pacific Purple' and 'UC157'. In contrast, the marketable yield of 'Pacific Purple' was lower than 'UC157'. The marketable yield of 'RG murasakishikibu Luce' was significantly higher than 'Pacific Purple' and equivalent to 'UC157'. The number of spears of 'RG murasakishikibu First' and 'RG murasakishikibu First' was equivalent to 'Purple Passion' and significantly less than 'UC157' (Table 2). No significant correlation was found between yield and number of spears (r=0.598).

Table 2. Marketable yield and number of spears of 'RG murasakishikibu First', 'RG murasakishikibu Lice', 'Pacific Purple' and 'UC157'.

Cultivar	Type	Marketable yield (kg ha^{-1})[a]	Number of spears ha^{-1}
RG murasakishikibu First	Purple	11984ab	283928a
RG murasakishikibu Luce	Purple	15099b	294752a
Pacific Purple	Purple	10149a	232501a
UC157	Green	14235b	475368b

[a]Average yield for 3 years from 2017 to 2019.
Mean separation within columns by Tukey's multiple range test (p<0.05).

Spear weight of 'RG murasakishikibu First' was equivalent to 'Pacific Purple' and heavier than 'UC157'. Spear weight of 'RG murasakishikibu Luce' was heavier than 'Pacific Purple' and 'UC157' (Figure 1). The spear weight of 'RG murasakishikibu First' was distributed from 10 to 70 g, with 40 g having the highest percentage. 'RG murasakishikibu Luce' ranged from 20 to 80 g, with 50 g having the highest percentage. 'Pacific Purple' ranged from 10 to 90 g, with 30 g having the highest percentage. 'UC157' ranged from 10 to 60 g, with 30 g having the highest percentage (Figure 2).

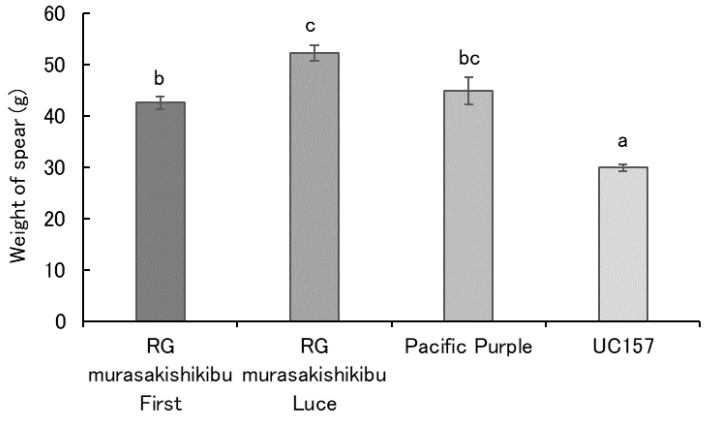

Figure 1. The weight of spears of 'RG murasakishikibu First', 'RG murasakishikibu Luce', 'Pacific Purple' and 'UC157'. Error bars indicate SE; mean separation within columns by Tukey's multiple range test (p<0.05).

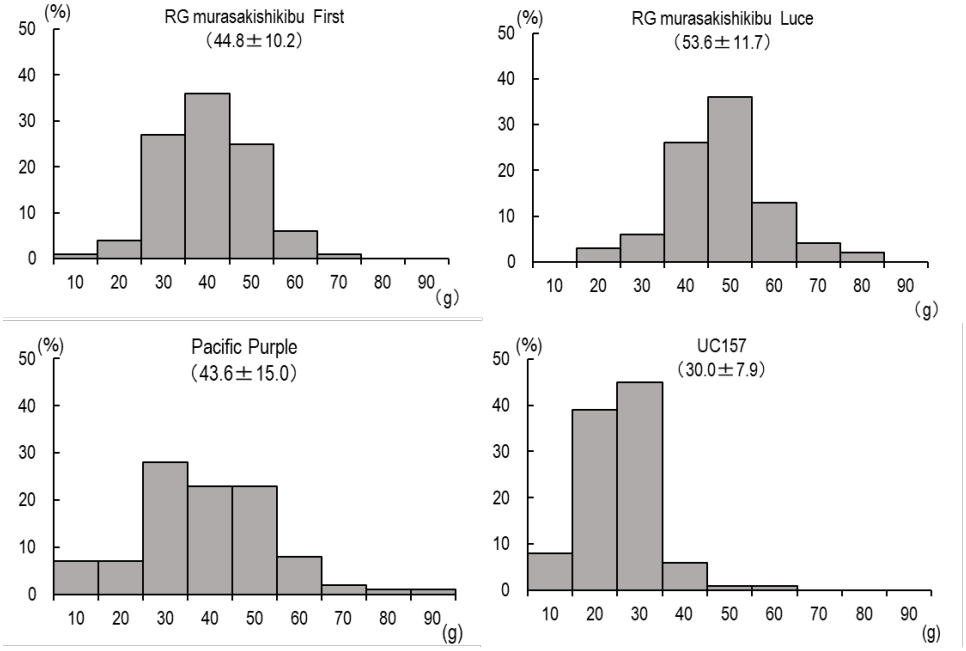

Figure 2. Distribution of spear weight of 'RG murasakishikibu First', 'RG murasakishikibu Luce', 'Pacific Purple' and 'UC157' in April, 2018 (mean ± SD, *n*=100).

The color of the spears of 'RG murasakishikibu First' had the same chromatic components a* and the same or a higher saturation c* than 'Pacific Purple'. The color of the spears of 'RG murasakishikibu Luce' was significantly higher in the chromatic component a* and in the chroma c* value than those in 'Pacific Purple' (Table 3).

Table 3. The spear color of 'RG murasakishikibu First', 'RG murasakishikibu Luce' and 'Pacific Purple'.

Cultivar	CIELAB coordinates[a]			
	L*	a*	b*	c*
RG murasakishikibu First	45.6a	4.6ab	1.9b	5.1b
RG murasakishikibu Luce	46.4a	5.4b	1.8b	5.9b
Pacific Purple	45.9a	3.6a	1.3a	3.9a

[a]L*: lightness, a* and b*: chromatic components, c*: chroma (brightness).
Mean separation within columns by Tukey's multiple range test ($p<0.05$).

Plant height, number of stems, stem diameter, growth index and height to the first branch of 'RG murasakishikibu First' and 'RG murasakishikibu Luce' were the same as 'Pacific Purple.' However, the two new cultivars had greater plant height, stem diameter, GI and height up to the first branch than 'UC157' (data not included).

DISCUSSION

'Purple Passion' and 'Pacific Purple' are purple cultivars developed using plants selected from 'Violetto D' Albenga' which is native to northern Italy. The mother plants were cultivated in Hokkaido (Benson et al., 1996; Falloon and Andersen, 1999). It has been reported that the yield of 'Purple Passion' in open-field culture in California was equivalent to that of 'UC157' (Benson et al., 1996). It was also reported that 'Pacific Purple' yields in open-field culture in Christchurch, New Zealand were higher than 'Purple Passion' and equivalent to 'UC157' (Falloon and Andersen, 1999). However, in the open field cultivation in Nagano and Fukushima prefectures, it is reported that the emergence of spring spears of 'Purple Passion' is slow and the yield is lower than that in 'UC157' (Motoki et al., 2011; Nii et al., 2011). In this

current study, 'Pacific Purple' showed the same tendency as the results reported previously (Motoki et al., 2011; Nii et al., 2011). These results suggest that purple asparagus cultivars bred in the United States and in New Zealand may not be suitable for the environments in the colder regions of Japan. The emergence of spring spears of 'RG murasakishikibu First' was earlier than that of 'RG murasakishikibu Luce' and 'Pacific Purple', and the yield was equivalent to 'UC157'. The emergence of spring spears of 'RG murasakishikibu Luce' was earlier than 'Pacific Purple', the yield was equal to 'UC157' and a spear weight was the heavier than both. Based on these results, 'RG murasakishikibu First' and 'RG murasakishikibu Luce' are cultivars that are suitable for cold regions in Japan. In particular, 'RG murasakishikibu First' exhibits the fastest emergence of spring spears among the purple cultivars examined, so it can be produced in combination with green asparagus.

'Purple Passion' can achieve the same yield as 'UC157' by increasing planting density (Motoki et al., 2011). Therefore, a yield equivalent to that of 'UC157' could be secured for 'RG murasakishikibu First' as well as for 'Purple Passion' by increasing the planting density. However, this would not be necessary for 'RG murasakishikibu Luce'. Furthermore, 'RG murasakishikibu Luce' could meet the needs of producers because it had a higher yield and a greater spear weight than the main cultivar, 'Pacific Purple'.

'Violetto D' Albenga' is a cultivar with inferior uniformity where the color of the spear can vary from purple to green. Consequently, 'Purple Passion' and 'Pacific Purple' were bred with the objective of producing more uniformly colored spears (Benson et al., 1996; Falloon and Andersen, 1999). The chromatic components a* and saturation c* of the spears of 'RG murasakishikibu First' and 'RG murasakishikibu Luce' were higher than those for 'Pacific Purple' and their saturation c* values were higher than for 'Purple Passion'. The spears of the two new cultivars were vivid purple and may, therefore, increase the sales value.

CONCLUSIONS

'RG murasakishikibu First' and 'RG murasakishikibu Luce' are suitable cultivars for cold production regions in Japan because of early emergence in spring, high yields, and the vivid coloration of their spears. However, these two new cultivars differed in these characteristics. By cultivating the two cultivars in combination, therefore, it would be possible to produce colored vivid purple asparagus in the spring under the conditions in Hokkaido. In future, the adaptability of these cultivars to Fusekomi forcing culture will be studied.

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Seasonal variation of sepal-petaloidy in F_1 progenies of double-flowered cyclamen

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Abstract

***Cyclamen persicum* is popular as a potted flower and its cold tolerant cultivars are used as garden plants. Recently, new double-flowered cyclamen cultivars with petaloid stamens or sepals have been bred by crossing. Our previous studies revealed that constant low and medium growth temperatures promoted the petaloidy of stamens. However, the effects of seasonal environmental changes, such as those associated with growth temperature and light conditions, on sepal-petaloidy of F_1 progenies was not assessed under actual cultivation conditions. Therefore, the seasonal variation of sepal-petaloidy in F_1 progenies of double-flowered cyclamen was investigated in this study. F_1 progenies produced by reciprocal crossing between single- and double-flowered cyclamens with petaloid-sepals were used. The seedlings obtained from eight cross combinations were cultivated using common methods under glasshouse conditions, from February to April 2019, during the flowering in each flower and within each plant. Degree of petaloidy, according to the sepal shape in each flower, was categorized into four levels. Additionally, a petaloidy index (PI) of each plant was calculated using the degree of petaloidy and the number of flowers. Based on the floral phenotypes of F_1 progenies, it was suggested that the double-flower trait was dominantly inherited. In double-flowered progenies, the degree of petaloidy showed variation where most of the flowers that opened in the early season expressed a lower degree of petaloidy than those flowers opened in the late season. The seasonal changes of petaloidy in F_1 progenies were expressed regardless of the cross combinations. However, the degree of petaloidy in several F_1 plants did not show significant seasonal differences between flowers in February and April during the flowering season. These results indicate that seasonal changes of petaloidy were regulated both by environmental and genetic factors.**

Keywords: *Cyclamen persicum*, environmental factor, floral morphology, growth temperature, light condition

INTRODUCTION

Cyclamen, which belongs to the *Primulaceae*, is a bulbous plant that originated from the Mediterranean region. *Cyclamen persicum* is popular as a potted flower. Its cold tolerant cultivars are also used as garden plants in European and East-Asian countries. Variation of flower shapes and colors is one of the reasons that makes the flower popular (Grey-Wilson, 2002; Takamura, 2006). Double-flowered cyclamen mutants with petaloid stamens and petaloid sepals were found and used for breeding. From these sources, new double-flowered cyclamen cultivars have been produced and are still being bred (Grey-Wilson, 2002; Murayama et al., 2011; Tanaka et al., 2013). Although the cultivars have been circulated commercially in markets, various degrees of petaloidy within and among plants is a problem for growers and consumers seeking a stable expression of double-flowers in the cultivars. *Cyclamen persicum* has a long flowering period in winter in the Mediterranean region and in East Asia (Grey-Wilson, 2002; Ishizaka, 2008). Thus, it was estimated that the species is affected by environmental factors from floral development to anthesis. Petaloidy (double-flower formation) is a genetic trait and is mainly controlled by genetic factors. However, the

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trait is also influenced by environmental factors. Previous studies revealed that constant low and medium growth temperatures (15 and 20°C) promoted the stamen petaloidy of cyclamen (Mizunoe and Ozaki, 2015). In another report, the degree of stamen-petaloidy in double-flowered cyclamen grown under alternating temperatures of 20/10°C (day/night) was similar to that under a constant temperature of 15°C (Mizunoe and Ozaki, 2017). In addition, seasonal changes in petaloidy have been recognized and these changes differed depending on the genetic background (Mizunoe and Ozaki, 2015). In the petaloid sepal type of double-flowered cyclamen, the effect of environmental factors on sepal-petaloidy was not assessed under actual cultivation conditions. Inheritances of petaloidy and responses to seasonal changes in environmental factors are important in F_1 progenies because cyclamen cultivars are maintained by seed propagation. Therefore, the seasonal variation of sepal-petaloidy in F_1 progenies of double-flowered cyclamen was investigated in this study.

MATERIALS AND METHODS

F_1 progenies produced by reciprocal crossing between single- and double-flowered cyclamens were used. Seeds obtained from eight cross combinations were treated with 50 mg L^{-1} gibberellic acid (GA_3) for five minutes, and then sowed into 200-cell plug trays filled with Metro-Mix 350 (Sun-Gro horticulture, Agawam, Massachusetts, USA). The trays were kept for germination at 15°C under natural daylight in the Phytotron of Kyushu University (Fukuoka, Japan). The seedlings were transplanted into 7.5-cm diameter plastic pots filled with Metro-Mix 350 and 12-cm diameter plastic pots filled with a soil mixture consisting of red clay soil, bora soil, leaf mold at a ratio of 3:3:4 (v/v/v), 3 and 6 months after sowing, respectively. Three months after transplanting (9 months sowing), the pots were moved to a glasshouse at Kyushu University. In the glasshouse, plant culture was conducted using common methods. The growth environment was set at the natural temperature from spring to autumn (April to November), and minimum temperature was kept above 10°C from winter to spring (December to March) in 2018 and 2019.

Temperature data in the glasshouse were collected using a temperature sensor, SLAW-WP01 (Smartlogic, Tokyo, Japan) from January to March 2019. Meteorological data before and during the flowering period were collected from a weather observation system (Climatec, Tokyo, Japan) consisting of a temperature sensor (C-HPT-5-JM), a pyranometer (CHF-SR20-JM) and a sunshine recorder (CIS-162). The sunshine recorder detected irradiation when the intensity of solar radiation was more than 0.12 kW. The data collection site was located in the same field as the glasshouse. The meteorological data were subjected to statistical analyses, the Tukey-Kramer and Steel-Dwass tests, using Statcel-the Useful Add in Forms on Excel, 4th edn (OMS publishing, Tokyo, Japan) developed by Yanai (2019).

Opened flowers of second year seedlings were sampled from February to April in 2019 during the flowering season. Degree of petaloidy, according to the sepal shape in each flower, was categorized into four levels: 0 = sepal-like, 1 = slight petaloidy, 2 = less than half of original-petal size and 3 = more than half of original-petal size. A Mann-Whitney's U test was performed for the degree of petaloidy between February and April using the addin forms as mentioned above. Additionally, a petaloidy index (PI) was calculated from the following formula using the degree of petaloidy and each number of the flowers that had been categorized into the four levels.

$$\text{Petaloidy index} = \sum_{k=0}^{3}(k * n_k) \ / \ \sum_{k=0}^{3} n_k$$

n_k: number of flowers, k: degree of petaloidy (k=0, 1, 2, 3).

RESULTS AND DISCUSSION

Based on the floral phenotypes (Figure 1), where most of F_1 progenies were double-flowered, it was suggested that the double-flower trait was dominantly inherited. In the double-flowered progenies, the degree of petaloidy showed seasonal changes where flowers that opened in late season expressed a higher degree of petaloidy than those that opened in early season (except for several single-flowered plants in 'Karakurenai' progenies). The

seasonal changes of petaloidy were not dependent on the combination of crosses; double-flowered F_1 progenies had this type of variation regardless of the reciprocal cross combinations between parent cultivars having either single- or double-flowers.

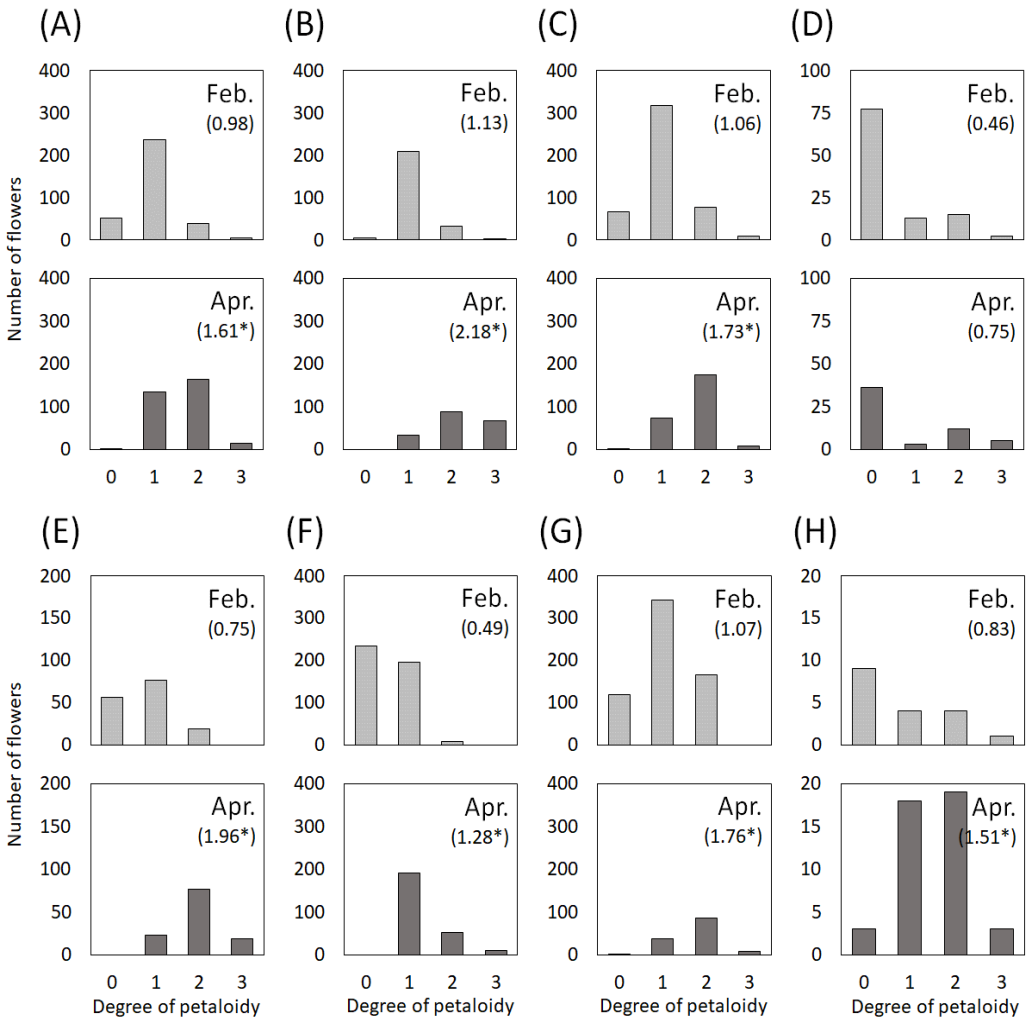

Figure 1. Seasonal changes of sepal petaloidy in each flower of F_1 plants. Degree of petaloidy was categorized into four levels: 0 = sepal-like, 1 = slight petaloidy, 2 = less than half of original-petal size, 3 = more than half of original-petal size. Cross combinations between a single-flower and a double-flower (A-D), a double-flower and a single-flower (E-H). 'K Middy Scarlet' × 'Classical Dress' (A), 'Metis Ponpon Mix' × 'Classical Dress' (B), 'Super Berano' ×' Classical Dress' (C), 'Super Berano' × 'Karakurenai' (D), 'Classical Dress' × 'K Middy Scarlet' (E), 'Classical Dress' × 'Metis Ponpon Mix' (F), 'Classical Dress' × 'Picora Shine Red' (G) and 'Karakurenai' × 'K Middy Scarlet' (H). Numbers in parentheses show mean values of degree of petaloidy in the month. Asterisk indicates significant increase from February to April according to Mann-Whitney's U tests at 5% level.

The petaloidy index (PI) of each plant, and the relationship between values in February and April, are shown in Figure 2. In most of the plants, PI values in April were higher than those in February as well as degree of petaloidy in each flower (Figure 1). Most of the plots were widely distributed in the upper-left side of the X=Y line of each accession. These variations in PI values meant that seasonal changes in the F_1 progenies were not originating

from any particular individual. In contrast, the degree of petaloidy in several F_1 plants, locating on the X=Y lines, did not show a difference in values between February and April through flowering season. These results indicated that sepal-petaloidy showed seasonal changes in most of the F_1 progenies, whereas several plants did not show these seasonal changes under the same environmental conditions. Thus, the degree of seasonal changes in sepal-petaloidy was not only influenced by environmental factors, but also by genetic regulation. It appears that the degree of seasonal changes, that is, the seasonal stability of petaloidy, was genetically regulated at individual levels. This study provides two important views for future breeding. The correct selection of genotype in double-flowered F_1 progenies is required and several observations of floral characteristics at regular intervals are necessary over a number of months. Furthermore, efficient breeding and stable production of double-flowered cyclamen will be achieved if F_1 plants showing stable PI values are selected.

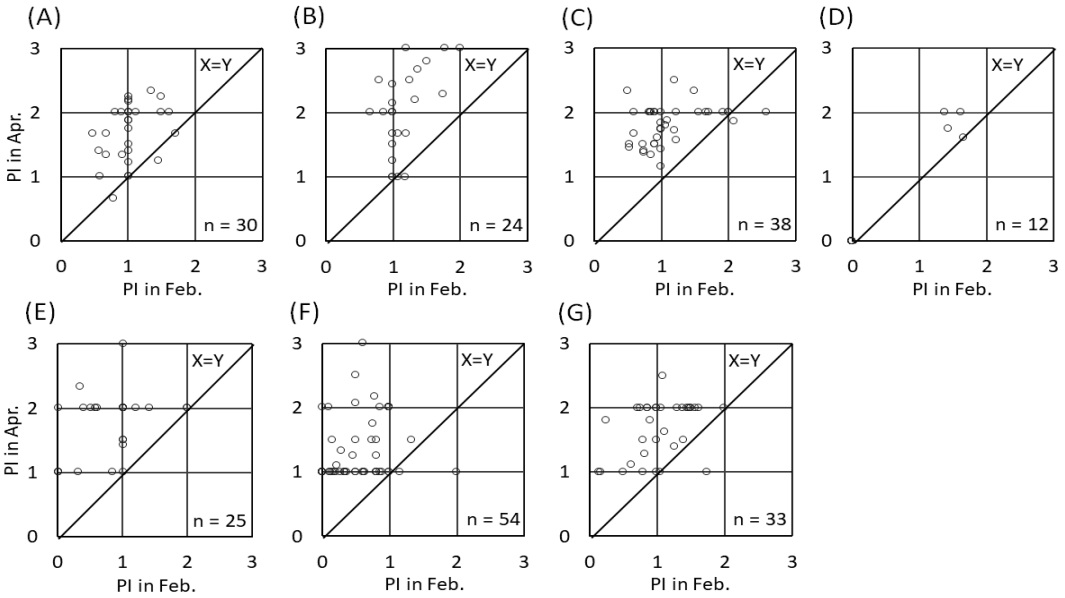

Figure 2. Seasonal changes of petaloidy index (PI) in F_1 plants. Cross combinations between a single-flower and a double-flower (A-D), a double-flower and a single-flower (E-G). 'K Middy Scarlet' × 'Classical Dress' (A), 'Metis Ponpon Mix' × 'Classical Dress' (B), 'Super Berano' × 'Classical Dress' (C), 'Super Berano' × 'Karakurenai' (D), 'Classical Dress' × 'K Middy Scarlet' (E), 'Classical Dress' × 'Metis Ponpon Mix' (F), 'Classical Dress' × 'Picora Shine Red' (G). Numbers shown at the lower right of squares indicate number of investigated plants. PI values of the cross combination between 'Karakurenai' and 'K Middy Scarlet' were excluded from this figure because of a low number of samples.

Floral morphogenesis was assumed to be influenced by environmental factors during floral bud development. Meteorological data from November 2018 to March 2019 were referred (Figure 3), since about three months were required from visible bud formation to flowering at 16 and 20°C, respectively (Oh et al., 2008). The transition of temperatures in field and glasshouse conditions were similar, although the actual temperatures were higher in the glasshouse. Temperature increased from January to March. For the flowers that opened in February, the environmental conditions from November to January might have contributed to petaloid sepal development. In that period, both temperature and the intensity of solar radiation gradually declined. However, these conditions gradually increased for the flowers that opened in April. In a previous report of petaloid-stamen formation in double-flowered cyclamen, constant low and medium growth temperatures (15 and 20°C) induced stamen petaloidy (Mizunoe and Ozaki, 2015). In the present study, it was suggested that sepal

petaloidy was induced by a low growth temperature, similar to that previously found for stamen petaloidy of double-flowered cyclamen. This is also similar to a temperature of about 17°C that has been shown to be appropriate for cyclamen (Yesson and Culham, 2006).

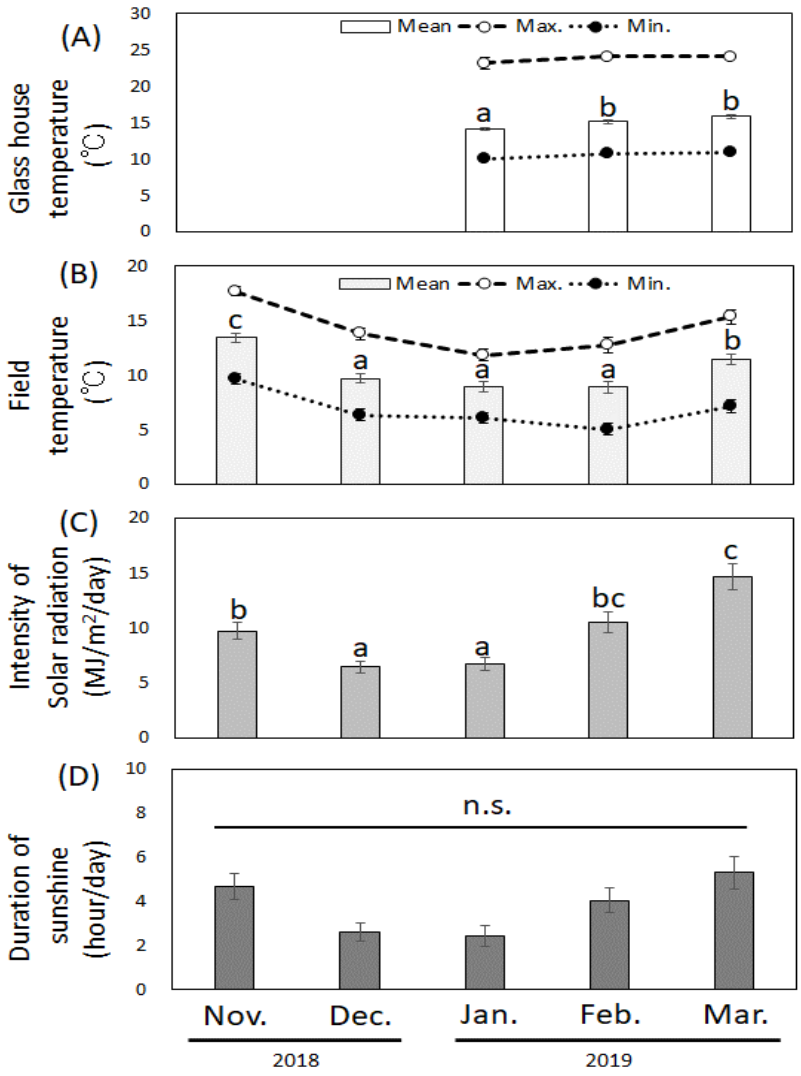

Figure 3. Environmental data from the experimental site during the flowering season. Glasshouse temperature (A), field temperature (B), intensity of solar radiation (C) and duration of sunshine (D). Duration of sunshine was measured when the intensity of solar radiation was more than 0.12 kW. Vertical bars indicate standard errors. Means followed by different letters represent significant differences according to the Tukey-Kramer test for temperature, and Steel-Dwass test for intensity of solar radiation among months at 5% level. No significant difference was detected by the Steel-Dwass test for duration of sunshine among months at the 5% level.

The intensity of solar radiation decreased from November to December and then increased from January to March (Figure 3). Light conditions such as light intensity, light quality and photoperiod were also reported to influence the number of flower buds, number of opened flowers, fresh and dry weights of petals at moderate levels for cyclamen (Heo et al., 2003; Villegas et al., 2006; Oh et al., 2008). These seasonal changes in light intensity were, therefore, likely involved in the seasonal changes of sepal-petaloidy. The duration of sunshine

did not show significant differences among the months of this study. Among other candidate factors that may have influenced sepal-petaloidy, mineral nutrition is a possibility. The total number of flowers in April was less than that in February (except for the cross combination of 'Karakurenai' and 'K Middy Scarlet'; Figure 1H). The flower development of F_1 progenies may had been influenced by a decrease in nutrient availability over the experimental period. Gillespie and Thomas (1983) reported that nitrogen application at medium to high levels promoted early flowering and increased the number of flowers per plant in cyclamen. In that report, a combined application of phosphorus and potassium improved flower quality, including flower size. It was considered, therefore, that a combination of these exogenous (environmental) factors influenced the endogenous status (physiological and epigenetic factors) of the plants. In *Arabidopsis*, Kiba et al. (2011) reported an interaction between nitrogen and the biosynthesis of plant growth regulators (auxin, cytokinin and abscisic acid). Similarly, phosphate starvation was closely related to downregulations of cytokinin and gibberellin biosynthesis (Franco-Zorrilla et al., 2005; Devaiah et al., 2009). It has also been reported that a gibberellin signal induces an upregulation of transcript levels of floral homeotic genes, *APETALA3*, *PISTILLATA* and *AGAMOUS* (Yu et al., 2004). From information in these previous studies, it was proposed possibility that growth temperature and/or light conditions influence endogenous factors, such as nutrition conditions and the concentrations of plant growth regulators, that impact on double-flowered plants and that they regulate petaloidy. The effects of environmental factors on double-flower formation have not been defined in many flowering species. Clarification of the correlations among exogenous factor, endogenous factor and petaloidy would provide new insights for understanding double-flower formation.

CONCLUSIONS

This study demonstrated that the degree of sepal-petaloidy varied during the flowering season in F_1 progenies originating from reciprocal crosses between single-flowered and sepal-petaloid double-flowered cyclamens. The results indicated that F_1 progenies of double-flowered cyclamen showed seasonal changes of sepal-petaloidy. Although the main factor influencing sepal-petaloidy could not be confirmed, the existence of seasonal changes suggested that environmental (exogenous) factors influenced the sepal-petaloidy. Seasonal changes in sepal petaloidy were different among accessions and plants, whereas the degree of petaloidy among several hybrids was stable during the flowering season. These results indicate that the seasonal changes in petaloidy were not only influenced by environmental factors but also by genetic regulation.

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Breeding for yellow vein mosaic disease resistance and export standard fruit quality in okra (*Abelmoschus esculentus* (L.) Moench)

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Abstract

Hybridization and selection of okra was carried out during 2016-2018 at the Kanchanaburi Agriculture Research and Development Center. Yellow vein mosaic disease (YVMD) resistant cultivars which possess export standard fruit quality were required. YVMD is a major problem for okra production as it has dramatically reduced yield and fruit quality. The pedigree selection method was applied and selection populations were obtained from hybrids between susceptible commercial cultivars (desire fruit quality) and YVMD resistant cultivars. The selection procedures were conducted in a disease outbreak field by growing selected lines and a susceptible cultivar ('PC03') in a 2:1 ratio. The progenies showed segregation characteristics in early generations but they became more stable after successive rounds of selection. In the F_6 generation, seven promising lines namely; KC5902-1-1-4-3-1, KC5915-2-18-15-20-10, KC5929-3-30-24-32-27, KC5930-2-31-28-38-31, KC5932-2-38-35-42-37, KC5944-2-54-44-46-38 and KC5950-1-60-55-52-40, were selected. They were completely resistant to YVMD in the disease outbreak field throughout crop production, while all 'PC03' plants showed disease symptoms 44 days after sowing (DAS). In addition, fruit quality was improved and met export standard criteria: five locules, green color, 7-12 cm fruit length, and downy pubescence. The desired plant height of 100-150 cm was obtained, along with 2-3 branches plant^{-1}, which leads to easy fruit harvesting. In addition, early flowering was observed at 35-42 DAS.

Keywords: *Abelmoschus esculentus* (L.) Moench., okra, hybridization, pedigree selection method, virus disease

INTRODUCTION

Okra (*Abelmoschus esculentus* (L.) Moench) is an important exported vegetable in Thailand. The main market is Japan which is over 95% of total exports. This market is strict on the standards for fruit quality. Young erect fruit with five locules, green to dark green color, 7-12 cm in length, and without defects from insects or diseases are in demand. In 2018, Thailand exported 2,829 t of fresh or chilled fruit and 1,835 t of frozen fruit earning 10 and 4 million USD, respectively (Office of Agricultural Economics, 2020). At present, okra production is not sufficient to meet the needs of the export market, partly due to the yellow vein mosaic disease, which was first recorded on okra in Thailand in 1995 and continues to the present day.

Yellow vein mosaic disease is known as okra vein yellowing disease in Thailand. It is caused by the *Okra yellow vein virus* (OYVV), which is classified in the *Geminivirus* group, and belongs to the genus *Begomovirus*. It is transmitted by tobacco whitefly (*Bemisia tabaci* Genn.) but is not capable of contact or seed transmission. In seriously infected areas, susceptible cultivars show disease symptoms about 18 days after sowing. The vein chlorosis becomes visible on young leaves and turns into an interwoven network of yellow veins. Moreover, curling of the leaf and top, yellow shoots, and yellow fruit are normally found. Seedlings and young plants express severe symptoms, abnormal, stunted growth, and reductions in yield

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(Anju Handa et al., 1993; Mazumder et al., 1996; Kittipakorn et al., 2000; Adthalungrong et al., 2011).

The YVMD is capable of infecting okra throughout the growing period. Yields of okra were decreased by 93.8, 83.6 and 49.3% when infection occurred at 35, 50, and 65 days after germination, respectively (Sastry and Singh, 1975). The critical period is between the seedling and young plant stages. Furthermore, the quality of fruit does not meet export criteria. An outbreak of YVMD is associated with the tobacco whitefly population, particularly during warm weather conditions (Mukhopadhyay, 2011).

In order to solve the problem of YVMD infection, a varietal improvement program was carried out. It succeeded with the release of a certified selection, named 'Phichit 1' (Adthalungrong et al., 2002). However, the OYVV in different planting areas has different virulence and the virus can often mutate, resulting in the loss of resistance in the improved selections. It is necessary, therefore, to consistently select, test, and develop resistant cultivars.

MATERIALS AND METHODS

Okra cultivars used in this study, for comparison and create the selective plots, consisted of three groups including: 1) 13 YVMD resistant lines derived from Indian cultivars namely: 'L09', 'L10', 'L11', 'M13', 'M14', 'M15', 'M16', 'M17', 'N18', 'N19', 'N20', 'N21', 'O22'; 2) seven selections with desired fruit quality characteristics that were derived from Japanese cultivars, namely 'K01', 'K02', 'K03', 'K04', 'K05', 'K06', 'K07'; and 3) a susceptible line that typically has severe YDMV damage, 'PC03'.

Crossing and selection procedures

Population creation was conducted by cross-breeding Indian cultivars with Japanese cultivars in 2015, creating 50 hybrids.

Selection of YVMD resistant hybrids/lines was on the basis that they complied with fruit export quality characteristics: viz., erect fruit with five locules, green to dark green color, 7-12 cm in length and without defects from insects or diseases. Pedigree selection started from the F_1 and continued to the F_6 generation by preparing plots of 13.5 m^2, each divided into three rows of 0.75×0.50 m spacing. The susceptible cultivar was planted in the middle row and the selection lines in the remaining two rows. Cultural practices were maintained under the Good Agriculture Practices guidelines for okra (Department of Agriculture, 2002). The selection plants were self-pollinated by covering the flowers with a paper bag one day before anthesis. The procedure was repeated until the lines were consistently resistant to YVMD and had the desired fruit quality.

RESULTS AND DISCUSSION

Selection of resistant cultivars with export quality fruit

The program of selection over the F_1-F_6 generations is shown in Table 1. The F_1 generation had a total of 50 hybrids, which included 1,228 plants, which were combined with the susceptible 'PC03'. Not all hybrids had an outbreak of YVMD, while 20.4% of the 'PC03' plants developed disease symptoms. The selection, therefore, focused primarily on good quality fruit. Two to eight plants per hybrid were selected from all hybrids, selfed, and bulked the seeds for F_2 selection.

In the F_2 population, it was found that the progeny were less resistant to YVMD – 22 lines showed resistance at more than 50% while nine were resistant at more than 80%. While 'PC03' suffered 100% infection by YVMD. Twenty-one lines with 2-5 plants each, for a total of 62 plants, were selected. Among these, first flowering occurred between 33 and 43 days after sowing (DAS) and 50% were between 37 and 47 DAS. The selected plants were selfed and seeds were collected for F_3 selection (Table 2).

In F_3 generation, with a total of 62 lines (1,428 plants), it was found that all lines were resistant to YVMD at more than 80% while 'PC03' was 100% infected. Eighteen lines with good quality fruit, having first flowering between 40 and 49 DAS with 50% flowering between 42 and 55 DAS were selected (Table 2). A total of 60 lines, 2-4 plants each were selected, selfed,

and seeds collected.

Table 1. Selection diagram for yellow vein mosaic disease resistant okra.

Selection period	Generation	Number of planting lines		Number of selected	
				Plants	Lines
Nov. 2015-Mar. 2016	F_1	50	Bulk	50	50
Jun.-Sep. 2016	F_2	50	⊗[a]	62	21
Oct. 2016-Feb. 2017	F_3	62	⊗	60	18
Apr.-Jun. 2017	F_4	60	⊗	60	18
Dec. 2017-Mar. 2018	F_5	60	⊗	45	15
May-Aug. 2018	F_6	45			7
Year 2019-2020					Farm trial

[a] ⊗ = self.

Table 2. Selection of okra lines F_2-F_6 for yellow vein mosaic disease resistance with export fruit quality.

YVMD resistance (%)	F_2		F_3		F_4		F_5		F_6	
	Progeny lines	Selected lines	Progeny lines	Selected lines	Progeny lines	Selected lines	Progeny lines	Selected lines	Progeny lines	Selected lines
0-50	28	2	0	0	6	0	0	0	5	0
51-60	3	1	0	0	1	0	0	0	1	0
61-70	4	3	0	0	1	0	0	0	3	0
71-80	6	6	0	0	7	1	0	0	3	0
81-90	5	5	6	0	7	1	0	0	0	0
91-100	4	4	56	18	38	16	60	15	33	7
Total	**50**	**21**	**62**	**18**	**60**	**18**	**60**	**15**	**45**	**7**

Selection in the F_4 (2,597 plants) and F_5 (2,636 plants) generations occurred with all of the 'PC03' plants being infected with YVMD. A total of 38 lines of the F_4 were resistant to YVMD at more than 90%. Only 16 lines, 2-4 plants each with the desired fruit characteristic for a total of 60 plants were selected. The selfed seeds were carried to F_5. Those that showed resistance to YVMD at more than 90% (Table 2), first flowering between 41 and 48 DAS, with 50% flowering between 45 and 50 DAS were selected. Self-pollination was carried out for the 45 selected plants and seeds were collected for the F_6 generation.

Most of the F_6 lines were resistant to YVMD at greater than the 90% level (Table 2). The other lines had slight symptoms of YVMD at the end of the growth period. In contrast, the susceptible 'PC03' first showed disease symptoms at 20 DAS and had completely succumbed at 44 DAS. Seven promising lines, which were completely resistant to YVMD and which had export fruit quality were selected. They were: KC5902-1-1-4-3-1, KC5915-2-18-15-20-10, KC5929-3-30-24-32-27, KC5930-2-31-28-38-31, KC5932-2-38-35-42-37, KC5944-2-54-44-46-38 and KC5950-1-60-55-52-40 (Figure 1). They also had good agriculture characteristics, plant height at 100-150 cm, 2-3 branches plant^{-1} which allow easy fruit harvest and were tolerant to lodging. First and 50% flowering were 33-42 and 35-43 DAS, respectively, except for KC5902-1-1-4-3-1 and KC5915-2-18-15-20-10 that had later flowering (Table 3).

Table 3. Characteristics of seven promising lines of selected F_6 okra, planted and evaluated during May-August, 2018 at Kanchanaburi Research and Development Center.

Code	Day to flowering (DAS[a])		R[b] (%)	Number of branches	Plant height	Fruit characteristics		
	Early	50%				Color	Pubescence	Pod
KC5902-1-1-4-3-1	71	78	100	2	Medium	Green	Downy	Wide
KC5915-2-18-15-20-10	53	56	100	3	Medium	Green	Downy	Long
KC5929-3-30-24-32-27	39	39	100	2	Medium	Green	Downy	Long
KC5930-2-31-28-38-31	33	35	100	2	Medium	Green	Downy	Long
KC5932-2-38-35-42-37	42	43	100	2	Medium	Green	Downy	Long
KC5944-2-54-44-46-38	42	42	100	2	Medium	Green	Downy	Long
KC5950-1-60-55-52-40	37	39	100	2	Medium	Green	Downy	Long
PC03	41	44	0	0	Short	Yellow	Downy	Long

[a]DAS: Day after sowing.
[b]R (%): Percentage of YVMD resistance.

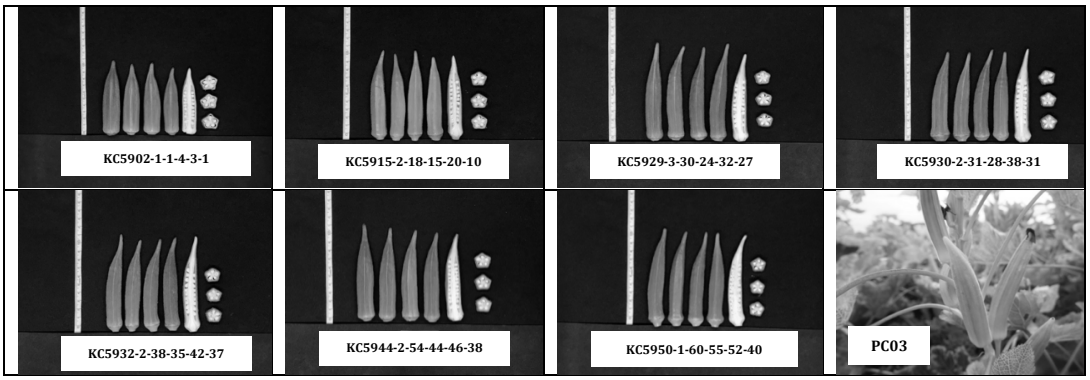

Figure 1. Photograph of seven selected F_6 okra lines resistant to YVMD and the susceptible 'PC03'.

In the early generations, fruit quality of the hybrids mostly resembled the Indian cultivars with a long slender shape. The YVMD resistant plants with desired fruit quality were selected despite the inclusion of some lines that had poor resistance. This was because fruit quality consisted of many characteristics, which were difficult to select so as to include all of the desired traits. The desired fruit characteristics were obtained in F_5-F_6 after intensive selection. Young erect fruit with five locules, green color, 7-12 cm length, along with a downy pubescence, and a wide pod, which resembled the Japanese cultivar, were observed in KC5902-1-1-4-3-1. A form with medium plant height, and 2-3 instead of one branch, was selected for ease of harvesting and for promising high yield.

It was easy to select for YVMD resistance because the trait was governed by one dominant gene (Jambhale and Nerkar, 1981) or two dominant genes (Pullaiah et al., 1998). The severity of YVMD infection during selection depended on many factors, including disease host, the presence of tobacco whitefly, environment, and the OYVV mutation. Thus in F_4-F_6, some lines showed a decline in resistance or developed the disease symptoms at the end of the growing period. Tobacco whitefly infestation was found similarly in all of the selection lines, as well as in the susceptible 'PC03'.

KC5902-1-1-4-3-1 and KC5915-2-18-15-20-10 had late flowering during F_6 selection. Okra is known as a short-day plant, that needs a day period of fewer than 12.30 h to induce the flower bud. Therefore, these two lines may be described as being short-day plant, while the others are not. In Thailand, a period of day length longer than 12.30 h occurs in late June. Therefore, these short day okra cultivars should not be planted during this period. Nevertheless, okra production for export to the Japanese market is normally from October to May, as production during this time is not possible in Japan. Yield trials are currently being

conducted in commercial fields at various locations in Thailand.

CONCLUSIONS

After six generations of selection, seven promising lines namely KC5902-1-1-4-3-1, KC5915-2-18-15-20-10, KC5929-3-30-24-32-27, KC5930-2-31-28-38-31, KC5932- 2-38-35-42-37, KC5944-2-54-44-46-38 and KC5950-1-60-55-52-40 were found to be completely resistant to YVMD infection in the field where there was disease pressure from the virus. The fruit quality of these selections is suitable for the export market, with fruit having five locules, green color, 7-12 cm length, and a downy pubescence.

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New hybrids of torch ginger as a cut flower in Thailand

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Abstract

Five torch ginger hybrids (*Etlingera elatior* × *Etlingera fulgens*) were planted at three locations (the Chiang Rai, Loei and Chanthaburi Horticultural Research Centers) during 2016-2019 to evaluate yield and flower quality in comparison with a commercial cultivar 'Dang Dok'. The four hybrids had export-suitable cup-shaped flowers, with dark red, red or red-pink bracts. The flowers were approximately half the size of 'Dang Dok', but with comparable peduncle length. Yields of these hybrid clones ranged from 129 to 404 flowers clump^{-1} year^{-1}, but varied by location depending on soil pH and the amount of exchangeable potassium. Clone #8 performed very well in all testing locations, yielding 244-361 flowers clump^{-1} year^{-1}, and was named 'Yala 1'. Vase life of the four hybrids, harvested at 50% flower opening stage, were 8.0-9.9 days at ambient conditions, similar to that of 'Dang Dok'.

Keywords: *Etlingera elatior*, *Etlingera fulgens*, cross pollination, clone, cup shaped inflorescence

INTRODUCTION

Torch ginger (*Etlingera elatior* (Jack) R.M. Smith) is a tropical plant in the *Zingiberaceae* family that is distributed throughout Southeast Asia. Different cultivars of torch ginger are widely grown as a landscape ornamental plant. The inner parts of the leafy shoot are used as a spice or vegetable in Southeast Asia and its natural fibers are used for textile production (Daochunad et al., 2015). Currently, torch ginger is utilized as a cut flower as well as an ornamental plant in Thailand. Planted areas for commercial cut flower production are located in Nonthaburi, Samut Sakhon, Kanchanaburi, Rayong, Chanthaburi and Krabi (Wannakrairoj, 2016). Export market includes the Middle East, Angola, Singapore, the USA, Japan, Spain and South Africa. A major problem with the export of cut torch ginger flowers is in regard to the provision of suitable packaging, because the flower is heavy and has an expanded head form (Jaranil, 1994; Wannakrairoj, 2016).

There are 15 closely related species founded in Thailand. Five species were used for a breeding program at the Yala Horticulture Research Center, in southern Thailand, including *Etlingera elatior* (Jack) RM Sm., *Etlingera fulgens* (Ridl) CK Lim., *Etlingera corneri* J. Mood et H.lbrahim, *Etlingera venusta* (Ridl) RM Sm. and *Etlingera maingayi* RM Sm (Sachati and Chansaeng, 2017). The program aims to create interspecific hybrids of torch ginger with small and light flower in a shape of a cup, suitable for export. Each clump should produce more than 100 flowers a year. Cross-pollination between lilly torch ginger (*Etlingera elatior*), and black tulip torch ginger (*Etlingera fulgens*) and the selection of hybrids was conducted between 2006 and 2014. *E. elatior* has head shaped flower, pink to fuchsia color, 9-15 cm in diameter, 72-115 cm in stem length, year-round flowering, and 50-80 flowers clump^{-1} year^{-1}. *E. fulgens* has cup shaped flower, bright red color, 4-5 cm in diameter, 27-35 cm in stem length, once-a-year flowering, and 50-120 flowers clump^{-1} year^{-1}. Five potential hybrids were obtained from the program. In this research, the five clones were tested at three locations with different climates in the upper part of Thailand, in order to find suitable clones that could be recommended to farmers.

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MATERIALS AND METHODS

The five torch ginger hybrids between *E. elatior* and *E. fulgens* with commercial potential were #2 ('Banyen' × 'Daengpa'), #3 ('Banyen' × 'Daengpa'), #7 ('Buachompu' × 'Dalhadam'), #8 ('Banyen' × 'Daengpa') and #9 ('Buachompu' × 'Dalhadam'). The rhizomes from the five hybrids, together with 'Dang Dok', a current commercial cultivar of *E. elatior*, were propagated by tissue culture. Young plantlets were transferred into the experimental plots when they were six-months-old and had six unfolded leaves.

All hybrids and 'Dang Dok' were planted in August 2016 using 2.5×3 m spacing at three locations (the Chiang Rai Horticultural Research Center (HRC), Loei HRC and Chanthaburi HRC). Soil properties and average temperatures of the three HRCs are shown in Table 1.

Table 1. Soil properties (0-30 cm depth) and average temperatures of Chiang Rai HRC, Loei HRC and Chanthaburi HRC in 2016.

	Chiang Rai HRC	Loei HRC	Chanthaburi HRC
pH (1:1 H_2O)	7.6	5.10	4.77
OM (%)	3.74	4.57	5.99
Avail. P (mg kg^{-1})	149	17	103.12
Exch. K (mg kg^{-1})	525	225	25.12
Soil texture	Sandy loam	Clay loam	Clay loam
Avg. min-max temperature (°C)	19.9-30.3	21.2-32.4	23.7-32.3

A randomized complete block design with four replications (24 plants each) was used. Each plant was grown in a 50×50×50 cm pit filled with 10 kg of compost. A fertilizer with a 16-16-16 formula was applied at rates of 30, 50, 80 and 120 g $clump^{-1}$ at 3, 6, 9 and 12 months after planting, respectively. Flower shape, color, diameter, weight, peduncle length and yield were recorded year-round from February 2018 to February 2019.

For assessment of postharvest performance, flowers were harvested at the 50 and 80% flower opening stages. Using a randomized complete block design, 12 flowers, 3 from each of 4 clumps at both development stages, were harvested. The flowers were kept at room temperature in glass vases filled with tap water. The vase life was determined when the bracts turned black.

RESULTS AND DISCUSSION

Shape and color

All of the torch ginger hybrids had a cup shaped flower that was similar to the female parent (*E. fulgens*). Bracts were aligned neatly while the commercial cultivar 'Dang Dok' (*E. elatior*) had a head shape and the bracts were spread like fingers. The advantage of a cup shaped flower is that it is easy to pack for transportation. The color of the bracts was red-pink, red or dark red. Hybrid #7 had vivid red bracts without a colored edge. The edge color of hybrid # 9 was green which was different from the other hybrids that had either a white or no edge (Table 2; Figure 1).

Table 2. Flower shape and bract color of torch ginger hybrids and 'Dang Dok'.

Hybrid no., name and parents	Flower shape	Bract color	
		Bract	Edge
2 - Yala 3 (Banyen × Daengpa)	Cup	Red (R45A)[a]	White
3 - Yala 4 (Banyen × Daengpa)	Cup	Red-pink (RP73B)	White
7 - (Banyen × Daengpa)	Cup	Vivid red (R47B)	None
8 - Yala 1 (Buachompu × Dalhadam)	Cup	Dark red (R53A)	White
9 - Yala 2 (Buachompu × Dalhadam)	Cup	Dark red (R53C)	Green
Dang Dok	Head	Red (R46B)	White

[a]Color code of the Royal Horticulture Society color chart.

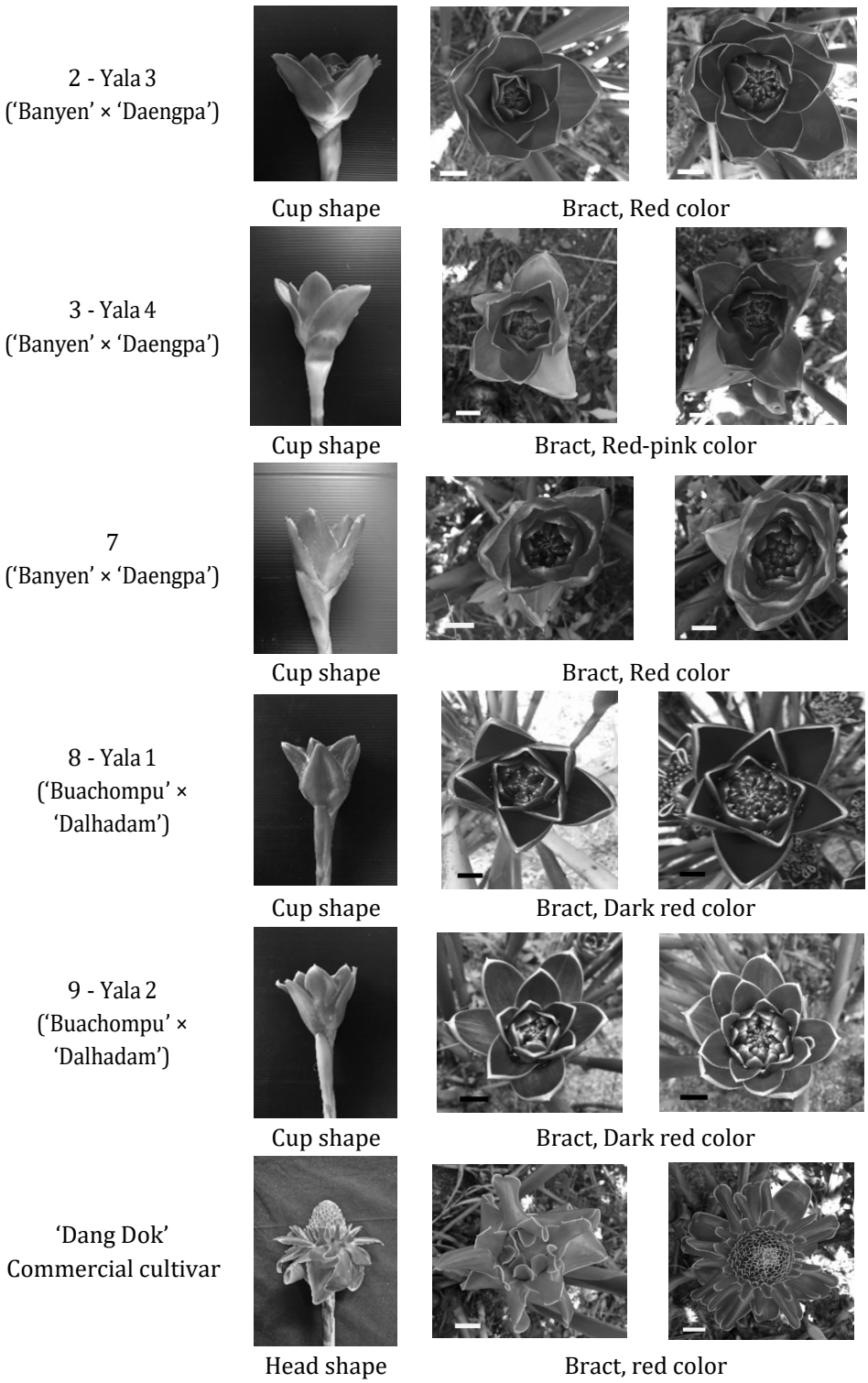

Figure 1. Flower shape side view (left), and top view at 50% (center) and 80% (right) opening stage of the torch ginger hybrids and of 'Dang Dok'.

Flower size and peduncle length

At all of the three growing locations, the flowers of each hybrid were comparable in

diameter, but were about half that of 'Dang Dok'. The peduncle lengths of most of the hybrids was similar to 'Dang Dok' in Chiang Rai, but were 20-55% shorter in the other two locations (Table 3).

Table 3. Flower size and inflorescence weight of torch ginger hybrids and 'Dang Dok' at the three research centers.

Hybrid no. and name	Chiang Rai HRC			Loei HRC			Chanthaburi HRC		
	Diameter (cm)	Weight (g)	Peduncle length (cm)	Diameter (cm)	Weight (g)	Peduncle length (cm)	Diameter (cm)	Weight (g)	Peduncle length (cm)
2 - Yala 3	8.9b	95.06e	86.00a	8.2b	97.57d	62.06b	7.8b	101.29c	49.30b
3 - Yala 4	8.7bc	132.33d	86.25a	7.9b	133.09bc	58.85b	7.6b	140.76b	49.0 b
7	7.3e	109.88e	74.25abc	nd	nd	nd	6.7bc	nd	50.06b
8 - Yala 1	8.1cd	177.86b	67.00c	7.2c	152.10ab	48.11b	7.4b	143.79b	43.85b
9 - Yala 2	7.9d	157.30c	70.75ab	7.2c	122.94 c	52.51b	5.9c	119.67bc	38.93b
Dang Dok	17.6a	224.55a	82.25ab	15.8a	166.25a	80.49a	17.7a	227.95a	69.44a
CV (%)	4.0	8.2	9.7	4.6	10.3	18.7	10.3	12.1	23.1

Averages in the same column followed by the same letter are not statistically different at 95% confidence level by DMRT.
nd = no data.

All of the torch ginger hybrids produced flowers year-round liked their female parent (*E. elatior*). Yield of each, reported as flowers per clump per year, was different at the three planting locations (Table 4). Clone #8 had the highest yields in most locations, indicating that it was adaptable to all types of weather and soil conditions. Hence it was named 'Yala 1'. All clones, except #7, had higher yields than the commercial 'Dang Dok' with more than 100 flowers clump^{-1} year^{-1}. Hence, they could be recommended to farmers for production in suitable growing areas all over Thailand. Clone #9 or 'Yala 2' could be recommended specifically for Chiang Rai, where the weather is cooler than at the other locations. Yield could be improved for 'Yala 2' in both Loei and Chanthaburi if the soil pH and potassium were raised to be less acidic. Since it is known that plants in the ginger family require high amounts of potassium for their growth (Nagarajan and Pillai, 1979; Rethinam et al., 1994), yield of all of the hybrids in Chanthaburi could be much improved if potassium fertilizer was added. Clone #3 produced quite well in Loei and should be tested further. Clone #7 had an attractive vivid red bract without edge color but yielded poorly in all three locations. Hence it could not be recommended for commercial production.

Table 4. Yield of torch ginger hybrid clones and 'Dang Dok' planted at the three Research Centers in 2018/19 season.

Clone no. name	Number of flowers per clump		
	Chiang Rai HRC	Loei HRC	Chanthaburi HRC
2 - Yala 3 (Banyen × Daengpa)	129.1bc	179.2bc	168.6ab
3 - Yala 4 (Banyen × Daengpa)	183.8b	243.4b	163.3ab
7 - (Banyen × Daengpa)	34.2d	6.0d	29.2c
8 - Yala 1 (Buachompu × 'Dalhadam)	347.8a	361.23a	244.3a
9 - Yala 2 (Buachompu × Dalhadam)	404.4a	129.5bc	212.0ab
Dang Dok	86.4cd	99.3c	138.5b
CV (%)	34.6	35.2	37.1

Averages in the same column followed by the same letter were not statistically different at 95% confidence level by DMRT.

Vase life was, in general, not different among the hybrids or between the hybrids and 'Dang Dok'. For those flowers harvested at 50% opening from Loei, 'Dang Dok' had a slightly longer vase life. The vase life at Chanthaburi HRC was shorter than that in either Chiang Rai or Loei HRC, due to the warmer weather during the evaluation period.

Harvesting torch ginger at the two different developmental stages directly affected vase life (Table 5). The vase life was 8-10 days at the 50% opening stage, when the true flower was

still hidden, as compared to only 3-6 days at the 80% opening stage, when the true flower had appeared. The diameter of the flowers at both developmental stages was not different. After harvest, the bracts of all hybrids did not open further, but turned brown and black at the end of the vase life period. Hence it is recommended that torch ginger hybrids are harvested at the 50% opening stage.

Table 5. The vase life of ginger hybrid clones and 'Dang Dok' grown at the three locations.

Clone number or name	Vase life (days)					
	Chiang Rai HRC		Loei HRC		Chanthaburi HRC	
	50%	80%	50%	80%	50%	80%
2 - Yala 3 (Banyen × Daengpa)	9.7a	6.1ab	8.6c	5.5a	8.0a	3.6a
3 - Yala 4 (Banyen × Daengpa)	9.4a	6.7a	8.7c	5.5a	8.4a	5.0a
7 - (Banyen × Daengpa)	6.8b	4.6c	nd	nd	7.7a	3.7a
8 - Yala 1 (Buachompu × Dalhadam)	9.5a	6.1ab	9.8b	6.0a	7.9a	3.3a
9 - Yala 2 (Buachomp × Dalhadam)	9.6a	6.4ab	9.9ab	5.7a	8.6a	4.7a
Dang Dok	10.2a	5.8b	10.6a	6.3a	8.5a	3.6a
CV (%)	5.8	8.0	5	10.4	8	48.6

Averages in the same column followed by the same letter are not statistically different at 95% confidence level by DMRT.
nd = no data.

CONCLUSIONS

Among the five hybrids derived from crosses between *Etlingera elatior* × *Etlingera fulgens*, and tested at three Horticultural Research Centers in upper Thailand, four could be recommended for commercial production throughout the country. They were more suitable for export than the current 'Dang Dok' cultivar, having cup instead of head shaped flowers. Yields were more than 100 flowers clump^{-1} year^{-1}. Flowers were approximately half the size of 'Dang Dok', but comparable in peduncle length and vase life. Hybrid #8 produced well in all growing locations, and was named 'Yala 1'. Other suitable hybrids were named 'Yala 2' to 'Yala 4'.

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DNA barcode for rambutan diversity in Thailand using chloroplast genome regions

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Abstract

Rambutan is one of the best-known fruits in Asia. Thailand is one of the largest producers of rambutan apart of Indonesia and Malaysia. The commercial rambutan, *Nephelium lappaceum* L., is considered closer to other Nepheliums, which are very difficult to distinguish from each other. Therefore, this study is to clarify the DNA barcode for diversity of rambutan using chloroplast genome regions (*psbA*, *trnL* and *rpoC*). Seventeen samples of rambutan were collected from the field at Chanthaburi Horticultural Research Center for analysis. Among these, 14 samples are classified as *N. lappaceum*. Six of the commercial cultivars and eight hybrid cultivars including 'Rongrien', 'Seechompoo', 'Seethong', 'Bangyeekhan', 'Namtankraud', 'Jaemong' and 'Pliew 1-8' were analyzed. The other three samples were Pulasan (*N. ramboutan-ake* (Labill.) Leenh.), *Nephelium* sp. No. 1 and *Nephelium* sp. No. 2. Extracted DNA samples were evaluated with 3 universal primers of *psbA*, *trnL* and *rpoC*. The result showed that using *psbA* primer separated the group of 'Seethong' and 'Pliew 2' ('Seethong' × 'Jaemong') and group of *Nephelium* sp. No. 2 from the others. Whereas *trnL*, *rpoC* and combination of three primers could not explicitly explain the diversity within rambutan. Our study will further examine more specific primers to confirm the relationships of the Thai rambutans. The genetic diversity of cultivated rambutan from this study will be used as a genetic database for development of a future breeding program.

Keywords: rambutan genetics, phylogenetic relationships, barcode sequencing

INTRODUCTION

The genus *Nephelium* is distributed throughout South-East Asia. *N. lappaceum* is known as rambutan. Rambutan is commonly cultivated in orchards and home gardens in Thailand, Malaysia, and Indonesia. Another species such as pulasan (*N. ramboutan-ake*) is a close relative and is rarely cultivated. However, fruits are sold in the local markets in Malaysia and Indonesia (Salma et al., 2015). *N. lappaceum* reveals a wide range of genetic variation within the species. The seedling progeny of *N. lappaceum* is heterogenous and shows a great variability in fruit characters. For instance, characteristics such as fruit size, shape, color, taste, thickness, juiciness, flavor and flesh adherence to the seed (Salma, 1986). Thailand has a high diversity of rambutan clones, including 'Rongrian', 'Seechompoo', 'Seethong', and 'Bangyeekhan'. However, the diversity of the *Nephelium* species has been not fully exploited. They are rapidly declining and in danger of being lost forever. Even though, Chanthaburi Horticultural Research Center, Department of Agriculture, has bred a new hybrid, 'Pliew 1'-'Pliew 8', the identification for each cultivar, especially from seedlings, is very difficult. Therefore, a study will be undertaken using a molecular technique, short DNA sequence to identify phylogenetic relationships.

DNA barcode is a technique used for identifying species using short orthologous DNA sequences. It is gradually being tested in many areas as a cost-effective tool for identifying and regulating pests, invasive and disease-carrying species, trade and sale of endangered species, and for many other species (Onuminya and Ogundipe, 2016). The goal of barcoding is that

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anyone, anywhere, anytime will be able to identify quickly and accurately any species whatever its condition (Stoeckle et al., 2005). This technology is being promoted by the International Consortium for Barcoding of Life (CBOL) to enable the rapid and inexpensive identification of the estimated 10 million species of organisms on earth. It has enormous benefits and brings huge gain to countries rich in biodiversity including: rapid species identification at any life stage or fragment, providing an insight into the diversity of life, quick and cheap identification of specimens as well as ability to control the movement of species across national borders (Stoeckle et al., 2005). Hence, the aim of this research is to explore the diversity of rambutan in Thailand with particular emphasis on identification of the plant samples using DNA barcode sequences which can be shared publicly.

MATERIALS AND METHODS

Plant materials

Rambutan (*N. lappaceum*) leaves were collected from Chanthaburi Horticultural Research Center, Tapon sub-district, Khlung District, Chanthaburi Province in Thailand longitude 12.5109°N; latitude 102.1695°E, at 44 m a.s.l. Leaf samples were collected from 17 species including 'Rongrien', 'Seechompoo', 'Seethong', 'Namtankraud', 'Bangyeekhan', 'Jaemong', 'Ngorkonson', 'Pliew 1' ('Seechompoo' × 'Rongrien'), Pliew 2 ('Seethong' × 'Jaemong'), 'Pliew 3' ('Seechompoo' × 'Seethong'), 'Pliew 4' ('Seechompoo' × 'Rongrien'), 'Pliew 5' ('Seechompoo' × 'Rongrien'), 'Pliew 6' ('Namtankraud' × 'Rongrien'), 'Pliew 7' ('Seechompoo' × 'Seethong'), 'Pliew 8' ('Seechompoo' × 'Seethong'), 'Pulasan' and unknown (*Nephelium* sp. No.1 and No. 2) for this study.

Taxon recording

Data were collected on leaf description and fruit taxonomy following International Plant Genetic Resources Institute (IPGRI). Details were recorded on shape and color of leaflet, and fruit.

DNA extraction and quantification

About 5 g of rambutan leaf was grounded into a fine powder using liquid N_2 with a mortar and pestle or with a grinding mill. The power was placed into a test tube containing an extraction buffer (1 mL CTAB: 2 µL β-mercaptoethanol) 7 mL, then incubated at 60°C for 30 min, gently mixing every by inversion every 10 min. After incubation, the sample was centrifuge at 4°C for 10 min at 8,000 rpm (Doyle and Doyle, 1987).

The 5,000 µL of supernatant was transferred to test tube with 5 mL of chloroform, Iso-Amylalcohol (24:1) and gently mixed for 10 min. Samples were centrifuged at 4°C for 10 min at 8,000 rpm. The 3,000 µL of supernatant was add to 1,800 µL of Isopropanol and 300 µL of 3 M NaOAC mix by inversion until DNA precipitates. The test tubes were placed in an ice box for 30 min. Samples centrifuged at 4°C at 8,000 rpm for 15 min. To the pellet, 500 µL 70% ethanol was added for washing and this step was repeated twice. Samples were then centrifuged at 4°C at 8,000 rpm for 5 min. The pellet and test tube dried at about 30-40°C for 2 h to ensure complete dryness. Then TE Buffer, 100 µL was added to the pellet and stored at -20°C.

PCR amplification and sequencing

For the polymerase chain reaction (PCR) technique, 100 µL Master mix solution with Go Tag Green Master Mix (Promega, US) reaction mixture was prepared. Each reaction contained 20 µL 5× buffer, 20 µL PCR buffer containing $MgCl_2$, 10 µL dNTP, 4 µL forward primer, 4 µL reverse primer, 0.6 µL GoTag, 47.4 µL distilled water and 4 µL DNA extraction in the Master Mix solution.

For DNA amplification, the amplification on the following regions: *RpoC, trnL, psbA* was done using primer Table 1. The reaction was programmed at initial temperature of 94°C for a duration of 5 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and 72°C for 5 min for the final extension.

Table 1. Primer used for each locus.

Locus	Primers	Direction	Sequences	Expected size (bp)
RpoC	rpoC_F	Forward	5' GGCAAAGAAGGAAGATTTCG 3'	485
	rpoC_R	Reverse	5' TGAGAAAACATAAGTAAACGA 3'	
psbA	psbA-trnH1	Forward	5' GTTATGCATGAACGTAATGCTC 3'	520
	psbA-trnH2	Reverse	5' CGCGCATGGTGGATTCACAATCC 3'	
trnL	trnL-trnF1	Forward	5' GGTTCAAGTCCCTCTATCCC 3'	465
	trnL-trnF2	Reverse	5' ATTTGAACTGGTGACACGAG 3'	

Analyzing the DNA

The extracted DNA was analyzed using 1.5% agarose gel and using Tris-boric acid/EDTA buffer (1X TBE). The electrophoresis was carried out with a constant voltage of 200 V for 30 min and stained with ethidium bromide and photographed with the gel documentation system visualized under UV.

Phylogenetic analysis

The PCR products were sent to the First BASE Laboratories (Apical Scientific Sdn Bhd, Malaysia) for sequencing after simple purified using a TIANquick Midi Purification Kit. For each marker, sequences were aligned with MUSCLE algorithm using MEGA7 program (Kumar et al., 2016). A reconstructed phylogeny for each marker determined using the maximum likelihood (ML) method. The phylogenetic trees were generated with 1,000 bootstrap replicates using the MEGA7 program.

RESULTS AND DISCUSSION

From the samples of rambutan collected from the field at Chanthaburi Horticultaral Research Center, 14 samples were identified as *N. lappaceum* L. by their botanical characteristics. This included six commercial and eight hybrid cultivars including: 'Rongrien', 'Seechompoo', 'Seethong', 'Bangyeekhan', 'Namtankraud', 'Jaemong' and 'Pliew 1-8'. The leaf and fruit characteristics of the rambutan samples are shown in Tables 2 and 3.

Table 2. The leaf characteristics of rambutan.

Samples	Leaf shape	Leaf apex	Leaf base	Color of mature leaf	Leaf phyllotaxy
Pliew 1	Elliptic	Acuminate	Cuneate	G137A	Odd pinnate
Pliew 2	Elliptic	Acute	Cuneate	G139A	Alternate
Pliew 3	Elliptic	Acute	Acute	G137A	Odd pinnate
Pliew 4	Elliptic	Acuminate	Cuneate	G137A	Odd pinnate
Pliew 5	Elliptic	Acute	Acute	G137A	Odd pinnate
Pliew 6	Elliptic	Acuminate	Cuneate	G137A	Alternate
Pliew 7	Elliptic	Acuminate	Cuneate	G137A	Odd pinnate
Pliew 8	Elliptic	Acute	Acute	G137A	Odd pinnate
Rongrien	Elliptic	Acuminate	Cuneate	G137A	Alternate
Seechompoo	Elliptic	Acuminate	Cuneate	G137A	Alternate
Seethong	Elliptic	Acute	Cuneate	G137A	Odd pinnate
Numtankraud	Elliptic	Acuminate	Cuneate	G137A	Alternate
Jaemong	Elliptic	Acuminate	Cuneate	G137A	Alternate
Bangyeekhan	Elliptic	Acuminate	Acute	G137A	Alternate

Descriptors for rambutan of International Plant Genetic Resources Institute (IPGRI, 2003)

The other three samples were comprised of Pulasan (*N. ramboutan-ake* (Labill.) Leenh.), *Nephelium* sp. No. 1 and No. 2. From the sequences generated the phylogenetic relationship of all 17 samples are shown in Figures 1-4. DNA barcodes for *rbcL*, *matK*, ITS, *trnH-psbA*, and *rpoC* have been used in several studies. In this study we amplified the DNA with those six

universal primers. However, this study found not noticeably results on *rbcL*, *matK*, and ITS. Therefore, the extracted DNA samples were evaluated on three primers, *psbA*, *trnL* and *rpoC*. Using the *psbA* primer, the phylogenetic analyses revealed the *Nephelium* sp. No. 2 was shown to be in the outer group. However, 'Seethong' and 'Pliew 2' were separated within the same group. Accordingly, 'Pliew 2' is designated as a hybrid of 'Seethong' × 'Jaemong'. Furthermore, the distribution of the other groups did not show any relationships. Additionally, the primers of *trnL*, *rpoc* and combination of three primers, could not explicitly explain the diversity of rambutan. Additionally, using DNA barcoding in the context of quality control is both a well and poorly regulated supply system. Standardization of protocols for DNA barcoding and DNA sequence-based identification are necessary before DNA-based biological methods can be implemented as routine analytical approach. These must be approved by the competent authorities for use in regulated procedures (Raclariu et al., 2018).

Table 3. The fruit characteristics of rambutan.

Samples	Fruit characteristics				
	Fruit shape	Fruit rind color	Base hair color	Tip hair color	Inner rind color
Pliew 1	Ovoid	YO21D	R47A	YG150B	Y4D
Pliew 2	Ovoid	YO21B	R46B	YG150B	Y4D
Pliew 3	Ovoid	YO17C	R47B	YG150B	Y8D
Pliew 4	Ovoid	YO21C	R47C	YG150B	Y8D
Pliew 5	Globose	OR34C	R47A	YG150B	Y8D
Pliew 6	Ovoid	Y7B	R50D	YG154B	Y4D
Pliew 7	Globose	YO21B	R50B	YG154B	Y4D
Pliew 8	Ovoid	YO21B	R52A	YG150C	Y8D
Rongrien	Ovoid	YO21A	R53B	YG149B	Y4D
Seechompoo	Ovoid	YO21C	R50A	R51A	Y8D
Seethong	Globose	O25A	R45A	YG149B	Y8D
Numtankraud	Globose	Y7A	Y3C	Y5B	Y4D
Jaemong	Oblong	O28A	R53C	R53C	Y8D
Bangyeekhan	Oblong	YO21B	R50B	R50A	Y8D

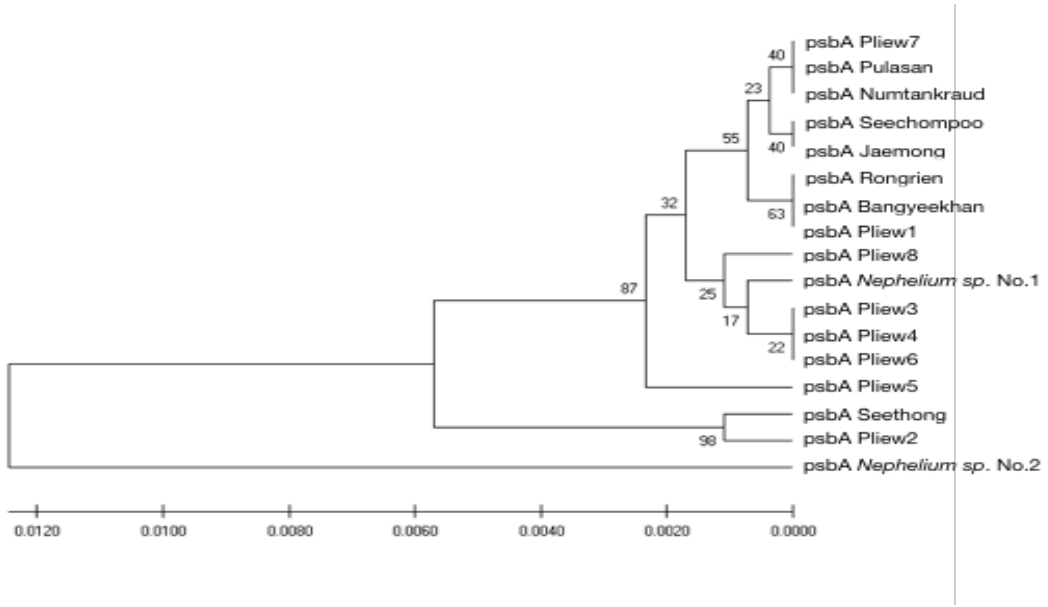

Figure 1. Phylogenetic relationships of 17 rambutans based on *psbA* barcode sequence data.

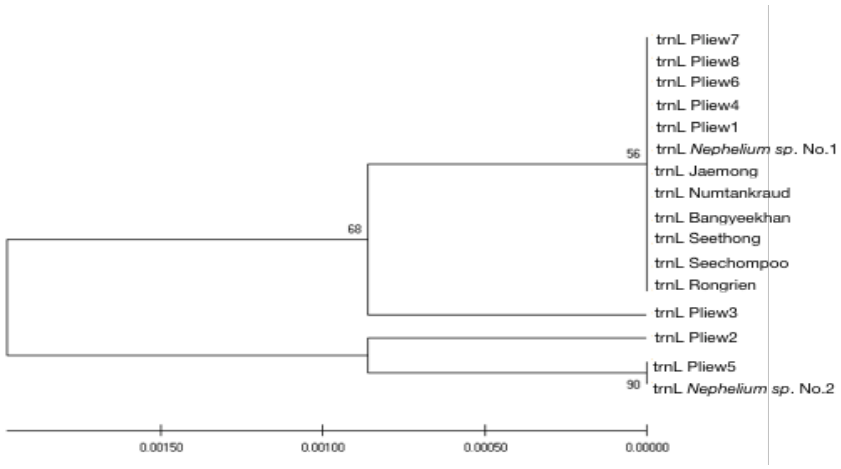

Figure 2. Phylogenetic relationships of 17 rambutans based on *trnL* barcode sequence data.

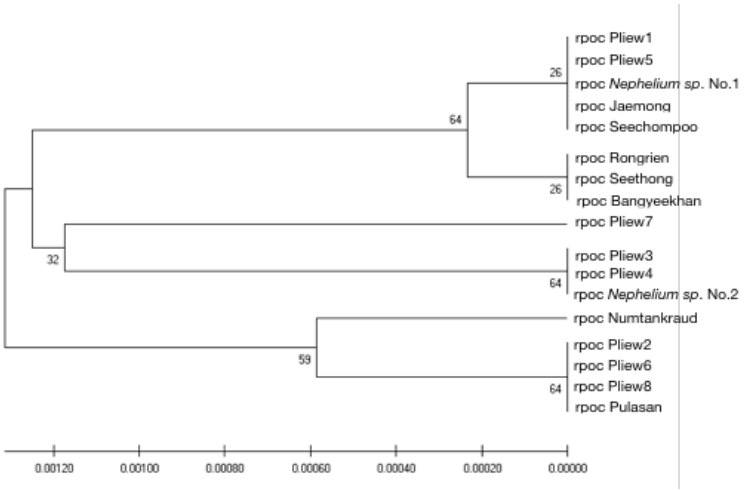

Figure 3. Phylogenetic relationships of 17 rambutans based on *rpoC* barcode sequence data.

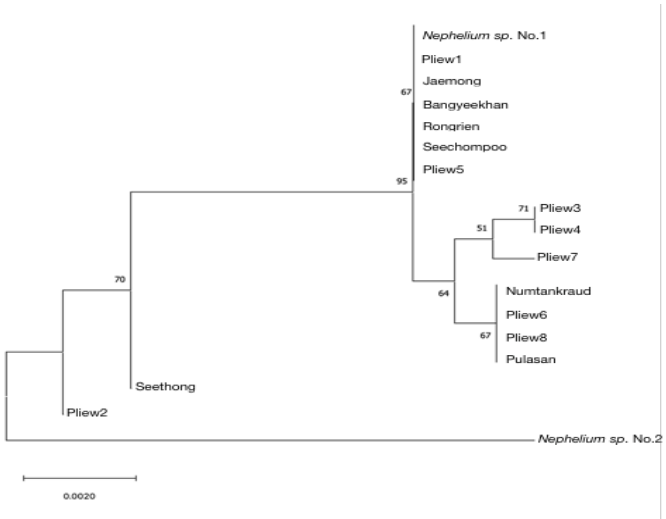

Figure 4. Phylogenetic relationships of 17 rambutans based on *psbA*, *trnL* and *rpoC* barcode sequence data.

CONCLUSIONS

The explanation of rambutan diversity using chloroplast genome regions (*psbA*, *trnL* and *rpoC*) is not clear. This study confirmed the need for further examination of different specific primers or other techniques such as GBS to confirm the relationships of Thai rambutan. The genetic diversity of cultivated rambutan shown by this study will be used as genetic database for a future breeding program.

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Identification of new durian cultivars in Si Sa Ket Province using microsatellite markers

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Abstract

Si Sa Ket Province has been recently recognized as a new durian planting area in Thailand. This study aimed to investigate genetic variation of local durian trees derived from open-pollinated seedlings. The genetic variation of 17 local durians and 12 commercial cultivars were analyzed using 26 microsatellite DNA markers that produced 151 alleles across all samples. The genetic relationship and dendrogram of these samples were analyzed by unweighted pair group method with arithmetic mean (UPGMA) using the NTSYS pc 2.1 program. The genetic compositions was analyzed using a STRUCTURE program. The results indicated durian samples had similar coefficients ranging from 0.51 to 0.82. All local durian cultivars were genetically different and different to the commercial cultivars. Together with leaf and fruit characteristic, five new cultivars were selected and registered.

Keywords: durian microsatellite primers, genetic variation, diversity, DNA analysis

INTRODUCTION

Durian (*Durio ziberthinus* Murray) is widely cultivated in countries located near the equator, including Thailand, Malaysia, Indonesia, Myanmar, the Philippines, Sri Lanka, India, Australia, and Papua New Guinea (Siew et al., 2018). Thailand has become a major durian producer. In 2019, the total production area was 116,236 ha, producing 1,017,097 t, averaging 8.7 t ha^{-1}. The major production areas are in the East and the South, covering 32 provinces. Thailand is now the largest durian exporter. In 2019, Thailand's total exports was 743,300 t, worth USD$ 1,600 million. Approximately, 25,987 t was in the form of frozen durian, valued at USD$ 167 million. In addition, 1,421 t was durian paste and dried durian, valued at USD$ 10.2 million. The main export cultivar is 'Monthong'. The main markets of fresh durian were China, Vietnam and Hong Kong. Additionally, the main markets for durian products were China, USA, Australia, Russia, Croatia and Hong Kong (Office of Agricultural Economics, 2019).

The Si Sa Ket Province, in the North-East of Thailand, has recently been recognized as a new durian planting region. The total area of 1,332 ha has been planted with a harvest area of 472 ha, producing 3,227 t of fruit (Office of Agricultural Economics, 2019). The suitable areas for growing durian are in three districts, including Kantharalak, Khun Han and Si Rattana. The soil series in this area is Packchong (Kt), rich in mineral nutrients and high organic matter (Nimkingrat et al., 2017). Therefore, durian from Si Sa Ket Province differs from those in other areas, in terms of their sweet taste, soft chewy texture and mild aroma. In 2018, Si Sa Ket Province registered a geographical indication (GI) for 'Monthong', 'Chanee' and 'Kanyao' durian, produced in the district of 'Lava Durian Si Sa Ket'. All fruit sold under this GI name, must be grown under good agricultural practice (GAP) in certified orchards. The GI grown durian also carries a QR code, to give consumers information and traceability, and is now in high demand (Nimkingrat et al., 2018).

Besides the asexually propagated 'Monthong' cultivar, other locally grown durian trees are from seeds of open-pollinated cultivars such as 'Chanee', 'Kanyao', 'Kradum' as well as 'Monthong'. Some of these trees produce good quality fruit and should be promoted. However, durian is mainly outcrossed and has a high rate of self-incompatibility (Morton, 1987). These durian trees differ in various morphological characteristics from their parents, making it

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difficult to characterize and identify. The diversity of durian has been studied and reported using molecular markers e.g., random amplified polymorphic DNA (RAPD) (Vanijajiva, 2011); sequence-related amplified polymorphism (SRAP) (Thinhuatoey et al., 2016); start codon targeted (SCoT) (Singhsilarak et al., 2018); simple sequence repeat (SSR) (Seubsuk et al., 2017; Siew et al., 2018) and inter-simple sequence repeat (ISSR) (Angeleina et al., 2019). This study aims to investigate the genetic identity of some local durians derived from open-pollinated seedlings using microsatellite markers. The genetically distinctive clone(s) with good fruit quality will be registered as new cultivar(s).

MATERIALS AND METHODS

Plant materials and DNA extraction

Durian trees of different cultivar including 12 commercial cultivars, as well as 17 unknown cultivars from open-pollinated seedlings, having fruit of good eating quality and high pulp to fruit ratio, were selected from the GAP certified orchards in Kantharalak and Khun Han District. Leaves from these trees were collected (Table 1). Genomic DNA was extracted using a modify CTAB method as described by Doyle and Doyle (1990). The DNA quantity and quality measured using a Nanodrop lite spectrophotometer (Thermo Scientific) and 0.8% agarose gel electrophoresis.

Table 1. List of commercial durian cultivars and unknown trees grown in Si Sa Ket.

No.	Cultivar name - Sources	No	Cultivar name - Sources
1	Chanee[a]	16	Un-Kantharalak
2	Mt-Khun Han	17	Lhongsalika-Khun Han
3	Kanyao[a]	18	Phuang Mani[a]
4	Mt-Khun Han	19	Kradum[a]
5	Un-Khun Han	20	Mt-Khun Han
6	Un-Khun Han	21	Ky-Kantharalak
7	Monthong[a]	22	Linlap-lae[a]
8	Un-Khun Han	23	Kop Suwan[a]
9	Nokyip[a]	24	Un-Kantharalak
10	Thong Nuanchan[a]	25	Un-Kantharalak
11	Un-Khun Han	26	Khun Non[a]
12	Cn-Khun Han	27	Salika[a]
13	Un-Kantharalak	28	Lhonglap-lae[a]
14	Mt-Khun Han	29	Un-Kantharalak
15	Un-Kantharalak		

[a]Asexually propagated tree. Other trees were derived from open pollinated seedlings of 'Chanee' (Cn), 'Kanyao' (Ky), 'Monthong' (Mt), and unknown (Un) mothers.

PCR amplification

Twenty-six microsatellite primers were selected for durian DNA amplification (Kaewsrisom et al., 2014; Santoso et al., 2017; Siew et al., 2018) (Table 2). The PCR reaction was carried out in a final volume of 10 µL containing 1X buffer S (Vivantis), 0.5 mM each of dNTP, 0.1 mM each of primer, 1 unit of *Taq* polymerase and 50 ng template DNA. Amplification was performed in a thermal cycler (Thermo Fisher Scientific) for 35 cycles after an initial denaturation at 95°C for 3 min. In each cycle, denaturation for 30 s at 95°C, annealing for 40 s at 58°C and extension at 72°C for 1 min after the 35th cycle with final extension of 5 min at 72°C. PCR product was analyzed on fragment analyzer automated parallel capillary electrophoresis system (Advanced Analytical).

Table 2. Microsatellite primers used for genetic analysis of durian.

No.	Primers name	Forward primer (5'→ 3')	Reverse primer (5'→3')
1	MS1CT-7	CAT GGA CAA GAA AGC GAT GA	TGG ATC AGA TGA ATC AGG TTG
2	MS1CT-9	CCC TAC GTT ACA TGA TGA TCC A	CCA TTT TGC TCC CTT ACT CTT C
3	MS1CT-16	TCC CCA GTT TTCGAC AGC AGT CC	GAC GTC GTT TTG GAA GGG TA
4	MS1CT-27	CAA TGC TTC CAG GTT TCC AT	CCT GGC AGG TTA TTT AT
5	MS1AAC-2	GAA AAA CTA AGC CCC CAA CC	ATG AAC ACC ACC ACC TCC A
6	MS1AAC-19	AGC CCA TTT GGT GCT GTA AT	AGC AAC CTC AGC CAT TGT
7	DZ01	AAT TCC ACA TGA CAC ACA GG	TCA TGC ATG TTG TAT GGC AG
8	DZ02	ACC TCC TCC CCA TTT CAC C	TGT TGA AGT CAT ACC TTT AGC C
9	DZ03	CTC TAA AAA GAA TGG GGA TAT TG	ATT CTG GAA CAA AAG TTA CAA AC
10	DZ04	TGC ATG TTT TGA AAA GTA CC	ATG GGG AAA AGA AAG TGA AG
11	DZ05	ACA CAT ACA CAA CTC ACC TC	ATG CCC GAT GAA ATT GTA AC
12	DZ06	ATG GGA TTT GGA TGA TGG GTT G	CGA CTC ACT ATA GGG CGA ATT G
13	DZ06-2	AGG TTG AAT TGA ACT GGG TTT TG	GCG GGA ATT CGA TTG ATG AG
14	DZ07	ACA CAC CAT CTT CCC TTT G	TGC ACA TGT TGT TTG TAT ATA TG
15	DZ08	ACA TAT ATA CAA ACA ACA TGT GC	GTC CAA TGA TGG AAA AAC TC
16	mDz3B71	GAT GGT GGA ATT GGT GGT GG	ATC GGC TCC AAC CCT TAA CT
17	mDz03F10	GGA CTA GAC AAC CAA GCA GAG	GCG TGG ACT ACT TCA AAC CC
18	mDz3B72	TGA ACG TTC TCC ACC CCT C	GAA GTT GGT TCC TTG CGG TT
19	mDz1C12	CGT TGT TGC CTG TCG GAT	CAC AAC CAT AGC ACC ACT CA
20	mDz03A31	TGT GGA GTC TTG TTC GGG AA	AGC AAC AAA CAG AAC CAC CG
21	mDz3G72	AGT TAA GGG TTG GAG CCG AT	TAC GTG TGA GGT CAA GCT GT
22	mDz4A6	AGA GAA GTT CGT TTG GAG CCA	ATC AAC ACC TGG CTT GAT CC
23	mDz03H9	AGC CTC CGT ATC TTT ACA TGT	CAT TCG ATG CTA CCA CAC CG
24	mDz6F06	GGT TAC AAC TTG CCC CAG TG	GAC CAC CAA CAC AAA CGG AA
25	mDz03A1	CGT GGA CTA CTT TTA TTG CAG AGG	CAA GTC CAT TCG TAT TGC CAT TTA G
26	mDz6A11	GCA CAA CCA TAG CAC CAC TC	TGT TAT TCT CGT GCC AAG CG

DNA analysis

DNA banding profiles were translated into binary data. The presence of band in each position was recorded as 1 and its absence as 0. Microsatellite bands for each primer were scored separately. Simple matching similarity coefficients among the 29 genotypes were calculated. A genetic relationship dendrogram was analyzed by UPGMA method using NTSYS pc 2.1 program and genetic compositions analyzed using a STRUCTURE program.

Leaf and fruit characteristic

The morphological characterization of the open-pollinated derived durian trees were recorded according to the plant germplasm database for durian (National Plant Varieties Protection Office, 2001). Characterization consisted of leaf shape, fruit shape, fruit apex, fruit shape spine, fruit weight, pulp proportion and pulp color.

RESULTS AND DISCUSSION

Out of 26 microsatellite primer pairs that were screened in this study, 21 primer pairs were successfully amplified into clear and reproducible bands. Five primer pairs were not successfully amplified, including DZ02, DZ06, DZ06-2, mDz3B72 and mDz3G72. A total of 151 alleles were scored across 21 loci, ranging from one to 15 alleles per locus with an average of 7.19 per locus. The minimum number of different alleles found at a locus was one in mDz03F10, while the maximum was 15 in mDz03H9. The polymorphism information content (PIC) for the 21 primers in this study varied from 0.607 for MS1CT-27 to 1.000 for mDz03F10. Amplification of 21 microsatellite loci onto 29 durian samples showed the PIC values were an average of 0.795 (Table 3).

Table 3. The number of alleles and PIC values of microsatellite markers in 29 durian samples.

Primer name	No. of alleles	PIC
MS1CT-7	6	0.680
MS1CT-9	12	0.897
MS1CT-16	8	0.851
MS1CT-27	5	0.607
MS1AAC-2	8	0.850
MS1AAC-19	6	0.820
DZ01	6	0.664
DZ03	10	0.866
DZ04	6	0.830
DZ05	8	0.867
DZ07	7	0.806
DZ08	9	0.837
mDz3B71	8	0.778
mDz03F10	1	1.000
mDz1C12	4	0.748
mDz03A31	4	0.695
mDz4A6	6	0.824
mDz03H9	15	0.853
mDz6F06	12	0.824
mDz03A1	7	0.765
mDz6A11	3	0.640
Total	151	-
Average	7.19	0.795

The DNA bands were scored and calculated for genetic similarity and cluster analysis. The dendrogram was constructed from the distance matrix based on simple matching coefficients. The dendrogram by UPGMA analysis, similarity coefficient ranged from 0.51 to 0.82. The major group contained 23 durian samples with similarity coefficient value close to 0.7. The next outsider to join a group with similarity coefficient value more than 0.68 was 'Khunnon' and 'No. 29'. The groups of an individual samples were 'No.15', 'Salika', 'Longlap-lae' and 'No.17' with similarity coefficient value less than 0.66. The highest similarity coefficient was found between 'No. 21' and 'Kradum' at 0.82. The lowest similarity coefficient was found between 'Kanyao' and 'No.17' at 0.51. Based on a cut-off point of 0.69 in similarity coefficient scale, the 29 durian samples formed one major cluster and five outsiders (Figure 1).

The genetic structure analysis illustrated that durian samples in the major group are diverse. The 17 open-pollinated derived durian trees differ in their genetic structure. A high level of genetic diversity among durian samples was observed in this study, partly due to outbreeding of the species. Group I was the large group. It indicated that the open-pollinating derived durian trees were related with commercial cultivars that provided genetic variability. The result of this study is similar to those of Seubsuk et al. (2017) and Pichakum (2018) that studied durian diversity in Nonthaburi and Chanthaburi Provinces using 14 and 16 microsatellite markers, respectively. The grouping by UPGMA method and dendrogram constructed of both studies and this study showed that 'Chanee', 'Kop Suwan' and 'Kradum' are genetically closed. In addition, the genetic grouping was not related to the morphological grouping, as reported by Hiranpradit et al. (1992). Durian cultivars that were morphologically classified into different groups, were found to be genetically similar, and vice versa.

In this study, the 17 open-pollinating derived durian trees are genetically different from the 12 commercial cultivars, and among themselves. They also had some morphological characteristics similar to the 12 commercial cultivars. Hence all of them could be registered as new cultivars. However, their parents could not be identified, since not all known cultivars were included in this study.

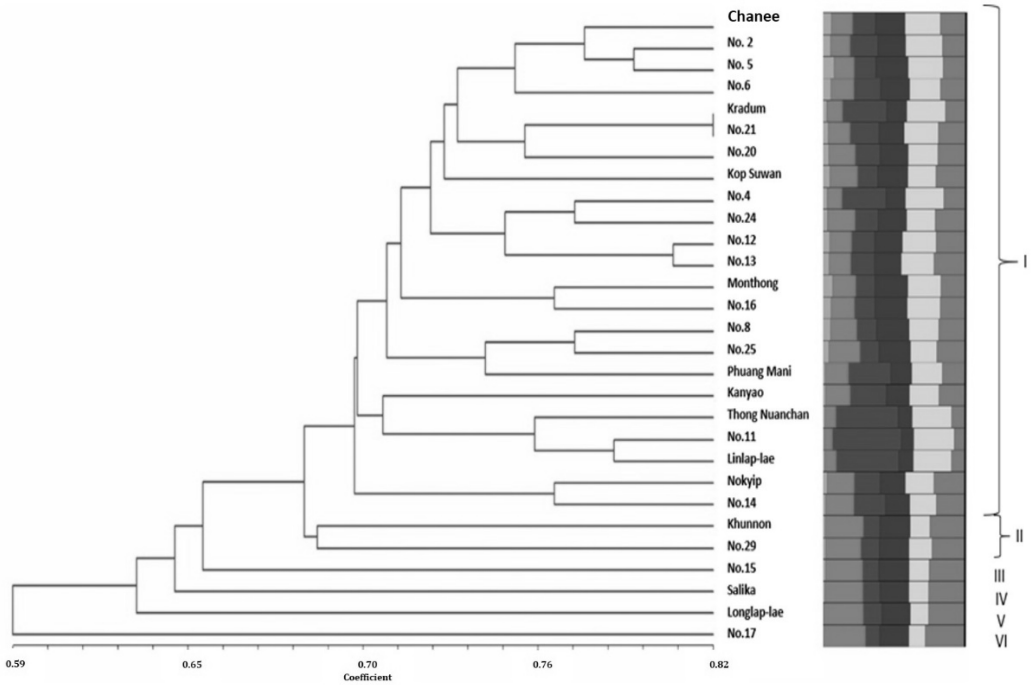

Figure 1. Dendrogram and genetic structure of 29 durian samples from 21 microsatellite makers using UPGMA analysis.

From the above genetic information together with leaf and fruit characteristic, five open-pollinated derived durian trees were selected. These included 'No. 2', 'No. 21', 'No. 4', 'No. 8' and 'No. 29'. Their leaf and fruit characteristics were listed in Table 4 along with their genetically closest commercial cultivars.

Table 4. Leaf and fruit characteristics of five open-pollinated derived durian trees and their genetically closest commercial durian cultivars.

Samples #/ cultivars	Leaf shape	Fruit shape	Fruit apex	Spine shape	Pulp color	Pulp (%)
No. 2	Oval-oblong	Rounded	Convex	Pointed-concave	Yellow (Y4B)	26.0
Chanee	Oval-oblong	Oval	Truncate	Pointed-convex	Yellow-orange (YO16B, C)	27.0
No. 21	Oval-oblong	Ovate	Pointed	Convex	Yellow (Y2C)	30.5
Kradum	Elliptic	Oblate	Depressed	Pointed-convex	Yellow (Y12B)	28.0
No. 4	Oval-oblong	Obovate	Truncate	Pointed-concave	Yellow-orange (YO15C)	31.0
Kop Suwan	Elliptic	Elliptic	Depressed	Hooked	Yellow (Y10B)	23.7
No. 8	Oval-oblong	Oval	Pointed	Pointed-concave	Yellow (Y10C)	30.5
Phuang Mani	Oval-oblong	Elliptic	Pointed	Pointed-convex	Yellow-orange (YO24B, D)	21.3
No. 29	Oval-oblong	Obovate	Truncate	Pointed-convex	Yellow (Y11A)	28.0
Khunnon	Elliptic	Oblate	Truncate	Convex	Yellow (Y 11C)	20.7

This study revealed that the five selected durian trees were clearly different in their morphology from their closest commercial cultivars. These will be registered as new cultivars and further studies on yield, disease resistance and eating quality conducted. Some of these will then be recommended to farmers. One cultivar deserved special attention, 'No. 4'. It has a relatively high pulp percentage with yellow orange color, and is more attractive to the consumer than those with the yellow colored pulp.

CONCLUSIONS

The genetic analysis of open-pollinated derived durian seedling trees and the

commercial cultivars in Si Sa Ket Province were analyzed using 26 microsatellite markers. Results showed that 17 open-pollinated derived durian trees were genetically different. The DNA bands were scored and calculated for genetic similarity and cluster analysis. Similarity coefficient ranged from 0.51 to 0.82. These durian samples were divided into one major group and five outsiders. Along with leaf and fruit characteristic, the five open-pollinated derived durian trees were selected and will be registered as new durian cultivars.

ACKNOWLEDGEMENTS

This work was supported by Si Sa Ket provincial Governor's Office in 2019. The authors are grateful to Dr. Suchirat Sakuanrungsirikul (Khon Kaen Field Crop Experiment Center) for her expert advice and equipment support throughout this project. The authors would also like to thank Mr. Tawatchai Subtira for lab assistance.

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The diversity of traditional Japanese and Taiwanese eggplant cultivars using SSR markers and fruit characteristics

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Abstract

Eggplant (*Solanum melongena* L.) originated in the eastern region of India and has been cultivated in Japan since 734 AD. A variety of fruit shapes such as round, ovoid, and long are found in Japan and Taiwan. In this study, we investigated the fruit shape and genetic diversity of 156 traditional Japanese and 30 traditional Taiwanese eggplant cultivars preserved in a Genebank Project in Japan. We analyzed their relationships and estimated their population structure. Fruit shape was classified based on 23 measurements obtained from image analysis using the Tomato Analyzer software. The principal component analysis suggested that the Taiwanese cultivars tend to have smaller fruit diameter and fruit length compared to the Japanese cultivars. In addition, we chose 10 SSR markers and genotyped by fragment analysis, and the UPGMA-method molecular to generate the phylogenetic tree. The phylogenetic tree was divided into two clades: Cluster I that included the cultivars from both regions and Cluster II that mainly included the Japanese cultivars. A STRUCTURE analysis was performed to estimate the population structure of the cultivars. As a result, four populations were identified. The Japanese cultivars were classified into Pop I, II and III, and the Taiwanese cultivars into Pop I and IV. In conclusion, for Japan and Taiwan there are many cultivars with a wide range of fruit shapes such from round to long with different genetic diversity. It is important to conserve these traditional cultivars.

Keywords: *Solanum melongena* L., eggplant, principal component analysis, SSR, genetic diversity, population structure

INTRODUCTION

Eggplant (*Solanum melongena* L.) is an annual plant belonging to the *Solanum* genus, *Solanaceae* family and is commonly attributed to eastern India (Vavilov, 1951). The earliest reference to eggplant in Japan dates to 734 AD. It is thought that the cultivars were brought in from China and Korea, and spread widely before the 8th century but the details are unknown. In Taiwan, it is believed to have been introduced from southern China before the 4th or 5th century. Some Taiwanese eggplant cultivars are similar in fruit traits to the Japanese eggplant cultivars. Recent studies have shown that China is considered one of the secondary diversity origins of eggplant (Hurtado et al., 2012). The traditional eggplant cultivars that originated in China later spread to other East and Southeast Asian countries, including Japan and Taiwan (Meyer et al., 2012). Assessing the diversity and relationships of the cultivars can help establish conservation strategies. The use of genetic resources in breeding programs can provide useful information for crop evolution. In this study, we analyzed fruit shape and the genetic diversity of traditional Japanese and Taiwanese eggplant cultivars. We attempted to elucidate the genetic background and infer the population structure of traditional eggplant cultivars with diverse fruit shapes.

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MATERIALS AND METHODS

Plant materials

We used 156 traditional Japanese and 30 traditional Taiwanese eggplant cultivars, managed by Genebank project of the National Agriculture and Food Research Organization (NARO) in Japan. Four plants of each cultivar were sown and the open-air cultivation carried out by staggered planting, 1 m between rows and 50 cm between plants in the university plots (39.799549N; 140.046035E).

Fruit shape characterization

At harvest, three fruit plant^{-1} collected about one week after the fruit set was confirmed. Fruit were washed, the calyx was removed, and the fruit cut longitudinally. The fruit sections scanned with a CanoScan LiDE220 scanner (Canon) at a resolution of 75 dpi. The scanned images were analyzed using Tomato Analyzer v. 4.0 software (Brewer et al., 2006; Rodríguez et al., 2010) to measure the fruit shape traits. A total of 23 fruit shape traits, corresponding to basic measurements (Perimeter, Area, Width_MH, Max_Width, Height_MW, Max_Height, C_Height), fruit shape index (fruit_Shape1, fruit_Shape2, C_fruit_Shape), blockiness (P_Blockiness, D_Blockiness, Triangle), homogeneity (Ellipsoid, Circular, Rectangular), asymmetry (Obovoid, Ovoid), and internal eccentricity (Eccentricity, P Eccentricity, D_Eccentricity, F_S_Index, ECC_Area_Index) evaluated.

Molecular characterization

Genomic DNA was extracted from the young leaves of each plant according to the protocols for DNA extraction kit DNA Suisui-P (Rizo Inc., Ibaraki, Japan). Forty-one SSR markers (Nunome et al., 2009) were selected and screened for polymorphisms using 125 Japanese cultivars. Ten SSR markers that produced clear bands, and showed polymorphism were selected for the polymorphism analysis. PCR reactions were performed in a 20-µL reaction mixture containing DNA 20 ng, Primer Mix 6.0 pmol, 2× Multiplex PCR Buffer (TaKaRa) 10 µL, and Multiplex PCR Enzyme Mix (TaKaRa) 0.1 µL. The Bar-Coded Split Tag (BStag) method (Konishi et al., 2015) was used to fluoresce PCR products using DNA primers with a 16-bp tag sequence added to the 5' end. Fluorescently labeled oligo primers corresponding were added to the tag sequence. The PCR conditions were as follows: one cycle of 2 min at 94°C five cycles of 30 s at 94°C, 30 s at 60°C (lowered by 1°C cycle^{-1}), 30 s at 72°C, 35 cycles of 30 s at 94°C, 30 s at 55°C, 30 s at 72°C, and then extended at 72°C for 7 min. GeneScan 500LIZ size standard was added to the PCR products and fragment analysis performed using a 3130xl Genetic Analyzer (Applied Biosystems). The genotype of each cultivar was determined from the detected fragment size and waveform.

Data analysis

Principal component analysis (PCA) was performed using statistical analysis software R from the fruit shape trait data of the Japanese and Taiwanese cultivars. The proportion of variance was calculated for each principal component and the relevant components were identified based on a cumulative proportion of 80%.

SSR data obtained using the analysis software GenAlEx v.6.5 (Peakall and Smouse, 2012) to calculate the SSR distance matrix between the cultivars. The molecular phylogenetic tree generated by the UPGMA method using the analysis software MEGA7 (Kumar et al., 2016). In addition, STRUCTURE v2.3.4 (Pritchard et al., 2000) used to estimate the population origin and the assignment of each cultivar to the population. Ten simulations performed of *K,* from 1 to 10 with the Length of Burnin period of 50,000 interactions and MCMC Reps after Burnin period of 10,000 iterations. In addition, 10 simulations of *K* from 2 to 10 with the Length of Burnin period of 75,000 interactions and MCMC Reps after Burnin period of 15,000 interactions were carried out. Furthermore. population structure analysis using the Δ*K* method (Evanno et al., 2005) was conducted. The frequently reported for *K* (the number of population structure) = 2 (Janes et al., 2017). However, since *K*=2, this does not allow us to focus on a subdivided population structure, especially for the lower population structures,

following the ΔK method. The most informative *K* was identified from the maximum value of the statistic *ΔK* at these simulations after *K*=2 in this study.

RESULTS

Principal component analysis for fruit characteristics

It was not possible to obtain fruit characterization data for some cultivars due to disease outbreaks. Presented below are the results of the principal component analysis of 130 Japanese and 28 Taiwanese cultivars. The first and second principal components accounted for 61.42 and 14.24%, respectively, of the variation in mean values in fruit characteristics for each cultivar (Figure 1). The first principal component was positively correlated with long fruit characteristics. This was determined by size (perimeter, area), length (Height_MW, Max Height, C_Height) and fruit index (fruit_Shape1, fruit_Shape2). Furthermore, fruit index was negatively correlated with round fruits characterized by fruit diameter (Width_MH, Max_Width) and egg-shaped fruits (triangle, ovoid). The second principal component was positively correlated with fruit shape index, blockiness, homogeneity, asymmetry, and internal eccentricity, and negatively correlated with basic measurement, which indicates the size of each cultivar. When the principal component score of each cultivar was projected onto a two-dimensional PCA plot, some Japanese cultivars were categorized as overlapping with Taiwanese cultivars (Figure 2). The Japanese and Taiwanese cultivars showed a similar distribution in the first principal component of fruit shape. In the second principal component of negative fruit size, the Japanese cultivars showed a wide distribution, while the Taiwanese cultivars showed a consistent positive correlation.

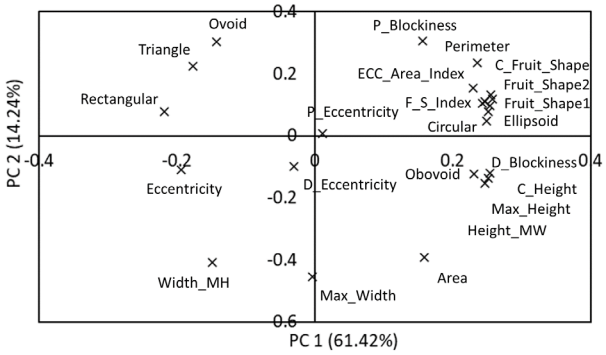

Figure 1. PCA relationship between fruit shape traits measured by Tomato analyzer.

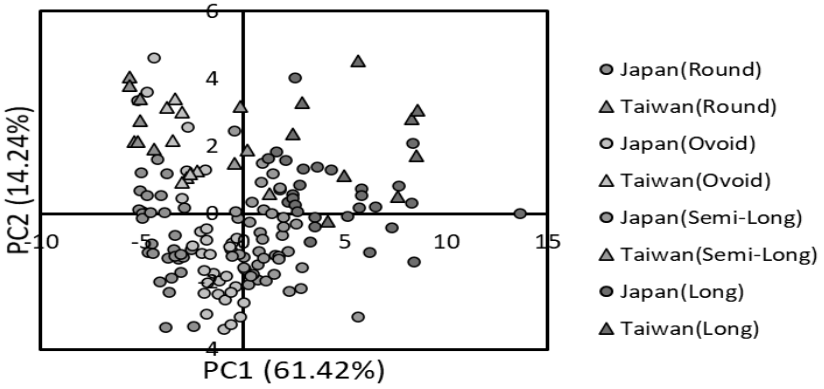

Figure 2. PCA fruit shape-based relationships between 130 traditional Japanese and 28 traditional Taiwanese eggplant cultivars.

Polymorphism of SSR markers

Shown in Table 1 are the number of alleles, heterozygosity, polymorphic information content (PIC) and their mean values for all 10 SSR markers. The number of alleles ranged from 6 to 20, with a mean value of 12.80. The mean number of alleles of the Japanese and Taiwanese cultivars were 11.70 and 7.30, respectively. The heterozygosity ranged from 0.03 to 0.21, with a mean value of 0.12. The mean heterozygosity of the Japanese and Taiwanese cultivars was 0.10 and 0.22, respectively. PIC ranged from 0.25 to 0.88, with mean value of 0.68. The mean PIC of the Japanese and Taiwanese cultivars was 0.64 and 0.66, respectively.

Table 1. The number of alleles, heterozygosity, and PIC for each SSR marker.

Locus	Linkage	No. of alleles			Heterozygosity			PIC		
		All	Japan	Taiwan	All	Japan	Taiwan	All	Japan	Taiwan
eme03H10	E_01	14	13	8	0.16	0.15	0.17	0.83	0.81	0.82
emj05M23	E_02	8	6	5	0.06	0.03	0.27	0.25	0.17	0.54
emk01J09	E_03	6	5	5	0.03	0.02	0.10	0.27	0.13	0.67
emh11I06	E_04	16	15	10	0.21	0.15	0.53	0.72	0.65	0.86
emh05H12	E_05	13	12	5	0.10	0.08	0.17	0.80	0.78	0.42
emf01O04	E_06	17	16	11	0.21	0.21	0.23	0.88	0.87	0.82
emf11K21	E_07	10	10	5	0.09	0.10	0.07	0.70	0.68	0.24
eme25D01	E_09	12	10	6	0.10	0.09	0.13	0.67	0.62	0.75
emh11L01	E_10	20	18	11	0.08	0.05	0.20	0.84	0.82	0.76
emf21K08	E_11	12	12	7	0.14	0.11	0.30	0.85	0.84	0.77
Mean		12.80	11.70	7.30	0.12	0.10	0.22	0.68	0.64	0.66

Cluster analysis and population structure analysis

A molecular phylogenetic tree was constructed by UPGMA from cluster analysis using 10 SSR markers. Except for some cultivars (TWN-020, JPN-139 and JPN-133), the phylogenetic trees are divided into two clades. Those classified as cultivars from both regions and those classified mainly as the Japanese cultivars (Figure 3). A STRUCTURE analysis was performed, and four populations were detected (Figure 4). Pop I consisted of 45 Japanese cultivars and 9 Taiwanese cultivars. No trend found for the fruit shape of the Japanese cultivars, but all Taiwanese cultivars had semi-long and long shape. Pop II consisted of 51 Japanese cultivars and Pop III consisted of 56 Japanese cultivars and one Taiwanese cultivar. There was no trend for fruit shape in these populations. Pop IV consisted of four Japanese cultivars and 20 Taiwanese cultivars. The Taiwanese cultivars tended to have fruit shape such as round and ovoid. These cultivars also had a hard peel and flesh, similar to *S. insanum* L.

In this study, four populations were identified between these Japanese and Taiwanese cultivars. We calculated the genetic differentiation (*Fst*) and number of migrants (*Nm*) between the populations based on these populations. *Fst* is an index of genetic differentiation between populations. It describes the moderator when the value of *Fst* was among 0.05-0.25 and high when *Fst* was greater than 0.25 (Wright, 1984). The *Nm* less than 1.0 is generally regarded as the threshold quantity beyond which, significant population differentiation occurs (Slatkin, 1987). As a result, moderate genetic differentiation occurred between each population, with the greatest degree of genetic differentiation between Pop III and Pop IV (*Fst*=0.192). The smallest was between Pop II and Pop III (*Fst*=0.052) (Table 2).

In contrast, the *Nm* results did not identify significant genetic differentiation in each population, indicating the occurrence of genetic flows in these populations (Table 2).

Table 2. The genetic differentiation (*Fst*; a) and the average number of migrants (*Nm*; b) between each population.

a)	Population	Pop I	Pop II	Pop III	Pop IV	b)	Population	Pop I	Pop II	Pop III	Pop IV
	Pop I	-	0.090	0.113	0.082		Pop I	-	3.605	4.567	3.683
	Pop II	0.090	-	0.052	0.174		Pop II	3.605	-	7.779	1.885
	Pop III	0.113	0.052	-	0.192		Pop III	4.567	7.779	-	1.402
	Pop IV	0.082	0.174	0.192	-		Pop IV	3.683	1.885	1.402	-

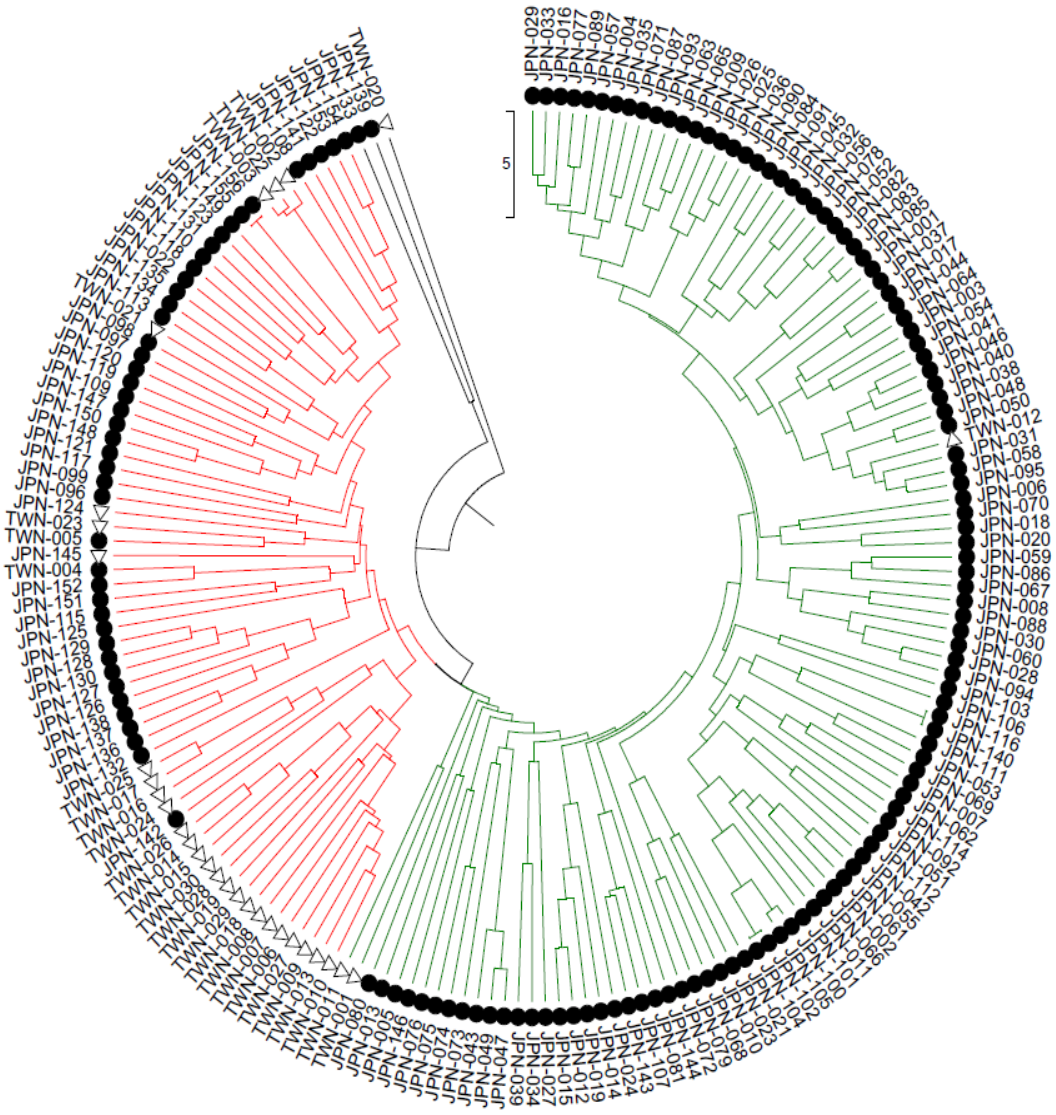

Figure 3. The molecular phylogenetic tree by UPGMA method of 156 traditional Japanese and 30 traditional Taiwanese eggplant cultivars (Red: Cluster I, Green: Cluster II, ●: the Japanese cultivars, △: the Taiwanese cultivars).

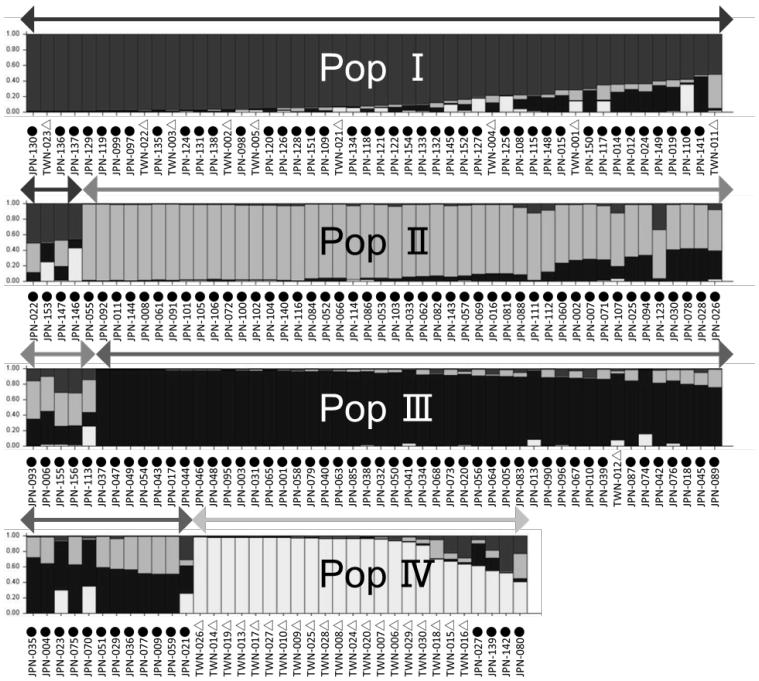

Figure 4. The population structure of 156 traditional Japanese and 30 traditional Taiwanese eggplant cultivars (*K*=4).

DISCUSSION

Fruit characteristics between traditional Japanese and Taiwanese eggplant

The results of the principal component analysis of the fruit shape showed that the Japanese cultivars have a wide distribution of the first and second principal components. On the other hand, the Taiwanese cultivars showed a wide distribution for the first principal component, but a positive correlation for the second principal component. The first principal component indicates fruit shapes such as round and long, suggests the Japanese and Taiwanese cultivars have a wide range of fruit shapes, including round, ovoid, semi-long, and long shapes. The second principal component was negatively associated with fruit size, suggesting the Taiwanese cultivars tended to be smaller than the Japanese cultivars. Specifically, the round Taiwanese cultivars tended to have smaller fruit diameter and fruit length than the round Japanese cultivars, while the long Taiwanese cultivars tended to have smaller fruit diameter than the long Japanese cultivars. In addition, comparing fruit shape, the distribution of round and ovoid Japanese cultivars was wider than the distribution of semi-long and long Japanese cultivars for the second principal component. Therefore, the round and ovoid Japanese cultivars have a range of small to large fruits.

The genetic differentiation

In population genetics, a situation in which the population is subdivided and the allele frequencies for each population differ describes it as “the population has a structure”. How strongly these population structures are expressed depends on the degree of heterozygosity (the proportion of heterozygous), which can be expressed quantitatively by the degree of reduction.

In this study, the means of the heterozygosity of the Japanese and the Taiwanese cultivars were 0.10 and 0.22, respectively, and lower in the Japanese cultivars than in the Taiwanese cultivars. Therefore, it was suggested that the population of the Japanese cultivars had greater inbreeding than the population of the Taiwanese cultivars. The *Fst* and *Nm* results showed moderate genetic differentiation in these populations, suggesting that this may have been caused by gene flow. In particular, the results suggest that Pop II and III may be nearly

genetically identical.

The relationships between genetic diversity and fruit shape diversity

The phylogenetic tree was divided into two clades by cluster analysis. The population structure analysis identified the four genetic populations. These revealed that the Japanese cultivars were in the Pop I, II and III, and the Taiwanese cultivars were in the Pop I and IV. There was no trend for fruit shape, such as round ovoid and long in each population of the Japanese cultivars. However, for the Taiwanese cultivars of Pop I and IV, they showed a tend to have a long and round type fruit, respectively. The long-type Taiwanese cultivars had purple peel and soft flesh while the round-type Taiwanese cultivars had green or light purple peel and hard flesh. On the other hand, although the relationship between population structure and fruit shape of the Japanese cultivar could not be confirmed, it is necessary to elucidate the genetic background of the diverse fruit traits of eggplant in the future. The morphological characteristics of these traditional cultivars, including the Japanese cultivars, vary widely, but differences in cultivation suitability and regional preferences are thought to have influenced the background of cultivation.

CONCLUSIONS

In this study, we investigated fruit characteristics, genetic diversity, and the population structure of traditional Japanese and Taiwanese eggplant cultivars. Fruit characteristics-based principal component analysis suggested that a variety of round to long shaped cultivars existed in Japan and Taiwan. However, the Taiwanese cultivars tended to have a smaller fruit diameter and length than the Japanese cultivars. The phylogenetic tree was divided into two clades by cluster analysis, and population structure analysis identified the four genetic populations. The Japanese cultivars were classified into Pop I, II and III, and the Taiwanese cultivars into Pop I and IV. The Japanese cultivars of each population had no trend for fruit shapes, while the Taiwanese cultivars of Pop I and IV showed they tended to have long and round types, respectively. In Japan and Taiwan, there are many cultivars with a variety of fruit characteristics and genetic diversity, and the preservation of these traditional cultivars will provide the basis for future breeding programs.

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Berberis hypoxantha C.Y. Wu ex S.Y. Bao (*Berberidaceae*), a new record for Vietnamese flora

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Abstract

In October-November 2015, a collaborative plant collection expedition was carried out in national parks of northern Vietnam. The staff of the Plant Resources Center (PRC) of the Vietnam Academy of Agricultural Sciences, Hanoi, and the US Department of Agriculture, Agricultural Research Service National Clonal Germplasm Repository in Corvallis, Oregon, participated. During the expedition, an unusual species of *Berberis* was observed. This species was found in Hà Giang Province in wet evergreen mixed forests on karst limestone between 1500 and 1600 m. Its range in Hoang Lien Son National Park, Lào Cai Province, was at elevations of 1500 to 2500 m, under a dense tree canopy mixture of broad leaved and semi-evergreens on upland reddish soils that were weathered from granite and eroded limestone. Subsequently, samples were examined and voucher specimens were prepared. The objective of this manuscript is to describe the species in preparation for potential addition to Vietnamese flora. This species had bright yellow flowers, evergreen leaves, and deep purple black fruits, but it differed from other *Berberis* in the region. Although it appears to belong to section *Wallichianae*, its lack of significant spines and the obscure veining of the leaves are a particular combination not recorded elsewhere in the section. The sample proved to be *Berberis hypoxantha* C.Y. Wu ex S.Y. Bao, previously known only from the type collection from Nanchang Gongshe, Xichou, Xian in SE Yunnan province, China, and is newly recorded from three locations in Hà Giang and two in Lào Cai province, Vietnam. Vouchers from Lào Cai province were deposited in the Hanoi National University Museum: HNU 022576 and HNU 022577.

Keywords: *Berberis*, *B. hypoxantha*, barberry, Hà Giang, Lào Cai, Vietnam flora

INTRODUCTION

Berberis L. (*Berberidaceae*) is a genus of simple-leaved evergreen or deciduous shrubs or rarely small tree-like bushes almost all of which have 1-5 (-9) nodal spines (Ahrendt, 1961; Adhikari et al., 2012; Pabon-Móra and González, 2012). The genus has two main centers of distribution: first across the Eurasian-land mass stretching from the Sierra Nevada of southern Spain to the Russian Far East and Japan and as far south as Sri Lanka and second Latin America with a concentration in the Andean spine (Ahrendt, 1961; Adhikari et al., 2012). The estimated number of species is around 400-500, the majority of which are found in China (Harber, 2012).

Historically two *Berberis* species have been recognized in Vietnam; *B. wallichiana* and *B. julianae* (Nguyên, 1998; Võ 2007). A "Revision of the *Berberis* of China and Vietnam" (Harber, 2020) argues that these are misidentifications and the species concerned are respectively *B. ferdinandi-coburgii* and *B. subacuminata,* both common in south Yunnan. In this paper, we report a third Vietnamese species *B. hypoxantha* as a new record for the flora of Vietnam.

All three Vietnamese species are members of section *Wallichianae* (Schneider, 1905, 1942; Chamberlain and Hu, 1985; Harber, 2017); a large section of some 100+ species with evergreen coriaceous leaves, single or fascicled flowers and black, dark blue or dark purple

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fruit. Species from this section are found over a wide area stretching from the eastern Himalayas to the East China Sea and Taiwan and south to Indonesia and the Philippines (Yu and Chung, 2014).

MATERIALS AND METHODS

In October-November, 2015, a collaborative plant collection expedition between the staff of the Plant Resources Center (PRC) of the Vietnam Academy of Agricultural Sciences, Hanoi, and the US Department of Agriculture, Agricultural Research Service National Clonal Germplasm Repository in Corvallis, Oregon, was carried out. Permission for the exploration was obtained from the Hoang Lien Son Nature Reserve, Lào Cai province, Vietnam. *Berberis* samples were examined and voucher specimens were prepared.

RESULTS AND DISCUSSION

Botanical description

Berberis hypoxantha C.Y. Wu ex S.Y. Bao, Bull. Bot. Res., Harbin 5(3): 6. 1985. TYPE: China. SE Yunnan, Xichou Xian, Nanchang Gongshe, 19 May, 1959, *Q. A. Wu 8025* (holotype, KUN 0160907, isotype KUN 0160906, WUK 0270468 image).

Shrubs, evergreen, to 3 m tall, mature stems purple, shiny, terete, densely verruculose; spines largely absent, sometimes 1-3, concolorous, 0.1-0.5 cm, weak. Petiole almost absent or to 4 mm, leaf blade abaxially pale green, adaxially dark green, elliptic, oblong-elliptic or elliptic-obovate 3-7×(1.25) 2-2.5 cm, leathery, abaxially sometimes pruinose, midvein raised abaxially, impressed adaxially, lateral veins inconspicuous or obscure on both sides, margin entire, sometimes undulate, rarely spinulose with 2-5 teeth on each side, base cuneate, apex rounded or acute, sometimes mucronate. Inflorescence a fascicle 4-20-flowered, pedicel 18-20 mm; bracteoles triangular 1.3-1.7×1-1.5 mm, flowers yellow. Sepals in 2 whorls; outer sepals ovate or elliptic ovate, 2.5-3×2-2.5 mm; inner sepals obovate or oblong ovate, 5-6.5×4-4.5 mm; petals: obovate to orbicular, 4.5-5.5×2-3.3 mm, base very slightly clawed, glands close together ca. 1 mm, apex distinctly notched. Stamens 3.5-4.0 mm, anther connective produced to ca. 0.5 mm, truncate. Pistil ca. 4 mm; ovules 1-3. Berry black or dark purple, narrowly obovoid or ellipsoid, 9-12×5-7 mm; style not persistent or persistent and short. (Figures 1 and 2).

Phenology

In China, *B. hypoxantha* was collected with immature fruit in May; its flowering season is unknown. In Vietnam, the species was collected flower in April-May, and fruit in October. Flowers and fruit were observed simultaneously in October and November.

Distribution and habitat

In China, *B. hypoxantha* had been only known from the type which was collected from a mountain slope at an unrecorded elevation in Xichou, Xian.

In Vietnam, the species was found in Hà Giang province in wet evergreen mixed forests on karst limestone between 1500 and 1600 m (Figure 3). Its range in Hoang Lien Son National Park, Lào Cai province, was at elevations of 1500 to 2500 m, under a dense tree canopy mixture of broad leaved and semi-evergreens on upland reddish soils that were weathered from granite and eroded limestone. These soils were nutrient poor, and contained iron and aluminum oxide. At those locations the dominant vegetation included birches (*Betulaceae*), walnuts (*Juglandaceae*), willows (*Salicaceae*), blueberry (*Vaccinium*) and rhododendron relatives (*Rhododendron*: *Ericaceae*), raspberry relatives (*Rosaceae*), chesnut (*Fagaceae*), false gingseng (*Araliaceae*), magnolia (*Magnoliaceae*), macassar kernels (*Brucea javanica*) and Himalayan yew (*Taxus wallichiana*).

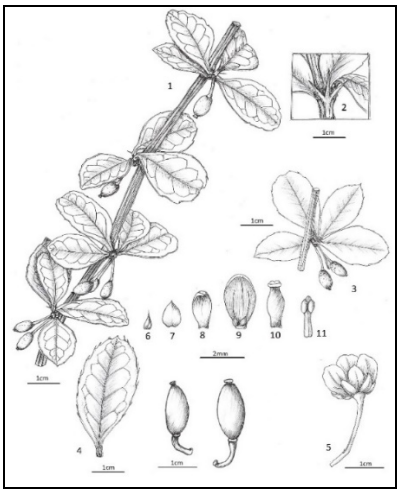

Figure 1. Drawing of *B. hypoxantha* C.Y. Wu ex S.Y. Bao. Branch (1), trifid spine (2), fruit (3), leaf with spinules (4), flower (5), bracteole (6), outer sepal (7), inner sepal (8), petal with anthers (9), pistil (10), anther (11) and fruit (12). Drawing by Ngo Duc Phuong.

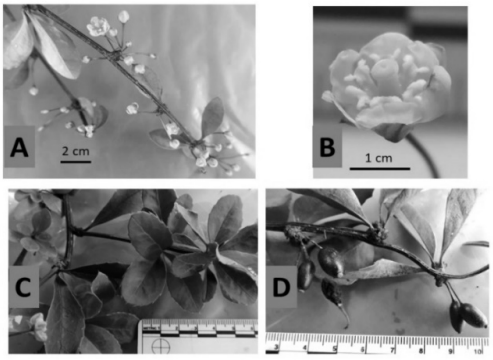

Figure 2. Images of *B. hypoxantha* C.Y. Wu ex S.Y. Bao. collected from Fansipan Mountain, Lào Cai province, Vietnam, October, 2015. Inflorescenses (A), flower closeup (B), branch with spinules on leaf margin, bracteoles, sepals (C) and fruits (D). Images by K.E. Hummer.

Figure 3. The dots indicate new localities of the distribution of *B. hypoxantha* C.Y. Wu ex S.Y. Bao in China and Northern Vietnam (Harber, 2020).

The protologue of *B. hypoxantha* was based entirely on the holotype and isotype at KUN (the isotype at WUK not being cited). All three of these specimens have only very immature fruit. The Vietnamese specimens cited below have made possible a much fuller description of the species than was given in C.Y. Wu's protologue and reproduced in Chinese in Bao (1997) and Ying (2001) and in English in Ying et al. (2011). The unidentified *Berberis* species from Vietnam referred to in Averyanov et al. (2002) is *B. hypoxantha* (Harber, 2020; Ying et al., 2011).

B. hypoxantha is an unusual species. Although it appears to belong to section *Wallichianae*, its lack of significant spines and the obscure veining of the leaves are a particular combination not recorded elsewhere in the section. Molecular phylogenetic analysis was carried out on various sections. *Wallichianae* species (Yu and Chung, 2014; Adhikari et al.., 2015). We hope to collaborate with further analysis on *B. hypoxantha* and other Vietnamese *Berberis* to determine their relationship to other species in the section.

Selected specimens

1. N Vietnam: Hà Giang province.

Dong Van District, Ho Quang Municipality, vicinity of Ta Xa village, 23.266667°N; 105.366667°E, 1550-1600 m, April 28, 1999, P. K. Loc, P. H. Hoang & L. Averyanov, CBL 1779 (HN, LE (2), MO 2331499, specimen on left of sheet, P P02313634); Yen Minh district, La Va Chai Municipality, vicinity of Ngan Chai village, 6 km east of Yen Minh town, 23.1167°N; 105.1334°E, 1500-1600 m, May 1, 1999, P. K. Loc, P. H. Hoang & L. Averyanov CBL 1888 (HN, LE, MO 2331500, P P02313635). Yen Minh district, La Va Chai Municipality, vicinity of La Va Chai Village, 23.116667°N; 105.133333°E, 1500-1550 m, October 9, 1999, N. T. Hiep, B. Q. Binh, L. Averyanov & P. Cribb NTH 3483 (LE).

2. Lào Cai province.

Fansipan Mountain, Hoang Lien Son Mountain Range, near main trail to summit, 2300 m, March 15, 2016, T.V. Tu & N.Q. Vinh, Hanoi National University museum: HNU 022576. No. 3, Ta Phin Commune, Sa Pa district, Lào Cai province. 22.418333°N; 103.839722°50E, 1536 m, HNU 022577.

CONCLUSIONS

The *Berberis* species observed from Hon Lien Son National Park, Lào Cai, Vietnam, has traits that match the description of *B. hypoxantha*. This species has bright yellow flowers, evergreen leaves, and deep purple black fruits. It differs from other *Berberis* in the region though it belongs to section *Wallichianae*. A big difference between this and the other species is the lack of significant spines and the obscure veining of the leaves. These traits are indicative of *B. hypoxantha*, formerly only known from Xian, China, and now observed to be present in Vietnam.

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Isolation and analysis of flavonoid 3'-hydroxylase (F3'H) genes from cyclamen 'Strauss'

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Abstract

Anthocyanins are the most abundant and primary flavonoid pigments, which produce a wide range of colors. Flavonoid 3'-hydroxylase (F3'H) is one of the key enzymes in anthocyanin biosynthesis and has been identified from a variety of ornamental plants. For isolating and charactering the anthocyanin related *F3'H* in cyclamen, three candidates of *F3'Hs* named as *STRF3'H1*, *STRF3'H2a* and *STRF3'H2b* were obtained from petals of *Cyclamen persicum* cultivar 'Strauss' (STR). Phylogenetic analysis and sequence alignment suggested that these putative STRF3'Hs belong to the CYB75B class of cytochrome P450 superfamily, containing the unique conserved motifs of F3'H enzyme. The prediction of transmembrane structure showed that STRF3'H2a and STRF3'H2b each had a transmembrane region, while F3'H1 did not. STR and the wild *Cyclamen persicum* (*C. persicum*), have different major anthocyanin components in petals. Since the synthesis of major anthocyanins in *C. persicum* and STR are closely related to F3'H and flavonoid 3',5'-hydroxylase (F3'5'H), the expression of these genes had been compared by real-time PCR. *F3'Hs* were expressed strongly in STR, weakly in *C. persicum*, while *F3'5'H* was opposite. The results implied F3'H is likely taking an active role in pigmentation in STR. The function will be further analyzed to verify the relevance between *F3'H* and STR flower color formation.

Keywords: anthocyanin, cloning, flower color, pigment

INTRODUCTION

The striking colors of most plants are attributed to the accumulation of flavonoids. Anthocyanins are an important group of flavonoids, which produce a wide range of colors to attract pollinators and seed dispersers, protect plants from UV radiation, participate in the synthesis of plant hormones, etc. (Huits et al., 1994; Winkler and Helentjaris, 1995). The biosynthetic pathway of anthocyanin is well established, many structural genes and regulatory genes have been cloned and applied to flower color breeding. In the whole biosynthetic pathway, F3'H and F3'5'H played crucial roles in determining the hydroxylation pattern of flavonoids. F3'H catalyze hydroxylation at the 3'positions of the B-ring to produce dihydroquercetin (DHQ) while F3'5'H catalyze hydroxylation at the 3', 5'positions of the B-ring to produce dihydromyricetin (DHM). That is, F3'H and F3'5'H determined the type of anthocyanin and the color of petals. Since *F3'H* and *F3'5'H* gene were first isolated from petunia in 1999 (Brugliera et al., 1999) and 1993 (Holton et al., 1993) respectively, many studies have isolated these two genes from different ornamental plants by homologous cloning technology and applied it to color improvement.

Cyclamen is one of the world's best-selling potted plants due to better ornamental traits and simple cultivation management. Most of the commercial cyclamen are obtained from a single wild type, purple flower *C. persicum* ($2n=2x=48$), through natural variation and the hybridization of mutants (Akita et al., 2018). In recent years, some genes related to flavonoid synthesis of cyclamen have been cloned (Akita et al., 2011; Kitamura et al., 2012). In 2010 (Boase et al., 2010) a full-length cDNA of *F3'5'H* (*CpF3'5'H*) had been isolated from *C. persicum* and the function also been characterized. But up to now the molecular and biochemical characterization of F3'H in cyclamen has almost not been described. Color mutants are good

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materials for studying the function of genes related to anthocyanin synthesis. The major anthocyanin components in STR petals has been changed from malvidin 3,5-diglucoside (Mv3,5dG, a major anthocyanin in *C. persicum*) to peonidin 3-*O*-neohesperidoside (Pn3Nh). Meanwhile, the flow of synthesize Pn3Nh and Mv3,5dG was partly controlled by F3'H and F3'5'H, respectively. To explore the relationship between F3'H and STR flower color formation, here we isolated STR*F3'H* genes obtained three ORFs, analyzed their sequences, compared the transcription level in *C. persicum* and STR. As far as we know this is the first report about *F3'H* isolated from cyclamen.

MATERIALS AND METHODS

Plant materials and extraction of total RNA

C. persicum and STR were grown in greenhouse facility at Saitama Institute of Technology. The petals of cyclamen were divided into two parts: a base part known as the 'eye' and all other part of the petal called 'slip'. Slips were sampled and immediately frozen in liquid nitrogen then kept at -80°C until required. Total RNA was extracted from the slips, following cetyltrimethylammonium bromide method.

Isolation of *F3'H-like* genes

First-strand cDNA was synthesized from the RNA extracted from slips using an oligo(dT)-anchor primer (5'-GAC TCG AGT CGA CAT CGA T_{17}-3') with reverse transcriptase according to the manufacturer's instructions (PrimeScript II 1st cDNA synthesis kit, TaKaRa). The degenerate primers were designed based on the conserved domains of plant F3'H proteins involved in anthocyanin accumulation. To isolate the putative *STRF3'H* full-length cDNA, 3'rapid amplification of cDNA ends (RACE) method and 5'-RACE method were carried out by using a 5'/3'-RACE 2nd Generation Kit (Roche, Germany). All polymerase chain reaction (PCR) products were cloned into the pTAC-2 Easy vector (BioDynamics Laboratory Inc., Japan), and the clones were sequenced by a DNA sequencer (Model 3500, Applied Biosystems) using the BigDye® Terminator ver. 3.1 Cycle Sequencing Kit (Applied Biosystems, MA, USA).

Multi-alignment analysis was performed by the Clustal W program, the deduced amino acid sequence of STRF3'H1 and STRF3'H2a, STRF3'H2b were aligned with other F3'H proteins that acquired from the DDBJ/GenBank DNA databases. Transmembrane domain was predicted by TMHMM (http://www.cbs.dtu.dk/services/TMHMM/). Phylogenetic trees were constructed using the Neighbor-Joining method with MEGA7.

Expression analysis of *STRF3'H* genes

To investigate the expression level of *F3'H* and *F3'5'H* genes between *C. persicum* and STR, we performed real-time PCR on the Eco™ Real-Time System with Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies). *STRF3'5'H* was cloned from *C. persicum* (GQ891056). The *eEF1a* genes were amplified as an internal control. Each experiment was repeated at least three times. The primers used for amplification were listed in Table 1.

Table 1. Primers used in this study.

Primer	Sequence (5'-3')	Primer	Sequence (5'-3')
C.perF3'5'H-FP	ATGGCACTAGACATAGTCTTGC	*STRF3'H2*-FPa	GAGGGAAGCTCACCGACACC
C.perF3'5'H-RP	TTAAGCAACATAGGCACTTGGG	*STRF3'H2*-FPb	GAGGCAAGCTCACCGACACT
STRF3'H1-FP3	TTGGTAGTTGGCCAAAACCG	*STRF3'H2*-RPa	TTCTCCGGCAATAGCCCCTCG
STRF3'H1-RP	TTAAGCCTGGTAAACTTCCTTAGCG	*STRF3'H2*-RPb	TCCTCCGGCAATAGCCCGTCT
STRF3'H1-RP5	CCGTGTTTCTGGTCACTTCC	*eEF1a*Fw	CTGGTGGTTTTGAGGCTGG
STRF3'H1-RP6	CCAACATTACACGTCCTAGC	*eEF1a*Rv	CTGGCCAGGGTGGTTCATGAT

RESULTS

Isolation and sequence analysis of *F3'H-like* genes from STR

Three ORFs (*STRF3'H1*, *STRF3'H2a* and *STRF3'H2b*) were isolated from slips of STR, deduced to encode 507, 517, 517 amino acids, respectively. The predicted molecular weights of these three amino acids were 56.1, 56.9, 56.9 kDa, and the calculated isoelectric point were 6.82, 7.03, 6.66, respectively. TMHMM online analysis of the amino acid sequences showed that both STRF3'H2a and STRF3'H2b have a transmembrane domain located at S_5 to T_{27}, the part of M_1 to P_4 located inside the microsomal membrane, K_{28} to V_{517} located outside. However, the prediction also showed that STRF3'H1 has no obvious transmembrane, and the entire peptide chain may be located outside membrane (Figure 1). A phylogenetic tree of several reported plant F3'H and F3'5'H protein sequences was constructed. STRF3'H1, STRF3'H2a, STRF3'H2b are clustered in the CYP75B clade together with other F3'Hs but further from cyclamen F3'5'H group in evolutionary distance (Figure 2).

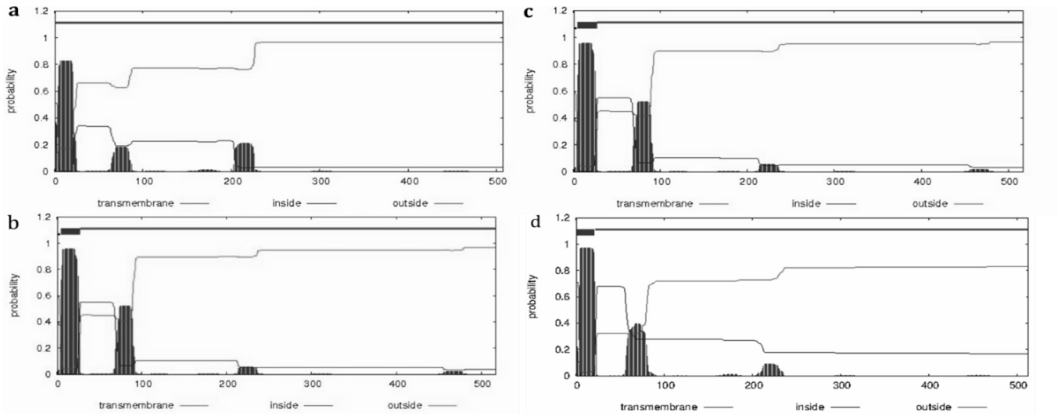

Figure 1. Predicted transmembrane structure domain of *STRF3'H1*(a), *STRF3'H2a* (b), *STRF3'H2b*(c) and *tt7*(d).

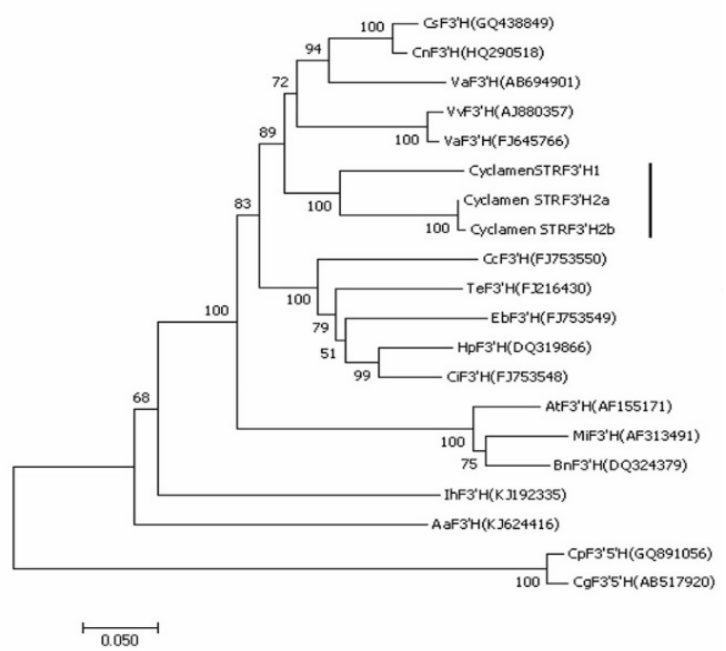

Figure 2. Phylogenetic tree analysis of STRF3'H and other published F3'Hs and F3'5'Hs. Vertical line indicates STRF3'Hs.

Comparison of the amino acid residues of *STRF3'H 1*, *STRF3'H 2a* and *STRF3'H 2b* with *Arabidopsis transparent testa 7* (*tt7*), showed 68, 66.4 and 66.2% identities, respectively. The amino acid sequence alignment revealed that the presumptive STRF3'H proteins contain several domains that highly conserved in plant F3'Hs. Three F3'H-specific conserved motifs, "VVVAAS", "GGEK" ($G_{417}GER_{420}$ of *STRF3'H1*), "VDVRG" ($A_{423}DVRG_{427}$" of *STRF3'H1*, $A_{433}DVRG_{437}$" of *STRF3'H2a,2b*) were found in STRF3'Hs amino acid sequences. In addition, four cytochrome P450-specific conserved motifs were also existed (marked by black line in Figure 3), the proline-rich region "PPGP", the heme domain "FGAGRRICAG", the oxygen-binding pocket "AGTDTS" and the E-R-R trinity for stabilize the core structure (Murakami et al., 1994; Werck-Reichhart et al., 2002). The phylogenetic tree and multiple alignment demonstrated that *STRF3'H1*, *STRF3'H2a*, *STRF3'H2b* encodes a flavonoid 3'-hydroxylase enzyme in planta.

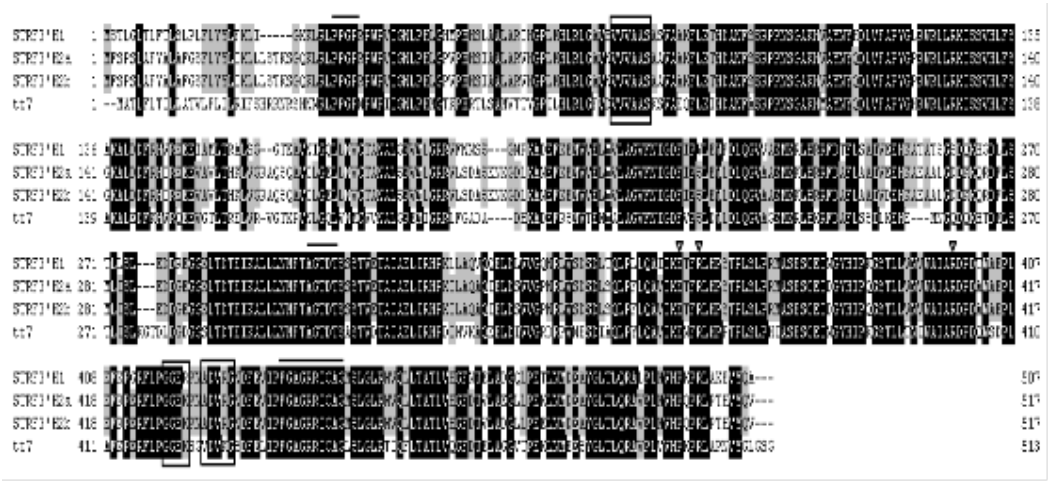

Figure 3. Multiple alignment of the deduced amino acid sequences of *F3'H1*, *F3'H2a*, *F3'H2b* from STR and *Arabidopsis TT7* (AF155171, 2000). The F3'H-specific motifs are marked with black boxes. The black lines indicate cytochrome P450-specific conserved motifs. Arrowheads indicate an E-R-R triad forming the pocket locking motif for the stabilization of the core structure.

Expression analysis of *F3'H* and *F3'5'H*

The expression pattern of *F3'Hs* and *F3'5'H* in *C. persicum* and STR were investigated by real-time PCR. From Figure 4, *F3'Hs* were abundantly expressed in STR, the relative expression of *F3'H1, F3'H2a, F3'H2b* in STR was about 25 times, 15 times and 18 times that of *C. persicum,* respectively. *F3'5'H* was expressed strongly in *C. persicum* about 33 times that of STR.

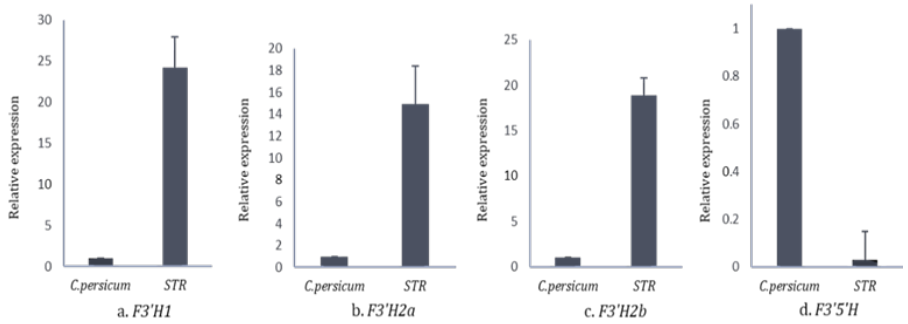

Figure 4. Real time-PCR analysis of *F3'H1* (a), *F3'H2a* (b), *F3'H2b* (c) and *F3'5'H* (d) transcription levels in *C. persicum* and STR. The *eEF1* gene was amplified as an internal control. The error bar represents the standard error.

DISCUSSION

This time we isolated three *F3'Hs* from STR, the homology between *STRF3'H2a* and *STRF3'H 2b* was 99.4%. *STRF3'H1* has 83% nucleotide sequence identity in the coding region to both *STRF3'H2a* and *STRF3'H2b*. By analyzing the assumed amino acid sequences of three *STRF3'Hs*, they all have the conserved motif specific to P450 and F3'H. "GGEK" is a unique motif to F3'H, an important feature that distinguishes them from their close relatives F3'5'H (Brugliera et al., 1999). But it presents as "GGER" in *STRF3'H1*, which were also found in *Vitis vinifera* (Castellarin et al., 2006), *Antirrhinum kelloggii* (AB547161) and other plants. Microsomal-type cytochrome P450 is a type of membrane protein that bounds to the membrane through the N-terminal transmembrane signal anchor domain to exert physiological functions (Murakami et al., 1994). The proline-rich region 'PPGP' at the N-terminal of F3'H, is considered to be the hinge motif necessary for P450 enzyme to anchor on the membrane. Although it was present in three STRF3'Hs, TMHMM speculated that F3'H2a and F3'H2b each have a transmembrane domain located at the 5th to the 27th amino acid, while F3'H1 may not. *Arabidopsis tt7* was also analyzed, the results were same as STRF3'H2a and STRF3'H2b (Figure 1). From amino acid sequence alignment, the N-terminal of F3'H1 was five amino acid residues less than that of F3'H2a and F3'H2b. Does this affect the anchoring of F3'H1 on membrane thus the structure and function? Actually, the functional analysis based only on the amino acid sequence is not very rigorous, and functional verification of F3'H is still needed.

The major anthocyanin components of *C. persicum* and STR were identified as the delphinidin-based blue/purple pigments and cyanidin-based red pigments, respectively. F3'H and F3'5'H are key enzymes at the branch of cyanidin and delphinidin synthetic pathway. It suggested that the red mutant of STR may be related to F3'H and/or F3'5'H. Previous studies have shown that F3'5'H is a key enzyme for synthesizing delphinidin and its derivatives (Holton et al., 1993). Loss of endogenous F3'5'H transcript will cause the flower color of cyclamen to change from purple to red/pink (Boase et al., 2010). According to the real-time PCR results, the expression level of *F3'5'H* in *C. persicum* was higher than that in STR. On the other hand, the red petals of STR contain a large number of Pn3Nh, and *F3'Hs* was expressed extensively in STR. *F3'H* is a vital structural gene of flavonoid anabolism, *Arabidopsis tt7* mutant that has no F3'H function exhibits a yellow seed coat, and the anthocyanin accumulation level of the mutant plant is lower compared to wild species (Schoenbohm et al., 2000). Overexpression of lisianthus *F3'H* gene in *I. nil* cultivar 'Violet' can change the flower color of violet that lacks F3'H function from red to blue (Takatori et al., 2015). Although to be determined, the high transcriptional level of *F3'H* and large accumulation of red pigment in STR suggested that F3'H may play an active role in STR flower color formation. However, the expression levels of these *STRF3'H* genes were also different. Further research is needed to determine which one is a functional.

CONCLUSIONS

In this study three *F3'H*-like genes (*STRF3'H1*, *STRF3'H2a*, *STRF3'H2b*) have been isolated and sequenced. *STRF3'Hs* was studied from the aspects of sequence alignment, conservative domain analysis, evolution analysis, expression pattern. These findings have laid a foundation for the in-depth analysis of F3'H function and the mechanism of cyclamen flower color formation in future research.

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Encoded chlorophyll degradation enzymes and gene expression in the exocarp during ripening of *Carica papaya* (L.) 'Krung', 'Khak Nual' and 'Khak Dum'

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Abstract

Papaya (*Carica papaya* L.) is a fast-growing fruit crop originating from Mexico and Central America. It is cultivated in many tropical and sub-tropical regions globally. In Thailand, papaya has been integrated into Thai cuisine and used as an ingredient in many food dishes and cosmetic products. Papaya fruit can be harvested either at mature green (for cooking) or color break (consumed as fresh fruit) stages. Although harvest time can be estimated from planting and bloom periods, harvest time varies due to cultivars and seasons. Thai farmers largely rely on fruit characters, skin texture and color as a harvest index. Papaya skin (peel or exocarp) is generally dark green when mature, indicating high levels of chlorophyll which is lost as fruit ripen. Among commonly grown papaya cultivars, 'Khak Dum', 'Pluk Mai Lie' and 'Khak Nual', the latter is commonly harvested and consumed at the mature green stage. Investigating changes in skin color during ripening, skin color was measured at 3 stages (mature, color break and ripe). Data collected were compared with the quantitative expression of five genes encoded enzymes in the chlorophyll catabolic pathway. The results showed the association of *RCCR* expression with skin color change. Chlorophyll degradation during ripening suggested skin color to be a good indicator for a fruit harvest index.

Keywords: fruit color, peel, flesh, papaya

INTRODUCTION

Papaya (*Carica papaya* L.) is one of the main horticultural crops of many tropical and subtropical regions globally. In 2018, most of the papaya production was concentrated in three countries, India, Brazil and Mexico (Food Agriculture Organization of the United Nations Statistics Division, 2020). In Thailand, papaya is an important fruit crop due to its short cultivation period and versatility for fresh use in food dishes. In 2018, Thailand cultivated 5,715 ha and produced 176,043 t of papaya (Food Agriculture Organization of the United Nations Statistics Division, 2020). The major commercial cultivars are 'Khak Dum', 'Khak Nual' and 'Pluk Mai Lai'. 'Khak Dum' and 'Pluk Mai Lai' are commonly consumed as ripe fruit. Papaya in Thailand has a sweet taste (10-14 °Brix) and good flavor. Conversely, 'Khak Nual' is used at mature green stages as an ingredient in Thai cuisine. Like most other cultivated fruits, papaya harvest time can be estimated days of fruit growth. However, there is considerable variation in harvest time based on cultivars, and seasonal growing conditions (Yang et al., 2010).

Another index that commonly used by papaya growers is the peel color. Coloration of the peel is due to the presence of two main pigments; chlorophylls that provide green color, and carotenoids, which are responsible for the characteristic coloration of mature fruit (Rodrigo et al., 2013). Pigments responsible for change in the peel color from mature green to yellow at the ripe stage is characterized by a rapid decrease in chlorophyll via the chlorophyll catabolic pathway.

Chlorophyll degradation is an important process in fruit ripening and senescence in

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plants. It is regulated by light and plant hormones such as ABA and ethylene (Zhu et al., 2017). Plants regularly dispose of damaged and or old senescent leaves. The nutrients from those leaves are transferred to new organs. Chlorophyll is broken down and colorless catabolites are stored in the vacuole. For fruit, chlorophyll degradation occurs during the ripening process. The degradation involves the removal of Mg^{2+} and phytol from the chlorophyll molecule by chlorophyllase which produces chrlorophyllide and pheophyllide. Pheophyllide is converted into pFCC by pheophorbide a oxygenase (PAO) and red chlorophyll catabolite reductase (RCCR). This is eventually converted to tetrapyrrolic ring (NCCs), a colorless molecule that stored in the vacuole. In order to determine the significance of papaya peel color changes and ripening responses, color changes during fruit ripening and expression study of genes encoded enzymes in chlorophyll catabolic pathway included chlorophyllase (CpC), pheophytinase (PPH), pheophorbide a oxygenase (Pao1), pheophorbide a oxygenase (Pao2) and red chlorophyll catabolite reductase (RCCR) were investigated in papaya 'Khak Nual'.

MATERIALS AND METHODS

Color measurement

Papaya peel and flesh color of 'Khak Nual' harvested based on flesh color at mature, color break and ripe stages (five fruit samples for each stage) were measured using a Minolta CR400 colorimeter (Tokyo, Japan). Color measurement was determined based on the value of L*, a*, b*, Chroma and Hue parameters. Parameter a* takes positive values for reddish color and negative values for the greenish color. Whereas b* takes positive values for yellowish color and negative values for the bluish color. L* is an approximate measurement of luminosity. Chroma is considered the quantitative attribution of colorfulness and used to determine the degree of difference of a hue in comparison to a gray color with the same lightness. Hue angle is considered the qualitative attribute of color, which is the property according to which each color can be considered as equivalent to a member of the gray scale, between black and white (Granato and Masson, 2010).

Expression studies of genes in chlorophyll catabolic pathway

The expression levels of genes encoded enzymes in chlorophyll catabolic pathway in 'Khak Nual' was compared with 'Khak Dum' (consumed at ripe stage) and 'Krung' (consumed at mature stage). Total RNA samples were isolated from papaya peel using the RNeasy Mini Kit (QIAGEN, USA). The gene expression was determined by quantitative RT-PCR analysis. Twenty-microlitre reaction contained 10 µL of 2x KAPA SYBR FAST qPCR master mix, 50 ng of total RNA, 0.4 µL of 10 mM primes, 0.4 µL of 10 mM dNTPs, 0.4 µL of 50x KAPA RT mix. Thermal cycling was 42°C for 5 min, 95°C for 5 min, 40 cycles of 95°C for 3 s, 50-62°C 30 s and 72°C for 20 s. Relative expression levels were calculated using the methods presented by Livak and Schmittgen (2001). Values were normalized against the expression level of the housekeeping gene actin.

RESULTS AND DISCUSSION

The changes in peel and flesh color during ripening are shown in Tables 1 and 2. For peel color, there was no difference in a* value (green color) at mature and color break stages, while a significant drop had occurred by the ripe stage. There were significant changes of b* (yellow color) value in all stages; 25.41±2.99 at mature, 30.76±4.72 at color break and 65.88±3.09 at the ripe stage. This suggests a significant decrease in chlorophyll content. This outcome is supported by a study of 'Sekaki' papaya by Ruslan and Roslan (2016). Comparing flesh color measurements, an increase in carotenoid content at color break and ripe stage was found. A raise in b* value was found, from 14.27±2.87 at the mature stage to 30.76±4.72 and 65.88±3.09 at the color break and ripe stages, respectively. These values were previously reported to be an indicator for carotenoid accumulation (Jing et al., 2105; Saengmanee et al., 2018). This result suggested that b* value is a good indicator for 'Khak Nual' to be harvested at the mature stage.

Table 1. Color measurement of papaya peel.

Papaya stage	L*	a*	b*	Chroma	Hue
Mature (M)	37.46±2.04c	-17.17±1.62a	25.41±2.99c	30.63±3.35c	124.01±1.06a
Color break (CR)	47.79±4.81b	-18.24±1.32a	30.76±4.72b	35.90±4.24b	121.13±3.63a
Ripe (R)	68.00±2.35a	-10.62±3.38b	65.88±3.09a	66.79±3.52a	80.94±2.49b

L*, a*, b*, Chroma and hue values were analyzed using analysis of variance (ANOVA) and Duncan's multiple range test (DMRT). Data within the same column followed by different letters are significantly different at p=0.05. a* represent negative values for the green color, whereas b* represent positive values for yellow color and negative values for the blue color.

Table 2. Color measurement of papaya flesh.

Papaya stage	L*	a*	b*	Chroma	Hue
Mature (M)	75.78±2.25a	-2.72±0.42c	14.27±2.87c	14.52±2.89c	101.00±1.22a
Color break (CR)	71.94±3.52b	11.41±5.69b	36.20±1.69b	38.35±1.81b	72.72±8.52b
Ripe (R)	61.44±1.46c	36.05±1.83a	44.13±1.55a	56.99±1.89a	50.77±1.48c

L*, a*, b*, Chroma and hue values were analyzed using analysis of variance (ANOVA) and Duncan's multiple range test (DMRT). Data within the same column followed by different letters are significantly different at p=0.05. a* positive values for red color and negative values for green color, whereas b* represent positive values for yellow color and negative values for the blue color.

The quantitative analysis of five genes encoded enzymes in chlorophyll catabolic pathway in papaya peel for the three cultivars during ripening were determined (Figures 1 and 2). Gene expression was highest in 'Khak Nual'. The expression of all five genes was highest at the ripe stage. Among the five genes, *RCCR* expression was highest. Comparing between 3 developmental stages, RCCR expression at ripe stage was the highest. Only *RCCR* expression in 'Khka Nual' continued raising during ripening (Figure 1). *RCCR* expression in 'Khak Dum' peaked at color break and then declined while the expression in 'Krung' declined at color break and then increased again at ripe stage (data not shown). The possible role of this enzyme was investigated. For apple, *RCCR* expression was inhibited with the use of 1-MCP and delayed apple ripening (Lv et al., 2020). For banana, peel color is the major indicator of ripeness. The presence of new fluorescent chlorophyll catabolites (FCCs) and non-fluorescent chlorophyll catabolites (NCCs) were reported by Moser et al. (2012). This is indicatives of the variation in chlorophyll degradation in leaves and fruit. The roles of other enzymes in chlorophyll catabolism were reported in litchi by Hu et al. (2019). Results in tomato, suggest different mechanisms of chlorophyll degradation for leaves and fruits. While PPH was a core hydrolase enzyme of leaves, other hydrolase(s) played a major role in fruit chlorophyll degradation (Guyer et al., 2014). Nguyen et al. (2014) reported that GLK2 (Golden2-like transcription factor) controlled chlorophyll content in fruit while GLK1 regulated it in leaves. Seifert et al. (2014) reported that chlorophyll breakdown can be measured non-destructively via spectral shift on fruit peel and positively correlated it to ripening of apple, mango, and tomato. In addition, the differential gene expression level of these genes was also found to contribute to peel color. In papaya, understanding the pigment changes may be useful for manipulating coloring, and better co-ordinate softening as well as establishing harvest guidelines.

CONCLUSIONS

The chlorophyll degradation in the peel of 'Khak Nual' papaya during ripening was studied. The results suggested a positive correlation of an increase in b* value (yellow color) with ripening and expression pattern of RCCR, an enzyme at the last step of chlorophyll degradation.

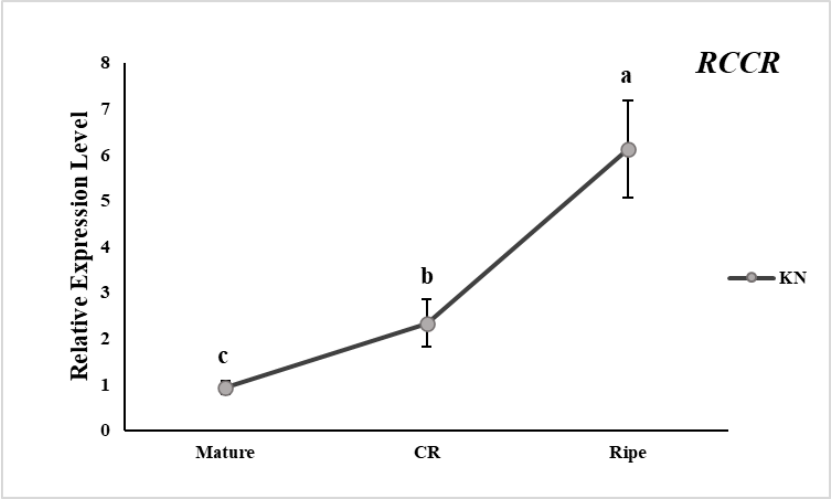

Figure 1. RCCR expression level during ripening of 'Khak Nual' (KN). As indicated, significant difference of means at p=0.05. Five replicates were analyzed per sample using quantitative RT-PCR.

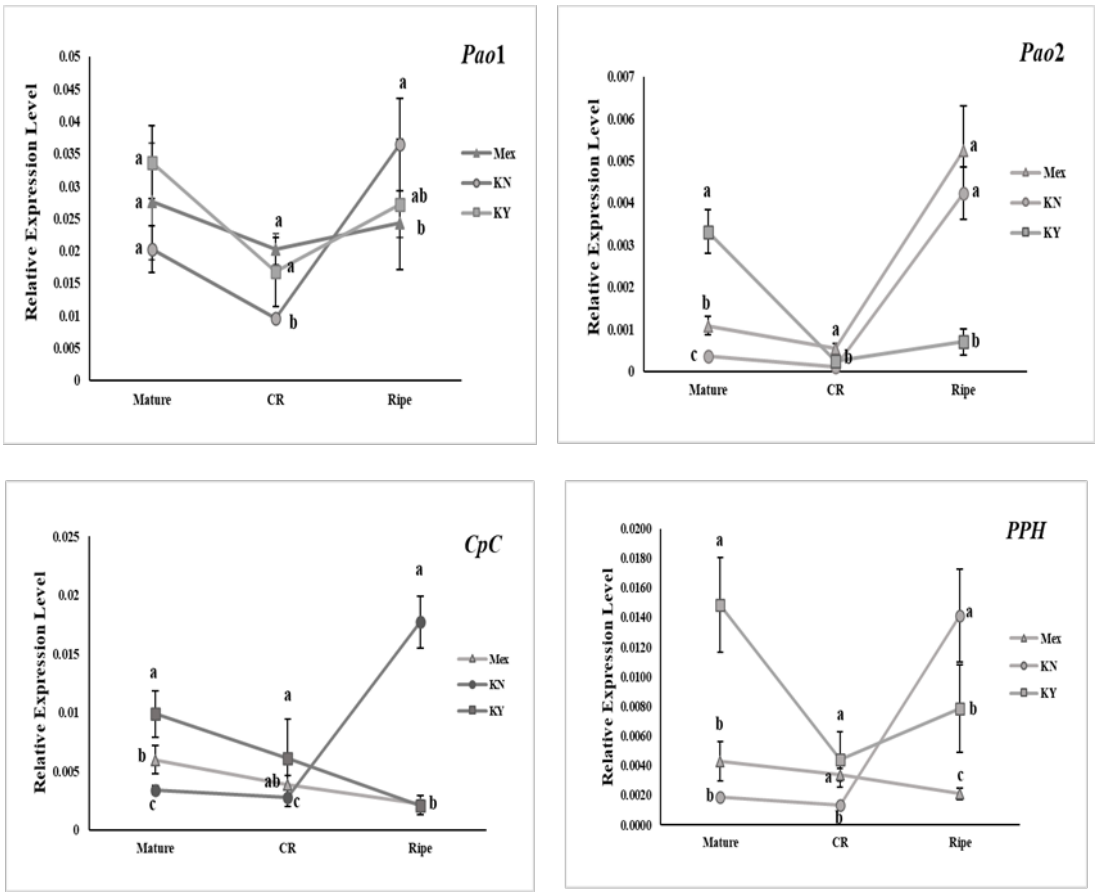

Figure 2. Expression studies of four genes encoded enzymes in chlorophyll catabolic pathway (*CpC*, *PPH*, *Pao1* and *Pao2*) during ripening of 'Khak Nual' (KN), 'Krung' (KY) and 'Khak Dum' (Mex). As indicated, significant difference of means at p=0.05. Five replicates were analyzed per sample using quantitative RT-PCR.

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Frequent reversion and continuous size variation in the small fruit mutant 'Totsutanenashi' persimmon

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Abstract

Fruit size is a commercially important trait of fruit tree species. In many cases, it is under polygenic control. Fruit size differentiation that occurs from bud sport mutants provides good samples for the elucidation of the complex molecular and genetic basis of fruit size determination and will benefit the development of breeding strategies that are focused on fruit size. 'Totsutanenashi' (TTN) persimmon, bearing small-sized fruit, is a bud-sport mutant of 'Hiratanenashi' (HTN), an important persimmon cultivar in Japan. The fruit of TTN is significantly smaller than that of HTN, making TTN a suitable subject for the study of fruit-size determination mechanisms. Recently, multiple reverse mutations leading to large fruit were found as bud-sports on different TTN trees. Additionally, large variations in fruit size were observed among different TTN branches. The variation was branch-dependent and the reverted fruits, which were larger than TTN fruit, were observed for two successive years in the same branch. A heritable factor was, therefore, suggested to be involved in the variation. Collectively, TTN and its revertants are appropriate plant materials for investigating possible genetic and epigenetic systems involved in fruit-size changes.

Keywords: bud sport, mutant, fruit size, revertant, physiology

INTRODUCTION

Fruit size is a commercially important fruit trait that influences consumer preference. Understanding fruit-size determination aids targeted fruit-size breeding and production. Fruit size is also an important food security issue because it is often related to final yield. Fruit size is controlled by both environmental and genetic factors, with the latter having a stronger impact as indicated by minor genetic changes having a dramatic influence on fruit development and, thus, final fruit size. Persimmon (*Diospyrous kaki* Thunb.) varies greatly in fruit size and shape. It is, therefore, a good research subject to study fruit-size control. Additionally, bud-sport mutants for fruit size are available in this species.

Bud-sport mutations found during cultivation are common sources of trait variation among horticultural crops (Azuma et al., 2009; Minas et al., 2015). Bud-sport mutants, natural mutations occurring on a single branch or spur in an orchard, provide valuable new characteristics while retaining the desirable qualities of the original parent plant (Foster and Aranzana, 2018). Thus, these mutants retain most of the parental genetic background and are considered desirable genetic materials to study the mutations responsible for novel traits (Otto et al., 2014). In tree fruit, somatic mutations that alter overall reproductive growth characteristics, such as fruit-set behavior, fruit color, size, shape, and maturity, have been found previously (Petit and Hampe, 2006). However, few fruit-size mutants in fruit trees, such as a large-fruit mutant in pear (*Pyrus communis* L.), 'La France' (Isuzugawa et al., 2014), have been reported to date.

'Totsutanenashi' (TTN) persimmon is a small-fruit bud-sport mutant of 'Hiratanenashi' (HTN), which is a major persimmon cultivar in Japan. The fruit of TTN is significantly smaller than that of HTN throughout fruit development (Yamane et al., 2008). TTN was first discovered in Masaharu Kondo's orchard in Sado, Niigata Prefecture and is now regarded as a cultivar of potential market interest, not only because of its small cute appearance but also

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because it has other appealing attributes, such as higher sugar content (Yamane et al., 2008). Both HTN and TTN are nonaploid and, therefore, ploidy change did not appear to be responsible for the small-fruit mutation (Yamane et al., 2008). Although fruit enlargement is the result of cell division and cell expansion, a reduction in the parenchymal cell number is the main factor causing the decreased fruit size in TTN during the early growth stage (Habu et al., 2016). Cytokinins are plant hormones that regulate cell division, and exogenous treatments with a synthetic cytokinin CPPU induce the enlargement of TTN fruit to normal size, implying the possible involvement of cytokinins in the small-fruit mutation (Habu et al., 2016; Naito et al., 2018).

In recent years, multiple reverse mutations affecting fruit size have been found in TTN trees and named as TTN-revertants (TTNRs) (Figure 1). TTNRs show variations in fruit size compared with HTN fruit and are therefore additional useful genetic materials for further studies on the fruit-size determination system in persimmon. In this study, fruit-size variance in a TTN tree was characterized. The data may supply fundamental information for future studies on, and the horticultural use of, TTN.

Figure 1. Image of TTN individual with a bud-sport mutated stem producing TTNR taken in October 2016.

MATERIALS AND METHODS

Persimmon cultivars HTN and TTN, as well as TTNRs, growing in the experimental orchard at Kyoto University, Kyoto, Japan (34°N; 135°E) were used in this study. A TTNR found in a commercial orchard in Gifu, Japan was multiplied by grafting on a seedling rootstock and planted in pots in the early spring of the 2015 growing season. Trees were grown in 25 or 60 L pots using standard management practice, including normal pruning, irrigation, and fertilization. All the measurements of growth characteristics were performed in 2016. The branches for fruit size analysis were selected randomly. Fruit diameters were measured for five fruits per genotype using calipers. Internode lengths of vegetative shoots were measured from the third to seventh internodes on three branches of uniform strength. The top three leaves were sampled for each genotype and scanned with a GT-9800F meter (Epson, Suwa, Japan). Leaf areas were measured from the scanned images using ImageJ software (Schneider et al., 2012).

Fruit size variation analysis was conducted using a single TTN tree planted in the field of the experimental orchard at Kyoto University in the 2018 and 2019 harvest seasons. The tree was a mature, large plant of approximately three meter height with an open-centered growth form. In the 2018 season, branches bearing larger fruit than the typical TTN fruit were labeled. During the experiment, the tree produced hundreds of fruit-bearing shoots and most of the shoots produced the typical TTN fruit. Two or three mature fruit were sampled per branch and weighed. The statistical analysis was performed using a one-way analysis of variance in SPSS. (v23.0.0.0).

RESULTS AND DISCUSSION

Phenotypic comparison of HTN, TTN and TTNR

Fruit sizes, leaf areas and internode lengths of HTN, TTN and TTNR plants are shown in Figure 2. The patterns of fruit enlargement were similar between HTN and TTNR (Figure 2A) while changes in the diameters of TTN fruit occurred in slower increments during the fruit developmental period, especially during the last stage of fruit growth, compared with HTN and TTNR (Figure 2A). The average TTN fruit diameter was less than half those of the HTN and TTNR fruit at the time of commercial harvest in 2016. The average leaf area and internode length of TTN were each significantly smaller than those of HTN and TTNR (Figure 2B, C).

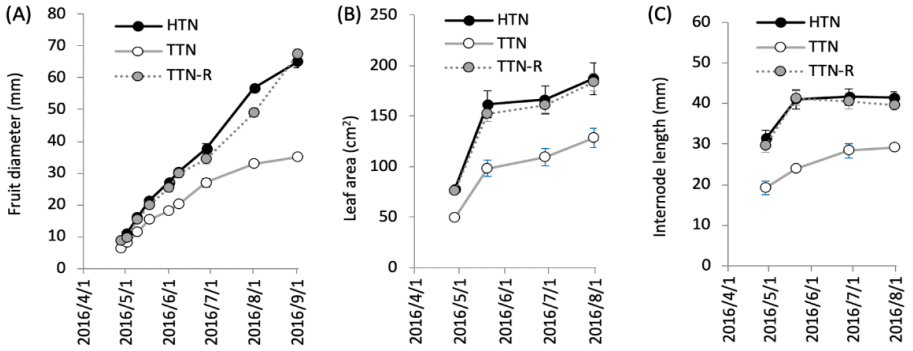

Figure 2. Comparison of fruit diameter (A), leaf area (B) and internode length (C) of HTN, TTN and TTN-revertant (TTN-R) in continuous development stages in 2016. The error bar represents standard error.

Fruit-size variation occurred on TTN branches after cultivation for two consecutive years

TTN branches bearing larger mature fruit than the typical TTN small sized fruit were numbered in the 2018 harvest season. One TTN branch bearing fruit similar in size to those on the other TTN trees was used as the control. The average fruit weight of the control group was 26.3 g, whereas the maximum and minimum average fruit weights of other branches were 78.8 g (No. 10 branch) and 34.8 g (No. 11 branch) (Figure 3). According to a one-way analysis of variance, the fruit weights of the No. 1, 2, 3, 7, 8, 10 and 14 branches were significantly different from that of the control group. The average fruit weights of the No. 2, 3 and 10 branches were ~2.5 times greater than that of the control. Although the weights of fruit on other branches were not statistically different from the control, it should be noted that there were fruit-size variations within branches. For example, on the No. 13 branch, the maximum fruit weight was 45.2 g, while the minimum was 33.3 g.

Increases in fruit-size in reversion events on TTN branches in two consecutive years

In the 2018 and 2019 harvest seasons, mature fruits of the No. 2, 10 and 15 branches were sampled (Figure 4). Compared with the typical TTN fruits, significantly larger fruits were produced on these branches. The reverted branches produced significantly larger fruit than the typical TTN fruit both in the 2018 and 2019 seasons, though relatively larger fruit was obtained in the 2019 season compared with the 2018 season (Figure 4). Based on the branch locations, these branches resulted from three independent reversion events in a single TTN tree. Because larger fruits than those from the typical TTN branches were produced in separate years in the same branches, it is suggested that the reversions were controlled by heritable factor(s). This indicates that TTN, as well as each TTNR line, provide unique materials that can be used to elucidate the genetic control of fruit size in persimmon.

Because consumers generally prefer a uniform fruit size, the fruit-size diversity within TTN may adversely affect the commercial value of the fruit from this cultivar. Different levels of branch-dependent size variation were observed in a single TTN tree (Figure 3), suggesting

the necessity for the further selection of stable small-fruit producing lines. The results also suggest the involvement of heritable factors that control fruit size in addition to those affected by the mutation from HTN to TTN. Some branches had potential reversion but it was not possible to confirm the stability of that reversion. However, at least three branches (No. 2, 10 and 15) were independently reverted in a single 3-m height tree, indicating a remarkably high frequency of the reversion events. This high frequency of the reversion events in different branches suggests that common loci underly the observed reversions, and that epigenetic control was most likely a reasonable explanation for the observed situation.

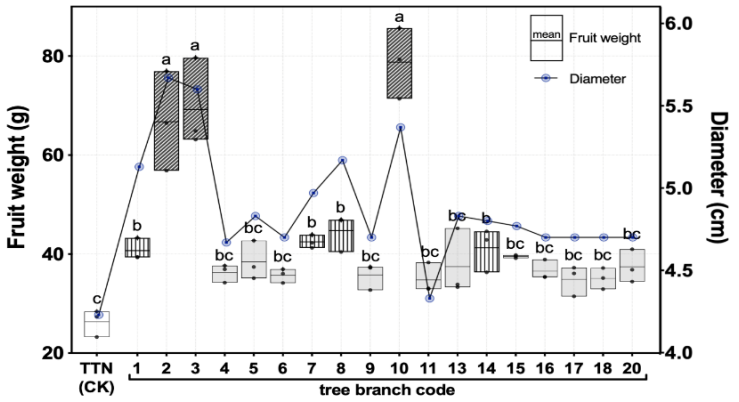

Figure 3. Fruit weight (g) and diameter (cm) of fruit in different branches on the TTN tree. The floating bars represent the fruit weight range, from minimum to maximum, of each branch, and the line inside each bar represents the mean value. Values with different letters (a, b, c) were significantly different according to the Tukey test at 0.05 level.

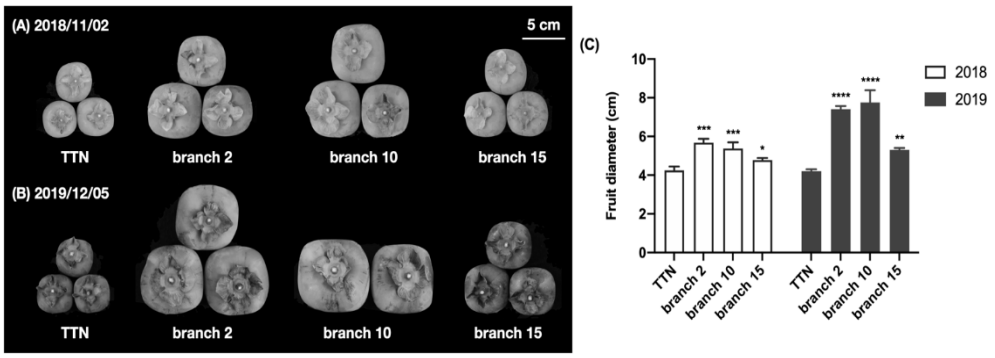

Figure 4. TTN fruit and revertant fruit in No. 2, 10 and 15 branches in the 2018 (A) and 2019 (B) harvesting seasons. (C) Comparisons of fruit diameter. Asterisks indicate significant differences of diameters compared within each year (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ordinary one way ANOVA). Each bar represents the mean ± SD of two or three biological replicates.

CONCLUSIONS

TTN is a small-fruit mutant of persimmon that originated from HTN, which has normal-sized fruit. Several reverse mutations, which resulted in fruit of varying sizes, were found on TTN trees. Reversions of not only fruit size but also of vegetative growth were observed, which suggests that these reversion events occurred through the same mechanism responsible for the mutation from HTN to TTN. It is worth noting that some of the TTN branches produced significantly larger but not fully reverted fruit. The significantly larger fruit sizes were observed in both 2018 and 2019 seasons in the same branch and, thus, may be conferred by

heritable factors. Epigenetic regulation may explain this situation, and further analysis of the mechanism behind the size variation is ongoing using high-throughput sequencing.

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Developing a successful micropropagation for *Albizia myriophylla* Benth.

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Abstract

***Albizia myriophylla* Benth. (Cha Em Thai) is a native medicinal plant in northeastern Thailand. It has a sweet flavor and its functions include resolving dental caries, aphthous ulcers and coughs. The purpose of this study was to create a clean culture for mass propagation of planting material. Initially, lateral buds were surface sterilized with 10 and 15% Clorox®, respectively. Results showed 40% of the sterilized material survival as explants. These buds were cultured on Murashige and Skoog (MS). After culturing for four weeks, the lateral buds develop white callus tissue (60%) and young shoots (30%). Furthermore, young seeds were sterilized with 10-15% Clorox®, then cultured on semi-solid Woody Plant Medium (WPM) or MS medium with or without BA. The results showed a significant difference at the $p<0.05$ level. The highest survival rate of 97.5% and a germination rate of 82.5% was achieved when cultured on MS medium with 4 mg L^{-1} BA. In addition, after surface disinfecting mature seeds with 3% hydrogen peroxide, they were cultured for three weeks in a semi solid liquid MS, containing 0, 2 and 4 mg L^{-1} BA. Furthermore, the MS medium received 0.1 mg L^{-1} NAA and 4 mg L^{-1} BA. The PGRs free WPM media were cultured for three weeks. Findings showed the highest survival rate of 70% was from seeds culture on semi-solid MS medium containing 2 mg L^{-1} BA. However, the highest germination rate of 62.5% was significantly different at the $p<0.05$ level when cultured in PGRs free liquid MS medium.**

Keywords: tissue culture, plant growth regulators, in vitro, medicinal plant, Cha Em Thai, native plant

INTRODUCTION

Albizia myriophylla Benth. (*Fabaceae*), locally known as "Cha Em Thai" is a medicinal native plant found in Assam, Bangladesh, Cambodia, East Himalaya, India, Laos, Malaya, Myanmar, Vietnam, and Thailand. The sweet flavoured *Albizia myriophylla* is traditionally used for dental caries and aphthous ulcer (Neamsuvan et al., 2012), irregular menstrual flow (Grumezescu and Holban, 2018) and has very strong antibacterial with cytotoxic activities (Joycharat et al., 2016). Phana Thai Traditional Medical Centre (Thailand) has a high demand for its use in raw material form, to develop new unique products. However, the availability of this plant is substantially deficient. Furthermore, it is propagated by seed and has a low natural germination rate due to its steely seed coat (Tropical Plants Database, 2020). It is also difficult to propagate via cuttings or grafting. Due to the difficulty in propagating *A. myriophylla*, a mass micropropagation method is being developed to establishing explants and to conserve this treasured plant. Plant tissue culture is routinely applied to propagate rare or economic plants. For instance, *Flemingia macrophylla* (Sirikonda et al., 2020), *Ceratonia siliqua* (Saïdi et al., 2019) and *Zhumeria majdae* (Fallah et al., 2019) were all successfully propagated, whereas *A. myriophylla,* has never been successful propagated. Furthermore, the different types and concentrations of plant growth regulators (PGRs) depend on cultural conditions, type of explant, and plant genotype (Gaspar et al., 1996). Benzyladenine (BA) not only plays a role in shoot morphogenesis during seed germination (Nikolić et al., 2006), but it is active in metabolism during all phases of germination from imbibition to radicle emergence and the start of seedling establishment (Nikolić et al., 2006; Stirk et al., 2005). Nevertheless,

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using BA for stimulating the rate of seed germination through the use of different concentrations for different plant species such as at 0.25 mg L^{-1} BA in *Farsetia macrantha* (Choudhary et al., 2020), and 10 µM in *Digitalis purpurea* (Patil et al., 2012), and 0.22-3.50 µM BA in *Lotus corniculatus* (Nikolić et al., 2006) has been undertaken. The establishment of sterilized explants for in vitro propagation can be beneficial for mass propagation, conservation, and sustainable use of *A. myriophylla*.

MATERIALS AND METHODS

An expert taxonomist performed the initial identification of the explant material used in this study.

Lateral bud culture

Lateral buds from young shoots of *A. myriophylla* were collected from the Don Ling Jao Phu Forest Park (Geographical coordinates; GC; 15.669471; 104.857603). Buds were cleaned with tap water and detergent, then sprayed with 70% ethanol. They were then soaked in sterilized water containing 15% of Clorox® with 1-2 drops of tween-20 for 10 min. The explants were then soaked in 10% Clorox with 1-2 drops of tween-20 and shaken for 15 min. The explants were then rinsed with sterilized distilled water three times for 5 min. The sterilized lateral buds were cultured on 0.7% agar MS medium (Murashige and Skoog, 1962) supplemented with 30 g L^{-1} sucrose at pH 5.8. Plants were placed in a culture room at a temperature of 26±1°C, with a photoperiod of 16 h at a light intensity of 38 µmol m^{-2} s^{-1} (provided by fluorescent daylight lamps). After one month, surviving explants and contamination percentage were recorded.

Immature seeds culture

Immature seeds embed in fresh, green, and unopened pods were collected from Sireeruckhachati Nature Learning Park, Mahidol University, Nakhon Pathom, Thailand (GC: 13.790095; 100.318333) (Figure 1A). The pods were soaked in 15% Clorox with 1-2 drops of tween-20 added for 5 min. Pods and seeds then soaked in a 10% Clorox with 1-2 drops of tween-20 added for 15 min. The pods were then rinsed with sterile distilled water for 5 min three times. Finally, the immature seeds were removed from the sterilized pods and cultured on 0.7% agar MS medium supplemented with 0, 2 and 4 mg L^{-1} BA and Woody Plant Media (WPM) medium. In addition, all media contained sucrose, 30 g L^{-1}, maintained at a pH of 5.8, and placed in a culture room at a temperature of 26±1°C with a photoperiod of 16 h and a light intensity of 38 µmol m^{-2} s^{-1} (provided by fluorescent daylight lamps). After culturing for two and three weeks, data collected and recorded on the percentage of contamination, survival of uncontaminated, and germination rates.

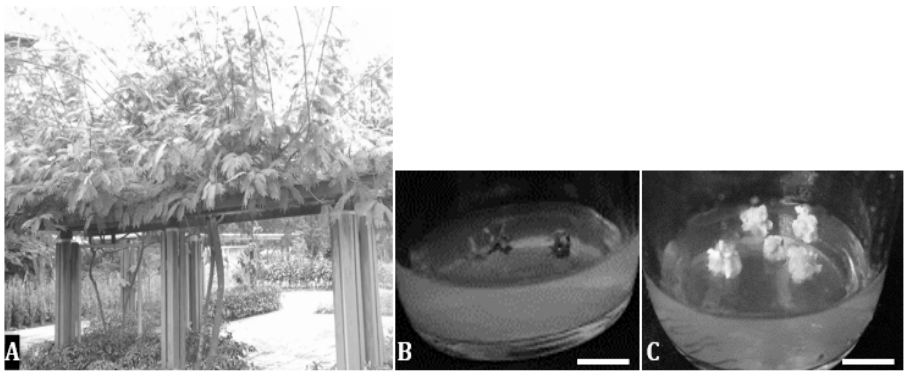

Figure 1. Plant material used for lateral bud culture of *Albizia myriophylla* at the "Sireeruckhachati Nature Learning Park" (A), lateral buds after surface sterilization and cultured for four weeks (B), and callus formation on new explant shoots after transferring to MS medium 4 mg L^{-1} BA (C) at six weeks (scale bar = 1 cm).

Mature seeds culture

In the case of mature seeds, dried, brown, and opened pods were collected from Sireeruckhachati Nature Learning Park, Mahidol University, Nakhon Pathom, Thailand. Initially, the dried seeds were separated from pods and soaked in 3% (v/v) of hydrogen peroxide for 5 min. The seeds were then rinsed in sterilized distilled water three times for 5 min each. The cleaned seeds were cultured on PGRs free-MS liquid medium. The semi-solid MS medium contained 0, 2 and 4 mg L^{-1} BA, with 0.1 mg L^{-1} NAA and 4 mg L^{-1} BA, and aPGRs free-WPM semi-solid medium (total six treatments). All of the media had 30 g L^{-1} of sucrose, 7 g L^{-1} agar and 400 µL of Plant Preservative Mixture (PPM) (PhytoTechnology Laboratories) as a biocide. Similar to what is used in a tissue culture system. Seeds and media were placed in a culture room at a temperature of 26±1°C, at a photoperiod of 16 h and light intensity of 38 µmol m^{-2} s^{-1} (provided by fluorescent daylight lamps). After culturing for one week, the seeds in the liquid medium were placed into a PGRs free-MS semi-solid medium for shoot elongation. This medium is similar to other treatments. Seeds were cultured for three weeks, and data collected on the percentage of contamination, survival of uncontaminated, and germination rates.

The immature and mature seeds culture experiments were performed in a completely randomized design with 10 replications per treatment, with 40 seeds per treatment. Data analysis consisted of analysis of variance (ANOVA) using the statistical software PASW Statistics 18. The results of the ANOVA, data from the qualitative factors were compared by the Duncan multiple range test (DMRT) ($p<0.05$).

In addition, seedlings from previous studies were subcultured onto semi-solid MS medium to increased the number of explants for further studies. an additional 20 matured healthy plantlets were tested for acclimatization through the following process. Firstly rooted plants were placed in a culture container at room temperature for a week. The lid of the bottle was slightly unscrewed. This allowed the plantlets time to adjust from the ex vitro state. The rooted plants were then transplanted into a plastic pot. Before transplanting the roots were washed with tap water to remove the agar. Plantlets were placed into a plastic pot with a medium consisting of soil:rice husk-charcoal:raw rice husk:coconut coir in the ratio of 1:1:1:1. The medium was moistened with water, covered with a transparent plastic bag and placed in a well lit area avoiding direct sunlight for two weeks. The plastic bag was then opened and plants grown on for additional two weeks. The plastic bag cover was removed and plants grown on for an additional week. The soil surface was watered once a day. The potted plants were then transferred to greenhouse under 50% sunlight for three weeks. After transplanting and acclimatization for nine weeks, the survival rate of the acclimatized plantlets was recorded.

RESULTS AND DISCUSSION

Lateral bud culture

After surface sterilization and culturing the lateral bud explants on semi-solid MS medium for two weeks, a 40% contamination rate and 40% survival rate was obtained. Compared to studies by Selvaskanthan et al. (2018) who showed surface sterilization using 10% (v/v) of Clorox® for 10 min, and 0.2% of $HgCl_2$ for 5 min resulted in 73% of uncontaminated green live cultures of immature axillary buds of *Gyrinops walla*. In addition, Arumugam et al. (2020) study of *Plectranthus amboinicus* included three main sterilisation steps. First gently brushing explants and then soaking in 70% (v/v) ethanol for 10 s, followed by soaking in a 40% (v/v) Clorox® for 15-20 min, resulted in 71.11% survival of uncontaminated explants. The use of Clorox® is usually reserved for used with lateral bud surface sterilization at various concentrations and time exposures depending on strength, age, and growing environment of the explants.

After one month, or results showed 30% of the lateral buds were developing young shoots, and 60% were starting to produce callus tissue. To induce shoots the explants were transferred to a MS medium containing 4 mg L^{-1} BA. However, after culturing for two weeks all explants developed callus tissue. Furthermore, after culturing for six weeks the callus

tissue did not develop any shoots (Figure 1).

Immature seeds culture

BA has a property to superintend growth and effect germination rates in a variability ways in diverse plants (El-Ghamery and Mousa, 2017). After surface sterilizing the fresh pods and culturing the separated immature seeds for three weeks, the contamination rate ranged between 2.5 and 12.5%. The highest epigeal germination rate was (82.5%) on MS medium supplemented with 4 mg L^{-1} BA. This was significantly different to all other treatments. The germinations rates of immature seeds when cultured on MS medium supplemented with 2 and 0 mg L^{-1} BA were 37.5 and 32.5%, respectively (Table 1). Likewise, Singh et al. (2019) showed BA can promote immature seed germination of *Artocarpus lakoocha.* Singh et al. (2019) studies found a maximum shoot regenerated of 7.23 shoots when cultured on MS medium with 4.44 µM BA. In *Pimpinella anisum*, the highest germination was related to the usage of BA compared to other plant hormones (Shahrajabian et al., 2019).

Table 1. Germination and contamination rate of *Albizia myriophylla* immature seeds on MS and WPM media for two and three weeks.

Medium	Contamination (%)	Survival[a] (%)	Total germinated seeds (%)	
			2 weeks	3 weeks
MS + PGRs free	7.5	42.5	17.5±0.35b	32.5±1.06b
MS + BA 2 mg L^{-1}	2.5	25.0	10.0±0.75b	37.5±1.88b
MS + BA 4 mg L^{-1}	7.5	97.5	62.5±0.99a	82.5±1.72a
WPM-PGRs free	12.5	47.5	15.0±1.18b	27.5±1.88b

The number (mean ± SD) followed by different letters indicate significant differences between groups at the $p<0.05$, analyzed by DMRT.

[a]Survival rate of uncontaminated seeds.

Results from this study showed the germination rate did not increase after culturing for four weeks. Therefore, the germinated seeds were transferred to a new hormone-free MS medium for an additional thee weeks of growth and shoot elongation (Figure 2E). Furthermore, nodes from these explants were subcultured and transferred onto MS to increasing the number of plantlets.

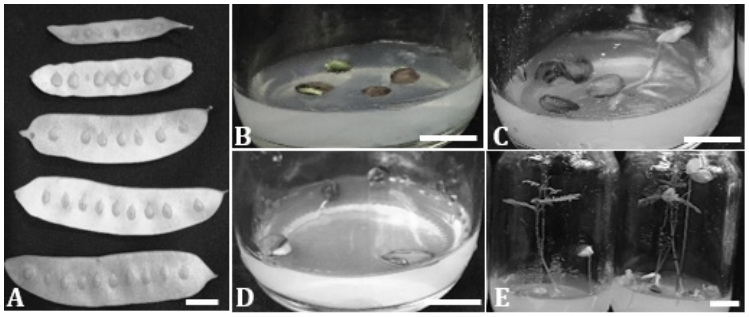

Figure 2. Images of immature seedpods, seed germination and seedling growth of *Albizia myriophylla* after culturing for three weeks; immature seeds in green pods (A), immature seed cultured on MS supplemented 2 mg L^{-1} BA (B), immature seeds cultured on MS supplemented 4 mg L^{-1} BA (C), immature seeds cultured on PGRs-free WPM medium (D), and seedling after being transferred to new PGRs-free MS medium (E) for three weeks (scale bar = 1 cm).

Mature seeds culture

The mature seeds of *A. myriophylla* were separated from opened pods, which lost their sterile condition. Putri et al. (2019) and Dorta et al. (2020) studies found explants immersed

or cultured in liquid medium containing PPM significantly reduced contamination rates. Therefore, PPM was added to the media to prevent fungial and bacterial growth. After three weeks on the medium with PPM, the seeds were cultured in a PGRs-free liquid MS medium. The uncontaminated rate ranged 10.0-47.5%. The highest survival rate, 70.0%, was obtained from culturing on semi-solid MS medium supplemented with 2 mg L^{-1} BA (Table 2; Figure 3). The first germination was monitored within one week after surface sterilization, earlier than observation for the immature seeds.

Table 2. Mature seed germination and contamination rates of *Albizia myriophylla* after cultured on MS and WPM media for one and three weeks.

Medium	Contamination (%)	Survival[a] (%)	Total germinated seeds (%)	
			1 week	3 weeks
Liquid MS + BA 0 mg L^{-1}	0	62.5	60.0±1.60a	62.5±1.58a
MS + PGRs free	10.0	47.5	25.0±1.03b	47.5±1.38b
MS + BA 2 mg L^{-1}	47.5	70.0	7.5±0.51c	22.5±0.83c
MS + BA 4 mg L^{-1}	32.5	65.0	7.5±0.83c	32.5±1.40bc
MS + NAA 0.1 + BA 4 mg L^{-1}	10.0	57.5	30.0±0.92b	47.5±1.30b
WPM-PGRs free	22.5	35.0	7.5±0.51c	12.5±0.74d

The number (mean ± SD) followed by different letters indicate significant differences between groups at the $p<0.05$, analyzed by DMRT.

[a]Survival rate of uncontaminated seeds.

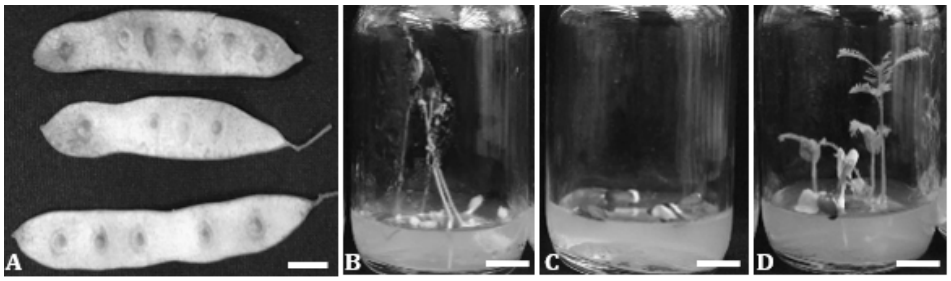

Figure 3. Mature seed germination and seedling growth of *Albizia myriophylla*; mature seeds in dried and brown pods (A), mature seeds germinating after culturing in a PGRs free liquid MS medium for one week and then transferred to PGRs free semi-solid MS medium for additional two weeks (B), mature seed germination after culturing on PGRs-free WPM medium for three weeks (C), mature seed germination after culturing for three weeks on semi-solid MS medium supplemented with 0.1 mg L^{-1} NAA and 4 mg L^{-1} BA (D) (scale bar = 1 cm).

After culturing for three weeks, the seed germination rates increased. The highest germination rate of 62.5% was recorded in the PGRs free liquid MS medium. This was followed by the semi-solid PGRs free MS medium reaching 47.5% seed germination rate. The semi-solid MS medium supplemented with 0.1 mg L^{-1} NAA and 4 mg L^{-1} BA also achieving 47.5% germination rate (Table 2; Figure 4). Generally speaking, the treatment results show the germination rate of mature seeds is lower compared to the immature seeds. The characteristic of mature seeds embeded in the dry opened pods (Figure 3A) were slightly small and varied in size compared to the immature seeds. This may affect the seeds absoluteness and viability. A possible reason is the accumulation of glycerol in dried-aged seed. Wiebach et al. (2020) study found glycerol in dried-aged seed was negatively correlated with the ability for seed germination in wheat and barley.

Not only does the seed developmental stage affect germination rate but also the sterilizing agent may also affect the germination rate. Dufková et al. (2019) reported lower concentrations of 10-100 mM of hydrogen peroxide encouraged seed germination, but higher

dosages (1,000-3,000 mM) inhibitive germination. *A. myriophylla* has a very hard seed coat when mature. If hydrogen peroxide was chosen for surface sterilization of mature seeds higher germination rates may have been achieved. Previous reports by Barampuram et al. (2014) and Labdelli et al. (2019) on cotton showed the best contamination-free and significantly improved germination rates were achieved after pretreating with hydrogen peroxide.

Figure 4. Growth and acclimatization of *Albizia myriophylla* after culturing for four weeks, the formation of creamy white callus tissue at the base of the node segment (A), rooted plantlets after transferred to ex vitro with their roots washed (B), rooted plants in a soil mixture, covered with a plastic bag for moisture control (C) and nine-week-old plantlet after acclimatization and transplanting (D) (scale bar = 1 cm).

Although, WPM is a specific medium for woody plants, germination rates on PGRs free WPM medium were very low both in both immature and mature seeds when compared to MS medium (Tables 1 and 2; Figure 3). In addition, the results of a study by Lewis et al. (2020) on seed germination and embryo rescue of *Asclepias tuberosa* using MS and 1/2 MS showed germination percentages of 94.88 to 97.43%. The use. WPM alone showed germination percentages of 82.05% (Lewis et al., 2020). The use of MS medium appears to be a more appropriate medium for seed germination of ash (*Fraxinus excelsior*) as reported in Rostami et al. (2019). Olomola et al. (2019) also reported good seedling shoot growth of *Tamarindus indica* using in MS medium. Besides, Asonibare et al. (2017) reported the chemical composition of MS medium was responsible for enhanced growth rates of *Piliostigma Thonningii*. MS medium also includes KI, KNO_3 and $CoCl_2$ which is responsible for promoting faster plant growth rates. This is achieved by enhancing effective shoot and root regeneration and KI, KNO_3 and $CoCl_2$ are deficient in the WPM medium (Olomola et al., 2019).

The node segments of seedlings subcultured onto semi-solid MS medium formed creamy white callus at the base of each explant after two weeks (Figure 4A). After nine weeks the results showed, after acclimatization and transplanting to ex vitro condition, the mature healthy plantlets had an 80% survival rate (Figure 4B-D).

CONCLUSIONS

Immature and mature seeds are recommended as suitable explants for in vitro propagation of *A. myriophylla*. The appropriate media for immature seeds was a semi-solid MS medium supplemented with 4 mg L^{-1} BA and for mature seeds a PGRs free liquid MS medium. These media provided the highest germination rates. The explants shoots formed roots on the PGRs free semi-solid MS medium. Furthermore, after 9 weeks the healthy rooted plantlets had a survival rate of 80% after acclimatization and transplanting. Therefore, the findings of this study will be used to further develop mass propagation techniques for *A. myriophylla* to ensure its conservation and sustainable use.

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The hormonal control of callus induction in 'GM256' × 'M9' progeny of different stem rooting ability

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Abstract

The objective of this research was to find an efficient protocol to improve callus induction in apple rootstocks. Concentrations and ratios of auxin and cytokinin were investigated on 'GM256' × 'M9' progenies having either low or high rooting ability. Specifically, leaves of plants with different stem cutting rooting abilities were cultured for 21 days on Murashige and Skoog (MS) semisolid medium containing 2, 3 and 4 mg L^{-1} thidiazuron (TDZ) in combination with 0.2, 0.35 and 0.5 mg L^{-1} 1-naphthaleneacetic acid (NAA) in the dark. Regeneration ability was positively correlated with stem cutting rooting ability. Easy-to-root progeny had a lower browning percentage and a higher callus formation percentage when compared with hard-to-root progeny. NAA was the main factor affecting hard-to-root progeny, while TDZ had a significant effect on easy-to-root progeny. Shoots were induced only in easy-to-root progeny. In conclusion, callus formation of progenies with both high and low rooting ability was promoted when cultured with 0.5 mg L^{-1} NAA + 2 mg L^{-1} TDZ.

Keywords: apple rootstocks, tissue culture, callus induction, rooting ability

INTRODUCTION

Apple (*Malus pumila* Mill.) is known for its nutritional qualities and is of significant economic importance in many countries, including China. Apple scions are usually grafted onto rootstocks to maintain trueness-to-type. Apple rootstocks play an essential role in regulating the environmental adaptability and growth management of apple trees. Apple rootstocks are commonly propagated using layering. However, this method is time-consuming and inefficient. Cutting propagation is broadly utilized for horticultural crops in nurseries. This benefits from the adventitious root (AR) formation property of stems (Tsafouros and Roussos, 2020). However, apple dwarfing rootstocks are generally considered difficult to propagate using cuttings and AR is a limiting factor in cutting propagation of apple rootstocks. Despite many physiological and molecular studies on root formation, the mechanisms underlying adventitious rooting are not completely understood. (Moriya et al., 2015; Li et al., 2018).

Adventitious rooting ability differs significantly in different species, and even different individuals in the same progeny show differences in rooting capacity (Xiao et al., 2014). For difficult-to-root species and individuals, tissue culture can relieve this problem.

The first report of in vitro regeneration from apple leaf explants was made by Liu et al. (1983) using apple seedlings. Several later reports have revealed critical factors affecting leaf regeneration in apple. They include nitrogen source and concentration, incubation conditions, leaf origin, leaf maturity, position on the stem, mode of excision, explant orientation, and the types and concentrations of different growth regulators (Yepes and Aldwinekle, 1994). Among all of these factors, plant growth regulators can both qualitatively and quantitatively influence in vitro culture and regeneration. The balance between auxin and cytokinin determines the state of differentiation and dedifferentiation (Skoog and Miller, 1957). In general, an intermediate ratio of auxin and cytokinin promotes callus induction (Ikeuchi et al., 2013). TDZ and NAA have been successfully and wildly utilized to promote regeneration from apple leaf

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explants.

Generally, optimum conditions for regeneration are not always the same for different genotypes within the same species (Modgil and Pathania, 2018). To date, however, there has been little discussion about the identification of suitable media for use with plant material having different rooting ability. It is necessary, therefore, to determine suitable protocols for propagating such material.

In this study, the rooting ability of softwood cuttings of 613 progenies derived from a 'GM256' × 'M9' cross were examined. These different progenies had significant differences in rooting capacity and, consequently, easy- and hard-to-root progeny were selected. The objective was to identify an efficient combination of key plant growth regulators that would promote callus formation for these plant materials with different rooting ability and to compare some of the differences in callus formation between these two groups of progeny.

MATERIALS AND METHODS

Plant material

This study used F_1 progenies consisting of 613 individuals derived from a cross between 'GM256' and 'M9' made in 2014. Trees were planted in 2019 in an orchard in Beijing, China. The research was carried out at the China Agriculture University, Beijing.

Evaluation of rooting ability from softwood cuttings

In spring, cuttings were taken from parent trees. The base of each cutting was immersed in 1,500 ppm indole butyric acid (IBA: 1.5 g dissolved in 600 mL 75% alcohol and then diluted with water to 1 L) for 40 s and inserted into an aperture disk filled with sand as a growing medium. The cuttings were cultured under a mist system. Approximately 30 days after planting, the rooting rate (RR) of each progeny was calculated as the number of rooted cutting divided by the number of planted cuttings. Two progenies with significant differences in RR were selected to be used in this study, DG1116 (easy-to-root, RR=100%) and DG1510 (hard-to-root, RR=24.07%) (Figure 1).

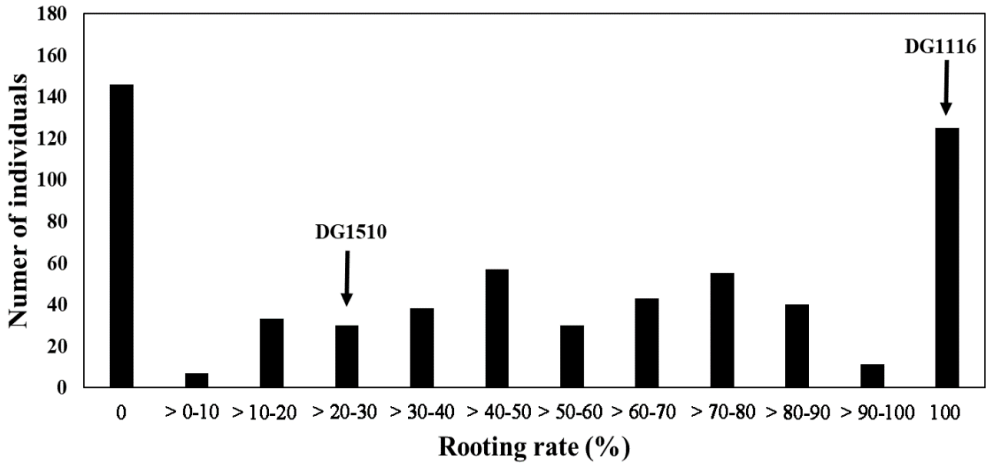

Figure 1. Distributions of the rooting rate of 613 individuals of the F_1 population derived from 'GM256' × 'M9'. Data were collected 30 days after stem cuttings were taken from the parent trees. DG1116 was selected from easy-to-root progenies which had a rooting rate of 100%; DG1510 was selected from hard-to-root progenies with a rooting rate in the 20 to 30% range.

Explant preparation

Young leaves from selected progenies were used as explant sources. Leaves were surface-sterilized by immersion in 75% ethanol for 10 s, followed by 8 min treatment in 0.1%

mercury bichloride solution. Samples were then washed five times with sterile distilled water. Leaves were cut into 1×1 cm leaf discs, which served as explants for the induction of callus formation.

Explants culture and callus induction

All sterilized explants were placed abaxial side down in Petri dishes containing full-strength MS medium supplemented with 10 g L^{-1} sucrose, 10 g L^{-1} glucose, and 4.5 g L^{-1} agar and with different combinations of plant growth regulators (Table 1). The leaves were cultured at 24±2°C in the dark.

Table 1. Different culture media tested for leaf regeneration.

Medium	Concentration of NAA (mg L^{-1})	Concentration of TDZ (mg L^{-1})
A1B1	0.2	2
A1B2	0.2	3
A1B3	0.2	4
A2B1	0.35	2
A2B2	0.35	3
A2B3	0.35	4
A3B1	0.5	2
A3B2	0.5	3
A3B3	0.5	4

Data collection and statistical analysis

For each treatment, three replicates were used with eight explants per replicate. The incidence of browning was recorded 12, 24, 48 and 72 h after inoculation. The amount of callus formation was recorded 21 days after inoculation. Following Gao et al. (2020), browning rate and callus induction rate were calculated as follows: 1) browning rate (%) = (number of browning explants/the total number of inoculated explants for each treatment)×100; 2) callus induction rate = (number of explants that formed callus/the total number of inoculated explants for each treatment)×100. Callus proliferation was scored by a grading system (Table 2). The total rate of callus development was calculated as follows: total percentage of callus = the ratio of level 1×25% + the ratio of level 2×50% + the ratio of level 3×75%. Data were processed using the SPSS 25.0 software program (SPSS Inc., Chicago, USA). The effect of treatments was tested by analysis of variance and differences among means were tested using the LSD multiple range test.

Table 2. Callus levels and description.

Level	Description
0	There was no callus on the leaf explants
1	There was 25% callus on the leaf explants
2	There was 50% callus on the leaf explants
3	There was 75% callus on the leaf explants
4	Callus covering the entire leaf explants

RESULTS AND DISCUSSION

Wound-induced browning

Browning occurs in the initial stages of tissue culture and can lead to the death of explants. Browning is mainly caused by oxidases, acting on phenolic acids to produce toxic compounds that lead to the formation of dark brown tissue. Auxin treatment has no significant effect on browning whereas citric acid, polyvinylpyrrolidone, and ascorbic acid can be used to avoid tissue browning (Dobránszki and Teixeira da Silva, 2013; Gao et al., 2020). Oxidase

activity has been shown to be significantly different between easy-to-root and hard-to-root apple rootstocks (Tsafouros and Roussos, 2020).

In this study, the explant browning rate differed significantly between the two progenies (Figure 2). Plant growth regulators did not affect the browning phenomenon (data not shown). The hard-to-root progeny had the highest browning percentage of 36.9%, followed by the easy-to-root offspring with 18.6% within 12 h. The browning percentage in hard-to-root progeny was higher than easy-to-root progeny at $p<0.05$ at all assessment times.

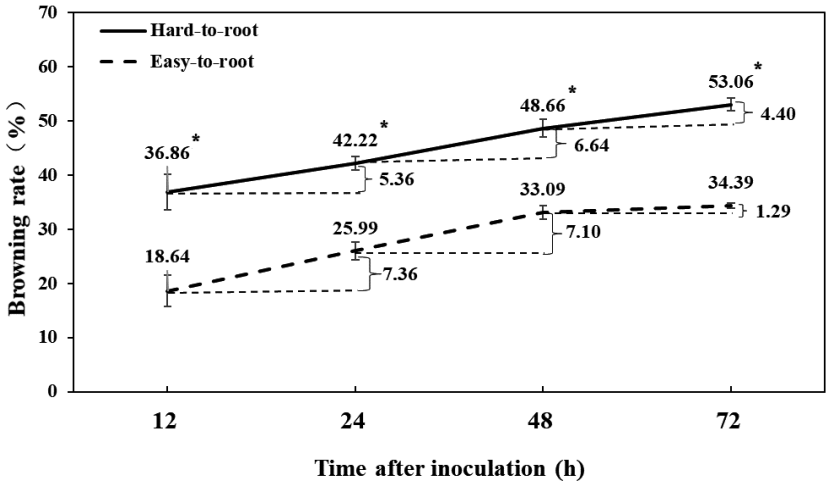

Figure 2. The browning percentage on explants from hard-to-root and easy-to-root progeny at 12, 24, 48 and 72 h after inoculation. Line bars represent the standard error (SE). * denotes statistically significant differences between the two progenies according to Student's t test at $\alpha=0.05$.

Callus induction

After 21 days of incubation, all of the growing media were able to induce the leaf explants to produce callus. However, differences between the two genotypes were significant. The-third-level of callus development (75% of the explant surface covered) in the easy-to-root progeny was 66% while in the hard-to-root progeny it was only 14%. The hard-to-root progeny had higher percentages in the first and second levels (25 and 50% coverage) – 43 and 43% vs. 8 and 26%, respectively (Figure 3). According to this classification criteria, therefore, the callus formation in the easy-to-root progeny was greater than that in the hard-to-root progeny.

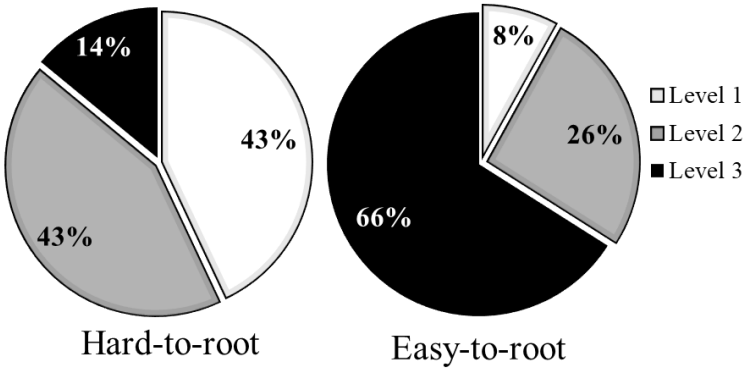

Figure 3. The proportion of callus in each level of two progenies with different rooting rate derived from 'GM256' × 'M9'. Data were collected after 21 days of tissue culture.

Regeneration has been shown previously to be different in different parts of a leaf. The regenerative capacity was higher from the middle part toward the base of the leaf (Welander, 1988). Moreover, it has been previously shown that leaf area significantly affected regeneration capability. For example, Tchoundjeu et al. (2002) reported that rooting percentages increased with increasing leaf area in *Prunus africana*. In this study, the different levels of callus formation occurred on leaves with the same area and under identical culture conditions so the differences observed must have been solely due to the differences in progeny.

Both easy-to-root and hard-to-root progeny had high callus formation in A3B1, while A1B2 produced the least callus formation in the hard-to-root progeny and A3B3 the least in the easy-to-root progeny. However, for the easy-to-root progeny, callus formation in A3B1 was not significantly different when compared with the other media, excepted to A3B3 (Figures 4 and 5). Previously, we found that a medium with 0.2 mg L^{-1} NAA + 2.0 mg L^{-1} TDZ was the most suitable medium for 'GM256', and a medium with 0.5 mg L^{-1} NAA + 4.0 mg L^{-1} TDZ was the most suitable for 'M9' (unpubl.).

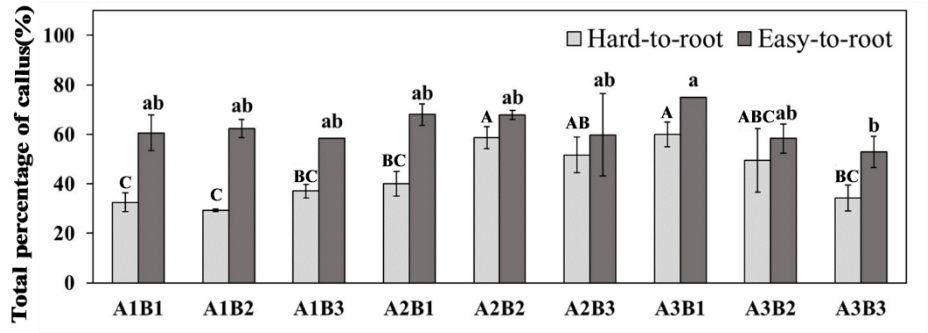

Figure 4. The total percentage of callus formation in nine combinations of TDZ and NAA with hard-to-root and easy-to-root progeny. Line bars represent the standard error (SE). The same letter above bars represents a non-significant effect ($p>0.05$), according to the LSD multiple range test. The capital letter for Hard-to-root bars and small letter for Easy-to-root bars.

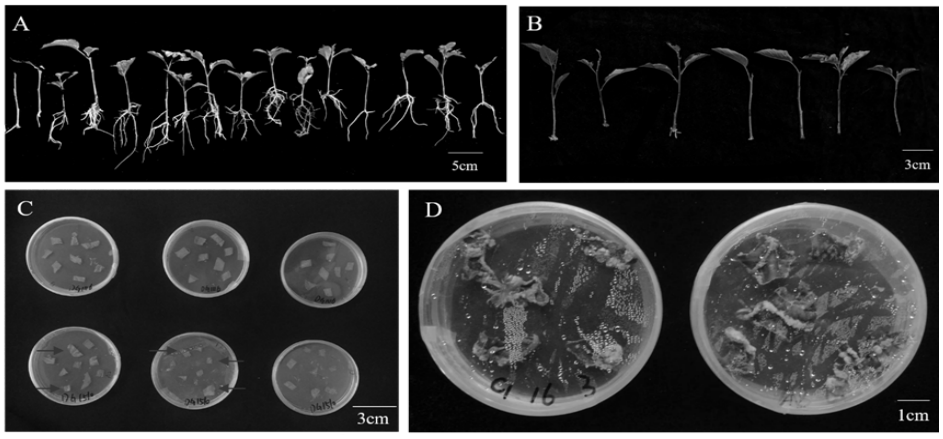

Figure 5. Phenotype of cuttings and leaf explants. Easy-to-root progeny among 613 individual hybrids from 'GM256' and 'M9', where the rooting rate was 100% in the rooting rate evaluation trial (A). Hard-to-root progeny, which have relatively low rooting rates among the same hybrid progenies (B). Leaf explants cultured on medium at 24 h after inoculation, arrows indicate browning on the leaf explants (C). Leaf explants cultured on medium 40 days after inoculation; arrow indicates the site where adventitious shoots have been induced from the callus (D).

According to the present results, therefore, genotype strongly affected callus formation. In order to study the effect of plant growth regulator on rooting ability, the significance of NAA and TDZ was analyzed (Table 3). NAA significantly influenced the first level callus formation and the total percentage of callus in hard-to-root progeny, whereas TDZ had no significant effect. In contrast, the second and third levels of callus formation in easy-to-root progeny were affected by TDZ, whereas NAA had no significant affect. There were significant interactions of TDZ and NAA with both easy- and hard-to-root progeny. The NAA×TDZ interaction was significant in terms of level 1 callus formation and the total percentage of callus in hard-to-root progeny. For easy-to-root progeny, the interaction was significant in level 2 of callus formation. Similar results have been reported by Ikeuchi et al. (2013).

Table 3. Probabilities of the effect of TDZ and NAA on callus formation.

Progeny	Callus	Source of variation		
		TDZ	NAA	TDZ×NAA
Hard-to-root	Level 1	ns[a]	***	**
	Level 2	ns	ns	ns
	Level 3	ns	ns	ns
	TP[b]	ns	**	*
Easy-to-root	Level 1	ns	ns	ns
	Level 2	*	ns	*
	Level 3	*	ns	ns
	TP	ns	ns	ns

[a]ns: there was no significant difference.
[b]TP: the total percentage of callus.
Significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Shoot regeneration

Adventitious shoots can be induced through callus either indirectly (Dufour, 1990) or directly without callus formation (Pawlicki and Welander, 1994). In this study, adventitious shoots were induced through callus. After 39 days ofinoculation, adventitious shoots had been induced by callus in the A2B1 and A3B2 treatment in the easy-to-root progeny. This is consistent with both easy- and hard-to root progeny exhibiting higher callus induction ability with A2B1.

CONCLUSIONS

NAA has a more important role in callus induction for hard-to-root progeny through promoting total callus formation, while TDZ tends to enhance callus induction with easy-to-root progeny.

A combination of 0.35 mg L^{-1} NAA plus 2 mg L^{-1} TDZ on MS medium (A2B1) was the most effective in stimulating callus formation of leaf explants from both in easy- and hard-to-root progeny.

ACKNOWLEDGEMENTS

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Effects of preharvest UV-B irradiation on anthocyanin biosynthesis in blueberry

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Abstract

The effects of preharvest UV irradiation on blueberry fruit development were investigated to explore the potential use of UV irradiation in blueberry production systems. Long-term UV-B treatments with environment-dependent dosing were applied to southern highbush blueberry (*Vaccinium corymbosum* interspecific hybrid), 'O'Neal' trees in a greenhouse, during the entire reproductive period. The experimental treatments included UV irradiation conditions representing an average winter day (low, 0.07 W m^{-2}), an average summer day (medium, 0.14 W m^{-2}) and 30% above that of an average summer day (high, 0.19 W m^{-2}). Compared with the control, the total anthocyanin content increased by 167 and 148% after the application of medium- and high-dose UV, respectively. Gene expression changes of anthocyanin biosynthesis-related structure genes, the MYB transcription factor, a UV receptor, and light-responsive genes were examined and different responses under different UV-B exposures were identified. Simultaneous upregulation of the MYB transcription factor, *VcMYBPA1*, with biosynthesis-related structure genes (such as *VcCHS* and *VcDFR*) in response to low- and high-dose UV-B suggests that *VcMYBPA1* may increase biosynthesis-related gene expression, thereby enhancing anthocyanin accumulation in fruits in response to UV-B. The results demonstrate the possible use of UV-B irradiation at doses of 0.14 and 0.19 W m^{-2} to improve blueberry anthocyanin accumulation.

Keywords: anthocyanin, blueberry, fruit maturation, MYB, UV-B

INTRODUCTION

There are three types of UV that can be defined by wavelength: UV-C (<280 nm), UV-B (280-320 nm), and UV-A (320-400 nm). UV-C can be absorbed by ozone in the stratosphere, while the atmospheric ozone layer cannot attenuate UV-B or UV-A. Compared with UV-A, UV-B with a shorter wavelength, has a more substantial biological effect. UV-B acts as an essential environmental factor in regulating plant phenology and growth regulation even though it only accounts for 5% of the ultraviolet rays that reach the Earth (Björn, 1996; Wargent and Jordan, 2013).

Numerous studies have shown that flavonoids are a class of substances that effectively absorb ultraviolet light to protect plants or tissues from DNA damage caused by UV-B exposure. Supplementing with UV-B can induce the biosynthesis of flavonoids, especially anthocyanins. Research on grapes (*Vitis vinifera*) (Martínez-Lüscher et al., 2014), apples (*Malus* × *domestica*) (Henry-Kirk et al., 2018), strawberries (*Fragaria* spp.) (Josuttis et al., 2010) and postharvest treatment research on blueberries (Nguyen et al., 2017) have shown that targeted UV-B treatment can effectively enhance the production of phenylpropanoid substances in highly nutritious fruits and vegetables.

Highbush blueberry (*Vaccinium corymbosum*) has become one of the most economically important fruit crops in current fruit markets because blueberry fruits contain high levels of anthocyanins which have positive effects on human health. In recent years, the extent (i.e., area) of blueberry cultivation has rapidly increased, especially in greenhouses in northeastern China. Compared with on-season fruit from field-grown plants, off-season greenhouse fruit contains lower concentrations of anthocyanins. These lower anthocyanin levels are the result

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of UV-B transmission being reduced in glass-glazed greenhouses and plastic houses because glass and plastic films absorb UV-B (Muñoz et al., 2008). Supplemental UV-B irradiation can potentially help increase anthocyanin accumulation in blueberry fruits; however, little is known about the effects of UV-B radiation on anthocyanin biosynthesis when UV-B irradiation is applied during the reproductive stage in the greenhouse. The purpose of this study was to clarify the effects of preharvest UV-B on blueberry anthocyanin accumulation.

MATERIALS AND METHODS

Plant materials and UV-B treatments

Two- to three-year-old, pot-grown shrubs of southern highbush blueberry (*V. corymbosum* interspecific hybrid) 'O'Neal' were used in this study. All plants were grown in a greenhouse kept at 25°C during the daytime and 20°C at night. The UV-B lighting was provided by SPWFD24UB1PB lamps (Panasonic, Osaka, Japan).

After 6-7 weeks of blooming, when fruits were at the S4 green stage as described in Zifkin et al. (2012), the long-term UV-B supplementation was applied as an environment-dependent dose to the *V. corymbosum* plants. Three UV treatments of seven h day^{-1} duration at three different levels were imposed; 1) a low-dose treatment with a UV dose representing an average winter day biologically effective dose (0.07 W m^{-2}); 2) a medium-dose treatment with a UV dose representing an average summer day (0.14 W m^{-2}); and 3) a high-dose treatment with a UV dose representing 30% greater than an average summer day (0.19 W m^{-2}) and an untreated control group. The UV-B value of each treatment, which represented biologically effective UV-B radiation, was calculated according to the generalized curve described by Caldwell (1968). Green fruits were harvested one week after the start of the UV-B treatment, pink fruits were collected one week after berry veraison, which is the S6 green stage as described in Zifkin et al. (2012), and S8 mature fruits were harvested when ripe. All fruit collections were made 2 h after the commencement of the UV treatment on each sampling day. Pericarp tissues were separated from the flesh, immediately frozen in liquid nitrogen and stored at -80°C until use.

Extraction and determination of anthocyanin composition and contents

Anthocyanins were extracted and detected as described by Irizumi et al. (2013). Frozen samples were crushed using a Multi-beads shocker (Yasui kikai, Osaka, Japan) and 0.2 g of frozen crushed fine powder was resuspended in 5 mL of 0.1% (v/v) HCl-methanol at 10°C for 40 min, then centrifuged at 13,000×g at 4°C for 15 min. The supernatant was subjected to high performance liquid chromatography (HPLC)-photodiode array (PDA). The chromatography column used in this study was the Wakopak Wakosil-II 5C18 RS (4.6×150 mm) and the relative contents of each anthocyanin were calculated based on peak area, and the area in pink fruits in the control group was used as a reference. The peak area of each anthocyanin component was calculated and used as relative content. The sum of all peak areas was used as the relative total anthocyanin content.

Total RNA isolation, cDNA synthesis, and analysis of transcript level by qRT-PCR

The total RNA was extracted from the peel of the pink stage fruit samples using the CTAB-KAc method and reverse-transcribed using the ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). Quantitative reverse transcription-PCR (qPCR) was conducted using a Light Cycler 480 (F. Hoffmann-La Roche, Basel, Switzerland) and a THUNDERBIRD® SYBR qPCR mix (Toyobo). The primers for anthocyanin biosynthesis-related genes, including *VcANS*, *VcCHS*, *VcPAL*, *VcDFR*, *VcF3'5'H*, *VcF3'H*, and *VcFLS*, and the putative positive transcription factor *VcMYBPA1*, were used as described in Inostroza-Blancheteau et al. (2014) and Nguyen et al. (2017) (Table 1). Light transduction genes *VcUVR8, VcCOP1-1, VcCOP1-2* and *VcHY5* were identified according to the GDV database of blueberry and the primers were designed by NCBI primer-blast tools. The qPCR for each gene was performed under the following conditions: 95°C for 5 min followed by 40 cycles of 95°C for 5 s and 58°C for 1 min. *Vc*GAPDH was used as the reference gene, as described previously (Zifkin et al.,

2012). The all transcripts levels obtained for each replicate were used for correlation analysis.

Table 1. Primer sequences of light transduction genes used for gene expression analysis.

Gene	Gene ID	Sequence 5'-3'	
UVR8	CUFF.6010.1	Forward primer	TGGTTATGGTGGCATGTGGAT
		Reverse primer	AACCTATGAGGCACAAGGTGG
HY5	CUFF.47089.1	Forward primer	GAGAGTACCGGAGATCGGC
		Reverse primer	TTTATCGGCCGGACTTCTGC
COP1-1	CUFF.28774.1	Forward primer	CGAACGCATGGCCTTCTTTG
		Reverse primer	GAATTGCCTGCGTTCATCCG
COP1-2	CUFF.8155.1	Forward primer	GCTCAGCAGACCACCATATCC
		Reverse primer	CCACTGAACACATGGAGTGGA

Statistical analysis

A randomized design was used for this study. Treatments consisted of five replicates, and all samples were analyzed in triplicate. The ANOVA statistical analysis for total anthocyanin content of the different treatments was completed using SPSS 19.0 (IBM Corp., Chicago, USA), values with the same letters within an experiment were statistically similar as determined by Duncan's multiple range tests (α=0.05). T-test was used to compare the difference of gene expression between control and each treatment, * and ** represent a significant difference at $p<0.05$ and $p<0.01$, respectively.

RESULTS AND DISCUSSION

Effects of UV-B light on anthocyanin accumulation in blueberry fruit

Plants have developed strategies to counteract the adverse effects of UV-B radiation. Phenolic and flavonoid secondary metabolites effectively defend the plant from harmful UV-B radiation (Martínez-Lüscher et al., 2014). Phenolic acid and flavonols are beneficial UV-B absorbing compounds that are accumulated mainly in epidermal cells to capture UV-B radiation. Moreover, as a complement to the role of the UV-B absorbing compounds, plant cells can generate antioxidants, such as anthocyanins, to scavenge the reactive oxygen species. In this study, no anthocyanins were detected in the green fruit of the control group, and a significant increase of anthocyanin was detected in all of the UV-B-treated groups (Figure 1a). The anthocyanin content of mature fruits in the control group was significantly lower than that in the medium and high-dose treatments (Figure 1b). Changes of anthocyanin in our long-term treatment show a similar trend to a post-harvest and short-term UV-B treatment (Nguyen et al., 2017). Our study and previous studies collectively demonstrate the vital role of UV-B for the enhancement of anthocyanin synthesis in blueberries.

Expression of anthocyanin biosynthesis genes, UV-B signal transduction gene and related MYB genes

The veraison period (pink-fruit stage) is the crucial period for anthocyanin synthesis in blueberry fruits. Figure 2 shows the gene expression of key anthocyanin biosynthetic genes in the peel tissue of pink blueberry fruits. This heat map (Figure 2) consists of relative values of qRT-PCR results for *VcFLS, VcPAL, VcCHS, VcF3'H, VcF3'5'H, VcDFR, VcANS, VcMYBPA1* and the UV-B transduction genes *VcHY5, VcCOP1*, and the UV-B photoreceptor protein *VcUVR8*. The results show that gene expression was affected by UV-B treatment. Compared with the control group, the expression levels of *VcPAL* and *VcFLS* significantly increased after UV-B exposure, while *VcCHS*, *VcDFR*, and *VcMYBPA1* only showed significantly higher gene expression levels in the low- and high-dose UV-B-treated fruits. In addition to these upregulated genes, *VcANS* and *VcF3'H* were negatively regulated by UV-B in this study. Among the genes related to UV-B signal transduction, expression of *VcHY5* was upregulated in all UV-B treatments, while for the *VcCOP1-1* and *VcCOP1-2* genes no significant changes occurred under low-dose UV-B exposure

but they were upregulated in the other treatments. In contrast to the other upregulated transduction genes, *VcUVR8* showed lower transcription abundance only after low- and high-dose UV-B treatments.

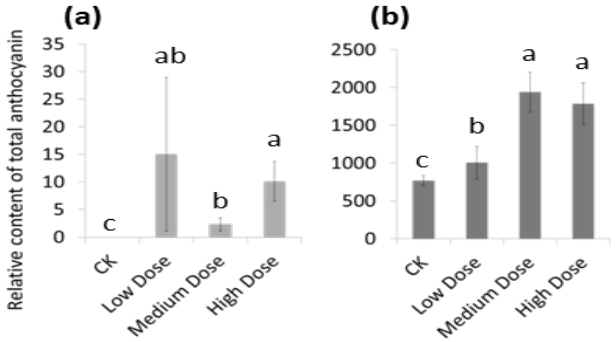

Figure 1. Effect of UV-B on the accumulation of anthocyanin in the green stage (a) and the mature stage (b) of developing blueberry fruit.

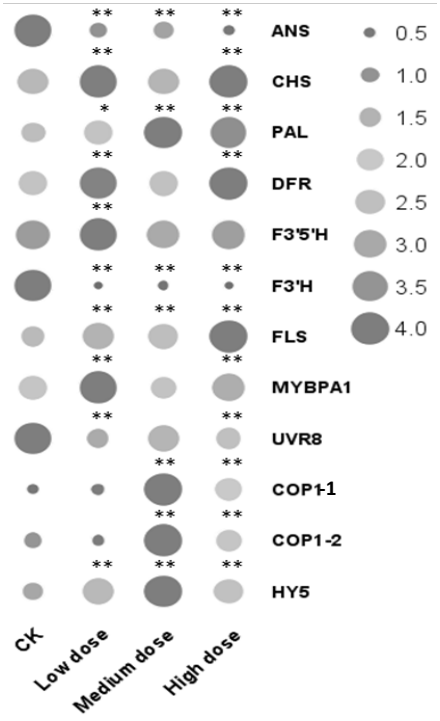

Figure 2. Heat map of anthocyanin biosynthesis, UV-B transduction, and MYB genes expression.

Previous studies have demonstrated the effect of UV on the phenylpropanoid biosynthetic pathway (Martínez-Lüscher et al., 2014; Henry-Kirk et al., 2018; Josuttis et al., 2010). However, the effects of UV-B on genes involved in anthocyanin biosynthesis during fruit development of blueberry have not been well characterized previously. In this study, we found that the expression of anthocyanin biosynthesis pathway-related genes to UV-B differed depending on the irradiation dose. This may be because the different anthocyanins accumulate with different molecular mechanisms under different UV doses.

The *MYBPA1* protein also plays an essential role in determining the effect of anthocyanins on the color of the blueberry during the ripening period (Zifkin et al., 2012). To

investigate the involvement of *VcMYBPA1* in anthocyanin accumulation in response to UV-B, a correlation analysis between all anthocyanin synthesis-related genes and *VcMYBPA1* was conducted (Figure 3). *VcMYBPA1* was highly correlated with *VcCHS* and *VcF3'5'H,* with correlation coefficients of 0.74 and 0.73, respectively. *VcPAL*, *Vc*F3'H, and *Vc*ANS were not highly correlated with VcMYBPA1, and *Vc*F3'H and *VcANS* showed a high positive correlation with each other (correlation efficient 0.95), which suggests that these two genes were regulated together by transcription factors other than *VcMYBPA1* in response to UV-B. The results of this correlation analysis indicate the possible transcriptional function of V*cMYBPA1* in the blueberry anthocyanin synthesis network.

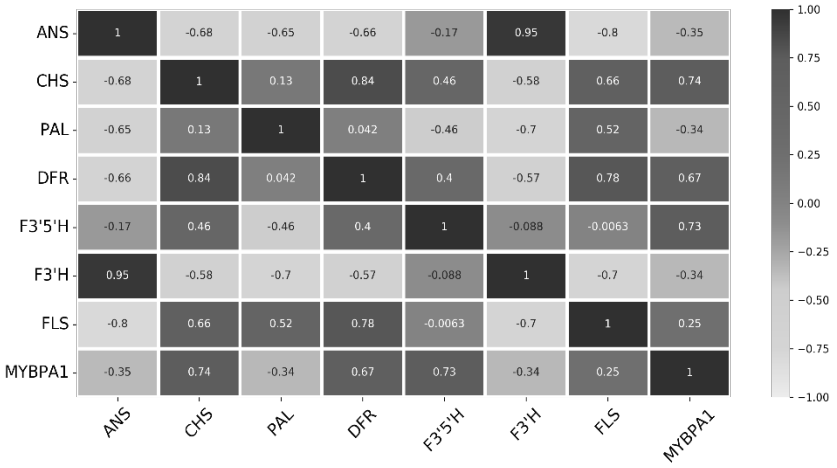

Figure 3. Correlation matrix between *Vc*MYBPA1 and anthocyanin synthesis-related genes. (ANS, anthocyanidin synthase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3'5'H, flavonoid 3'5'-hydroxylase; F3'H, flavanone 3'-hydroxylase; FLS, flavonol synthase; MYBPA1, proanthocyanidin-related myeloblastosis transcription factor; PAL, phenylalanine ammonium lyase).

In conclusion, supplementing with 0.14 and 0.19 W m^{-2} UV-B in the greenhouse could improve anthocyanin biosynthesis in green and mature 'O'Neal' fruits via an increased expression of the genes related to anthocyanin biosynthesis and UV-B signal transduction. Our study demonstrates that UV-B enhances anthocyanin accumulation at the mature stage, which suggests the possible use of UV-B irradiation to improve fruit quality and produce fruits with greater potential for human health benefits.

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Different light spectra and intensity level effects on vegetative growth and antioxidant content of coriander

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Abstract

The main purpose this paper is to study effects of red and blue colored lighting and light intensity on coriander growth. LEDs tubes were constructed using three light spectral combinations with different light ratios of red (660 nm) and blue (447 nm). Light ratios were 10:4, 10:2 and 10:1 with two photosynthetic photon flux density (PPFD) values of 150±10 and 250±10 µmol m^{-2} s^{-1}. Daylight was used as the control treatment. Findings showed coriander growth characteristics cultured under LED lights is enhanced compared to a daylight environment. Using LED with higher PPFD resulted in better growth parameters. At a PPFD value of 150 µmol m^{-2} s^{-1}, the high red per blue ratio increased height, number of leaf and shoot of coriander compared with the lower red per blue ratio. However, the low red per blue ratio at PPFD value of 250 µmol m^{-2} s^{-1} increased number of leaf and shoot of coriander. A greater dry weight was obtained when grown under the LED light source compared to coriander grown under a daylight environment. In addition, antioxidant activity was increased under the higher blue light levels. However, comparing the antioxidant activity based on a 1-g dry mass, coriander grown under daylight had a higher antioxidant activity compared to all LED light treatments.

Keywords: *Coriandrum sativum*, LED light, red and blue light ratios

INTRODUCTION

Light is the main environmental factor affecting plants physiological processes. The light spectra affects plant photosynthesis, growth and phytochemical concentrations. The red and blue light spectrum affects how plants synthesis responds via chlorophyll a and b (Moss and Loomis, 1952). The red and blue light spectrum may have either a positive or negative effect depending upon the ratio of red and blue light. For example, a high red light exposure increased the fresh and dry weight in shoots and roots of lettuce (Son and Oh, 2015). Studies by Kang et al. (2016) and Shimizu et al. (2011) found increases in lettuce leaf length, leaf width and shoot to root ratio, as well as fresh and dry weight. In contrast, Wang et al. (2016) reported on changes in leaf area from exposure to high levels of red light. However, high blue light levels were found to increase chlorophyll content (Son and Oh, 2015; Kang et al., 2016; Wang et al., 2016). A study by Kook et al. (2013) supports findings that high blue levels increases leaf width and length, leaf area, fresh and dry weight were increased. Furthermore, Naznin et al. (2016) found an increase in antioxidant content in coriander, but reported a decrease in the height of lettuce, while Chen et al. (2016) reported a decrease in stem elongation.

Light intensity also has a positive effect on phytochemical accumulation in plants. Zhang et al. (2018) reported high photosynthetic photon flux density (PPFD) increases growth rates and antioxidant levels in lettuce. This research focuses on increasing antioxidant content in coriander. Wong and Kitts (2006) study found that antioxidant activity can decrease oxidation, restrain cancer, and enhance immunity in the human body.

The aim of this study is to determine the effects of red and blue light ratios with two different PPFD levels on the vegetative growth characteristics and antioxidant content in

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coriander compared with coriander grown in daylight conditions, growth and antioxidant. Results were compared with daylight.

MATERIALS AND METHODS

Plant culture

Seeds of coriander were sown into soil under a daylight environment. Approximately 17 days later seedlings had 1-2 leaves and were 4-5 cm in height. The seedlings were then root-washed, wrapped with sponge sheets, and place into a plastic seedling pot. Pots were placed into a nutrient film technique (NFT) hydroponic set-up. The electrical conductivity (EC) and pH of the nutrient solution was controlled at 2.5-3 mS cm^{-1} and 5.5-6, respectively. Plants were exposed to an ambient air temperature of 25-30°C and relative humidity of 65-85%. These seedlings were irradiated with different spectral light treatments and harvested 45 days after sowing (DAS).

Lighted treatments

Light treatments consisted of LED light sets using three different spectral combinations. These combinations comprised of two monochromatic LEDs with a peak wavelength of 447 nm blue LED (B) and 660 nm red LED(R). The three different light treatments combinations ratios were R:B = 10:4, 10:2 and 10:1 (Figure 1). These light combinations were constructed within T8 LED light tubes using 66 LED chips per tube. The photosynthetic photon flux densities (PPFD) levels were 150±10 and 250±10 µmol m^{-2} s^{-1}. The day night photoperiod was 16 and 8 h, respectively. The control treatment was normal daylight and night conditions (Figure 2).

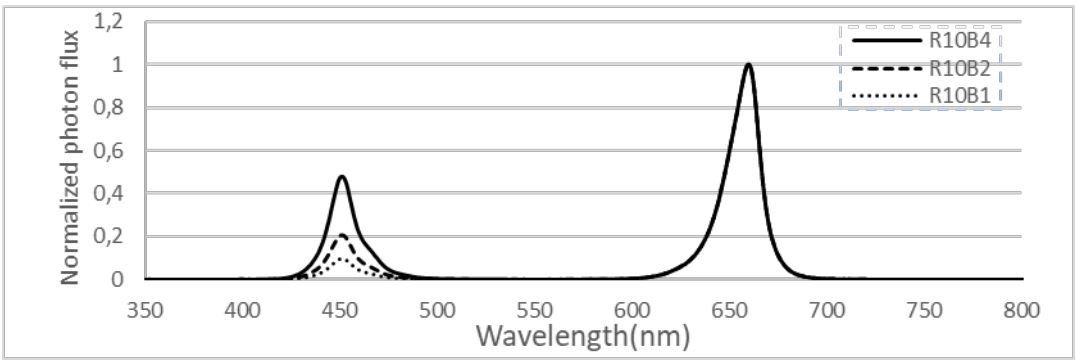

Figure 1. Normalized photon flux of 660-nm red (R) and 447nm blue (B) light wavelengths with three different combinations consisting of R:B=10:4, R:B=10:2 and R:B=10:1.

Growth characteristics

The vegetative growth characteristics height, was measured from base (soil level) to the leaf tip of the highest leaf. The leaf and shoot number was counted, and the shoot and root fresh weight was recorded at 45 days after sowing (DAS). The shoot and root dry weights obtained after drying in an incubator at 65°C for 48 h.

Antioxidant parameters

The antioxidant activity was obtained using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay (Zhu et al., 2006). One mL of dry coriander extracted of a known concentration unit of mg mL^{-1} was added to the prepared DPPH oxidation of a 0.1 mM concentrate in 1 mL of 95% ethanol solution and allowed to react for 30 min. The absorbance wavelength for each sample was measured at 517 nm by spectrophotometer (Model Thermo Scientific GENESYS 10S). The standard solution prepared using Trolox (1, 2, 4, 6, 8 and 10 mg mL^{-1}). The antioxidant capacity (mg Trolox equivalents g^{-1}) of the coriander samples determined using the standard curve. The total antioxidant activity (mg Trolox equivalents

plant^{-1}) obtained by multiplying the dry weight plant^{-1} of coriander and antioxidant capacity.

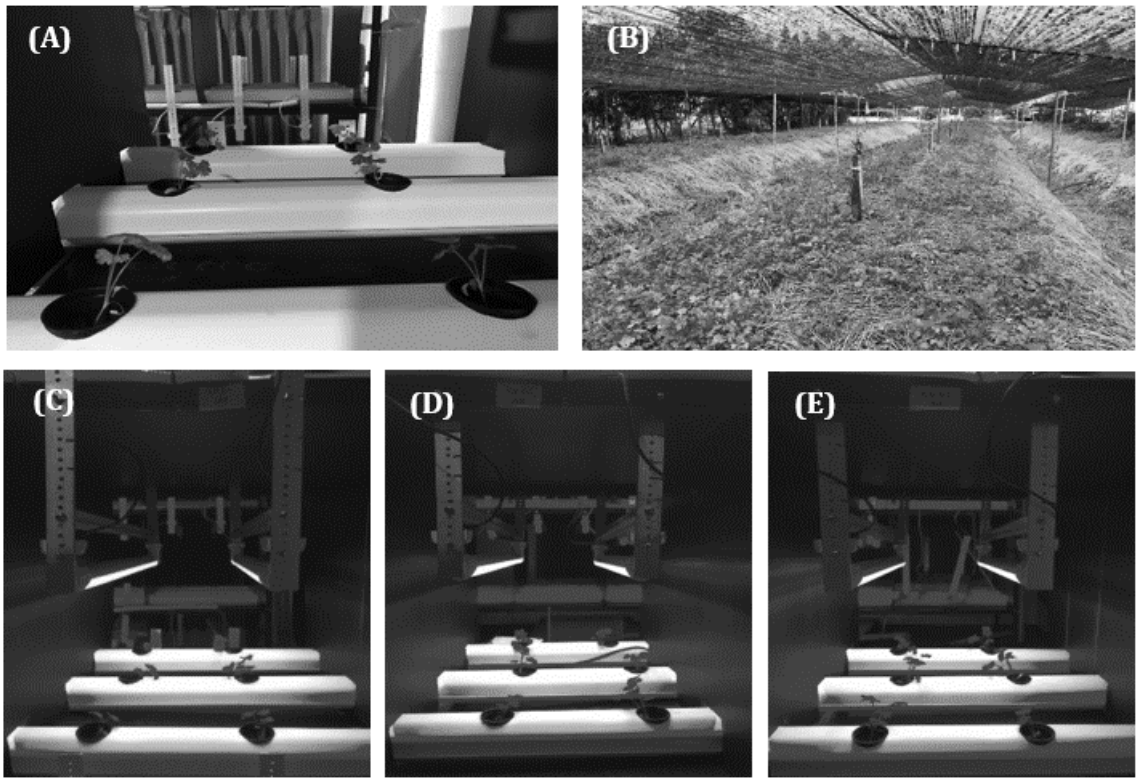

Figure 2. Coriander cultured under red and blue LED light combinations compared with daylight. Coriander in a NFT gully (A), daylight treatment (B), R:B=10:4 (C), R:B=10:2 (D) and R:B=10:1 (E).

RESULTS AND DISCUSSION

Growth parameters

The result showed coriander grown under LED lights at both PPFD levels, had improved vegetative growth compared to the daylight treatment. The coriander under the R:B LED light treatments, 10:4, 10:2, and 10:1 were 1.27, 1.64 and 1.56 times, respectively, taller than the daylight treatment. At the highest PPFD treatment R:B ratio of 10:2, the coriander was the tallest. At the lower PPFD level, the coriander height also increased due to the increase in the red level. Coriander grown under R:B ratios of 10:1 and 10:2 were 1.34 and 1.32 times taller than under daylight. However, the height of coriander under R:B ratio 10:4 was only slightly taller than the daylight treatment (Figure 3). Naznin et al. (2016) study reported high red spectrum levels increased the height of coriander compared to coriander under lower red levels. Chen et al. (2016) found increasing of the blue light levels decreased the height of plants. Lowest leaf number was observed in coriander grown under daylight compared to both PPFDs treatments. At the 250 µmol m^{-2} s^{-1}, R:B 10:2 ratio, the coriander produced the greatest number of leaves. The leaf number was 4.1 times greater than coriander grown under daylight. By increasing the red proportion of the light source caused an increase in leaf number. Conversely, increasing the blue portion of the light source decreased leaf number at lower PPFD level. However, leaf number under LED treatments were 1.54-4.1 times higher than that of daylight (Figure 4A). Chen et al. (2016) and Son and Oh (2015) reported that red light helped increase leaf number compared the blue light level at 135 µmol m^{-2} s^{-1}.

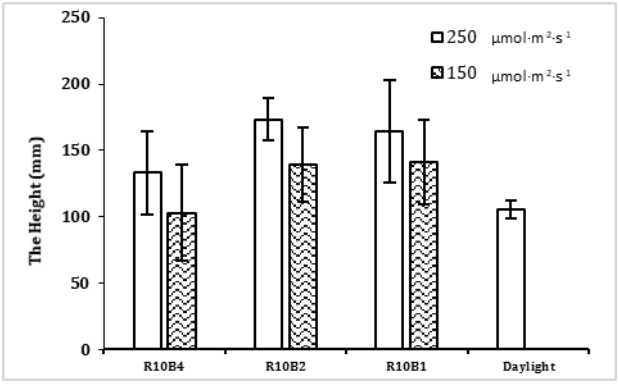

Figure 3. The height of coriander under different light treatments using three R:B light spectral combinations and two different PPFD levels at 150 and 250 µmol m^{-2} s^{-1}. Vertical bars indicate standard errors (*n*=6).

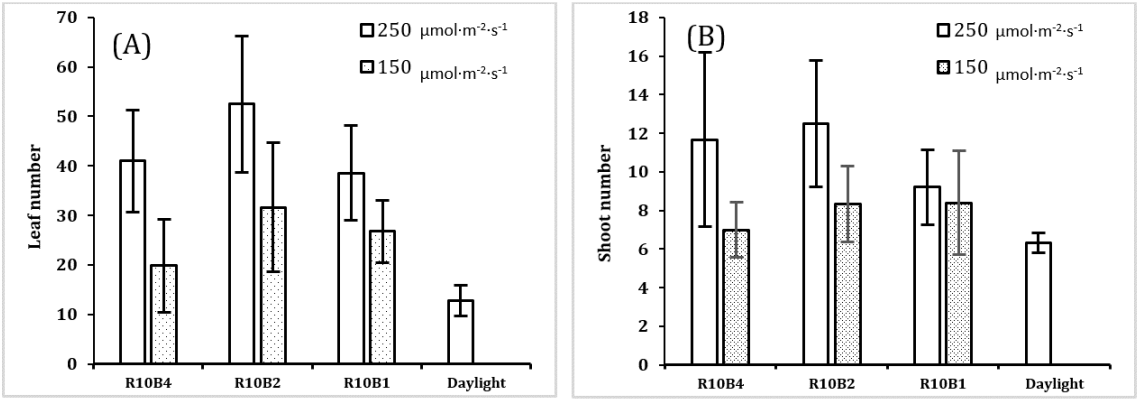

Figure 4. Leaf number (A) and shoot number (B) of coriander under different light treatments using three R:B light spectral combinations and two different PPFD values at 150 and 250 µmol m^{-2} s^{-1}. Vertical bars indicate standard errors (*n*=6).

Coriander growing under daylight had the lowest shoot number compared to LED light treatments. At 250 µmol m^{-2} s^{-1}, coriander under R:B ratio 10:2 had the highest shoot number. However, at 150 µmol m^{-2} s^{-1}, the higher red-light levels resulted in increased coriander shoot numbers. Therefore, shoot number under LED treatments was about 1.10-1.97 times higher than the daylight treatment (Figure 4B). In Naznin et al. (2016) a R:B ratio of 10:1 had the highest shoot number and increasing the red light levels caused shoot number to decrease continuously at 120 µmol m^{-2} s^{-1}.

Figure 5 shows the fresh and dry weights of coriander were significantly increased by using LED light compared to the day light. Comparing the daylight treatment to red and blue light combinations showed the fresh weight to be between 4.4 and 21.51 times greater and the dry weight 4.69-21.82 times greater. At both PPFD intensity levels, the highest fresh and dry weights for coriander was obtained from the R:B ratio 10:2. Furthermore, at the 250 µmol m^{-2} s^{-1} level no significant change in fresh and dry weight when red light levels were increased or decreased. Previous research reported that 660 nm red light increased fresh weight of lettuce compared to white light (Chen et al., 2016) and red LED increased the dry weight of plants compared to blue LED light (Son and Oh, 2015).

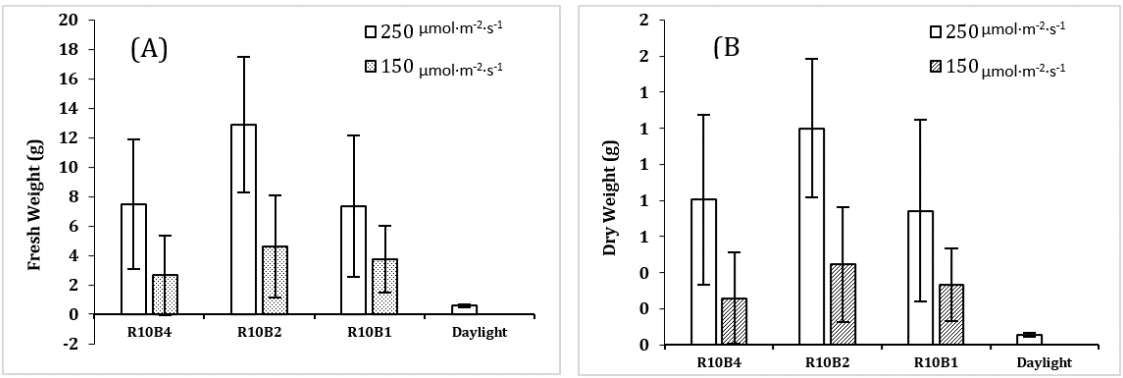

Figure 5. Fresh weight (A) and dry weight (B) of coriander under different light treatments using three R:B light spectral combinations and two different PPFD values at 150 and 250 µmol m^{-2} s^{-1}. Vertical bars indicate standard errors (*n*=6).

The total antioxidant activity of coriander grown under daylight had lower antioxidant activity $plant^{-1}$ than those under LED lights. The highest antioxidant activity was obtained from coriander under R:B ratio of 10:2. Furthermore, antioxidant activity was reduced when the R:B ratio was 10:1 and 10: 4 (Figure 6A). Coriander grown under high PPFD had more total antioxidant activity than the lower PPFD. The trend for total antioxidant activity followed the trend of dry weight of coriander. Nevertheless, the antioxidants activity 1 g^{-1} dry weight of coriander grown under daylight was higher than the LED light treatments (Figure 6B). This may be from the positive effect of light outside the spectrum range of photosynthetic active radiation (400-700 nm). The antioxidant activity values were no different at both PPFD values. Furthermore, at 250 µmol m^{-2} s^{-1}, the antioxidant activity was reduced by increasing red light level. The effect on antioxidant activity by the light spectra was similar to previous research. For instance, Son and Oh (2015) reported the highest antioxidant was found in lettuce grown under the highest blue light combination. Naznin et al. (2016) also found that the highest antioxidant activity for coriander grown under the highest blue light combination.

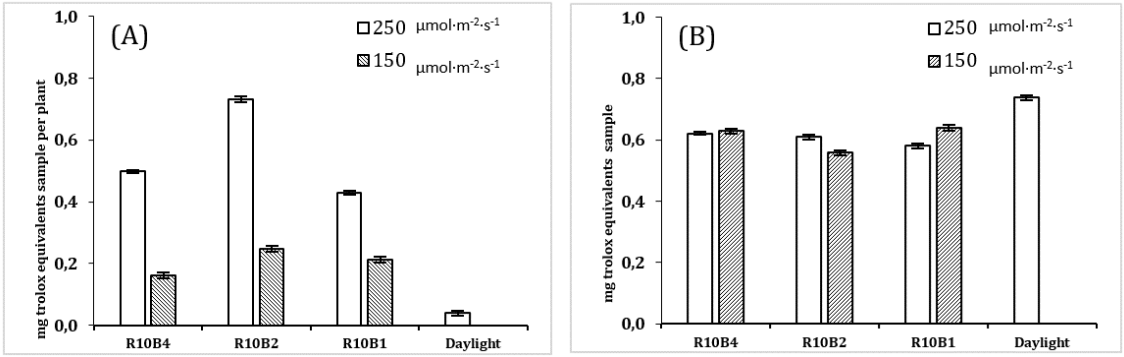

Figure 6. Total antioxidant activity (A) and antioxidant activity 1 g^{-1} of dry weight (B) of coriander under different light treatments using three R:B light spectral combinations and two different PPFD values at 150 and 250 µmol m^{-2} s^{-1}. Vertical bars indicate standard errors (*n*=3).

CONCLUSIONS

This study investigated the effects of red and blue light spectra on growth and antioxidant activity in coriander cultivated under different light spectra and intensities compared to daylight. It was found that LED lights has a greater effected on the growth of coriander than daylight. Under high LED light levels, the PPFD increased growth and total antioxidant activity compared to lower PPFD light levels. The light spectral combination of

R:B ratio 10:2 at 250 μmol m^{-2} s^{-1} produced the highest biomass compared to the other light treatments. By Increasing the red light levels resulted in increased height and shoot number. Increasing the blue light levels increased the leaf number, fresh and dry weight of coriander. Increased blue light levels, enhanced the accumulation of antioxidant activity. However, the daylight treatment resulted in the highest antioxidant activity g^{-1} dry weight.

ACKNOWLEDGEMENTS

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High temperature enhanced zinc and water content in inflorescences and shoot tips of longan (*Dimocarpus longan* Lour.)

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Abstract

Recently, global warming induced climate change in several regions of the world, is leading to yield loss in agriculture sectors, especially fruit crops. Subtropical fruit trees are one of the most sensitive species affected by rising temperatures, especially longan (*Dimocarpus longan* Lour.). Improvements on basic knowledge about increasing temperature effects on water content and zinc in inflorescences and shoot tips improved longan fruit production. Inflorescences and shoot tips of 'Poung Tong' longan were collected and the cutting area was dipped in water and carried by plastic bag to prevent water loss before placing into an artificial incubator at 40°C ambient temperature with air circulation for 30 min. The control treated samples were kept at room temperature. Analysis of the zinc content in inflorescences and shoot tips was carried out by precision detection of inductively coupled plasma mass spectrometry (ICP-MS). Findings showed the zinc content was 30.39% above the control treatment in the inflorescences and the zinc content in the shoot tips incubated under the high temperature condition (40°C ambient temperature) was 15.06% above the control treatment. In addition, the water content was also translocated into the inflorescences and the shoot tips when incubated under 40°C. The amount of water increased by 7 and 9% over the control treatment in the inflorescences and the shoot tips, respectively. The changes in water content, within the smaller branches of the inflorescences, scanned by electron microscope (SEM), supports the rapid translocation ability of zinc to accumulated in the inflorescence.

Keywords: longan, high temperature, zinc, water content

INTRODUCTION

Global warming and increasing temperatures in the world environment are a major crisis creating a barrier for fruit production. This is especially true for flowering and fruit set, leading to yield loss in fruit crops (Benlloch-González et al., 2018, 2019). High temperature (>35°C) and prolonged heat stress of plants, cause physiological and biochemical adaptations in defense mechanisms. Depending on the developmental stages of growth, plant species and types of plant tissue involved, a depletion in productivity may result, especially under severe high temperature conditions (Bita and Gerats, 2013). During a plant's growth and development, zinc has been identified as an essential micronutrient. In higher plants, zinc plays an important role in regulating various plant functions such as detoxication of reactive oxygen species (ROSs) and plant growth structures, especially in fruit crops (Marschner, 1995; Gupta et al., 2016). In addition, Zn plays a key role in a plant's tolerance to several abiotic stresses such as drought (Ma et al., 2017). Furthermore, zinc has been shown to improve plant growth characters, including root and shoot traits, when grown under severe drought stress

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(Gadallah, 2000). Under high temperature situations, Akter and Islam (2017) indicated that zinc was remobalised and improved plant crop development in wheat. Tao et al. (2018) reported that zinc regulated the protein yield trait, and grain weight in wheat under heat stress. Plant water status is a very sensitive parameter to an increase of ambient temperature. Oscillating temperatures under high temperature regimes caused plant functions to change. This is dependent on the developmental stage of a plant structure, especially in peach flower buds (Yooyongwech et al., 2008a). Sasaki et al. (2015) found that xylem vascular bundles and water transport tissue relate the function of zinc movement in the active growth tissue of rice.

One of Thailand's economically important fruit crop, 'Poung Tong' longan, was studied to determine the effects of 40°C temperatures on changes of zinc and water content in inflorescence and shoot tips.

MATERIAL AND METHODS

Plant material and treatments

Plant tissue of 'Poung Tong' longan was used for this experiment. The inflorescences and leaf shoots were collected from an orchard located in the Samut Sakhon Province, central region of Thailand. The cut area was placed in water in a plastic bag and sealed to reduce water loss. After collecting, about 10 cm of the inflorescences (contained 70% flower bud and 30% full boom) were prepared. Approximately 15 cm of a single shoot, fully expanded leaves to the end of the shoot, were kept in water. The samples were placed in an artificial incubator at 40°C with air flow circulation for 30 min. Control samples were kept at room temperature. In addition, samples placed in an ambient air temperature at 40°C without air circulation for 30 min and observed. The relative humidity was about 10±3 and 53±3% in the incubator and the room, respectively. All plant samples taken were kept at -20°C until the microelement analysis.

Water content and stem diameter determination

According to the heat treatment, water content was calculated by weight basis. The following equation was used for water content (%) = 100×(fresh weight – dry weight)/fresh weight. In addition, the diameter of the inflorescences (1-2 cm) and leaf shoot tips at the end were measured using digital Vernier calipers. Furthermore, the xylem zone, 1-2 cm at the end of each shoot tip was observed by scanning electron microscope (SEM) for small branchlet sized inflorescences. A stereo light microscopy (SLM) was used for the leaf shoot tip. The relationships of the water content percentage and the diameter of tip samples were calculated as a ratio, modified from Cao et al. (1999) research.

ICP MS analysis

Zinc and calcium microelement analysis was conducted using a modified method from Jajda et al. (2015) research. Powder from the 1-2 cm end of the tip (0.3-0.5 g) were digested using nitric acid and microwaved in Titan MPS, PerkinElmer, Germany. Supernatants were filtrated, zinc and calcium analyzed using a ICP-MS (NexION 350X, PerkinElmer, USA).

Experimental design and statistical analysis

The experiment was designed as a completely randomized design (CRD) with three replications (n=3). Error bars in each figure represented ±SE. The mean values were compared using Tukey's HSD (>2 treatments), t-test (only two treatments) and analyzed by SPSS software.

RESULTS

Increasing temperature has been well established as having a negative effect on plant growth and development, especially fruit crops. In this study, ambient temperature at 40°C with or without air circulation showed a trend for accumulation of zinc in the inflorescence. Furthermore, zinc in 40°C ambient temperature with air circulation was increased by 30.39% over the control treatment. Similarly, an increase in zinc content in the shoot tips was

recognized in the high temperature conditions of 40°C with air circulation. On the other hand, zinc content in the shoot tips under the 40°C was slightly enhanced by 15.06% over the control treatment (Figure 1B). Calcium content in each treatment of inflorescences and shoot tips remained unchanged (Figure 1C, D). Calcium has been reported as having a low translocation rate in plant tissues, leading to high concentration in the root tissues (da Cruz et al., 2019).

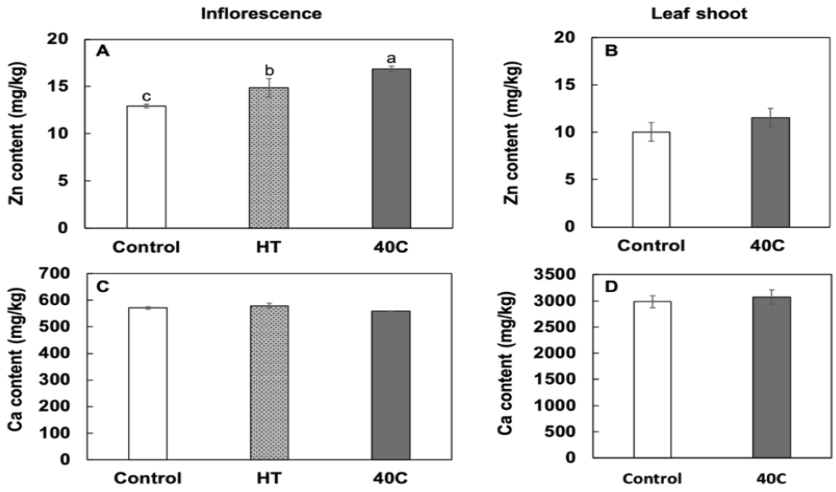

Figure 1. Zinc (Zn) and calcium (Ca) content in the inflorescences (A, C; different letters in each bar represent significant different at p≤0.05 using Tukey's test) and shoot tips (B, D; * represents significant different using *t*-test at p≤0.05) of 'Poung Tong' longan, incubated under control, heat temperature of 40°C without air circulation (HT) and 40°C with air circulation (40°C) for 30 min. Error bars in each column represent ±SE.

Water content in both inflorescences and shoot tips under the 40°C ambient temperature condition compared to the control treatment was 6.97 and 8.98% greater in the inflorescences and leaf shoot tips respectively (Figure 2). Furthermore, the diameter of flower branchlet and leaf shoot tip was 1.15 and 2.34 mm larger, respectively (Figure 3A). The ratio of water content in the inflorescences was higher than in the leaf shoot tips (Figure 3B). The xylem vascular tube was also apparent in the smaller branchlet of the inflorescences than in the stem of the leaf shoot (Figure 4).

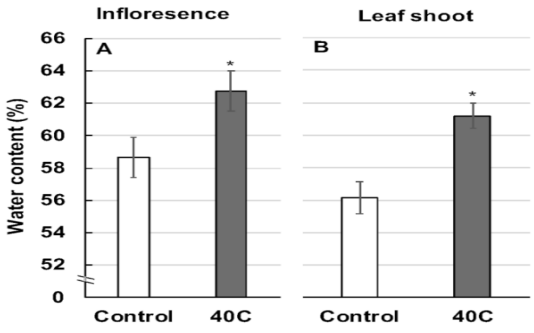

Figure 2. Water content in the inflorescences (A) and shoot tips (B) of 'Poung Tong' longan, under control and 40°C ambient temperature with air circulation (40°C) for 30 min. * represents significant different using *t*-test at p≤0.05. Error bars in each column represent ±SE.

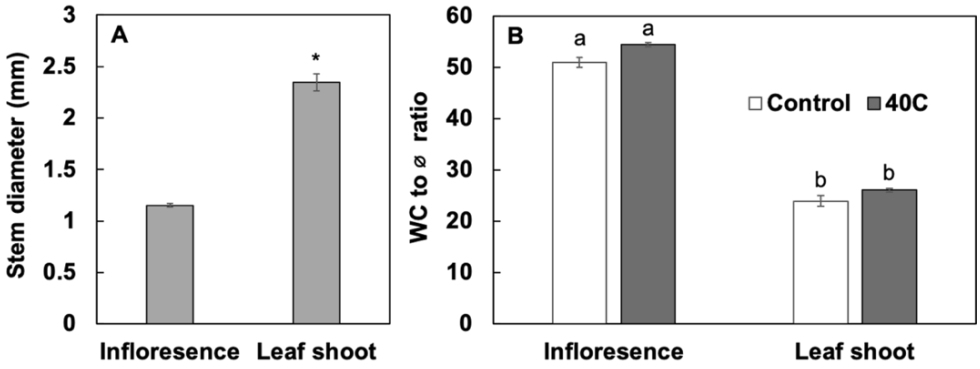

Figure 3. Inflorescence branchlet and shoot tip diameters (A; * represents significant different using *t*-test) and ratio of water content and diameter in the inflorescences and shoot tips (B; Different letters in each bar represent significant different at p≤0.05 using Tukey's test) of 'Poung Tong' longan, under control and 40°C ambient temperature with air circulation (40°C) for 30 min. Error bars in each column represent ±SE.

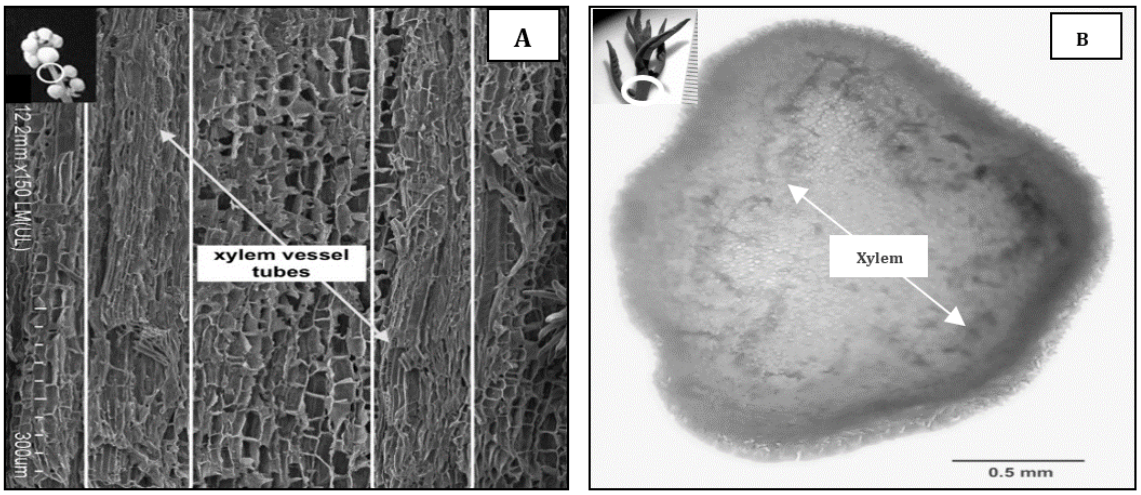

Figure 4. Xylem vessel zone in SEM long section of inflorescence branchlet (A) and SLM cross section of leaf shoot tips (B) in 'Poung-Tong' longan (a circle on of the top of the A and B indicated the location of the branchlet and the shoot tip for the xylem observation).

DISCUSSION

Due to environmental changes and negative effects of heat on agricultural plant production, a focus on potential discovers and the best solutions to mitigate against these changes could lead to yield maintenance or improvement (Bita and Gerats, 2013). Investigations into zinc content in inflorescences and the leaf shoot tips of longan 'Poung Tong' under high temperature stress (40°C ambient temperature) conditions was undertaken. Our findings confirm previous studies conducted on *Triticum aestivum* L. 'Acalou' under heat stress, which also found zinc concentrations differed in various plant parts. Stage of development also affected the zinc concentration and evidence showed zinc increased during maturation stages for the spike, shoot, and root under heat stress (Dias and Lidon, 2009). Furthermore, the zinc content in *Phaseolus vulgaris* was reported to be the highest in young tissue (40.1 mg kg^{-1} in the actively growing parts) in relation to leaf and stem tissue (Viets et al., 1954). Dias and Lidon (2009) suggested that zinc accumulation under heat stress

conditions may regulate membrane permeability. This permeability may be due to the mechanisms within the water channels, *PIPs*, genes, and water mobility into flower buds under increasing temperature (Yooyongwech et al., 2008b). Moreover, water enrichment in plant tissues under high temperature stress is related to an enhancement of hydraulic conductivity of plant cell membrane due to the reduced viscosity of water translocation (Akter and Islam, 2017).

In addition, in the stem of peach, a study by Cao et al. (1999) found the smaller the stem diameter the higher the percentage of water content. During active development of plant organs, including flower meristems, enhanced accumulating water and zinc, especially under 40°C ambient temperature conditions is possible. Sasaki et al. (2015) suggested that the zinc is required at high levels in plant development zones, such as the meristem and the elongation zone. The uploading of water through transpiration flow via xylem vascular tissues assists in zinc accumulation. Additionally, zinc-translocation detected by X-ray fluorescence spectroscopy in *Phaseolus vulgaris* demonstrated a decline in velocity and a concentration gradient from root-to-shoot. This suggested that radial mobility of zinc occurred through the xylem transport mechanisms (da Cruz et al., 2019). Furthermore, an increase of zinc is reported to be link with heat tolerance in wheat (Akter and Islam, 2017). In the case for longan, it is possible that the greater zinc levels under high temperature conditions may be a mechanism of heat protection, especially in the inflorescence.

In conclusion, the trends of zinc and water content increasing in the tips of the excised inflorescences and the shoot of 'Poung Tong' longan under the 40°C temperature condition was established by this study. However, the regulation on water content and zinc level in the active growing parts using high temperature incubation should be investigated further.

ACKNOWLEDGEMENTS

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6-Benzylaminopurine application enhances petal coloration of pink flowered chrysanthemums

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Abstract

During growing seasons with high-temperatures, anthocyanin accumulation in petals is low, resulting in poor coloration of the inflorescences in pink flowered chrysanthemums. To restore anthocyanin accumulation, 6-benzylaminopurine (BA) at 50 or 100 mg L^{-1} was applied to pink flowered 'Pelican' chrysanthemums at constant temperatures of 20, 25, and 30°C. BA-treated flowers displayed deeper pink petals at 25°C having intermediate L* and C* values compared with those at 20 and 25°C without BA. The amounts of two main anthocyanins, Cy 3-6"-MMG and Cy 3-3", 6"-DMG, in the BA-treated flowers were higher than in the control flowers at 25°C. A higher concentration of BA (100 mg L^{-1}) produced higher anthocyanin accumulation compared to that at 50 mg L^{-1} BA. The highest anthocyanin accumulation, which was significantly different from the control, occurred when BA was applied during the petal appearance to vertical stage (the petal elongated to the vertical position). However, accumulation was lower when BA was applied after petal expansion. This study demonstrates for the first time that applying BA is effective in enhancing coloration and pigmentation in chrysanthemum petals under elevated temperature conditions at 25°C.

Keywords: anthocyanins, BA, cytokinin

INTRODUCTION

Chrysanthemum is one of the most important ornamental plants in world flower markets. All-year-round cut chrysanthemum production is well-established through the use of photoperiodic control (night break) and under protected cultivation. Markets demand a stable supply of cut flowers of uniform quality. However, abnormal growing conditions have increasingly occurred world-wide in recent years, especially regarding the occurrence of high temperatures. Temperatures beyond the optimal temperature range for the cultivation of chrysanthemum can cause delays in flowering time and poor petal coloration (Nozaki and Fukai, 2008).

The main pigments in chrysanthemum flowers are anthocyanins and carotenoids. Pink and red flowered chrysanthemum genotypes accumulate cyanidin 3-O-(6"-O monomalonyl-glucopyranoside) (Cy 3-6"-MMG) and cyanidin 3-O-(3", 6"-O-dimalonyl-glucopyranoside) (Cy 3-3", 6"-DMG). High temperatures decrease the accumulation of the main anthocyanins, resulting in poor petal coloration (Nozaki et al., 2006b). Reduced anthocyanin accumulation under high temperature conditions has been reported in many flowers, including roses (Dela et al., 2003), chrysanthemums (Nozaki et al., 2005), and lilies (Lai et al., 2011), confirming the universal nature of this response. Furthermore, the accumulation of anthocyanins is dependent on the stage of petal development, and the rapid increase in accumulation in the early developmental stage of the petals is most sensitive to high temperature conditions (Puangkrit et al., 2018).

Many plant growth regulators have been investigated to determine their role in the control of anthocyanin biosynthesis. For example, gibberellins (Weiss et al., 1995), abscisic acid (Jeong et al., 2004; Katayama-Ikegami et al., 2016), jasmonate (Shan et al., 2009; Peng et al., 2011), and 6-benzylaminopurine (Ji et al., 2015) have been shown to be involved in regulating anthocyanin accumulation. Cytokinins are important regulators in many aspects of

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plant growth and development including nutrient mobilization, cell division, and shoot formation (Binns, 1994; Deikman and Hammer, 1995). Exogenous cytokinin application has been shown to induce anthocyanin accumulation in carrot suspension cell cultures (Ozeki and Komamine, 1986), *Arabidopsis* seedlings (Deikman and Hammer, 1995), and apple callus cultures (Ji et al., 2015).

6-Benzylaminopurine (BA) is an asynthetic cytokinin that has been used as an authorized agro-chemical in a number of different applications in commercial horticulture. BA has been shown to promote anthocyanin accumulation in plant tissue (Deikman and Hammer, 1995), and in detached petals of *Rosa hybrida* (Nakamura et al., 1980) and *Impatiens balsamina* L. (Klein and Hagen, 1961), indicating that BA may be a candidate for practical chemical control of petal coloration in summer production of cut chrysanthemum flowers. However, there appears to be no published reports on the effect of BA on anthocyanin accumulation in chrysanthemum petals. In this study, we demonstrate that BA can be used for improvement of petal coloration in pink flower chrysanthemums under high temperature conditions as a practical cultivation technique.

MATERIALS AND METHODS

Plant materials

Pink flowered chrysanthemum (*Chrysanthemum morifolium* Ramat.) 'Pelican' was used in this study. Rooted cuttings of 'Pelican' were planted in 15-cm plastic pots with a masa soil (volcanic soil):manure (3:1) substrate. The plants were grown in a greenhouse maintained at a minimum temperature of 13°C until the flower bud break stage, then transferred to growth chambers kept at constant temperatures of 20, 25, and 30°C with natural irradiation. Commercial liquid fertilizer (Hyponex, N-P-K=6-10-5) was applied weekly. Three pots were used in each treatment. Three ray florets from an inflorescence were sampled randomly (total 9-ray florets) for petal color evaluation and three were used for pigment analysis.

BA treatment

A commercially available BA solution (3% 6-benzylaminopurine (BA) with surfactant, Kumiai Chemical Industry Co. Ltd.) was diluted with distilled water to a rate of 50 or 100 ppm BA. The BA solutions were sprayed on the flowers every two days in the morning after the ray florets appeared.

Petal color measurement

Petal color was determined by measuring the lightness (L*), chromatic component a* which represents the degree of green and red contrast, and chromatic component b* which represents the degree of blue and yellow contrast using a colorimeter (NR-3000; Nippon Denshoku Industries Co., Ltd., Japan). Chroma was calculated using the equation $C^* = (a^{*2}+b^{*2})^{1/2}$.

Pigment analysis

Anthocyanins analysis was conducted following Puangkrit et al. (2018). Briefly, dried samples were immersed in acidified methanol and kept at 4°C for 24 h. The extracts were then dried and re-dissolved in acidified methanol. Filtered extracts were injected into an HPLC equipped with two Cosmosil $5C_{18}$AR-II columns (4.6 mm i.d. × 50 mm and 4.6 mm i.d. × 250 mm). Solvent A (1.5% H_3PO_4 in H_2O, v/v) and solvent B (1.5% H_3PO_4, 20% CH_3COOH, and 25% CH_3CN in H_2O, v/v) were used for liner gradient elution. The two main anthocyanins were identified by comparing their retention times at 520 nm with those of standard compounds. The content of the anthocyanins was defined as the area within each anthocyanin pigment peak and normalized as 1 g of fresh weight (g^{-1} FW). The concentrations of Cy 3-6"-MMG and Cy 3-3",6"-DMG at 25°C in the control treatment was defined as 100, and values in different treatments were expressed as relative content values ± SE (n=3). The relative values were subjected to a Tukey's multiple range test.

Experiment design

1. Experiment 1 – effect of BA on coloration and pigmentation of pink flowered chrysanthemum.

To evaluate the effect of BA on petal color, BA (100 mg L^{-1}) was sprayed every two days on the inflorescences of plants kept at 25°C until the outer petals (ray florets) opened at the horizontal position (total six spray applications). Control plants (water spray application) were kept at 20, 25, and 30°C. Petals were harvested on the day after the last spray application, measured for petal color, and dried for pigment analysis. All treatments consisted of three plants.

2. Experiment 2 – effect of petal development stage-specific BA application on pigmentation of pink flowered chrysanthemums.

Petal pigmentation in pink flowered chrysanthemum is dependent on petal developmental stage (Puangkrit et al., 2018). Both 50 and 100 mg L^{-1} BA were applied for different terms based on the developmental stages of the inflorescences at 25°C (Figure 1). The application terms were as follows: Term 1, bud break (top of the inflorescence opened and the thin membrane ruptured) to petal appearance; Term 2, petal appearance to petals elongated to the vertical position; Term 3, petals expanded vertical to the horizontal position. Each treatment consisted of three plants. BA was applied every day during each term. Petals were harvested for pigment analysis seven days after petals had elongated to the vertical position.

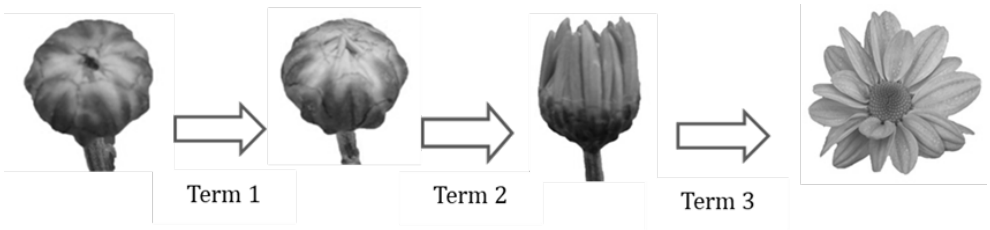

Figure 1. Developmental stages of inflorescence and related treatment terms.

3. Experiment 3 – effect of BA on pigmentation of pink flowered chrysanthemums at 20 and 30°C.

To confirm the effect of BA under both higher and lower temperature conditions, concentrations of 50 and 100 mg L^{-1} BA were applied to the inflorescences of plants kept at both 20 and 30°C every day from bud break to the stage when petals were expanded to the horizontal position. Each treatment consisted of three plants.

Statistical analysis

All the experiments were designed using a completely randomized design (CRD) and were subjected to an analysis of variance (ANOVA). The mean values were subjected to a Tukey's multiple range test (post-hoc comparisons) and an alpha level of $p<0.05$ was used for acceptance of the null hypothesis to determine significant variables.

RESULTS

Experiment 1 – effect of BA on coloration and pigmentation of pink flowered chrysanthemums

Flowers exposed to a higher temperature displayed a pale petal color with higher L* and lower C* values (Figure 2). The flowers treated with BA at 25°C displayed deeper pink petal color compared with the control flowers at 25°C. The L* and C* values of BA-treated flower were intermediate to those of control flowers kept at 20 and 25°C. BA-treated flowers had petals with curved ends, resulting in smaller-diameter inflorescences.

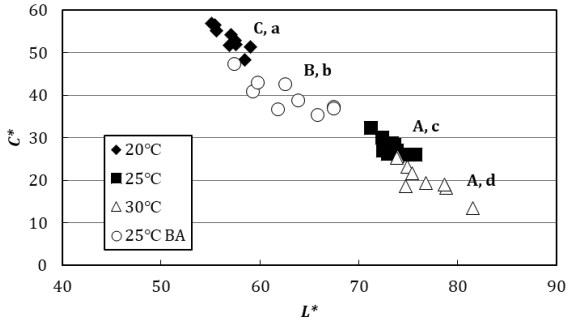

Figure 2. Effect of temperature and BA on petal color of pink flowered chrysanthemums (*n*=9). The different letters in each treatment are significantly different ($p<0.05$), uppercase letters are L* values, and lowercase letters are C* values.

The two main anthocyanins, Cy 3-6”-MMG and Cy 3-3”, 6”-DMG, were detected, as shown previously in other pink flowered chrysanthemums (Nozaki et al., 2006a). The pigment concentrations were low at 30°C compared with those at 20 and 25°C (Figure 3). The anthocyanin concentration in the BA-treated flowers at 25°C was intermediate of those that were untreated at 20 and 25°C. BA treatment did not change the components of the main anthocyanins in the petals. These results indicate that petal coloration and anthocyanin accumulation in the chrysanthemum petals is temperature dependent, and that BA treatment enhances anthocyanin accumulation in the petals of chrysanthemum ‘Pelican’.

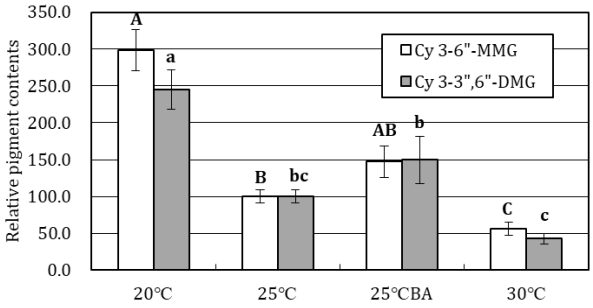

Figure 3. Effect of temperature and BA on pigmentation of pink flowered chrysanthemums. Plants were kept at 20, 25 and 30°C until inflorescences opened. BA (100 mg L^{-1}) was sprayed every two days until the outer petals (ray florets) opened to the horizontal position. The values of Cy 3-6”-MMG and Cy 3-3”, 6”-DMG at 25°C were defined as 100, and vertical bars indicate ± SE (*n*=3). Values with different letters within each pigment are significantly different ($p<0.05$).

Experiment 2 – effect of petal development stage-specific BA application on pigmentation of pink flowered chrysanthemums

To determine the optimal inflorescence developmental stage for the application of BA for enhancing anthocyanin accumulation, different doses of BA were applied at specific inflorescence development stages. Because each term required different days to complete, BA application times were different, namely, three times in Term 1, four times in Term 2, and seven times in Term 3, for a total of 14 times. A higher concentration of BA (100 mg L^{-1}) produced higher anthocyanin accumulation compared with those at 50 mg L^{-1} BA (Figure 4).

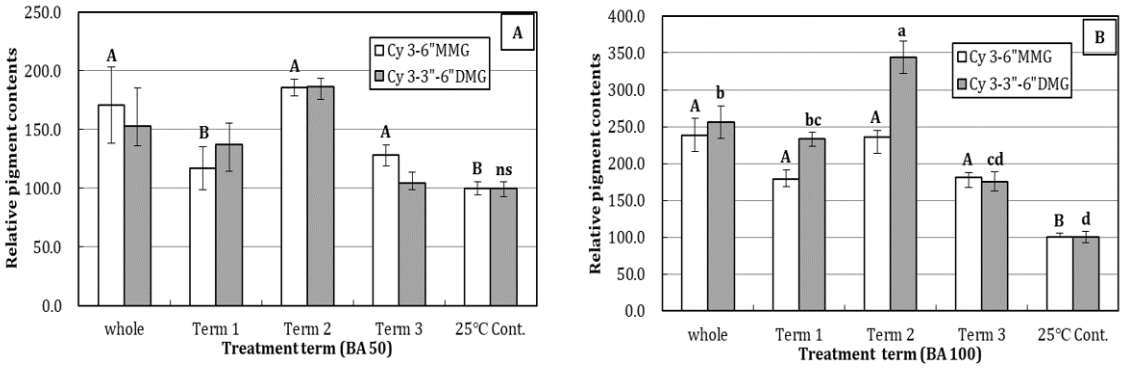

Figure 4. Effect of petal development stage-specific BA application on pigmentation of pink flowered chrysanthemums. BA 50 mg L^{-1} (A), 100 mg L^{-1} (B). For Terms 1, 2 and 3, refer to Figure 1. Whole is all terms combined and 25°C Cont. is a water control treatment. The values of Cy 3-6"-MMG and Cy 3-3", 6"-DMG at 25°C and sprayed with water (control) were defined as 100, and vertical bars indicate ±SE (*n*=3). Values with different letters within each pigment are significantly different ($p<0.05$).

Anthocyanin content was more than double that of the control when 100 mg L^{-1} BA was applied in Terms 1, and 2, and over all of the periods. The highest anthocyanin accumulation was found when BA was applied in Term 2, that is, in the time from petal appearance to the vertical stage. The effect of BA on anthocyanin accumulation was a little lower when applied in Term 3 compared with the other treatments. The same trends were observed at 50 mg L^{-1} BA. The results showed that the most effective BA application term was during the period of petal (ray floret) development. Inflorescence size was a little smaller when BA was applied, but no malformation of petals was observed when BA was applied in Terms 1-3 (Figure 5).

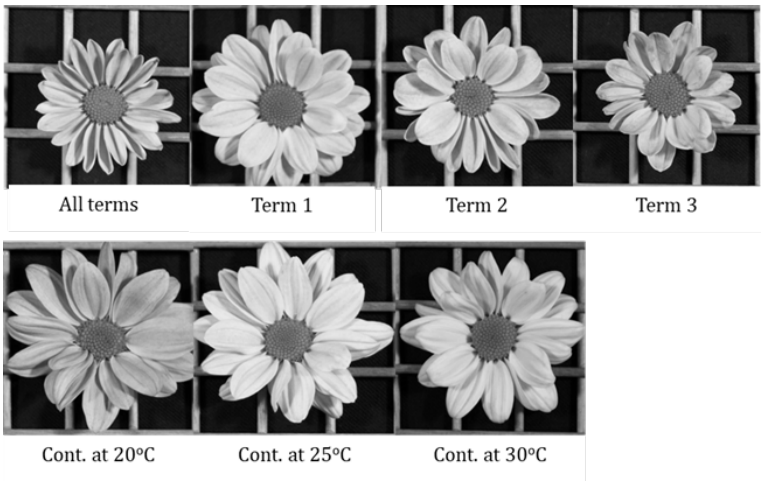

Figure 5. Flowers applied BA at different petal developmental stages. BA concentration was 100 mg L^{-1} for Term 1, 2 and 3 (Figure 1).

Experiment 3 – effect of BA on pigmentation of pink flowered chrysanthemums at 20 and 30°C

To confirm the effect of BA and temperature on pigmentation, rates of 50 and 100 mg L^{-1} BA were applied to flowers at constant temperatures of 20 and 30°C. The anthocyanin accumulation in the untreated petals kept at 20°C was more than three times higher than that at 25°C (control) (Figure 6). A further increase in anthocyanin accumulation was observed when the petals were treated with either 50 or 100 mg L^{-1} of BA at 20°C. Applying 100 mg L^{-1}

BA produced higher anthocyanin accumulation compared with the inflorescences treated with 50 mg L^{-1} BA at 20°C. In contrast, there was no effect of BA application on anthocyanin accumulation at 30°C (Figure 6B). The anthocyanin accumulation in the petals kept at 30°C was much lower than at 25°C, even when the inflorescences were sprayed with 100 mg L^{-1} BA.

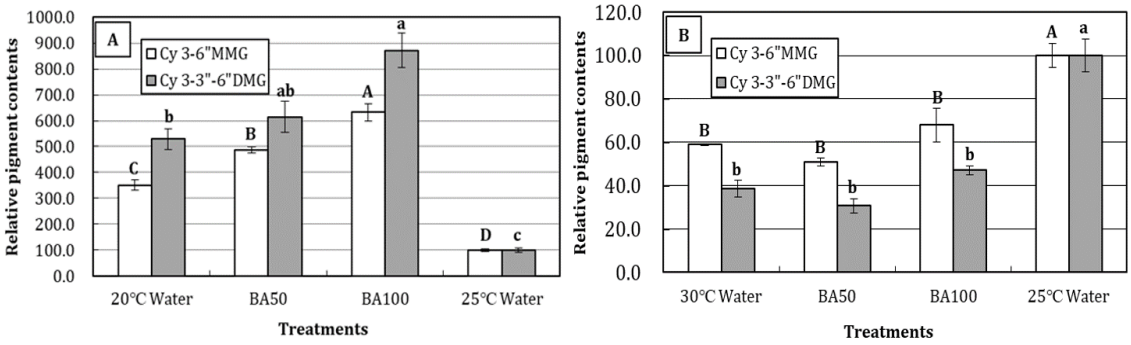

Figure 6. Effect of BA on pigmentation of pink flowered chrysanthemums at 20 and 30°C. Treatment at 20°C (A), Treatment at 30°C (B). The values of Cy 3-6"-MMG and Cy 3-3", 6"-DMG treated at 25°C and sprayed with water was defined as 100, and vertical bars indicate ±SE (*n*=3). Values with different letters in each pigment were significantly different ($p<0.05$).

DISCUSSION

The relationship between temperature and anthocyanin accumulation has been well documented in many horticultural crops because reductions in anthocyanin at elevated temperatures is a serious problem in some fruit and flower production (Dela et al., 2003; Katayama-Ikegami et al., 2016; Lai et al., 2011; Mori et al., 2005). The present results showed that the accumulation of anthocyanin in the petals of pink flowered chrysanthemum 'Pelican' decreased in parallel with an increase in temperature. Temperature-dependent decreases in petal coloration and pigmentation have been recognized in other genotypes of chrysanthemum (Huh et al., 2008; Nozaki et al., 2006a). The temperature-dependent decrease is a quantitative reaction because there is no critical temperature for the reaction, suggesting that anthocyanin synthesis is downregulated biochemically according to the elevation in temperature. Lowered gene expression of key enzymes in the anthocyanin biosynthetic pathway under high temperature conditions has been reported in chrysanthemum (Huh et al., 2008; Puangkrit et al., 2018).

The present results showed that applying BA restores petal coloration and pigmentation in chrysanthemum petals (ray florets) at 25°C. The effect is BA-dose dependent and application-stage specific (Figure 4). The optimal developmental stage for application to the inflorescences was Term 2, that is, during the period from petal appearance to the stage when petals are elongated into the vertical position. This is the period when pigment accumulates most rapidly in the petals of chrysanthemum (Puangkrit et al., 2018). This finding suggests that BA increases anthocyanin biosynthesis in chrysanthemum petals similarly to that reported in cultured cells and calluses (Ozeki and Komamine, 1981; Ji et al., 2015). It has been reported that BA application brought about upregulation of phenylalanine ammonia lyase 1, chalcone synthase, chalcone isomerase and dihydroflavonol reductase in *Arabidopsis thaliana* (Deikman and Hammer, 1995). However, the mechanism by which BA enhances anthocyanin biosynthesis remains unclear. The present study also showed that application was less effective in Term 3 even though BA was applied more times during that term (7 times of application) than in the other terms (3- and 4-times). It is thought that the decrease in anthocyanin concentration in later-stage flowering is due to higher degradation rather than synthesis of anthocyanin (Puangkrit et al., 2018). This assumption suggests that BA was not effective in suppressing anthocyanin degradation in the petals. The increase in anthocyanin accumulation by BA was observed in the petals kept at both 20 and 25°C but did

not occur at 30°C. This result might be due to an increase in degradation at 30°C. Hence the results overall support the conclusion that BA enhances anthocyanin biosynthesis but does not suppress degradation.

The present study shows that applying BA is effective in improving coloration and pigmentation in chrysanthemum petals under elevated temperature conditions up to around 25°C. This result can be applied practically to achieve stable and high-quality cut chrysanthemum flower production in high temperature seasons.

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Changes in cell wall polysaccharides and quality of strawberry fruits from summer to autumn

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Abstract

Two strawberry cultivars, 'Summer Tiara' (a hybrid of a June-bearing cultivar and an ever-bearing (EB) cultivar) and 'ES-10' (an EB cultivar), were grown from July to November, 2015 at Yamagata Shonai Agricultural Technique Improvement Research Office, Japan. Changes in the fruit quality in cell wall polysaccharides, as affected by climacteric conditions, were compared. The fruit of 'Summer Tiara' was heavier overall than that of 'ES-10'. The total sugar concentration increased as the growing temperature decreased, and there was no difference in the concentration between the two cultivars. 'ES-10' was characterized by a higher reducing sugar concentration and a lower sucrose concentration than those of 'Summer Tiara'. In contrast to the total sugar concentration, the polyphenol concentration was greater at higher growing temperatures, especially in 'Summer Tiara'. Fruits harvested in summer were softer than those harvested in autumn, and such a fruit softening was closely associated with the solubilization of pectin and hemicelluloses. The fruit of 'ES-10' were firmer than those of 'Summer Tiara', because of the higher uronic acid concentrations in the ionically-bound pectin fraction. 'Summer Tiara' was characterized by a higher phenol: acetic acid:water (PAW)-soluble solid concentration than that of 'ES-10' which means that a larger amount of the solubilized pectin polymers in 'Summer Tiara' were transferred into the PAW-soluble fraction.

Keywords: cell walls, pectin, polyphenols, soluble sugars, summer to autumn production

INTRODUCTION

Strawberry fruit has a high market demand in Japanese markets during the period from summer to autumn (July-November), but that demand during this period has mainly been met by imported fruit. Summer to autumn strawberry production (SASP) in Japan is difficult mainly because of high temperatures, especially in the middle to southern regions of Japan (Yamasaki, 2013). Consequently, northern Japan has an advantage for SASP because of its cool summer. However, fruit production using June-bearing (JB) cultivars has some limitations, such as the failure of floral induction and rapid fruit softening after harvest (Wada, 2014). Ever-bearing (EB) cultivars or hybrid cultivars obtained from EB and JB crossing are, therefore, used for SASP, because of better floral induction and longer shelf life than that in JB cultivars (Yamasaki, 2013).

The sweetness of strawberry fruit is determined by the amount and composition of soluble sugars, mainly consisting of glucose, fructose, and sucrose (Nishizawa et al., 2002), whose variation may depend on season even though they often show a large seasonal change (Ruan et al., 2013). The firmness of strawberry fruit decreases under high-temperature conditions (Bourne, 1982), and such fruit softening often coincides with the modification of primary cell walls and degradation of the middle lamella, including increases in pectin solubilization and the depolymerization of xyloglucan (Santiago-Doménech et al., 2008). In contrast, polyphenolic substances that are known as anti-oxidative substances in strawberry fruit, often increase as the growing temperature increases (Kawanobu et al., 2010). Therefore, the strawberry fruit from EB cultivars, with its high polyphenol concentration, may be of sufficient merit to off-set its reduced market value due to the softening and low sugar

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concentration.

In this report, two promising strawberry cultivars, 'Summer Tiara' and 'ES-10', that were bred in Yamagata Prefecture, were grown from July to November, and the seasonal changes in the fruit quality and cell wall polysaccharides were compared. 'Summer Tiara' is a hybrid of 'Selva' (EB) and 'Benihoppe' (JB) and 'ES-10' is a hybrid obtained by crossing the two EBs cultivars, '03-84-42' and 'Miyagi-Natsuharuka' (female and male, respectively). The objective of this study was to evaluate the possibility of SASP in northern Japan.

MATERIALS AND METHODS

Plant materials

On March 26, 2015, seedlings with 2-3 unfolded leaves of 'ES-10' and 'Summer Tiara' were grown in a plastic film greenhouse (20×6.4 m) at Yamagata Shonai Agricultural Technique Improvement Research Office, Yamagata, Japan. The seedlings were set in planting beds (10 cm depth × 20 cm width × 15 m length) containing coco-peat. The planting pattern comprised 24 cm-spaced double rows (66,000 plants ha^{-1}). A mixed fertilizer comprising $N:P_2O_5:K_2O$=13.5:9.9:20.2 (Tank Mix F and B, OAT Agrio, Tokyo, Japan) at EC0.6-0.8 dS m^{-1} using a hydroponic system was applied to the beds. A sample of 20 ripe fruits per cultivar was randomly harvested monthly from July to November.

Fruit firmness

The firmness of the fruit (external firmness) was measured using a firmness meter (53207 fruit penetrometer, T.R. Turoni, Forli, Italy) by penetrating a 2-mm flat plunger into the equatorial region of the fruit (Nishizawa et al., 2002). The skin was then removed up to 2- to 3-mm depth, and a firmness of the cross-section of the flesh was also measured (internal firmness).

Preparation of receptacles

Four fruits were combined and five replicate sub-samples were prepared. The combined fruits were then lyophilized at -50°C, roughly homogenized in a mill, and achenes were removed and discarded. The remaining portion of the receptacle samples were powdered in a mortar and used for chemical analyses.

Soluble sugars

Soluble sugars in a 0.1 g sample of the powdered sample were extracted using 80% EtOH. Glucose, fructose, and sucrose in a 20-µL sample of the solution were enzymatically analyzed using a micro-plate reader (BT-MQX200, Viento, VT, USA) (Nishizawa et al., 2002).

Polyphenols

Polyphenolic substances contained in a 0.5-mL ASS solution were also measured using the Folin-Ciocalteu method, and the results were expressed as mg of the gallic acid equivalent.

Cell walls

Cell wall compositions of the fruit were analyzed following Lara et al. (2004). Briefly, a 0.2-g powdered sample was homogenized with 8 mL of phenol:acetic acid:water (2:1:1, w/v/v) (PAW), and shaken for 12 h at ambient temperature. After centrifugation of the homogenate at 3,000 *g* for 15 min at 5°C, the pellet was resuspended in 8 mL distilled water and centrifuged again. The PAW and water wash supernatants were dialyzed exhaustively using spectra pore membranes (6,000-8,000 MW cut-off, Spectram Medical, CL, USA) against distilled water for two days at 5°C, and brought to 25 mL with distilled water. The dialysate was centrifuged at 20,000 *g* for 20 min to sediment out the precipitate formed during dialysis. The supernatant was recovered, lyophilized at -50°C, and weighed (PAW-soluble solid, PSS). The pellet obtained after PAW extraction and water wash was subsequently washed twice in acetone, lyophilized at -50°C and weighed to determine the yield of crude cell wall materials (CWM). Cell wall polysaccharides in the CWM fraction were sequentially extracted with

distilled water, 50 mM sodium acetate in 50 mM cyclohexane-1, 2-diaminetetra-acetic acid (CDTA) (pH 4.5), 50 mM Na_2CO_3 containing 26 mM $NaBH_4$, and 4 M KOH. Both uronic acid (UA) and neutral sugar (NS) concentrations in each fraction were measured colorimetrically using a spectrophotometer (UV-1200, Shimadzu, Kyoto, Japan) (Nishizawa et al., 2002).

Statistical analysis

The data were subjected to an analysis of variance, and the means between cultivars were compared with Student's t-test using statistical software (Excel statistics ver. 5, Esumi, Tokyo, Japan).

RESULTS AND DISCUSSION

Growing temperatures and fruit fresh weight

The mean temperature in the greenhouse was 26-27°C during summer (July-August), but decreased thereafter, reaching 11°C in November (data not shown). SASP in Japan is limited mainly because of high summer temperatures, which accelerate fruit softening and inhibit flowering, especially in JB cultivars (Wada, 2014). Therefore, cultivation of EB cultivars or hybrid cultivars of EB and JB has been attempted during this period (Yamasaki, 2013). One of the disadvantages of EB cultivars is smaller fruit size compared with JB cultivars. In our study, the fruit weight of 'Summer Tiara' (6.9-11.6 g) was higher than that of 'ES-10' (5.9-7.5 g), indicating that 'Summer Tiara' was a more promising cultivar than 'ES-10' in terms of fruit size (Table 1). However, the fruit weight of 'Summer Tiara' still remained as much as 44-75% lower than that reported for the major JB cultivar, 'Otomegokoro' (16 g), which has been grown in the same area during winter to spring (Sugawara and Maruyama, 2006).

Soluble sugars and polyphenols

The fruits of EB cultivars are often less sweet than those of JB cultivars (Ogiwara et al., 1998). Although the total soluble sugar concentration did not significantly differ between the two cultivars, the concentrations of each sugar component differed between the two cultivars (Table 1). The reducing sugar: sucrose ratio in EB cultivars has previously been shown to be generally higher than that in JB cultivars (Ogiwara et al., 1998). In our study, the reducing sugar:sucrose ratio of the female parent ('Selva') (EB) of 'Summer Tiara' was 3:1 (Ferreyra et al., 2007) while that of the male parent ('Benihoppe') (JB) was 3:2 (Nishizawa et al., 2009) when they were grown in summer. Since 'ES-10' is the hybrid of the two EBs, it is suggested that the difference in sugar components between the two cultivars will be due to the difference in the genetic composition of the parents (Ogiwara et al., 1998).

In contrast to the soluble sugars, the polyphenol concentration in strawberry fruit often increases at higher growing temperatures (Ferreyra et al., 2007). Although the polyphenol concentrations of 'Summer Tiara' responded well to rising temperatures (13 mg g^{-1} DW in July), those in 'ES-10' remained at low levels, especially during summer (8-9 mg g^{-1} DW during July and August) (Table 1), indicating that 'Summer Tiara' is more tolerant of the high temperature conditions.

Firmness and cell wall polymers

The firmness in both the fruit and flesh firmness of the two cultivars increased over the duration of the study, but those of 'ES-10' were significantly higher compared to 'Summer Tiara' throughout the growing period (Figure 1).

Table 1. Changes in fruit fresh weight and soluble sugar and polyphenol concentrations of strawberry fruits harvested monthly from July to November.

Cultivar	July	August	September	October	November
Weight of receptacle (g)[a]					
Summer Tiara	11.6	6.9	8.2	7.9	9.2
ES-10	7.5	6.9	5.9	6.7	7.3
Significance	**	ns	*	*	*
Soluble sugars (mg g^{-1} DW)[b]					
Glucose					
Summer Tiara	176	175	169	167	147
ES-10	219	222	202	197	173
Significance	**	**	**	*	*
Fructose					
Summer Tiara	261	276	223	215	178
ES-10	285	296	282	255	222
Significance	*	*	**	*	**
Sucrose					
Summer Tiara	169	187	259	319	346
ES-10	87	130	173	243	279
Significance	**	**	**	**	**
Total					
Summer Tiara	606	641	658	700	672
ES-10	592	648	657	696	674
Significance	ns	ns	ns	ns	ns
Reducing sugar:sucrose ratio					
Summer Tiara	2.6	2.4	1.5	1.2	0.9
ES-10	6.4	4.0	2.8	1.9	1.4
Significance	*	**	**	*	*
Polyphenols (mg g^{-1} DW)					
Summer Tiara	13	12	11	9	8
ES-10	9	8	11	8	7
Significance	**	**	ns	ns	*

[a,b]Data are the means of 20 fruit and 5 subsample replications, respectively.
Significance: *p<0.05, **p<0.01, ns = not significant.

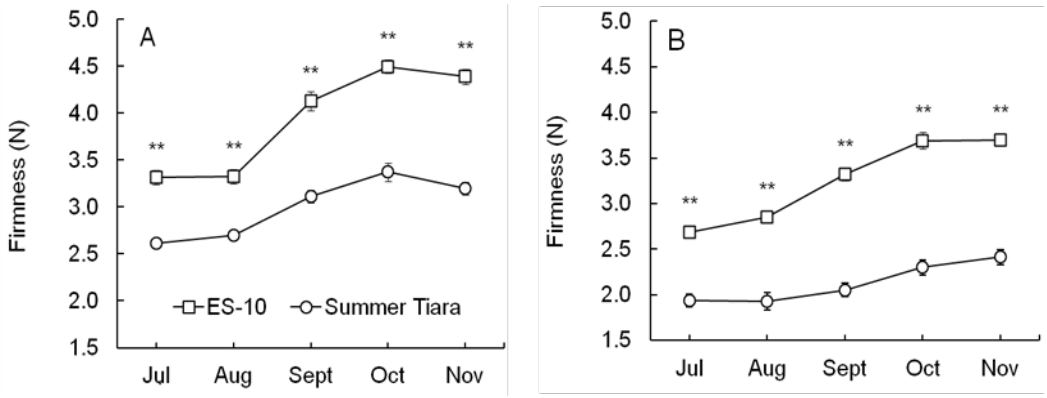

Figure 1. Changes in the firmness of strawberry fruits harvested monthly from July to November. Fruit firmness (A) and flesh firmness (B). Each point represents a mean of 20 replicates ± SE. **Significant at p<0.01 using student t-test.

The integrity of cell wall polymers is often the most important factor in determining the firmness of strawberry fruit (Santiago-Doménech et al., 2008). For instance, the softening during fruit maturation is associated with an increase in the cell wall polysaccharides that are involved in the water-soluble pectin fraction, whereas their decrease is involved in the ionically- and covalently-bound pectin fractions (Rose et al., 1998). Such an inverse relationship in the pectin fractions is considered to be the result of the increased solubility of pectin polymers, especially in the middle lamella and in primary cell walls of cortical parenchyma (Lara et al., 2004). A similar decrease in cell wall polysaccharides is also found in the hemicellulosic fractions as well at the full ripe stage (Trainotti et al., 1999). The degradation of cell wall polysaccharides is accelerated at higher growing temperatures, resulting in fruit softening (Barnes and Patchett, 1976).

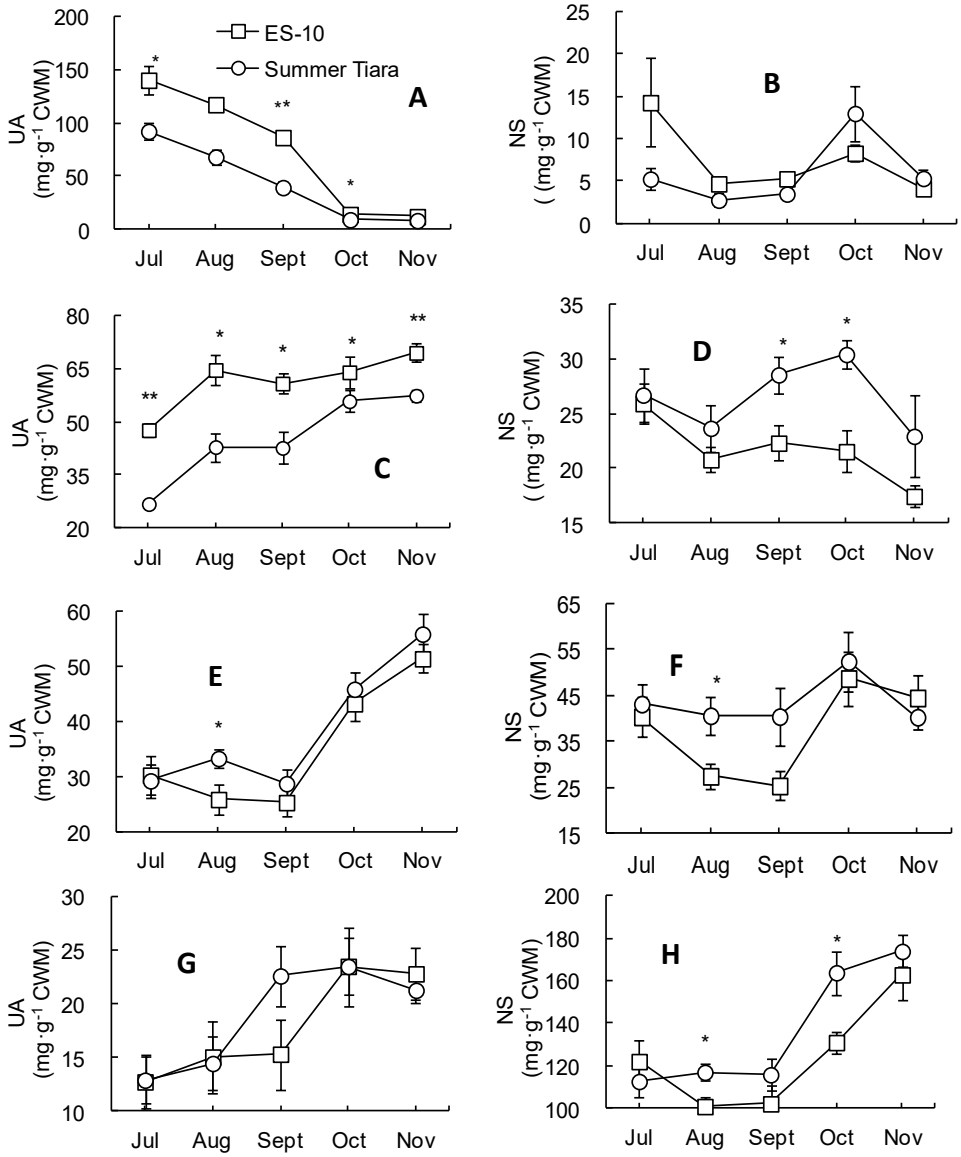

Figure 2. Changes in uronic acid (UA) and neutral sugar (NS) contents among cell wall polymers of strawberry fruits harvested monthly from July to November. Water- (A, B), CDTA- (C, D), Na_2CO_3- (E, F) and KOH- (G, H) soluble fractions. Each point represents a mean of five replicates ± SE. *, **Significant at $p<0.05$ or 0.01, respectively, using student *t*-test; all other differences were non-significant.

The results of cell wall polysaccharides analysis in our study were also in good agreement with those of previous studies. The UA concentrations in the CDTA- and Na_2CO_3-soluble fractions increased from summer to autumn (Figure 2C, E), respectively. A similar increase in the cell wall polysaccharides was also found in the NS in the KOH-soluble fraction (Figure 2H). These results show that the fruit firmness increased as the fruit growing temperature decreased due to the increased integrity of the cell wall polymers.

The UA concentration in the CDTA-soluble fraction, which was thought to be the main chain of ionically-bound pectin polymers, was higher in 'ES-10' than in 'Summer Tiara' (Figure 2C). This result efficiently explains the reason why the firmness of 'ES-10' was significantly higher than that of 'Summer Tiara' (Figure 1). The higher total NS: total UA ratio in 'Summer Tiara' than in 'ES-10' (Figure 3) also suggests that the depolymerization and loss of UA occurred in the main galacturonan chains (Rosli et al., 2004).

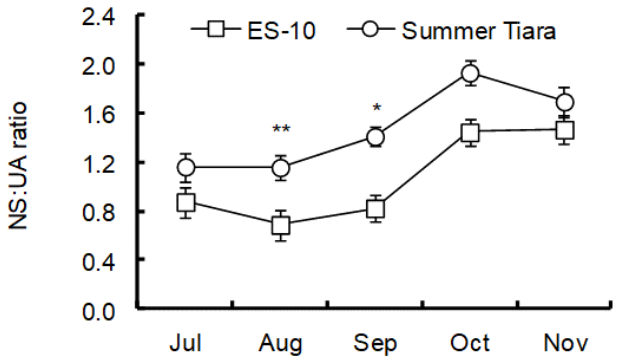

Figure 3. Changes in the total neutral sugar (NS) and total uronic acid (UA) ratio of strawberry fruits harvested monthly from July to November. Each point represents a mean of five replicates ± SE. *, **Significant at $p<0.05$ or 0.01, respectively, using student t-test; all other differences were non-significant.

In contrast, the UA concentration in the water-soluble fraction, which was thought to be the depolymerized pectin fraction released from the ionically- and/or covalently-bound pectin fractions, was higher in 'ES-10' than in 'Summer Tiara' (Figure 2). Differences in the cell wall concentrations in the other fractions, except for the UA in the Na_2CO_3-soluble fraction, were mostly non-significant between the two cultivars or higher in 'Summer Tiara' than in 'ES-10'. These conflicting results between the cell wall concentration in each fraction and the firmness may be explained by the CWM:PSS ratio (Figure 4) because a large amount of solubilized cell wall polymers in ripening strawberry fruit is transferred into the PAW-soluble fraction as well as the water-soluble fraction (Santiago-Doménech et al., 2008). Although the total structural materials (PSS+CWM) did not differ significantly between the two cultivars (Figure 4C), the PSS concentrations in 'Summer Tiara' were significantly higher than those in 'ES-10' (Figure 4A), while the CWM concentrations in 'ES-10' were mostly higher than those in 'Summer Tiara' (Figure 4B). As the result, the CWM:PSS ratio was higher in 'ES-10' than in 'Summer Tiara'. These results suggest that the solubility of the cell wall polymers is higher in 'Summer Tiara' than in 'ES-10', and that the solubilized cell wall polymers were mainly transferred into the PAW-soluble fraction in 'Summer Tiara'.

CONCLUSIONS

Although both 'ES-10' and 'Summer Tiara' can be used for summer to autumn strawberry production in northern Japan, 'ES-10' is more appropriate because of higher fruit firmness. However, both the sweetness and firmness of summer-grown strawberry fruit are still lower than those of autumn-grown ones, resulting in a lower market value, irrespective of the cultivars. Such shortcomings might be compensated partially, by the higher polyphenol concentration under the higher temperature conditions, especially in 'Summer Tiara'.

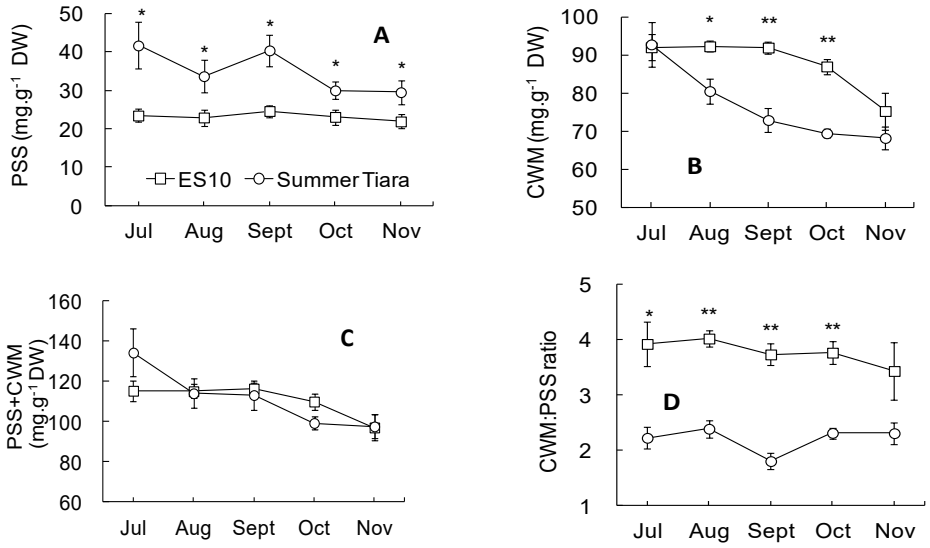

Figure 4. Changes in cell wall materials of strawberry fruits harvested monthly from July to November. PAW-soluble solid (PSS) (A), crude wall materials (CWM) (B), PSS+CWM (C) and CWM:PSS ratio (D). Each point represents a mean of five replicates ± SE. *, **Significant at $p<0.05$ or 0.01, respectively, using student t-test; all other differences were non-significant.

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Optimum strategies for the control of root-zone temperature to promote early-stage growth of chili pepper in soilless culture using an intelligent approach

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Abstract

In controlled environment agriculture it is crucial to control environmental factors optimally during cultivation in order to promote plant growth. One of the essential manipulating factors for promoting plant growth is root-zone temperature. In this study, the optimal strategies for root-zone temperature control in hydroponic cultivation were investigated for promoting the early-stage growth of chili pepper plants (*Capsicum annuum* L.). An optimal control strategy was examined using an intelligent control technique based on the plant speaking approach concept. This technique consists of a neural network model to identify the dynamic responses of plant growth as affected by root-zone temperature and a genetic algorithm to search for the optimal set-point of root-zone temperature. The experiment was conducted inside a controlled environment growth chamber during 60 days of observation. A non-destructive and continuous plant weight measurement system based on a load cell was developed for measuring the dynamic response of plant growth as affected by the change of root-zone temperature. Five data sets, which consisted of five dynamic responses of plant growth as affected by the dynamic root-zone temperature regimes within the range of 15-37°C, were used for system identification. Through simulation, using an identified neural network model, the optimal control strategies of root-zone temperature that maximized plant growth were then estimated using a genetic algorithm. The result showed that neural networks are useful in identifying the dynamic responses of plant growth; then, optimization using a genetic algorithm indicated that the dynamic control method with a daily control interval could offer the most effective way to promote the early-stage of plant growth of chili pepper.

Keywords: hydroponic, dynamic modeling, system identification, dynamic optimization, neural networks, NARX, LSTM, genetic algorithm

INTRODUCTION

Pepper is one of many significant worldwide crops that are sensitive to temperature stress (Erickson and Markhart, 2002). Besides air temperature, pepper is also sensitive to root-zone temperature (RZT) (Aloni et al., 1992). RZT has long been recognized to play an essential role in the growth of plants through the regulation of nutrient and water uptake processes, which directly affect plant growth (Kawasaki et al., 2013). In cultivation methods that use controlled root-zones, such as used in hydroponics, RZT can, therefore, be used as a factor for manipulating plant growth. Through the hydroponics technique, growers can flexibly control root-zone environments to meet the optimal conditions that enable the plant to grow to its maximum potential (Sambo et al., 2019). Consequently, determining the optimal set point for RZT control during cultivation in a hydroponic system could lead to an improvement in the growth of pepper plants.

Hashimoto (1989) stated that optimal crop cultivation conditions should be achieved by monitoring the physiological status of the plant because the physiological status of a plant

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during cultivation varies with time and is significantly affected by changes in environmental factors. This approach is widely known as a "speaking plant approach" (SPA). However, it is not easy to realize optimization of plant growth because eco-physiological processes during plant development are complex with its strong nonlinearity, time-delays, and time-variation (Yin and Struik, 2010). Therefore, to achieve effective control for plant growth, an approach that deals with such a complex system is necessary.

As the application of computers is increasingly widespread, a computational approach is starting to be used for dealing with such a complex system. Such approaches use highly sophisticated algorithms to improve control accuracy. Hashimoto (1980) introduced an intelligent control system for use in a plant production system. The recent development of intelligent control techniques, based on the speaking plant approach, has been successfully implemented in an intelligent greenhouse for commercial crop production (Nishina, 2015).

To date, studies of temperature control techniques for crop production in a controlled environment system have been conducted mainly for shoot-zone temperature (Kawasaki and Yoneda, 2019). However, there is a lack of research on the development of methods for control of RZT. Studies into the search for optimal RZT for maximizing plant growth are limited to the static control method (Kawasaki and Yoneda, 2019), and little is known about the application of a dynamic control that is based on an intelligent approach. Therefore, in this study, we examined an intelligent control technique based on the concept of the speaking plant approach to search for the optimal strategies of control of RZT to promote the early-stage growth of chili pepper plants in hydroponic cultivation.

MATERIALS AND METHODS

Intelligent control technique

In a controlled environment cultivation system, the early-stage growth of a plant is critical in determining the future stages of plant growth. Strong early-stage growth is needed to produce early and prolific fruiting (Bosland and Votava, 2012). Hence, optimal control during early-stage growth is critical. In this study, the improvement of plant growth was focused on achieving optimally control of RZT by considering the physiological status of the plant during cultivation. Figure 1 shows a block diagram of the proposed intelligent control system for optimizing RZT based on the speaking plant approach (Hashimoto, 1989; Morimoto et al., 1996). It mainly consists of three processes: eco-physiological measurement, system identification and the search of the optimal RZT set-points.

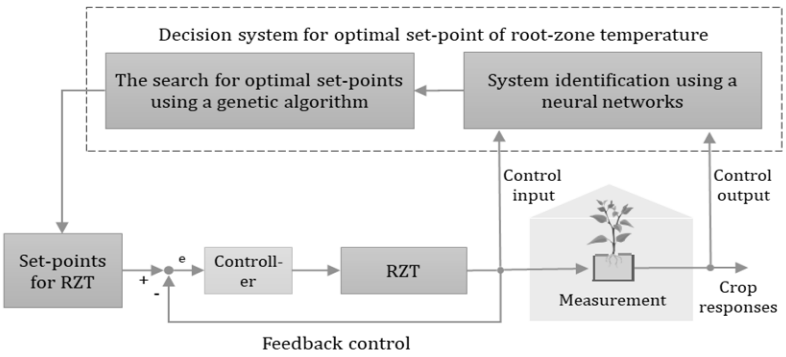

Figure 1. A block diagram of the intelligent control system for optimizing RZT based on speaking plant approach.

Plant materials and measurement

Seeds of chili pepper (*Capsicum annuum* L. 'Takanotsume Togarashi'; Takii Seed Ltd., Japan) were germinated for 14 days at 26°C room temperature. After 35 days from sowing, the seedlings were transplanted into the measurement system. The experiments were

conducted in a controlled environment growth chamber (2.5×2.5×2.0 m; NK System, Nippon Medical & Chemical Instruments Co., Ltd., Japan) where artificial lights were used as the source of light. The plants were grown in 12 h constant photoperiod at 270 μmol m^{-2} s^{-1} PPFD, measured at the base of the growth chamber. Temperature and relative humidity were controlled at 25/20±1°C and 55/70±5% RH, respectively. A total of 15 chili pepper plants were grown in a deep floating technique hydroponic system, with the nutrient solution controlled at 2.3±0.2 dS m^{-1}, and the dissolved oxygen level was maintained with the application of an air bubble generator. Figure 2 shows the RZT control system consisting of a cooling water circulator and a water heater to control the RZT independently from air temperature. Five different RZT regimes, in the range of 15-37°C, were randomly applied to the plants in order to obtain adequate information about the dynamic response of plant growth to RZT. During the early-stage growth, since only the vegetative growth could be observed, the plant growth responses to RZT were estimated by measuring the fresh weight of the plant using an automatic non-destructive plant weight measurement system based on a micro load cell (CZL635, loads up to 5 kg and 0.05% precision; Phidgets Inc., Canada), as shown in Figure 2 (Aji et al., 2020). However, because the plant roots were in the nutrient solution, the measured weight only represented the weight of the plant shoots (Hu et al., 2018).

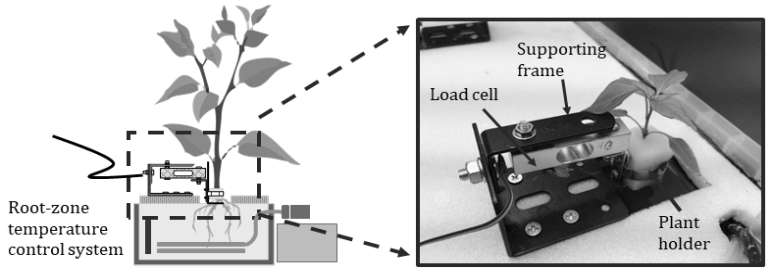

Figure 2. The non-destructive plant weight measurement system for measuring the response of plant growth and the independent RZT control system.

Neural network for system identification

Two types of neural network architecture were evaluated to identify the dynamic responses of plant growth as affected by RZT: a nonlinear autoregressive network with exogenous input (NARX) (Siegelmann et al., 1997) and long short-term memory (LSTM) network (Hochreiter and Schmidhuber, 1997). Both architectures were chosen due to their advantages in identifying a nonlinear time-series system and for their effective use in a control system (Mohd and Aziz, 2016; Wang et al., 2017).

In this study, a single-input, being the RZT (T_k) and single-output, being the responses of plant growth (W_k), were used for identification (k: sampling time). For NARX, the architecture of the network is shown in Figure 3a. In this network, the responses of plant growth were approximated using a feed-forward neural network from the input of the present value of RZT (T_k), historical data of RZT (T_{k-d}) and the historical data of the response of plant growth (W_k) (Aji et al., 2020). For the LSTM network, the architecture is shown in Figure 3b. This was a form of recurrent neural networks (RNN) with an LSTM layer where each cell in this layer was consisted of a forget gate, an update gate, and an output gate. This LSTM layer is crucial in dealing with long-term dependency in a dynamic system. A dropout layer was added to the networks to prevent the model from overfitting.

For evaluating the accuracy of the identified model, the data for identification were divided into three independent data sets, being a training, a validation, and a test data set. The data split was essential to provide an unbiased and robust evaluation of the final model (Kuhn and Johnson, 2013). The performance of the identified model was measured using root mean squared error (*RMSE*), mean absolute percentage error (*MAPE*), and the coefficient of determination (R^2). A program based on the Matlab® Deep Learning Toolbox™ R2019a

(MathWorks® Inc.) was created to develop the model.

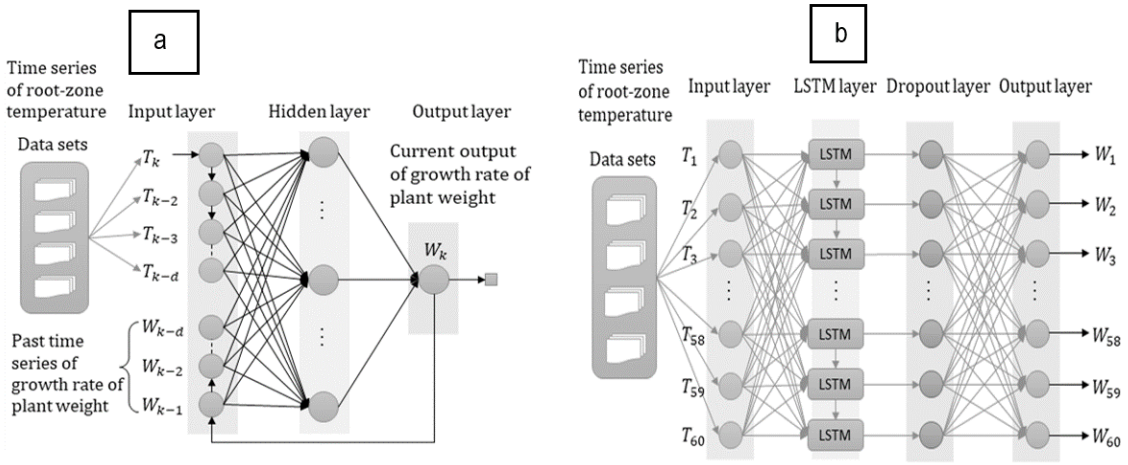

Figure 3. Proposed NARX networks with three layers, single-input of time-series RZT, single-output time-series of growth rate in plant weight, and time-delay procedure for system identification (a). Proposed LSTM networks with additional dropout layer for system identification (b).

A genetic algorithm for optimization

The genetic algorithm was used to search for the optimal RZT condition, which maximizes plant growth through the neural network model simulation. The optimization solution is determined by the sequences step of an evolutionary concept. In the genetic algorithm, a candidate solution T_k was represented as an individual. Figure 4 shows a flowchart of the genetic algorithm (Yang, 2010). For realizing the genetic algorithm optimization, a program based on the Matlab® Global Optimization Toolbox™ R2019a (MathWorks® Inc.) was created.

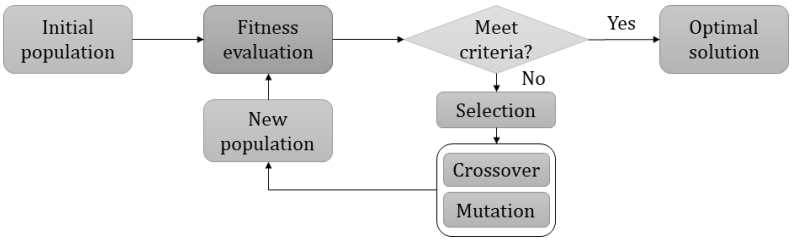

Figure 4. Flowchart of the optimization using a genetic algorithm.

Figure 5 illustrates the simulation method for finding the optimal RZT through genetic algorithm optimization (Morimoto and Hashimoto, 2009). For realizing the optimization, the control process was divided into l-steps. In this study, four different numbers of steps (l=1, 3, 6 and 45) were applied to examine the effect of the length of the control interval on plant growth. As the length of the whole control process was 45 days, the length of each step interval was 45 days or constant, 14 days, 7 days or 1 day, respectively.

Since the objective for optimization in this study was to maximize the growth in plant weight during the early-stage growth of the plant by controlling RZT, the objective function $f(T_k)$ was given by the integration of the growth rate, measured in plant weight W_k, during the control period ($1 \le k \le N$). Then, the optimization problem was to determine the l-steps set-points of RZT, where the RZT was constrained by the range of 15 to 30°C, as this was considered to be the limitation of the model. Therefore, the objective function can be written

as: maximize: min (min $f(T_k) = \sum_{k=1}^{N} W_k$); subject to: 15≤$T_k$≤30°C.

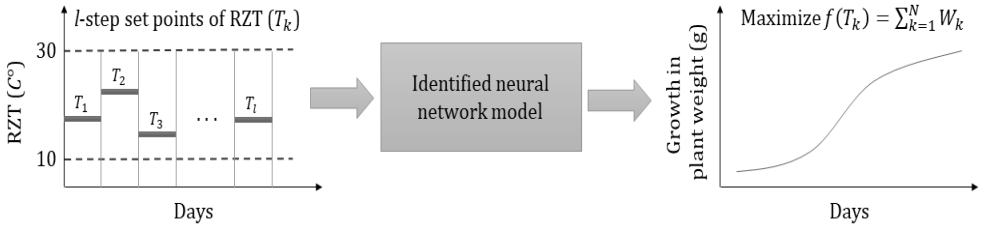

Figure 5. Method for finding optimal control (the combination of *l*-step) of RZT that maximizes the growth in plant weight.

RESULTS AND DISCUSSION

Dynamic response of plant growth to RZT for model identification

Figure 6 shows five types of daily growth measured as the growth rate in plant weight, as affected by the change of RZT obtained during 60 days of measurement in hydroponic cultivation. The measurement period corresponds to the early-stage growth (vegetative growth) of the chili pepper plant. The data for each pattern was obtained from the average values of three chili pepper plants. The measured data were used for system identification of the dynamic response of plant growth as affected by RZT using neural networks. In this study, the response of plant growth was represented by the growth rate in plant weight as it measured the change of plant weight over time, which offers a sensitive measurement of the dynamic response of plant growth as affected by RZT (Aji et al., 2020). Therefore, the growth rate in plant weight was used as the output variable.

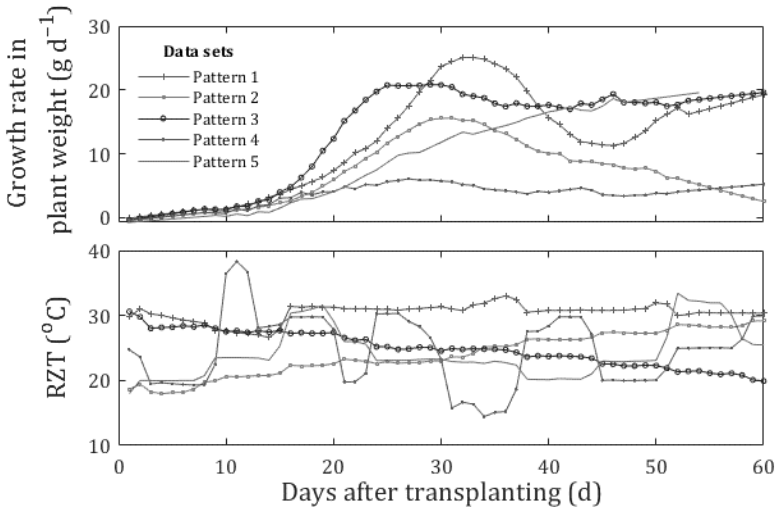

Figure 6. Five types of dynamic changes in the growth rate in plant weight as affected by RZT, obtained during measurement in hydroponic cultivation.

Neural network identification results

Based on cross-validation results, it was found that the NARX network outperformed the LSTM network. However, in general, both proposed networks performed well with promising accuracy. The performance of the model also can be seen in Figure 7a, where the estimated dynamic response calculated from the NARX and LSTM network model, and the observed response for the growth rate in plant weight were close. Even though the LSTM network is more well-known for time series problems, for this particular problem, the NARX network performed slightly better. Thus, the NARX network was chosen for model

identification.

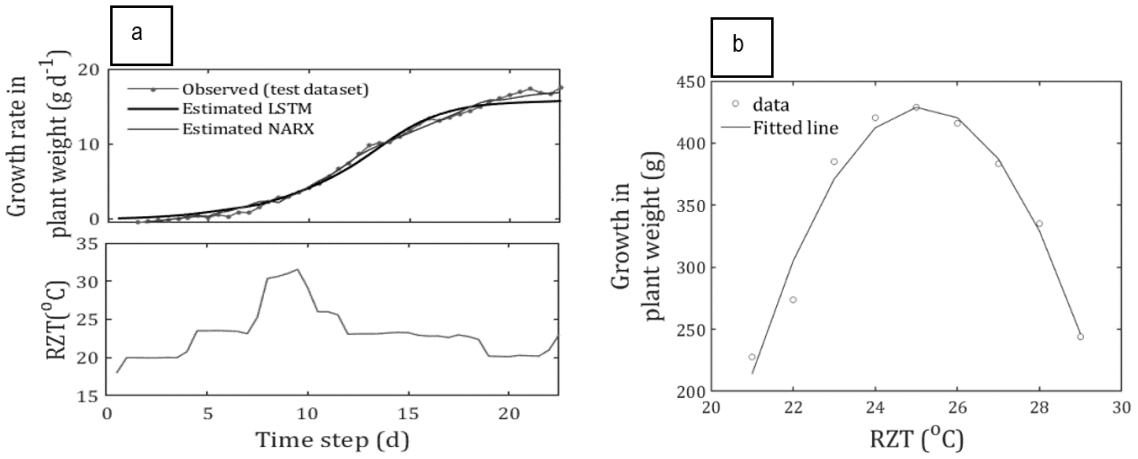

Figure 7. Comparison of estimated response, calculated from the NARX and LSTM networks model and the observed response (test data set) (a). The estimated static relationship between the growth in plant weight and RZT, obtained from simulation using the NARX network model (b).

Figure 7b shows the estimated static relationship of RZT and the growth in plant weight obtained from simulation using the identified neural network model, where the input RZT variable was set constant. It can be seen that the relationship between RZT and the growth in plant weight was a strongly nonlinear and that it peaked in the range of 24 to 26°C. This result is consistent with previous studies which reported that the optimal range of RZT for pepper cultivar was 24°C in terms of maximum shoot dry weight (Bosland and Votava, 2012) and no more than 25 to 27°C in terms of yield (Díaz-Pérez, 2010). This result suggests that the identified neural network model is reliable for predicting the dynamic response of plant growth as affected by RZT.

Estimated optimal control results

In Figure 8a, the lower graph shows the estimated optimal values of RZT obtained from the optimization process using the genetic algorithm under four different lengths of step interval. The middle and upper graphs show the growth rate in plant weight obtained from the neural network model simulation and the growth in plant weight as the integration of the growth rate in plant weight, respectively. From these results, the optimal value for the constant RZT set point was at 24.9°C. This setpoint is consistent with the simulation result in Figure 7b. However, the static control method generated the lowest plant growth at the end of the control period, as shown in Figure 8b. In contrast, the highest plant growth weight was generated by the dynamic control method with a daily step control interval. This type of optimal solution consists of a series of RZT set points, which gradually change the setting within the 18 to 29°C range during the control period. From the simulation using the neural network model in Figure 8a, this control method generated the fastest response in the plant growth rate at the beginning of the control period and was then able to maintain the rate at the highest level until the end of the control period. Consequently, this control method could produce the highest yield in terms of plant fresh weight at the end of the control period (45 d), as shown in Figure 8b. In general, the identified optimal control strategies of RZT in this study indicated that a dynamic control method of RZT, that changes flexibly based on plant responses, could offer a better way to promote plant growth than a static control method during the initial stage of growth. These results are also in agreement with the concept of the speaking plant approach proposed by Hashimoto (1989), as mentioned above.

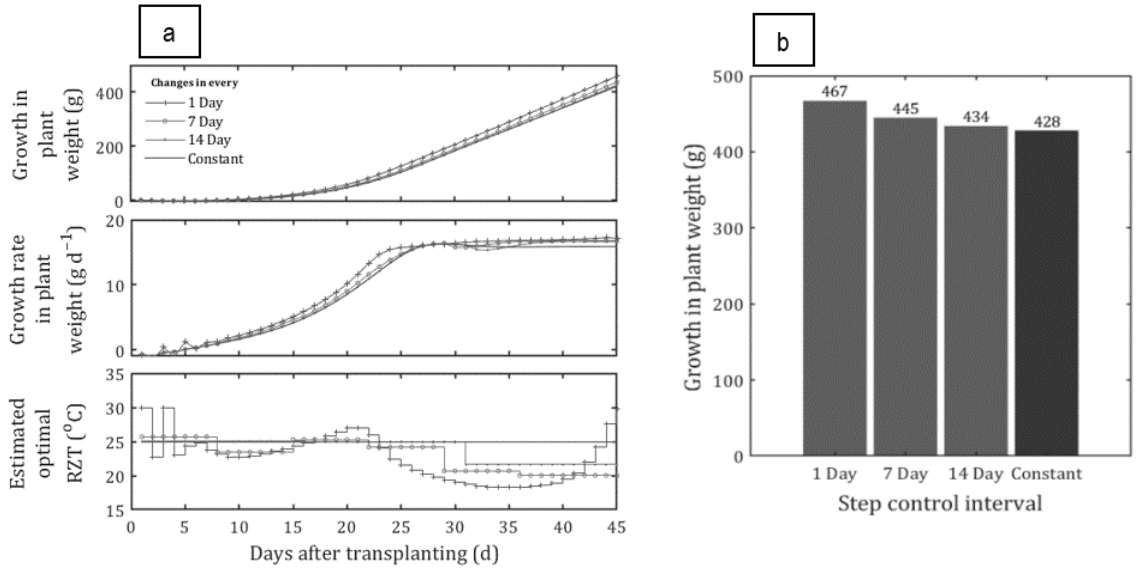

Figure 8. Estimated optimal control performance of plant growth in hydroponics as affected by RZT under four different lengths of the step interval, obtained from simulation and genetic algorithm optimization (a). Estimated plant weight of the chili pepper plants at the end of the control process (45 days after transplanting) (b).

CONCLUSIONS

In this study, the search for optimal control strategies for RZT to promote the early-stage growth of chili pepper plants in hydroponic cultivation was carried out using an intelligent approach. The NARX network showed a better performance than the LSTM network. The networks could be useful in identifying and predicting the dynamic response of plant growth, as affected by RZT, with promising accuracy. Through simulation and optimization using a neural networks model and a genetic algorithm, the optimal solution was estimated. It suggests that the dynamic control method (on a daily basis) could offer the most effective way to promote the early-stage of plant growth of chili pepper.

ACKNOWLEDGEMENTS

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Drought tolerance induced by a combination of abscisic acid and abscinazole in apple seedlings

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Abstract

The effects of abscisic acid (ABA) and abscinazole (Abz-E3M), an inhibitor of ABA 8'-hydroxylase, on drought stress were examined in apple seedlings. ABA, Abz-E3M, and a combination of both (ABA+Abz) were sprayed onto seedlings. The water potential, malonaldehyde (MDA) and proline concentrations, antioxidant enzyme activities such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and peroxidase (POD), endogenous ABA concentrations, and expressions of the *9-cis-epoxycarotenoid dioxygenase* (*MdNCED1*) gene were analyzed. Drought conditions reduced water potential (Mpa) in the leaves but increased proline accumulation, ABA concentrations, and *MdNCED1* expression. The activities of SOD, CAT, APX, and POD in ABA+Abz or ABA treated leaves increased at 4 DAT after the imposition of drought conditions. The ABA+Abz treatment maintained water potential and reduced proline concentration but increased 2,2-diphenyl-1-picryldrazyl (DPPH) radical scavenging activity, SOD, and APX activity. ABA+Abz had a stronger effect than the application of ABA alone on water potential (ABA+Abz at -1.76 MPa; ABA at -1.95 MPa), ABA concentrations (ABA+Abz of 867.3 µg kg^{-1} FW; ABA of 175.1 µg kg^{-1} FW) and proline concentrations (ABA+Abz of 0.52 nmol kg^{-1} FW; ABA of 0.68 nmol kg^{-1} FW at ABA). The results suggest that the combined ABA+Abz treatment increased endogenous ABA concentrations, maintained leaf water potential and increased antioxidant activities, and as a result may induce tolerance against drought stress.

Keywords: apple, abscisic acid, proline, water potential, antioxidant enzyme activity

INTRODUCTION

Drought stress triggers the accumulation of reactive oxygen species (ROS), which, in-turn affects redox homeostasis and results in oxidative stress as shown by a heightening of lipid peroxidation (Aroca, 2012). The closure of stomata is a spontaneous physiological reaction that enables a reduction of evapotranspiration losses under drought stress. Upon the perception of drought stress, plants initiate the accumulation of proline and soluble sugars for protection from damage during such conditions (Athar and Ashraf, 2009). Multiple antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), are induced under drought stress to scavenge the excess ROS (Yang et al., 2012).

ABA is a plant hormone that accumulates under various stresses including drought. High concentrations of ABA can quickly induce stomatal closure to minimize transpiration losses. It can also mitigate stress damage by promoting the synthesis of multiple osmotic protective substances, and by activating multiple defense response systems (Shafi et al., 2011). Previous research has shown that the improvement of antioxidase activity under abiotic stress is regulated by endogenous ABA (Guajardo et al., 2016). Thus, high concentrations of endogenous ABA could be beneficial in protecting plants against drought. It has been reported that pretreatment with ABA increases the endogenous ABA concentrations in wheat seedlings and enhances drought tolerance (Bano et al., 2012).

Our previous research has shown that using Abz-E2B, an inhibitor of ABA 8'-hydroxylase (the key enzyme in ABA metabolism), prevented drought damage by effectively increasing endogenous ABA accumulation in apple seedlings (Kondo et al., 2012). Similarly,

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Abz-E3M was an even more practical and effective inhibitor, increasing the ABA concentration in both rice and maize (Takeuchi et al., 2016). However, the effect of exogenous ABA combined with Abz-E3M has not been studied on plants are water stressed. Therefore, in this study, ABA and a combination of ABA and Abz-E3M were applied to apple seedlings to study their mitigation effects against drought.

MATERIALS AND METHODS

Plant materials and treatments

Ninety-day-old 'Tsugaru' apple (*Malus domestica*) seedlings were used in the study. Germinated seeds were sown into plastic trays (26×52×6 mm, 72 holes) containing moist vermiculite and turfy soil with a volume ratio of 1:2. About 0.5-1.0 L of water was applied to the bottom of each tray every morning to keep the soil moist. Commercial Hyponex® solution (Hyponex Japan Co., Osaka, Japan) was added as liquid fertilizer at 14-day intervals. All seedlings were grown in a greenhouse covered with polyvinyl film at Chiba University (35.78°N; 139.90°E). The greenhouse environments were monitored and regulated. The average daily temperature was 25-30°C during the day and 15-20°C at night, under natural light (with an average daily photosynthetic photon flux density (PPFD) of 500 μmol m^{-2} s^{-1}), and with an average relative humidity of 45-65% during the day and 65-75% at night. Drought was imposed by withholding water and nutrient solution from the growing trays. Uniform seedlings were selected and then divided into three groups of 140 seedlings. Three treatments were performed under drought conditions as follows: T1 = application of 100 μM ABA; T2 = application of 100 μM ABA +100 μM Abz-E3M; T3 = application of distilled water (ddH_2O) (control). The leaves were sprayed with ABA, ABA plus Abz-E3M or ddH_2O solutions containing a surfactant (0.5% of Approach BI® (Maruwa Biochemical Co., Tokyo, Japan)), and allowed to dry naturally. Mature, fresh leaves were collected at day-0 (before chemical and drought treatment), day-2, and day-4 after the start of the drought treatment. They were frozen with liquid nitrogen and then kept at -80°C until analysis.

Measurement of the water potential of apple seedling leaves

Leaf water potential measurements were performed at 4:00 am (before dawn) each time, to obtain stable and optimal data, as described by Sales et al. (2017). Samples (three replicates) were transferred to a sample cup and their water potential was immediately measured using a WP4-T water potential meter (Decagon Devices Inc.; Pullman, WA, USA).

Measurement of lipid peroxidation

The amount of malonaldehyde (MDA) produced in the leaves was measured to assess the level of lipid peroxidation. MDA concentrations were determined using the spectrophotometric method described by Heath and Packer (1968). The OD_{450}, OD_{532}, and OD_{600} were recorded with a UV-VIS spectrophotometer (2J1-0010, HITACHI, Japan) and three replications were performed per test group.

Measurement of proline concentrations

The proline concentrations were measured using the method described by Sarker et al. (2005). Three replications per test group (0.5 g fresh weight $sample^{-1}$) were each homogenized with 10 mL sulfosalisylic acid 3% (w/v) and centrifuged at 10,000 rpm for 15 min. The absorbance of the supernatant was measured at 520 nm using a spectrophotometer (Hitachi U-2910).

DPPH antioxidant assay

The antioxidant DPPH activity test was performed according to a modified version of the method described by Sales et al. (2017). Fresh samples of 0.5 g (three replications) were mixed with 25 mL of methanol and homogenized. The samples were measured with a spectrophotometer at 515 nm. The results were compared with the Trolox standard curve at 25-800 μM.

Antioxidative enzyme activity assay

The enzymatic activities of SOD, POD, CAT, and APX in the leaves were determined using the methods described by He et al. (2014). Sample (1 g) of leaf tissue was homogenized in a 50 mL tube with 15 mL phosphate buffer (50 mM, pH 7.8) containing 2% (w/v) polyvinylpyrrolidone and 0.5 mM EDTA in three replications. After centrifuging at 4°C for 15 min at 15,000 rpm, the supernatant was used to determine enzymatic activity. The activity of SOD was measured using the nitroblue tetrazolium reduction method described by He et al. (2014). Each unit of SOD activity was calculated using the amount of enzyme required for 50% inhibition. The activity of POD was determined based on the methyl catechol method of oxidation using H_2O_2. The change of OD_{560} was recorded for 3 min at 60-s intervals using a spectrophotometer. The CAT levels were determined by reading the decrease in absorption for 180 s at 240 nm. The activity of APX was assayed by documenting the decrease in absorbance at 290 nm of ascorbate within 1 min.

Quantitative analysis of endogenous ABA

ABA methods of extraction and analysis were the same as described by Sales et al. (2017). Fresh sample (1 g) was homogenized with 20 mL extraction solution comprised of 80% (v/v) methanol, 0.1% L (+) ascorbic acid (Kanto Chemical Co., Tokyo, Japan) and 0.1% butylated hydroxytoluene (BHT; 2,[6]-di-tert-butyl-p-cresol; Sigma-Aldrich Co., St. Louis, MO, USA), using 0.2 µg ABA-d_6 as an internal standard. The solution was purified using high-performance liquid chromatography (HPLC; flow rate, 1.5 mL min^{-1}; detection at 254 nm) that was equipped with an ODS-Mightysil RP-18 column (250×4.6 mm i.d.). The solution was methylated using ethereal diazomethane and finally analyzed using gas chromatography-mass spectrometry for ion monitoring (GC-MS-SIM; model QP5000; Shimadzu, Kyoto, Japan).

Expressions of the *MdNCED1* gene

Total RNA was extracted using the cetyltrimethylammonium bromide (CTAB) method of Kondo et al. (2012) and cDNA was synthesized using the ReverTra Ace® qPCR RT Master Mix and gDNA Remover (Toyobo Co. Ltd., Osaka, Japan). Quantitative real-time PCR analysis was performed. The *ubiquitin* gene was used as a reference gene, for which the primer sequence was designed as follows: forward: 5'-TCGCTGGAAAGCAGCTCGAAGA-3', reverse: 5'-GCTTTCCGGCAAAGATCAGACG-3'.

The *MdNCED1* gene primer was referenced to Sales et al. (2017), and used as follows: forward: 5'-GTATCACGTCCAAATCACTGAACC-3', reverse: 5'- ATTTGAGGTATGGCTTCTGAACG-3'.

The results were analyzed using the $2^{-\Delta\Delta CT}$ method (Yang et al., 2012) and three replicates were performed for each set of treatments.

RESULTS

Effects of exogenous ABA and Abz on water potential and MDA concentrations

The water potential was the lowest in the control group at 4 DAT (Figure 1A). In contrast, that of the ABA+Abz group was higher than that in the other groups. Drought stress increased the MDA concentrations, but this effect was noticeably weakened by the ABA+Abz group (Figure 1B).

Effects of exogenous ABA and Abz on proline concentrations

The proline concentration was highest in the control group at 4 DAT, but in the ABA+Abz group, it was the lowest at 4 DAT, with a decrease of 45.92% as compared to the control group (Figure 2).

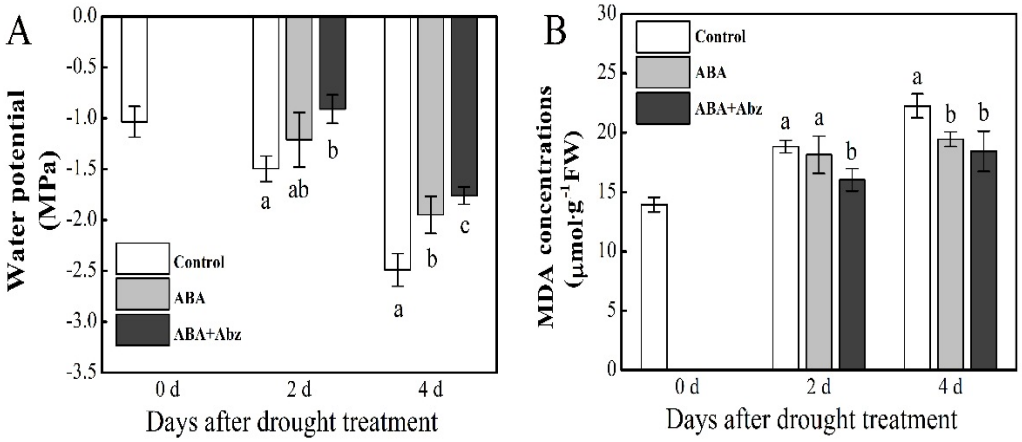

Figure 1. Changes of water potential (A) and MDA concentrations (B) in apple leaves under drought stress. Data are the mean ± SE of three replications. Different letters indicate significant differences by Tukey-Kramer test at p≤0.05.

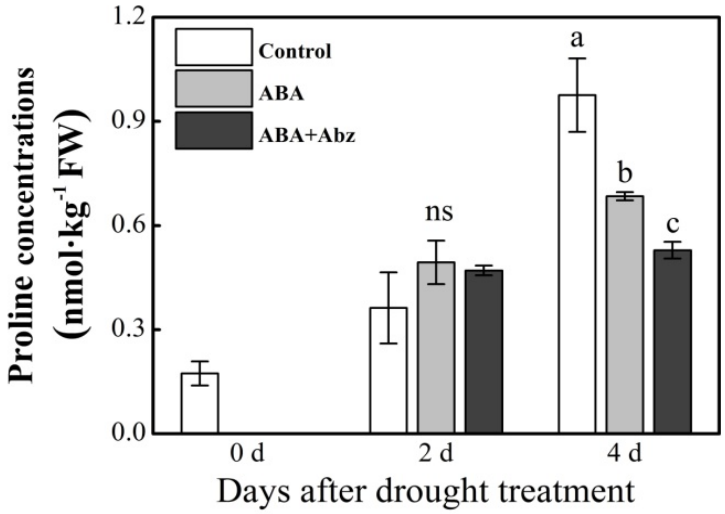

Figure 2. Proline concentrations in apple leaves under drought stress conditions. Data are the mean ± SE of three replications. Different letters indicate significant differences by Tukey-Kramer test at p≤0.05.

Effects of exogenous ABA and Abz on antioxidant and enzyme activity

The DPPH radical scavenging activity in the ABA+Abz group was significantly higher than in other groups at 2 DAT (Figure 3). At 4 DAT, the ABA+Abz and ABA treatments were higher than in the control group. Similarily to the DPPH activity, the activity of the antioxidant enzymes SOD, POD and APX in the control group increased at 2 DAT but decreased significantly at 4 DAT (Figure 4A, B, D). In contrast, the activity of the ABA+Abz or ABA groups was not reduced at 4 DAT in SOD, CAT or APX. In fact, the activity CAT increased at 4 DAT under drought conditions (Figure 4C).

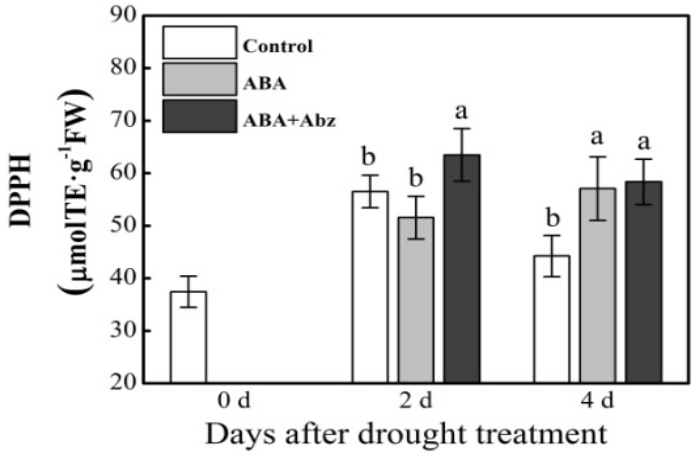

Figure 3. DPPH radical scavenging abilities in apple leaves under drought stress conditions. Data are the mean ± SE of three replications. Different letters indicate significant differences by Tukey-Kramer test at p≤0.05.

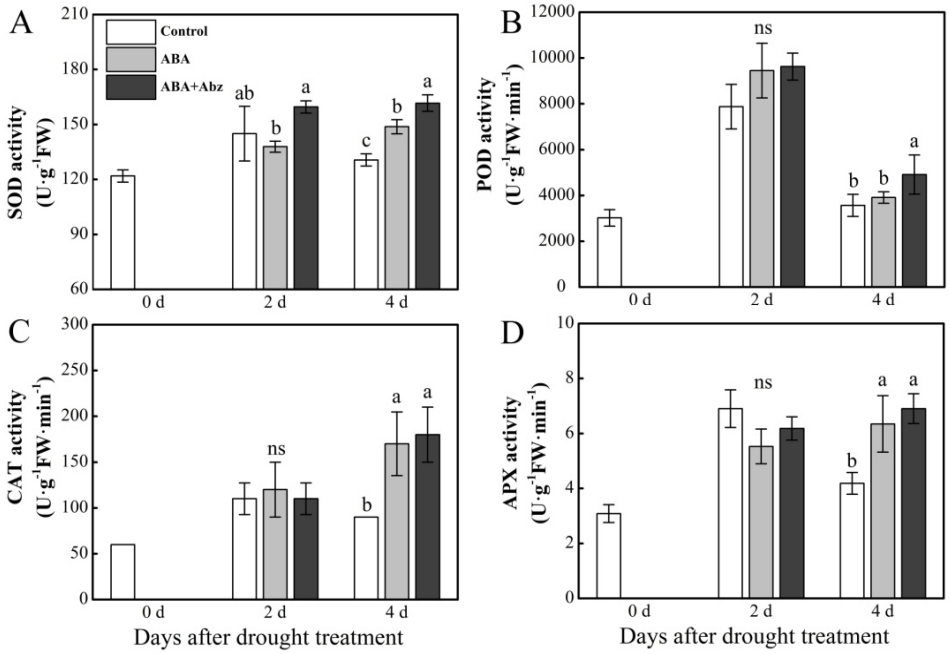

Figure 4. Antioxidant enzyme activities of SOD (A), POD (B), CAT (C) and APX (D) in apple leaves under drought stress. Data are the mean ± SE of three replications. Different letters indicate significant differences by Tukey-Kramer test at p≤0.05.

Effects of exogenous ABA and Abz on endogenous ABA concentrations and *MdNCED1* gene expression

Endogenous ABA concentrations in the control group at 2 DAT were nearly triple the levels found on day-0 (Figure 5A). Concentrations in the ABA+Abz group further increased by 2.32- and 4.60-fold at 2 and 4 DAT, respectively, in comparison to the control group. Although the concentration of endogenous ABA in the ABA group increased dramatically at 2 DAT, no significant difference between the ABA group and control group were found at 4 DAT. The expression of the *MdNCED1* gene in all three groups was induced at 2 DAT and increased further at 4 DAT (over 25-fold), with maximum expression in the control group.

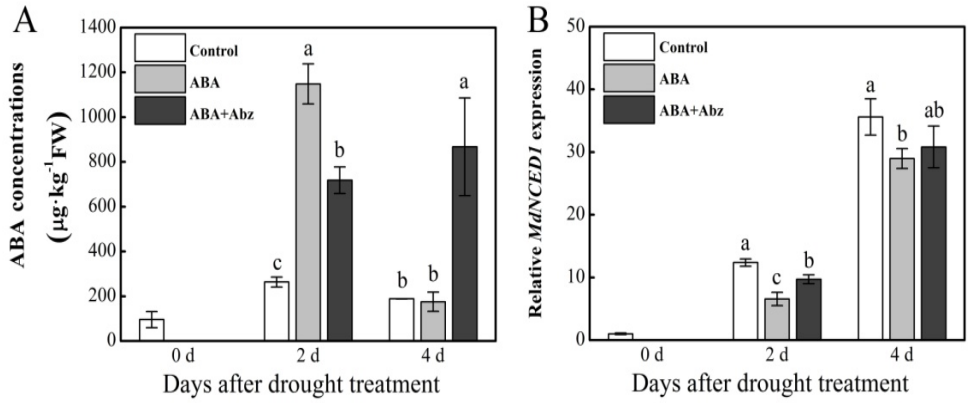

Figure 5. Endogenous ABA concentrations (A) and the expressions of the *MdNCED1* (B) gene in apple leaves. The experimental values were plotted compared with the control (*ubiquitin*) values. Data are the mean ± SE of three replications. Different letters indicate significant differences by Tukey-Kramer test at p≤0.05.

DISCUSSION

Sruamsiri and Lenz (1986) found that in strawberries, there is a water potential threshold of -1.7 for wilting and -2.5 MPa for irreversible drought effects. Our experiment showed similar results, with water potentials of -1.46 and -2.49 MPa at 2 and 4 DAT. It was observed that ABA and ABA+Abz groups maintained higher water potentials in the leaves as compared to the control group and that the ABA+Abz group maintained the highest values. It was deduced that ABA induces stomatal closure rapidly to reduce water loss. ABA and ABA+Abz groups quickly increased the endogenous ABA concentrations at 2 DAT, and the ABA+Abz group maintained higher ABA concentrations, providing a greater tolerance against drought stress.

MDA, which is a product of lipid peroxidation, reflects the degree of cell membrane damage by oxidative stress (Aroca, 2012). In our study, seedlings subjected to drought showed an elevated level of MDA, indicating that there was severe oxidative damage being caused by the drought conditions. Proline manifested similar results, as judged from the increased concentrations. It is regarded as a membrane stabilizer and an essential molecule for plant tolerance and recovery from environmental stresses (Zhong et al., 2020). The ABA and ABA+Abz groups inhibited the elevations of both MDA and proline concentrations, implying that there was less injury to the seedlings under the imposed drought conditions by maintaining the stability and functionality of the cell membranes. A lower MDA concentration was also observed in maize and wheat seedlings with ABA application under adverse conditions. Proline concentrations generally increase with increases in stress levels. In our study, the proline concentrations decreased in the ABA and ABA+Abz groups, which may reflect lower stress damage under drought conditions. ABA may prevent lipid peroxidation by inhibiting excess ROS produced under drought stress.

ROS, such as superoxide radicals ($O_2^{\bullet-}$) and H_2O_2, directly and indirectly damage enzymes, biological membranes and cellular components, causing severe oxidative damage in plants. SOD is a critical antioxidant that converts $O_2^{\bullet-}$ into O_2 and H_2O_2. Although ROS can play a key role as a signaling molecule, its excess accumulation can be toxic, and should be eliminated for the proper functioning of CAT, POD and APX (Yang et al., 2012). In our study, the activities of SOD, CAT, and APX in the ABA and ABA+Abz treated leaves increased after the application of drought conditions, but only at 4 DAT. POD activity at 4 DAT in the ABA treatment was not different from the control group. Thus, it is likely that the combined activities of CAT, APX and SOD played a critically protective role in scavenging the $O_2^{\bullet-}$ and H_2O_2 during the stress conditions. Simultaneously, radical scavenging abilities were also shown by DPPH at 4 DAT. Our results, therefore, indicate that both ABA and ABA+Abz were able to enhance antioxidant activities in apple seedlings under drought conditions.

In this study, the endogenous ABA concentrations increased rapidly at 2 DAT in both the ABA and ABA+Abz groups but the increase was not sustained and reduced markedly at 4 DAT in the ABA group. Abz-E3M is the inhibitor of ABA 8'-hydroxylase, which inhibits ABA catabolism, and thus is likely the reason that a higher ABA concentration was maintained at 4 DAT. Though the expression of the *MdNCED1* gene also increased at 2 and 4 DAT, it was lower than in the control treatment. It was deduced that ABA and ABA+Abz groups did not need the increase of *MdNCED1* expression because the stomata were closed quickly (Sales et al., 2018), and also the high endogenous ABA concentrations inhibited its synthesis.

CONCLUSIONS

Both the ABA and ABA+Abz groups mitigated the stress damage caused by drought conditions. Application of a combination of ABA and Abz inhibited the degradation of endogenous ABA, maintained endogenous ABA concentrations, stabilized leaf water potential and increased antioxidant activities, resulting in an induced tolerance to drought stress.

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Transpiration rates of eight-year-old mango 'Nam Dok Mai Si Thong' in well-watered conditions

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Abstract

The optimal water supply to a plant is based on its requirement. This research investigates the transpiration rates of the mango cultivar 'Nam Dok Mai Si Thong' for an irrigation regime based on compensation of water loss by transpiration. The experiment was conducted on eight-year-old mangoes planted on a farmer's property located in the Ban-Had District, Khon Kaen Province, Thailand. Characteristics of the four-tree experimental were 3.5-4.0 m canopy width and 31.5-36.9 cm trunk girth. The xylem sap flux density was measured by xylem sap flow, using the transient thermal dissipation (TTD) method. Sap flow probes were inserted into the trunk at 60 cm above the soil level. The data collected by a CR1000 data logger every 30 min, as was the soil water status and climate data. The investigation took place from April to August 2019. The soil water status results showed well-watered conditions with a volumetric soil water content greater than 0.18 m^3 m^{-3}. The daily sap flux density ranged from 24 to 37 L dm^{-2} h^{-1}. The tree transpiration calculation rates were 20-32 L $tree^{-1}$ d^{-1}. A relationship between vapor pressure deficit (VPD) and water loss exhibited a plateau correlation. This occurred when the average daily VPD and maximum VPD ranges were 0.43-2.84 and 1.37-5.98 kPa, respectively. Results suggest that mango transpiration is regulated by extreme air evaporative demand conditions and the potential value of water supply for eight-year-old mangoes should be roughly 32 L $tree^{-1}$ d^{-1}.

Keywords: xylem sap flux density, soil water content, vapor pressure deficit

INTRODUCTION

Mango (*Mangifera indica*) is one of Thailand's many exported fruits. The cultivar 'Nam Dok Mai Si Thong' is popular for both international and domestic consumption. Commercial mango orchards are expanding in northeastern Thailand. However, production of premium-grade mango requires sound agricultural management practices. Farming practices, such as pruning, nutrition and water management have a direct effect upon fruit yield and quality. Optimum water supply, has been shown to regulate fruit yield, size, and quality (Spreer et al., 2009; García-Tejero et al., 2010; Lu, 2013; Morgan et al., 2014; Nagaz et al., 2020).

Lu (2013) remarked that water management is critical to successful mango cultivation. Maintaining water balance through optimum irrigation practices protects against product quality defects and ultimately yield loss. One such technique, the transient thermal dissipation (TTD) method, evaluates xylem sap flow and provides a means to measure water loss by transpiration (Do and Rocheteau, 2002; Isarangkool Na Ayutthaya et al., 2010). Generally, tree transpiration is a field-operational variable, dependent upon soil moisture and climatic conditions (Lu, 2002, 2013; Isarangkool Na Ayutthaya et al., 2019). However, very few studies on optimum irrigation practices have been carried out on the mango cultivar 'Nam Dok Mai Si Thong' planted in northeastern Thailand. This study was designed to evaluate tree transpiration rates of the mango cultivar 'Nam Dok Mai Si Thong' under various climates under well-watered conditions. The goal of our work was to adapt the newly acquired information on tree transpiration for optimum irrigation management.

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MATERIALS AND METHODS

Plant material

The experiment was conducted on the mango plantation, cultivar 'Nam Dok Mai Si Thong', located in the Banhat District, Khon Kaen Province, Thailand. Four eight-year-old mango trees at the fruit-setting stage were selected for investigation into the transpiration rates from April to August, 2019. The average trunk girth was 31.5-36.9 cm with an average canopy diameter of 3.5-4.0 m.

Climate data

A Hobo Pre v2 data logger (Onset Computer Corporation, Bourne, MA, USA) recorded half-hourly values of air temperature and relative humidity. Vapor pressure deficits (VPD) were calculated according to specifications determined by Allen et al. (1998).

Sap flux density measurement

Xylem sap flux densities (Js) were measured through the transient thermal dissipation (TTD) method (Do and Rocheteau, 2002; Isarangkool Na Ayutthaya et al., 2010). This method is a modification of the continuous thermal dissipation method (Granier, 1985). The TTD is a cyclical method of heating and cooling to assess the transient thermal index over a 10-min rise in temperature within a half-hour period. The half-hourly sap flux densities (Js; L dm^{-2} h^{-1}) were calculated according to the non-species-specific calibrations as assessed by Isarangkool Na Ayutthaya et al. (2010).

Probe settings

A dual probe is inserted into the trunk of each of the four sample trees at approximately 60 cm above soil level, at the north azimuth. The probe was positioned in the outermost ring of the xylem. Protection from direct solar radiation and rainfall was accomplished via a deflector in the trunk area of the tree containing the probe. The probes were connected directly to a CR 1000 data logger (Campbell Scientific, Leicester, UK).

Tree transpiration calculation

Tree transpiration (E_{tree}), as a unit of a liter per day (L d^{-1}), was calculated according to Equation 1. This method neglected tree water storage, ET (L d^{-1}).

$$E_{tree} = Js\_daily \times sapwood\ \ area \quad (1)$$

Soil water content

Soil water content was monitored via CS616 Water Content Reflectometer (Campbell Scientific, Leicester, UK). Soil probes were installed at the soil surface at 80-100 cm from the main trunk. Soil water content data were recorded every 30 min through the CR1000 data logger.

Data analysis

Data analyzed using a one-way analysis of variance (ANOVA) (SPSS 11.5, SPSS Inc., NY, USA). Comparisons of means performed using Duncan's multiple range test.

RESULTS AND DISCUSSION

Environmental conditions

This study was conducted from April to August, 2019 during the off-season fruit-setting period for mango production in Banhat District, Khon Kaen Province, Thailand. Climate data revealed the highest maximum temperature (>40°C) and lowest minimum relative humidity (30%) occurred at the end of April (Figure 1A, B). The average temperature and relative humidity were 24.3 to 33.7°C and 53.1 to 85.6%, respectively. The VPD, which was calculated from both temperature and relative humidity variables. These showed a decreasing trend

throughout the five-month period (Figure 1C).

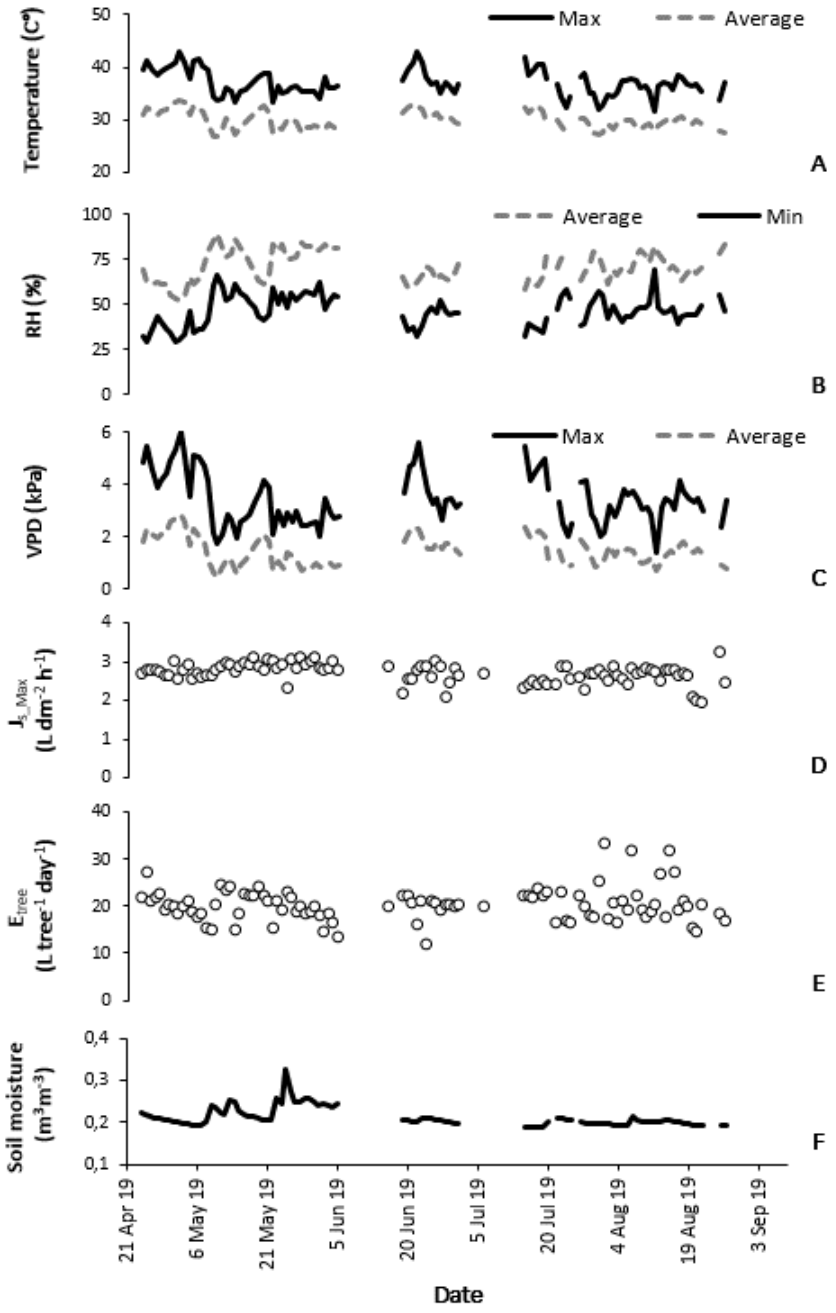

Figure 1. Changes in temperature (A), relative humidity (B), vapor pressure deficit (VPD) (C), sap flux density (J_s) (D), tree transpiration (E), and soil moisture (F) in mango cultivar 'Nam Dok Mai Si Thong' during April to August 2019.

Soil water content

The volumetric soil water content (greater than 0.18 m^3 m^{-3}) (Figure 1F) appeared to be stable, as the farmer regularly irrigated the trees. The soil water content data were indicative of mango trees under well-watered conditions.

Sap flux density and tree transpiration

The maximum sap flux density (J_{s_max}), according to VPD values were stable with little fluctuation (Figure 1D). However, tree transpiration (E_{tree}) was significantly altered (Figure 1E) ranging from 1.94 to 3.21 L dm^{-2} h^{-1} and 11.60-33.13 L d^{-1}, respectively, due to the effects of a variable climate. The fluctuation of E_{tree} caused by evaporative demand, regulated the trees water transport system (Isarangkool Na Ayutthaya et al., 2011, 2019).

Relationship between water transport and VPD

The relationships between maximum sap flux density (J_{s_max}) with maximum VPD ($VPD_{\_max}$) and average VPD ($VPD_{\_aver}$), are shown in Figure 2A, B. These VPDs show a stable value or plateau correlation when the $VPD_{\_max}$ and $VPD_{\_aver}$ ranged from 1.37 to 5.98 and 0.43-2.84 kPa, respectively. This evidence indicates sunny and dry conditions throughout the experimental period. Under extreme atmospheric drought, mangoes are relative stabile with minimal water loss. This may be a function of the stomata, which prevents excessive water loss through the leaves (Meinzer et al., 1997). An analysis of the relationship between J_{s_max} and both temperature and relative humidity, shows a plateau correlation similar to that of the VPD (Figure 2C, D).

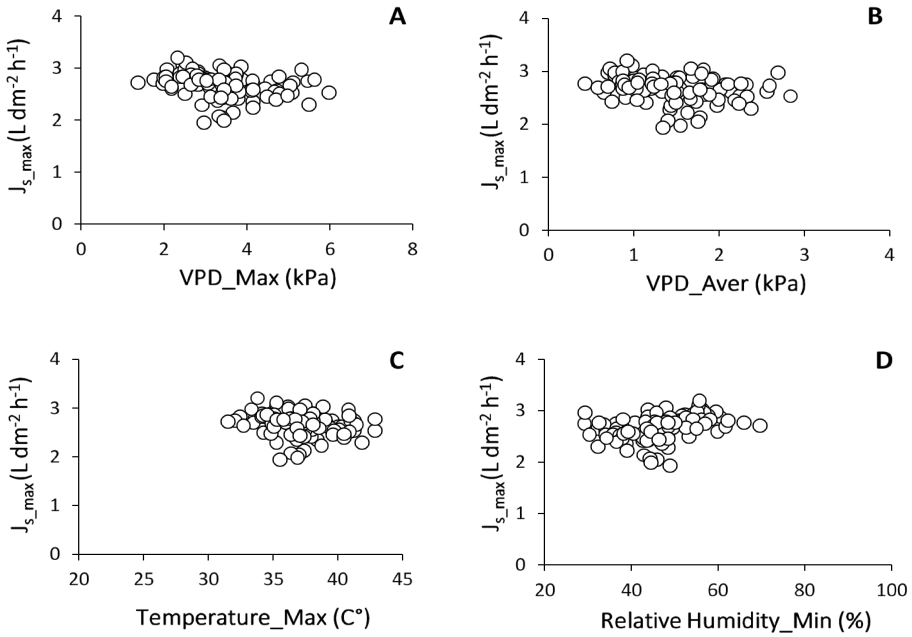

Figure 2. Relationships between maximum sap flux density (J_{s_max}) with maximum vapor pressure deficits ($VPD_{\_max}$, A), average VPD ($VPD_{\_aver}$, B), maximum temperature (C), and minimum relative humidity (D).

Figure 3 plots the relationships between E_{tree} with $VPD_{\_max}$ and $VPD_{\_aver}$, temperature, and relative humidity. These plots differed significantly, indicating the mangoes' ability to control water loss in the case of atmospheric drought.

Diurnal vapor pressure deficit (VPD) and sap flux density (Js)

To obtain a greater understanding of the diurnal VPD and J_s. Plots nanostructured under three conditions of extremely high VPD, middle VPD and low VPD (Figure 4). For each period, the diurnal pattern is expressed by two sample days. The results showed was no significant difference in the diurnal Js patterns between the extremely high VPD condition (Figure 4A, B) and middle VPD conditions (Figure 4C, D). However, the low VPD condition, in the narrow bell curve of diurnal Js (Figure 4E, F), indicating that Js was inhibited by a low evaporative demand factor (Isarangkool Na Ayutthaya et al., 2019). Notably, no changes in the J_{s_max} were observed

during the midday period.

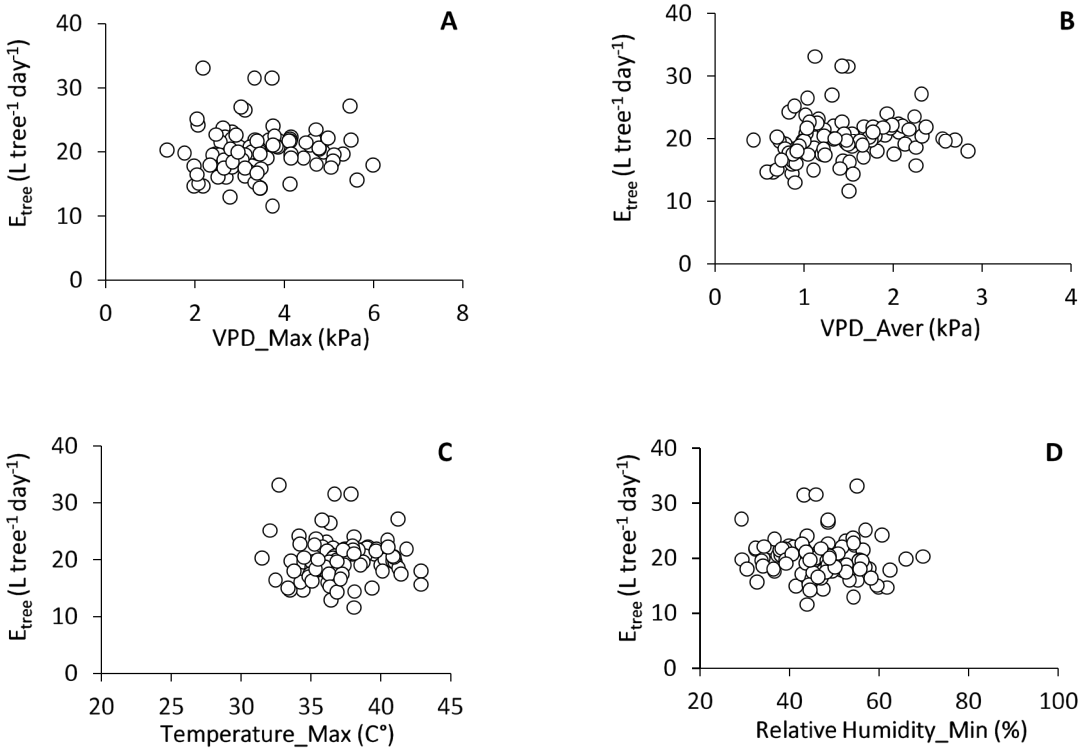

Figure 3. Relationships between tree transpiration (E_{tree}) with maximum vapor pressure deficits (VPD_{max}, A), average VPD (VPD_{aver}, B), maximum temperature (C) and minimum relative humidity (D).

Environmental effects on water transport

Comparisons of J_{s_max}, J_{s_daily}, and E_{tree} in the three evaporative demand conditions, showed that low evaporative demand reduced J_{s_daily} and E_{tree}, but did not affect J_{s_max} (Figure 5). Generally, the evaporative demand condition affects each of these parameters (Meinzer et al., 1997; Nicolás et al., 2008; Isarangkool Na Ayutthaya et al., 2011). Because our investigation analyzed transpiration in sunny conditions, further research is needed to evaluate lower evaporative demand conditions, particularly on cloudy and rainy days. We determined, however, that the water requirement for mango trees in a no-limit evaporative demand condition, should be greater than 20 L d^{-1} (Figure 5C).

CONCLUSIONS

Our study on the mango cultivar 'Nam Dok Mai Si Thong' under a soil water status (value greater than 0.18 m^3 m^{-3}, indicating well-watered conditions), showed the E_{tree} values of the eight-year-old mango trees averaged 20-32 L tree^{-1} d^{-1}. The E_{tree} evaluations showed fluctuations according to the evaporative demand, yet no effect to J_{s_max} was observed. Low VPD conditions regulated E_{tree} and depressed the diurnal J_s pattern into a narrow bell curve.

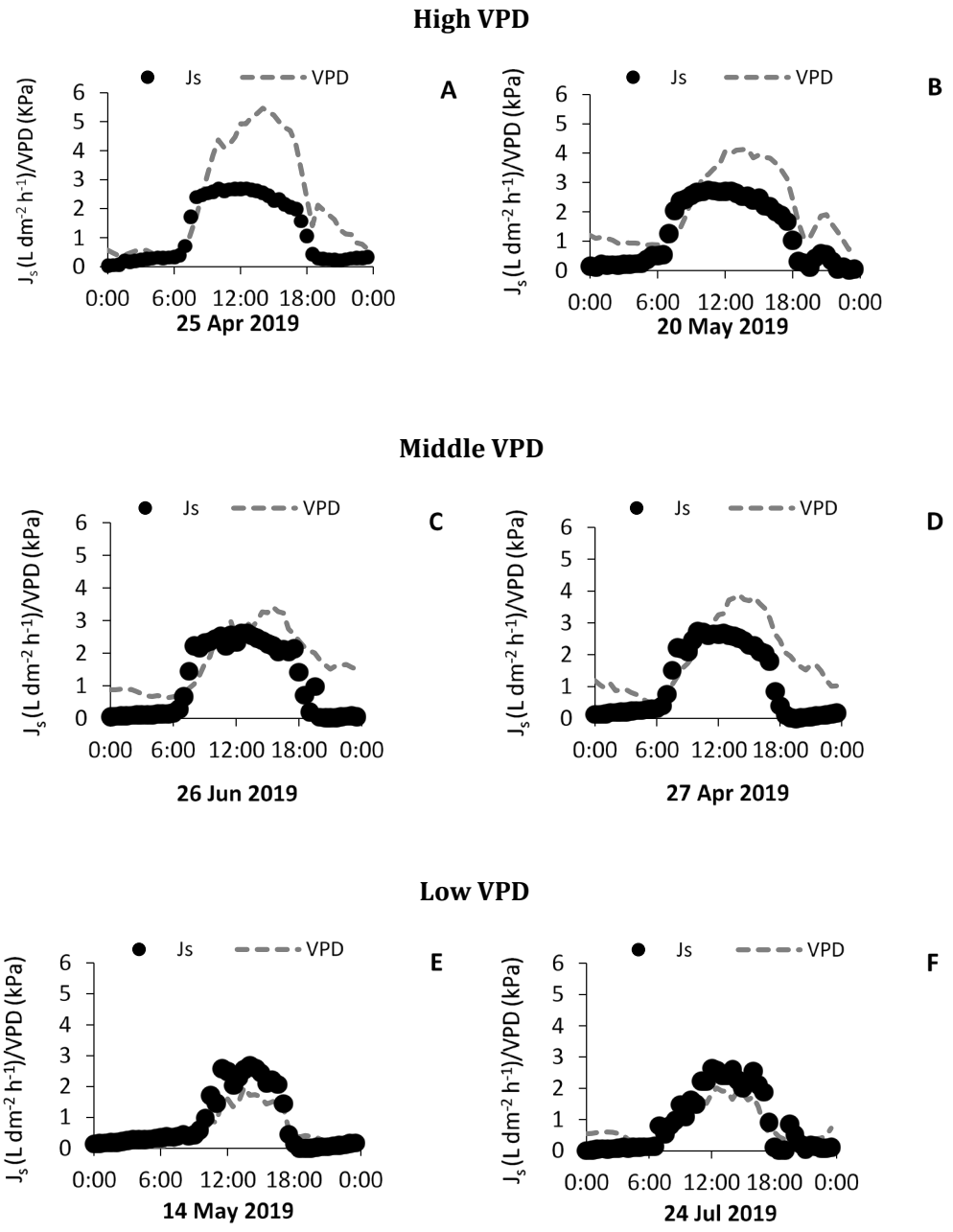

Figure 4. Diurnal patterns of vapor pressure deficit (VPD) and sap flux density (J_s) in three evaporative demand conditions: high VPD (April 25, 5.46 kPa (A) and May 20, 4.14 kPa (B)); middle VPD (June 26, 3.44 kPa (C) and April 27, 3.89 kPa (D)); and low VPD (May 14, 1.98 kPa (E) and July 24, 2.04 kPa (F)).

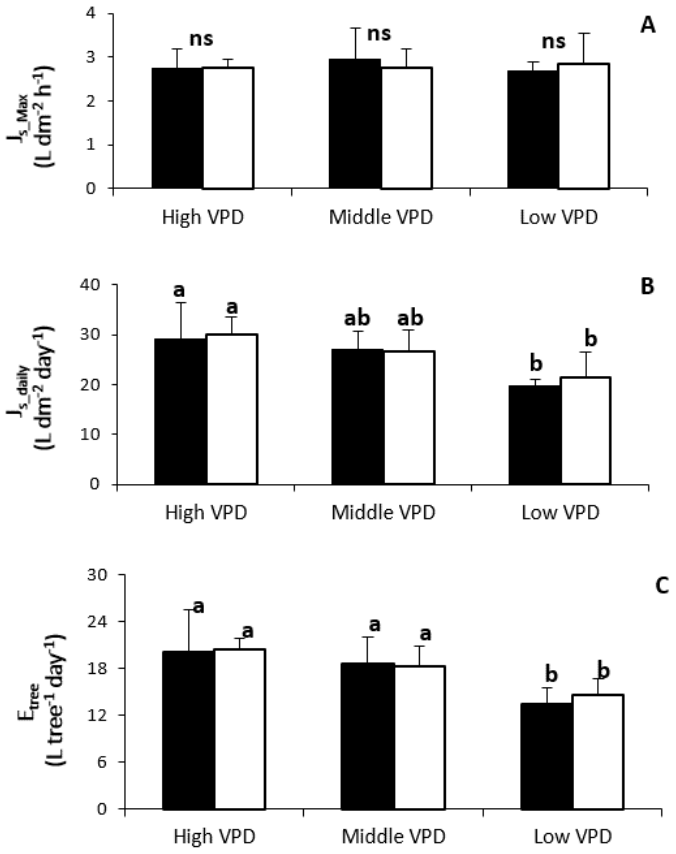

Figure 5. Comparisons of maximum sap flux density (J_{s_max}) (A), daily sap flux density (J_{s_daily}) (B), and tree transpiration (E_{tree}) (C) in three evaporative demand conditions: high VPD; normal VPD; and low VPD. The black and white bars indicate the first and second sampled days, respectively. Error bars represent the standard deviation. Different letter on the bars indicate significant difference and ns = non significant.

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Physiology and promotion of seed germination in ornamental peach

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Abstract

The establishment of efficient seed treatments for early germination and seedling growth is required to shorten the time that seedlings are in the nursery and to shorten breeding cycles. In our previous studies, the following has been revealed: 1) a 7-day period of rinsing in water combined with chilling at 5°C for four weeks promoted the germination rate in seeds of 'Yaguchi' peach more than where two days of rinsing were combined with four weeks of chilling; 2) the abscisic acid (ABA)-related genes (*NCED1*, *ABA 8-hydroxylase*, and *PP2C*) and stress response genes (*EID1*, *DREB2CA*, and *LEA D-34*) were involved in seed germination; and 3) the treatment of fluridone, an inhibitor of ABA biosynthesis, could promote germination of seeds chilled at 5°C for two weeks, however, the seedlings showed dwarfism and had lesions on the leaves. In the study reported here, seeds were treated with procaine hydrochloride, an inhibitor of DNA methylation, and later with fluridone to investigate the involvement of DNA methylation in seed germination under conditions with insufficient chilling. The procaine treatments that were used did not alleviate dwarfism or lesion development but significantly increased the SPAD value of the leaves on the seedlings. Preventing leaf yellowing by procaine is beneficial in the case of forced germination using fluridone, although the mechanisms of interaction between procaine and fluridone are unknown. Further study will be necessary to identify the involvement of epigenetics in both the dwarfing process and in lesion development in the seedlings grown from the seeds that are exposed to insufficient chilling.

Keywords: ABA, dormancy, epigenetics, fluridone, procaine, SPAD

INTRODUCTION

Peach (*Prunus persica* Batsch) is widely planted as a fruit tree but is also used as an ornamental plant for use as a garden tree or for its cut branches. It is a model species in the *Rosaceae* family for functional genomics research due to several distinct advantages, including a short juvenile phase (Arús et al., 2012).

Dry seeds of most temperate trees do not germinate and grow until they are imbibed followed by stratification at around 5°C (Stokes, 1965; Hartmann et al., 1997). Chilling treatments, therefore, increase germination percentage, and, for example, lead to the production of normal seedlings for *Prunus persica* 'GF305' (Martínez-Gómez and Dicenta, 2001), *Prunus avium* (Jensen and Eriksen, 2001), black mulberry (*Morus nigra* L.) (Koyuncu, 2005) and *Prunus persica* L. Batsch 'Big Top' (Leida et al., 2012). Abscisic acid (ABA) and gibberellin (GA) are known to play important roles in controlling seed dormancy and germination (Kucera et al., 2005; Finkelstein et al., 2008; Nonogaki et al., 2014).

In a previous study (Pawasut et al., 2010), ABA synthesis in the embryonic axes during chilling was believed to affect the varietal characteristics of seed dormancy, and rinsing seeds for >two days could remove sufficient ABA to allow seed germination with minimum chilling in three peach cultivars. In a transcriptomic study, based on RNAseq analysis, a set of genes was shown to be differentially expressed during seed dormancy in 'Yaguchi' peach (Worarad et al., 2017a, b). A seed rinsing treatment for seven days followed by four weeks chilling downregulated genes involved in ABA synthesis, catabolism and signaling pathways, which

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eventually suppressed ABA activity and consequently promoted germination and seedling growth compared with those from seeds rinsed for only two days (Worarad et al., 2017a, b). The results suggested that ABA-related genes (*NCED1*, *ABA 8-hydroxylase*, and *PP2C*) and stress response genes (*EID1*, *DREB2CA*, and *LEA D-34*) were involved in seed germination (Worarad et al., 2017a, b).

In 'Hokimomo' peach, rinsing seeds for seven days slightly increased the germination rate and significantly increased seedling height (Worarad et al., 2017a). The expression of genes *NCED1*, *ABA 8'-hydroxylase* and *GA2-oxidase* tended to decrease after seeds were rinsed for seven days and chilled for four weeks. Transcript levels of *LEA-D34* or *dehydrin*, a gene that is associated with drought stress (Yakovlev et al., 2008), could also be involved in seed dormancy (Worarad et al., 2017a).

Moreover, fluridone, an inhibitor of both carotenoid and ABA biosynthesis, can be an alternative method to promote germination by controlling ABA content and its metabolism, and consequently changing expression of certain ABA- and dormancy-related genes including *ABA-hydroxylase 3*, *EID1*, *LeMADS,* and *LEA D-34,* even under insufficient chilling conditions (Worarad et al., 2017b). For example, fluridone significantly increased the germination rate of 'Yaguchi' seeds from 0 to 71.4% after two weeks chilling, i.e., under conditions with insufficient chilling. All seedlings in both of the fluridone concentrations investigated, however, showed morphological lesions on all of the leaves (Worarad et al., 2017b).

DNA methylation changes lead to qualitative alterations in both gene expression and chromatin structure and are also involved in gene regulation during developmental phases (Bitonti et al., 2002), including early embryo and seed development (Lohe and Chaudhury, 2002). There is a requirement for the presence of the repressive histone protein, H3K27me3, in the silencing of seed development genes (Zhang et al., 2012). A switch model has been reported, which involves a change from tri-methylation at the 4th lysine residue of the histone H3 protein, where (H3K4me3)-associated is active, to trimethylation of histone H3 lysine 27, where (H3K27me3)-associated is the repressive transcription state, in development genes during seed germination (Molitor et al., 2014). Thus, it is assumed that epigenetics is involved in the formation of morphological lesions in the peach leaves that have been treated with fluridone under low chilling conditions.

In perilla, it has been reported that treatment with 5-azacytidine, a DNA demethylating reagent, induced flowering of an absolute short-day plant under long days (Kondo et al., 2006). Procaine, which is another DNA-demethylating agent, produces a 40% reduction in 5-methylcytosine DNA in human breast cancer cells (Villar-Garea et al., 2003). Procaine can also demethylate densely hypermethylated CpG islands, such as those located in the promoter region of the RAR beta 2 gene, restoring gene expression of epigenetically silenced genes (Villar-Garea et al., 2003).

In this study, procaine was used with fluridone to treat 'Yaguchi' peach seeds under insufficient chilling conditions and the growth of seedlings was observed in an attempt to understand the association of epigenetics, including DNA methylation, on seed germination and seedling formation.

MATERIALS AND METHODS

Plant materials

Fully ripened 'Yaguchi' peaches were collected from the peach flower garden of Koga Kubou Park in Koga City (36°10'N; 139°42'E), Ibaraki Prefecture, Japan, in late-October 2019. After the skin and flesh were removed and washed, endocarps were carefully cracked in a vice to take out the seeds.

Treatments of DNA demethylation agent and ABA inhibitor

Seeds were soaked in 0.5% sodium hypochlorite and rinsed four times using sterile water. Seeds were then transferred into Petri dishes containing two-layers of filter paper moistened with either sterile water, or 0.1, 1.0, 10 or 100 mM procaine hydrochloride (Sigma-Aldrich, Japan), a DNA demethylating agent, for two weeks under dark conditions at 20°C. Ten

seeds in three replications were used for each treatment. Subsequently, the same seeds were again soaked in 0.5% sodium hypochlorite and rinsed four times using sterile water, and then transferred into Petri dishes containing two-layers of filter paper moistened with 100 µM fluridone (Wako Yakuhin Co. Ltd., Japan). The plastic Petri dishes were sealed with parafilm and kept in the refrigerator at 5°C for two weeks. The seeds were then sown in a cell tray with a peat-based growing medium (Super mix A, Sakata Seed Co., Japan). After 30 days, lesions scores, height and numbers of nodes of each seedling were determined. The severity of lesions was assessed using a scale following Zigas and Coombe (1977). The germination percentage and SPAD (chlorophyll content) were determined after 31 days. SPAD value was measured using a SPAD-502 m (Konica Minolta Inc., Japan).

Statistical analysis

Polynomial contrast tests were performed between concentrations of procaine hydrochloride and seedling growth parameters. Data of lesion scores were subjected to ANOVA, and significant differences of the means were evaluated using Tukey's HSD test when the treatment was significant in the ANOVA. T-test or Fisher's protected LSD test was performed to compare the data ($p<0.05$).

RESULTS AND DISCUSSION

In this study, germination percentage varied from 53.3 to 81.0% and there was a significant difference between 0.1 and 10 mM procaine hydrochloride treatments, an inhibitor of DNA methylation (Figure 1). A cubic relationship ($p<0.07$) was found between procaine concentrations and germination rate using the polynomial contrast tests (Figure 1). This might have been due to the low germination rate at 10 mM, however, no significant promoting effect on germination was observed.

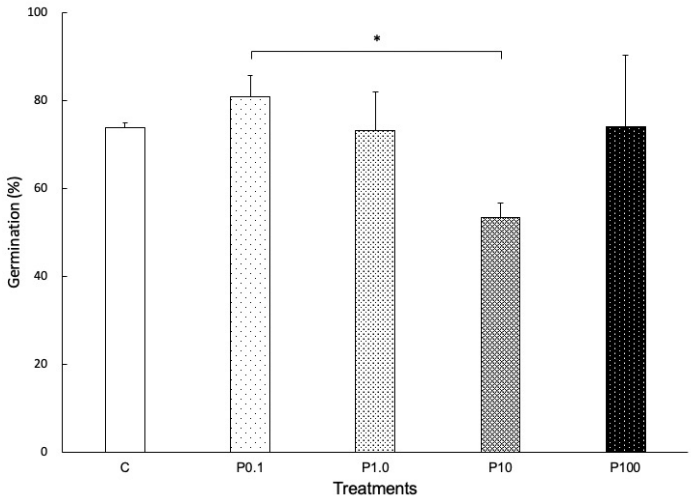

Figure 1. Germination rate of seeds treated with procaine hydrochloride (P) at 0. 0.1, 10 and 100 mM at 20°C for two weeks followed by 100 µM fluridone at 5°C for two weeks. Vertical bars indicate SE (n=3). A cubic relationship ($p<0.07$) was found between P concentrations and germination rate by polynomial contrast tests. * indicates significant difference by t-test ($p<0.05$). No improvement in germination due to the P treatment was determined.

Plant height of seedlings ranged from 9.8 to 12.1 cm and the number of nodes ranged from 14 to 16 (Figure 2). These data agree with our previous findings where 71.4% seeds germinated and the seedlings were 8 to 14 cm in height and had 13 to 17 nodes 30 days after sowing in the treatment with fluridone that had been chilled for two weeks (Worarad et al., 2017b).

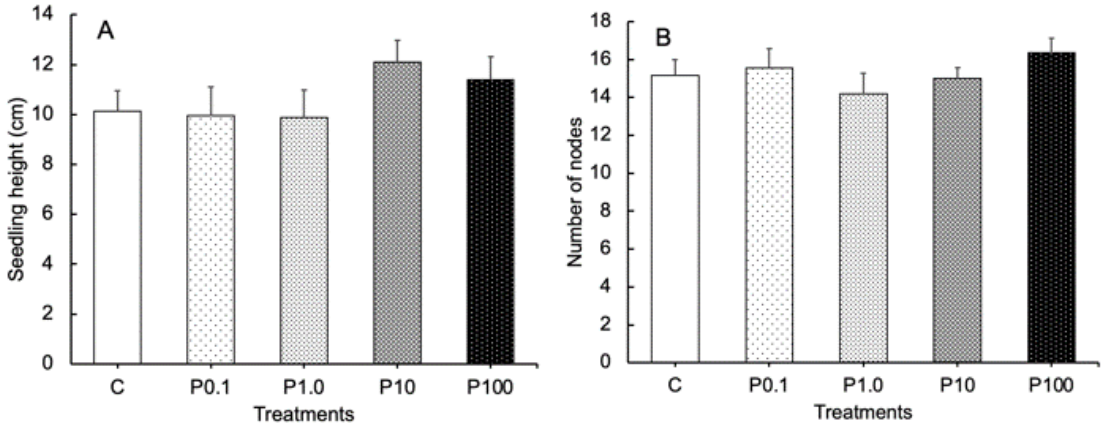

Figure 2. Plant height (A) and number of nodes (B) in seedlings grown from the seeds treated with procaine hydrochloride (P) at 0. 0.1, 10 and 100 mM at 20°C for two weeks followed by 100 μM fluridone at 5°C for two weeks. Vertical bars indicate SE (*n*=9-20). No significant relationships were found between P concentrations and the growth parameters using polynomial contrast tests.

Treatment with procaine hydrochloride did not alleviate the lesion scores, except that the most severe lesion score 5 was slightly decreased by the 10 and 100 mM procaine treatments (Figure 3). Procaine treatments linearly raised the SPAD value of seedling leaves (Figure 4). The 10 mM procaine treatment especially significantly increased the SPAD value from 31.4 to 36.9 (Figure 4).

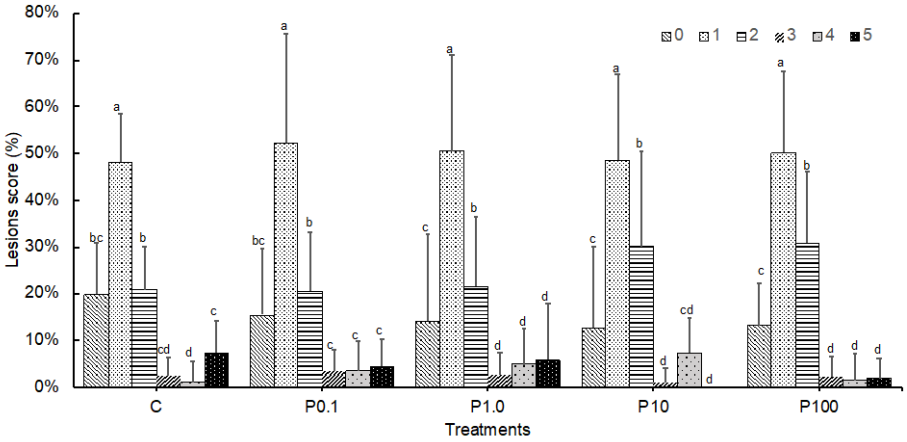

Figure 3. Lesion scores (%) of leaves on seedlings from the seeds treated with procaine hydrochloride (P) at 0. 0.1, 10 and 100 mM at 20°C for two weeks followed by 100 μM fluridone at 5°C for two weeks. Lesion scores were classified into a 0 to 5 scale according to Zigas and Coombe (1977). Vertical bars indicate SE (*n*=9-20). Different letters among lesion scores at each concentration of P indicate significant differences by the Tukey-Kramer test ($p<0.05$).

In seeds, epigenetic processes are involved at the endosperm development stage (Lohe and Chaudhury, 2002) and the repressive histone mark H3K27me3 is required for the silencing of seed developmental genes (Zhang et al., 2012). In perilla, treatment with 5-azacytidine, a DNA demethylating reagent, induced flowering of the short-day plant under long days (Kondo et al., 2006). Procaine, which is a DNA-demethylating agent (Villar-Garea et

al., 2003), can demethylate densely hypermethylated CpG islands, such as those located in the promoter region of the RAR beta 2 gene, restoring gene expression of epigenetically silenced genes (Villar-Garea et al., 2003). Therefore, we hypothesized that procaine treatments affected the growth and lesion score of leaves in the peach seedlings by impacting on demethylation of some specific sites.

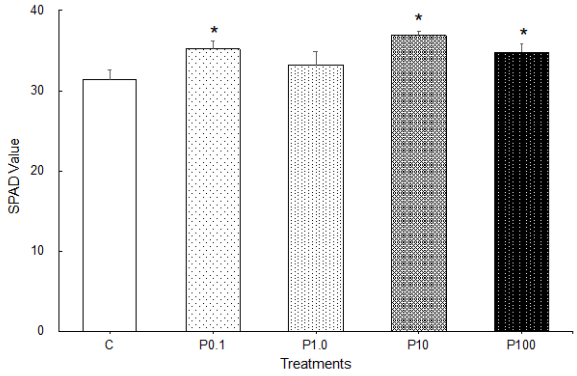

Figure 4. SPAD value of leaves of seedlings from seeds treated with procaine hydrochloride (P) at 0. 0.1, 10 and 100 mM at 20°C for two weeks followed by 100 µM fluridone at 5°C for two weeks. Vertical bars indicate SE (*n*=9-20). A linear relationship ($p<0.024$) was found between P and SPAD using polynomial contrast tests. * indicates significant differences between control and treatments at $p<0.05$ using the Fisher's protected LSD test.

The procaine treatments significantly increased the SPAD value of leaves on seedlings (Figure 4) where there had been insufficient chilling hours, i.e., only two weeks in this study. However, contrary to expectations, the procaine treatments did not alleviate the lesion scores of the peach seedlings (Figure 3). Further study of DNA methylation levels will be necessary to define the mechanism of dwarfing and lesion development in seedlings from seeds which have had insufficient chilling.

Fluridone was used in this study as an inhibitor of ABA. However, it was originally used as an inhibitor of carotenoid biosynthesis including the synthesis of pigments involved in photosynthesis. Leaf yellowing was, therefore, observed in the seedlings grown from the seeds that had been forced using fluridone treatments (Worarad et al., 2017b). Consequently, prevention of leaf yellowing through the use of procaine is beneficial where germination is forced using fluridone. Nonetheless the mechanisms involved in the interaction of procaine with fluridone are unknown.

CONCLUSIONS

Combined treatments of procaine, an inhibitor of DNA methylation, with fluridone, an inhibitor of ABA synthesis, did not promote germination of seeds chilled for only 2 weeks and did not affect either plant height or lesion development but did alleviate leaf yellowing. The involvement of epigenetics in seed germination and in the development of seedlings is unclear. Analysis of DNA methylation in the embryonic axes is needed to confirm whether epigenetics is related to seed germination and seedling growth.

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Growth, yield and postharvest quality of ampalaya (*Momordica charantia* L.) under protected cultivation in response to different pruning techniques

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Abstract

Bitter gourd or ampalaya (*Momordica charantia* L.) is one of the most consumed vegetables in the Philippines due to its nutritional and medicinal values. Therefore, its production must be increased through appropriate cultural management practices, such as pruning. This study was conducted to evaluate the growth, yield and postharvest qualities of ampalaya as influenced by different pruning techniques. The experiment was laid out in simple randomized complete block design with three replications and four treatments; T0) no pruning; T1) pruning the main stem at the 3rd node; T2) pruning the main stem at the 7th node; and T3) laterals within the first 0.5 m of the main vine pruned. Results revealed that removal of lateral vines within the first 0.5 m of the main vine significantly enhanced the reproductive stage as expressed in the number of days to first staminate and pistillate flowers appearance and node number bearing at the first flower. Furthermore, the vegetative characteristics: such as main vine length, fresh weight of vines and herbage yield, fruit number, heaviest weigh of marketable fruit and total fruit yield of 61.29 t ha^{-1} was obtained by pruning all lateral vines at 0.5 m from the base. Therefore, this is best pruning technique for ampalaya.

Keywords: flowering characteristics, fruiting characteristics, canopy management, yield capacity

INTRODUCTION

Bitter gourd or ampalaya (*Momordica charantia* L.) is a tropical and subtropical vine of the family *Cucurbitaceae*. It is widely grown in Asia, Africa and the Caribbean for its edible fruit. It originated in India and was introduced into China in the 14th century (Bagchi, 2005). Early cultivation of bitter gourd was recorded in the ayurvedic text written in Indian Sanskrit from 200 to 2000 BC. by members of the Indo-Aryan culture. In India, over 300 words describing cucurbits are found in the Sanskrit texts (Decker-Walters, 1999). Fruit of bitter gourd is a good source of carbohydrates, proteins, vitamins, minerals and have the highest nutritive value among cucurbits (Desai and Musmade, 1998; Miniraj et al., 1993). Bitter gourd extracts possess antioxidant, antimicrobial, antiviral, ant hepatotoxic and antiulcer genic properties while also having the ability to lower blood sugar levels.

For consumption, fruit are cooked with other vegetables. Gourds may be stuffed, stir-fried, or added in small quantities to beans and soups to provide a slightly bitter flavor. However, in most instances for food preparation, they are blanched (parboiled) or soaked in salt water before cooking to reduce the bitter taste. In addition, bitter gourd fruit may be dehydrated, pickled, or canned. Young shoots and leaved are also cooked and eaten as leafy vegetables. Furthermore, leaf and fruit extracts are used in the preparation of tea (Tindall, 1983).

In the Philippines, vegetable production of bitter gourd is in the top ten most consumed vegetables. However, per capita consumption of vegetables in the country is far below the recommended daily intake of 200 g of vegetables necessary to ensure a sufficient vitamin and

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micronutrient supply for the human body (FAO/WHO, 1992). The total land area of the Philippines is 299,404 km^2 or approximately 30 million ha. It is composed of 7,107 islands with 11 taking up about 95% of the total land area. The remaining 5% consists of small islands and islets of the total land area, forestry utilises 65%, agriculture about 33%, inland fisheries 0.44% and settlements and open land account for about 0.04% (Moog, 2006). To compensate for the countries growing population an increase in agricultural production is a must. New agricultural and management practices must be tested and practices modified. One very important cultural practice to test, is pruning to enhance productivity and quality.

Pruning is a horticultural and silvicultural practice involving the selective removal of certain parts of a plant, such as branches, buds, or roots to regulate its size (Soule, 1985). Pruning for bitter gourd is done in order to control the size of your plant, train to climb trellis, improve light and air flow around the plant and improve the quality and size of your harvest.

After harvest one of the most critical phases in maintaining produce quality is the postharvest handling phase. Postharvest handling including cooling, cleaning, sorting and packing are used to meet marketing requirements (product specifications). Once harvested, vegetables and fruits are subject to the active process of senescence. Numerous biochemical processes continuously change the original composition of the harvested product until it becomes unmarketable. Postharvest shelf life is typically determined by objective methods that determine the overall appearance, taste, flavour, and texture of the commodity (Kader, 2002). Postharvest shelf life is important in order to satisfy the needs of the consumers.

In the Philippines, although there are known advantages of pruning, many farmers still fail to adapt this technology due to the lack of knowledge and failure to recognize such advantages. Moreover, few studies of pruning in relation to its productivity impact and postharvest shelf life have been conducted on bitter gourd. There were few discoveries related to pruning but with different varieties, location or with different pruning techniques. This study aims to evaluate the response of ampalaya to different pruning techniques, determine the effects of different pruning techniques on post-harvest product quality of ampalaya and analyse the cost and returns on ampalaya production as influenced by different pruning techniques.

MATERIALS AND METHODS

Location of the study

The study was conducted at the Vegetable Experimental Area of Department of Horticulture, College of Agriculture and Food Science, Visayas State University, Visca, Baybay City, Leyte (10.7444°N; 124.7921°E).

Treatments and experimental design

The experiment was a randomized complete block design (RCBD) with three replications and eight samples in each treatment plot. The area of each treatment plot was 5 m^2 (1×5 m). Alleyways of 0.5 m between replications were provided to facilitate farm operations and management as well as data gathering. The four treatments were as follows: T0) no pruning; T1) pruning the main stem at the 3rd node; T2) pruning the main stem at the 7th node; and T3) laterals within the first 0.5 m of the main vine pruned.

Cultural management

The experimental area was ploughed and harrowed before planting. Seeds of ampalaya 'Galaxy F_1' were used in the experiment. Drip irrigation system was installed after transplanting. Trellising net with a height of 2 m was established one week after transplanting.

Ampalaya fruits were bagged with plastic cellophane, right after the flowers change colour from bright yellow to pale yellow. Fruits were harvested when fully developed or when their colour change from dark to light green and the ridges are far apart when fruit become more prominent. After harvesting, the fruit was classified as marketable or non-marketable. Marketable fruits are those that are free from disease, insect damage and mechanical injuries. Non-marketable fruits are diseased, small fruit and exhibit mechanical injuries.

Data collection

The number of days to first male and female flower, node number bearing the first male and female flowers, root length, stem girth, length of vines, fresh weight of roots and leaves were determined. Also, herbage yield (t ha^{-1}) was determined.

The fruit size, weight of marketable and non-marketable fruits, the number of marketable and non-marketable fruits and total fruit yield were also determined.

The visual quality rating included, the physical appearance of ampalaya using a visual quality rating (VQR) system. VQR evaluation was on a 9-1 scale, 9 equalling excellent, field fresh or no defect, 7 was good with minor defects, 5 was fair with moderate defects, 3 was poor with serious defects and limited marketability, 2 was limited edibility, and 1 was non-edible.

Postharvest measurements included cumulative weight loss, the initial weight and the weight of the fruit measured every other day. Sample plants per treatment were used to test the firmness of ampalaya. Determination using the finger-feeling and firmness score. Firmness index was evaluated on a 1-4 scale, 1 is firm or hard, 2 is first perceptible softening, 3 is moderately soft, and 4 is ripe-soft.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) to determine the level of significance. Tukey's test or honest significant differences (HSD) was used to compare the significant differences among treatment means.

RESULTS AND DISCUSSION

Horticultural characteristics

The flowering characteristics of ampalaya were significantly affected by the degree of pruning. The number of days to first male flower (emergence) and the node number bearing the first male flower were significantly different (Table 1). Ampalaya plants pruned at the 3rd node (T1) were the earliest to bear male flowers, averaging 29 days after transplanting compared to plants pruned at the 7th node (T2) which averaged 30 days after transplanting. Furthermore, removal of all laterals at 0.5 m from the base (T3) delayed the emergence of male flowers, averaging 34 days after transplanting.

Table 1. Pruning treatment effects on the flowering characteristics of ampalaya.

Treatments	No. of days to first flower		Node no. bearing the first flower	
	M	F	M	F
T0 – no pruning	31.63b	34.96	5.19c	10.32 bc
T1 – main stem pruned at 3rd node	29.88c	32.46	11.04a	13.96 a
T2 – main stem pruned at 7th node	30.00bc	34.17	7.75b	12.88 ab
T3 – laterals within the first 0.5 m of the main vine pruned	34.59a	37.96	3.78c	9.10 c
CV (%)	2.71	7.80	12.32	14.30

Means within the same column followed by a common letter and/ or without letter designation are not significantly different from each other at 5% level of significant.

Furthermore, the number of days to first female flower emergence was not significantly affected by the degree of pruning. Consistently, early female flower emergence was observed in plants pruned at the 3rd node (T1) averaging 32 days from transplanting. The last to bear female flower was T3, averaging 38 days from transplanting. The number of days to first male and female flowers is supported by the report of John (2004), indicating that male flowers normally are formed ahead compared to the female flowers.

Vegetative characteristics

Vegetative characteristics of ampalaya vines, such as length of roots, stem girth, fresh

weight of the roots and leaves are presented in Table 2. The results showed significant difference in the length of vines and subsequent fresh weight of vines. T3, attained the longest vine length, averaging 4.11 m. This is comparable to T0 no pruning. Correspondingly, weight of vines was heaviest at T3 with 0.68 kg per harvestable plot.

Table 2. Pruning treatment effects on the vegetative characteristics of ampalaya.

Treatments	Length (cm)		Stem girth (cm)	Fresh weight (kg 3.6 m^{-2})			Herbage yield (t ha^{-1})
	Roots	Vines		Roots	Leaves	Vines	
T0 – no pruning	30.54	3.69ab	1.02	0.23	0.39	0.54b	2.60b
T1 – main stem pruned at 3rd node	34.88	2.87b	1.13	0.25	0.29	0.39c	1.91c
T2 – main stem pruned at 7th node	38.00	2.76b	1.08	0.19	0.38	0.41c	2.17c
T3 – laterals within the first 0.5 m of the main vine pruned	38.35	4.11a	1.29	0.16	0.41	0.68a	3.05a
CV (%)	19.30	13.89	8.66	24.21	15.14	12.08	8.55

Means within the same column followed by a common letter and/ or without letter designation are not significantly different from each other at 5% level of significant.

Herbage yield as the total weight of the entire above ground plant portion within the harvestable area per plot was determined after the last harvest. Significantly, highest herbage yield of 3.05 t ha^{-1} was obtained from T3 (lateral vines 0.5 m from the base were removed). This was followed by T0 no pruning (which contain all the laterals and main shoot). The two pruned treatments, T1 and T2 had the lightest herbage.

Yield and yield components

The number and weight of marketable fruits were significantly affected by the different pruning techniques (Table 3). Ampalaya plants pruned to T3 (all lateral vines 0.5 m from the base were removed) produced highest number of fruits, averaging 51 fruits per treatment plot. However, this is not significantly different from the control treatment. Furthermore, T3 produced the heaviest marketable yield $plot^{-1}$. This may be attributed to the supportive effect of more available fertilizers to fewer lateral vines and fruits $plant^{-1}$. This enabled production of heavier, wider and longer fruits (Table 4). In addition, the removal of all laterals within 0.5 m from the base reduced the number of branches that are potential sink of nutrients that are not needed for development of reproductive laterals. This result supports findings of Sadras and Denison (2009) that the ratio between source and sink activities, results from the balance between the organ assimilate demand for growth and maintenance and the whole-plant assimilate supply through photosynthesis or reserve mobilization. The ratio between organ assimilate demand and assimilate supply at the plant scale has been found to be one of the main factors affecting plant growth and development (Marcelis et al., 2004; Mathieu et al., 2009).

Table 3. Pruning treatment effects on yield components of ampalaya.

Treatments	No. of fruits		Weight of fruits		Total fruit yield (t ha^{-1})
	Marketable	Non-marketable	Marketable	Non-marketable	
T0 – no pruning	37.33ab	3.00	14.98b	0.67	43.49b
T1 – main stem pruned at 3rd node	21.67c	1.33	8.25c	0.21	23.50c
T2 – main stem pruned at 7th node	33.33bc	3.33	12.15bc	0.46	35.02bc
T3 – laterals within the first 0.5 m of the main vine pruned	51.00a	1.00	21.89a	0.17	61.29a
CV (%)	21.42	18.20	16.05	11.29	16.33

Means within the same column followed by a common letter and/ or without letter designation are not significantly different from each other at 5% level of significant.

Table 4. Pruning treatment effects on the cumulative weight loss (%) of ampalaya.

Treatments	Weight loss (%)		
	Day 2	Day 4	Day 6
T0 – no pruning	13.73	19.20	24.50
T1 – main stem pruned at 3rd node	13.69	18.75	23.27
T2 – main stem pruned at 7th node	13.57	18.56	23.16
T3 – laterals within the first 0.5 m of the main vine pruned	13.64	18.65	23.27
CV (%)	8.16	6.74	5.97

Means within the same column followed by a common letter and/ or without letter designation are not significantly different from each other at 5% level of significant.

Correspondingly, total fruit yield in t ha^{-1} was significantly high for the T3. This result proves the beneficial effect of pruning 0.5 m from the base, resulting in highly productive growth and yield of bitter gourd compared to the other pruning treatments. The yield achieved may be ascribed to elimination of some vine laterals that would otherwise compete for nutrients, water and light interception. This further supports Palada and Chang (2003) study findings of factors needed for the optimum growth and development of the plants.

Postharvest characteristics

The pruning treatment did not significantly affect the percent weight loss for bitter gourd. However, a constant increase in the percent weight loss from day 2 to day 6 of storage at ambient conditions is shown in Table 4. Metabolic activity in fresh fruit and vegetables continues for a short period after harvest. The energy required to sustain this activity comes from the respiration process (Mannapperuma et al., 1991) which supports our findings. Respiration involves the oxidation of sugars to produce carbon dioxide, water and heat. At harvest, some fruits and vegetables continue to ripen. During this process, the respiration rate increases in bitter gourd.

Different pruning techniques showed no significant effect on the visual quality rating of bitter gourd (Table 5). At day 2 all treatments were considered fresh and marketable as per the VQR rating. All treatments were above 5 (fair, defects moderate). However, on day 6 all bitter gourd fruit from the pruning treatments were considered to be poor quality. The control and T1 appeared to be almost decomposed and non-marketable.

Table 5. Pruning treatment effects on the visual quality rating of ampalaya.

Treatments	Visual quality rating			
	Initial	Day 2	Day 4	Day 6
T0 – no pruning	9	6.83	5.00	3.00
T1 – main stem pruned at 3rd node	9	6.67	5.17	2.83
T2 – main stem pruned at 7th node	9	5.67	5.17	3.50
T3 – laterals w/in the first 0.5 m of the main vine pruned	9	7.33	6.17	5.17
CV (%)		16.16	23.18	38.81

Means within the same column followed by a common letter and/ or without letter designation are not significantly different from each other at 5% level of significant.

The firmness of bitter gourd fruit was not significantly affected by the different pruning techniques (Table 6). However, numerical differences were noted. Initial data showed T3 produced firmer fruit that were comparable with the control treatment. In addition, final testing showed that the removal of all lateral vines at 0.5 m from the base produced firmer fruit. Furthermore, T3 fruit were comparable with T1, main stem pruned at 3rd node and T2, pruned at the 7th node. No significant difference was observed on the shelf life of bitter gourd due to the different pruning techniques. However numerical differences were observed. Removal of all lateral vines 0.5 m from the base resulted in the longest shelf life, 7 days after harvesting. Pruning treatment T1, pruned at the 3rd node and T2, pruned at the 7th node were

comparable to the control treatment. However, T1, pruned at the 3rd node had the shortest shelf life.

Table 6. Pruning treatment effects on the physical characteristics of ampalaya.

Treatments	Shelf life	Firmness (kg f)	
		Initial	Final
T0 – no pruning	5.82	1	3.45
T1 – main stem pruned at 3rd node	4.67	1	4.08
T2 – main stem pruned at 7th node	5.00	1	3.47
T3 – laterals within the first 0.5 m of the main vine pruned	7.00	1	3.04
CV (%)	10.58		12.61

Means within the same column followed by a common letter and/ or without letter designation are not significantly different from each other at 5% level of significant.

CONCLUSIONS

Pruning ampalaya significantly enhanced the vegetative growth in terms of vine length, fresh weight, herbage yield and reproductive growth that advanced the appearance of staminate flowers. Yield and yield components of ampalaya were enhanced by removing all laterals, 0.5 m from the base of the plant. This pruning technique was also the most profitable technique as it generates the highest net return. Postharvest characteristics of ampalaya under different pruning techniques were comparable. Thus, the pruning treatments did not affect the postharvest quality of ampalaya.

It is recommended to prune ampalaya by removing all lateral vines 0.5 m from the base of the plant. Furthermore, there is a need to conduct a similar study to confirm the effect of pruning using other ampalaya cultivars. In addition, a similar study may need to be conducted at different elevations to further confirm the effects of the recommended pruning technique.

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Chilling and heating accumulations impact bud burst and flowering of 'KU-PP2' peach tree

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Abstract

Responses to chilling accumulation and heat requirement are crucial factors for peach growing in forcing culture. However, the performance of 'KU-PP2' peach trees under forcing conditions has not been elucidated. In this study, we investigated the effects of chilling accumulation and heat requirement on breaking dormancy and flower development and the correlation between these factors over two consecutive seasons. 'KU-PP2' peach trees in containers were transferred to a phytotron after being exposed to different chilling periods (250, 500 and 750 h). The air temperature in phytotron varied at 15, 20 and 25°C. Bud bursting and flowering were monitored every three days. The results show that the duration of chilling exposure reduced the time and heat required to induce bud break and flowering. At 250 chilling hours (CH), 'KU-PP2' trees required more than 10,000°C growing degree hours (GDH) for flower bloom, while at 500 and 750 CH, they required between 4,000 and 9,000°C GDH. The highest temperature accelerated budburst in peach trees with low- and mid-chilling exposure but delayed and prolonged the flowering period in trees with high chilling accumulation. Moreover, the heating temperature was also negatively correlated with the size of reproductive organs, pollen germination, and fruit set in both seasons. The interaction between chilling exposure and heating temperature affected final bud bursting, flower size and fruit set. These results suggested that inadequate chilling accumulation and excessive heat in forcing culture might have a negative effect on flowering and yield of 'KU-PP2'.

Keywords: *Prunus persica*, dormancy, chilling requirement, heat requirement, forcing

INTRODUCTION

For temperate plants, bud dormancy, which is a critical period in the phenology cycle, influences the quality and quantity of budburst, flowering, and fruit set (Beauvieux et al., 2018). Naor et al. (2003) stated that budburst is affected by two temperature-dependent factors: chilling accumulation and heat requirement. After chilling accumulation is fulfilled by exposure to low temperatures, warm temperature is required to satisfy the heat requirement for inducing budburst, anthesis, and foliation (Saxe et al., 2001; Heide, 2003; Polgar et al., 2014). Inadequate chilling or excessive heating negatively affect dormancy completion and bud breaking. For example, exposure to higher temperatures during bud break in grapevines delayed the anthesis, decreased flower number per inflorescence (Buttrose and Hale, 1973) and induced ovule fertility, resulting in fewer fruits per cluster (Ebadi et al., 1995).

Under natural conditions in Japan, the peach harvesting period extends from mid-June to early September. Therefore, it must compete with melons, watermelons, and grapes. Forcing culture is one method to prolong the peach harvesting season. Beppu et al. (2015) demonstrated that low-chilling peaches grown under forcing conditions could be harvested as early as late April. This indicated that forcing culture could produce very early season peaches, especially when fresh field grown fruit are not yet available. Demand is extremely high for early market fruits, which command very favorable prices for the grower. 'KU-PP2', a yellow-fleshed peach with a low chilling requirement, produces excellent yields and high sugar content. It was bred from a cross between low-chilling (200 Chilling hours; CHs) and

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Japanese high chilling (1,000 CH) cultivars and released by Kagawa University in 2016 (Manabe et al., 2015). Our goal is to introduce 'KU-PP2' into forcing culture for early peach production with high quality fruit. Increased knowledge about the relationship between chilling accumulation, heat requirement, and phenological cycles under forcing conditions is necessary for establishing the appropriate cultivation management practices.

In this study, we examined the influence of chilling accumulation and heat requirement, as well as the interaction between these factors on bud break and flowering in 'KU-PP2.' We aim to determine the optimal time to start heating and the optimal forcing temperature. Based on information obtained on chilling accumulation, heat requirement, date of bud bursting, flowering date, and fruit set, we designed a heating program that is optimal for breaking dormancy and hasten the flowering period.

MATERIALS AND METHODS

Plant materials

This study was conducted across two consecutive seasons (2016/2017 and 2017/2018) at the experimental orchard, Kagawa University, Japan. Nine healthy plants of 'KU-PP2' peach, which were grafted on 'Tukuba 1 Gou' rootstock and planted in containers (52 (L) × 30 (W) × 36 (H) cm), were selected for this study. All plants were exposed to a natural chilling temperature (<7.2°C) for fulfilling their chilling requirement. Each of the trees received a unique combination of a chilling duration of 250, 500 or 750 CH, followed by forcing air temperature of 15, 20 or 25°C in the phytotron.

Observation on flower and leaf bud break

Ten new current-year shoots were randomly selected for monitoring budburst. These shoots were pruned to a mean length of 20 cm. The progression of floral and leaf bud break for each shoot was observed every three days until 120 days after the onset of forcing. The emergence of sepals and leaf buds in the green tip stage, were considered to be budburst. The full bloom date was recorded when at least 50% of flowers had opened.

Evaluation of flower morphology, pollen germinability, and fruit set

At anthesis, five flowers were taken from each plant. Flower weight, pistil length, and ovary width were measured. From these flowers, pollen grains were collected to test the pollen germination on agar-sucrose medium (15% sucrose + 1% agar). Pollen germination was observed after three hours of incubation at 25°C. The pollen grains were counted as germinated when the pollen tube length reached the diameter of the pollen grain. Four weeks following full bloom, the number of fruit set per branch was counted. The fruit set rate was calculated as a percentage of the total opened flowers.

Calculation of chilling hours and heat requirement

Air temperature in the experimental orchard was measured at 1.5 m above the ground surface and recorded by the data recorder. Chilling hours (CHs) were calculated by the chilling hour (<7.2°C) model, in which the number of hours during which the air temperature decreases to below 7.2°C are counted (Weinberger, 1950).

Heat requirement was calculated and expressed as the number of growing degree hours (GDH) (Richardson et al., 1975) that accumulated from the onset of heating until the rest completion date, during which at least 50% of buds had reached bud burst stage. The lower and upper threshold temperatures for peach bud development are 4.5 and 25°C, respectively. The GDHs were calculated by deducting 4.5°C from each hourly temperature between 4.5 and 25°C and all temperature over 25°C, considered to be 25°C. Consequently, the highest heat accumulation for one hour was 20.5 GDHs.

Statistical analysis

The experimental data analyzed by standard procedure of analysis of variance (ANOVA). The percentage data transformed to arcsine $\sqrt{x/100}$ before being subjected to ANOVA. The

means of each treatment were compared and separated by Duncan's multiple range test (DMRT) using SPSS version 14 for Windows.

RESULTS AND DISCUSSION

Our observations over two consecutive flowering seasons showed that the pattern of floral budburst and flowering were not different. Therefore, all data expressed as mean values of both years.

Budburst and flowering

Comparison of the timing of budburst at each chilling exposure showed the period from the onset of heating to the first bud swelling of 'KU-PP2' trees was shortened by increasing the chilling exposure. Prolonged dormancy symptoms occurred in all forcing temperature treatments applied after 250 CH. Budburst of a tree exposed for 250 and 500 CH, occurred at 21 and 15 days after onset of heating, respectively. This duration decreased to 9 days as the exposure to chilling reached 750 CH (Figure 1). Weinberger (1950) described prolonged dormancy in peach cultivars grown under mild winter conditions in North American. These symptoms occurred due to inadequate chilling, resulting in poor bud break, lack of flowering synchrony and delayed anthesis.

Moreover, this study indicated that the impact of forcing temperature on budburst is depended on the chilling exposure. The excessive forcing temperatures negatively affected the timing and percentage of budburst at 750 CH (Figure 1). Typically, dormancy and reproductive performance of temperate fruit trees are influenced not only by chilling exposure, but also by the forcing temperature, which had a significant impact on flower bud development, budburst, pollination, and fruit set (Melke, 2015). All stone fruit species are sensitive to elevated temperatures during dormancy that may delay bud break and induce abnormal flower development. The floral bud does not complete its development until late winter or even until the beginning of bud swell. Therefore, the floral bud is sensitive to high temperatures during its development (Erez, 2000). However, the high temperature accelerated budburst and enhanced bud break rate in the 250 and 500 CH treatments (Figure 1). This suggested that the higher forcing temperatures could compensate for insufficient chill exposure.

Figure 2 shows the average cumulative flowering percentage expressed in terms of heat accumulation. Insufficient chilling exposure delayed the flowering period in 'KU-PP2' in this study. The first flower of the 250 CH treatment opened at 30 days after the onset of forcing, while for 500 and 750 CH, the first flower opening occurred at 21 and 9 days after the onset of forcing. Previous studies confirm that delayed foliation is a typical symptom of insufficient winter chilling (Byrne and Bacon, 1992; Erez, 2000). Regarding budburst, the effect of forcing temperature on flowering varied with chilling exposure. For 500 and 750 CH treatments forced at 25°C, the timespan from initial bloom to full bloom occurred over a longer span than that of the trees forced at lower temperatures. In contrast, the period from initial bloom to full bloom of the 250 CH treatment was reduced by increased forcing temperature. This result indicated that the excessive forcing temperature prolonged the flowering period in the 'KU-PP2' exposed to 500 and 750 CH. Under elevated temperature conditions, the development of floral buds varied, leading to non-uniform budburst and asynchronous flowering. Craufurd and Wheeler (2009) reported that high forcing temperatures improved the flowering rate to some extent. At shorter chilling exposures, high temperature forcing improved the final rate of flower opening; the final flowering rate of a tree that was exposed to 250 CH increased from 60 to 90% when the temperature was increased from 15 to 25°C. However, high temperature forcing retarded the flowering rate in 500 and 750 CH treatments.

Figure 3 shows the different patterns of average heat accumulation during seasons 2016/2017 and 2017/2018 in each phytotron. The prolonged chilling exposure reduced the heat requirement for flowering in 'KU-PP2'. Conversely, the buds that experienced inadequate chilling, required higher forcing temperature to reach full bloom than buds that experienced adequate chilling. For example, 'KU-PP2' peach trees, which had been exposed to 750 CH, required heating of 4,000 GDH. In contrast, the heat requirement of a tree exposed to 250 CH

increased to 10,000 GDH. In essence, the duration from the onset of forcing to full bloom decreased when the chilling exposure increased. Many studies have demonstrated that the time and heat requirements for peach bud break and flowering decrease with the increase in chilling accumulation (Pawasut et al., 2004; Okie and Blackburn, 2011; Maulión et al., 2014).

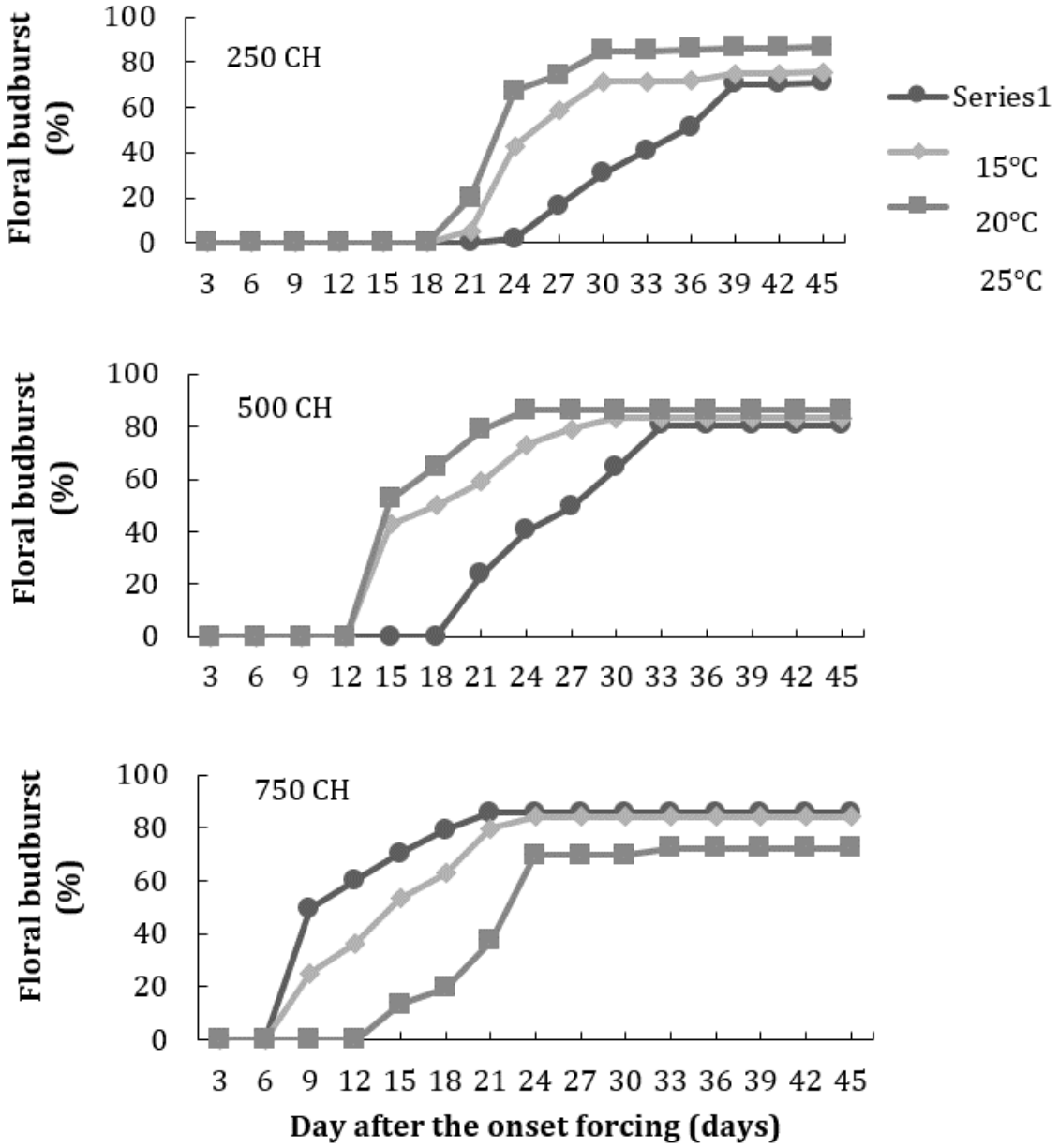

Figure 1. Effect of chilling accumulation and heating temperature on cumulative flower bud bursting of 'KU-PP2' peach trees in consecutive seasons (2016/2017 and 2017/2018). After exposure to chilling for 250, 500 and 750 CH. Trees were then exposure to different forcing temperatures (15, 20 and 25°C). Budburst was determined every three days after the onset of forcing.

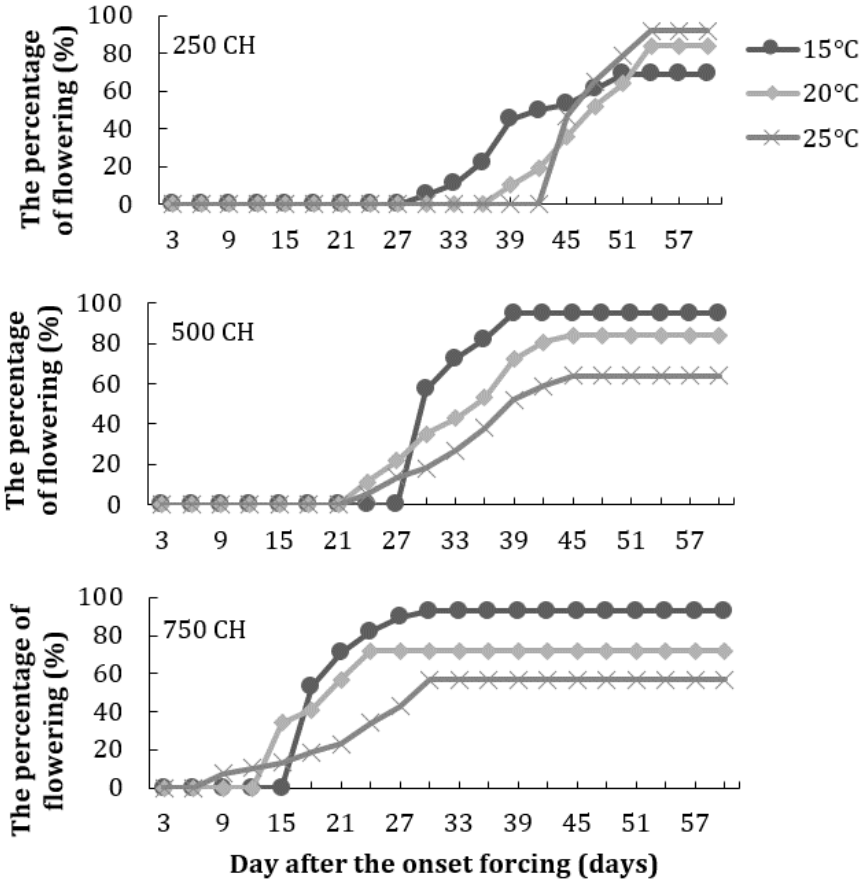

Figure 2. Effect of chilling accumulation and heating temperature on cumulative flowering of 'KU-PP2' peach trees in consecutive seasons (2016/2017 and 2017/2018). Flower opening was determined every three days after the onset of heating. The full bloom date was recorded when at least 50% of flowers had opened.

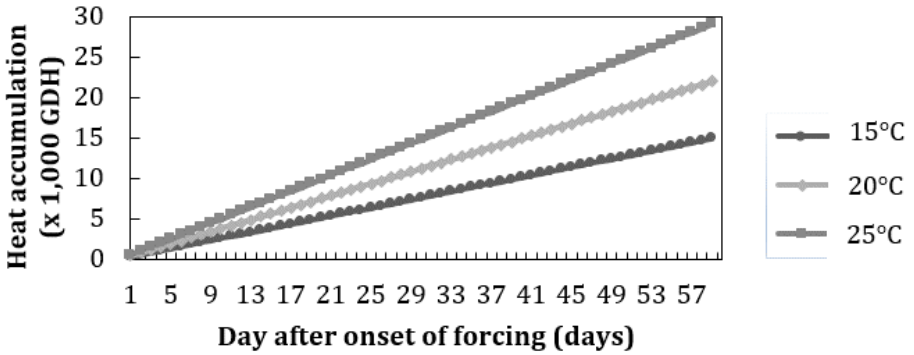

Figure 3. The average daily heat accumulation (Growing degree hours (GDH) from base temperature 4.5°C) from the onset of heating for two consecutive seasons (2016/1017 and 2017/2018). Each point represents one day.

Flower morphology, pollen germinability, and fruit set

Limitation of the chilling accumulation influenced flower quality and fruit set in 'KU-PP2' peach cultivar were reflected in less developed reproductive organs and reduced fruit

set. Compared to the mid- and high chilling exposure, the low chilling exposure (250 CH) had the smallest reproductive organs size and poor fruit set (Table 1). The average pistil length, ovary width, pollen germination, and fruit set rate of 250 CH treatments were 12.71 mm, 1.24 mm, 15.47%, and 30.3%, respectively. Similarly, the reproductive organs size, pollen germinability, and fruit set may also have been suppressed by the excessive forcing temperature. For example, as shown in Table 1, flowers that were forced at 25°C had the lowest flower weight, shortest pistil length, and smallest ovary size. Additionally, the lowest rate of pollen germination and fruit set occurred at 25°C for all chilling exposure treatments. The pollen germinability and fruit set markedly decreased from 52 to 14% when the air temperature increased from 15 to 25°C. The decrease in fruit set at high temperatures likely occurred due to the reduced flower development and underdeveloped reproductive organs that induced lower fertilization among flowers.

Table 1. Effects of chilling accumulation and heating temperature on morphological characteristics of flower, percentage of pollen germination, and fruit set in 'KU-PP2' for each treatment during two seasons (2016/2017 and 2017/2018).

Chilling hours	Forcing temperature (°C)	Flower weight (mg)	Pistil length (mm)	Ovary width (mm)	Pollen germination (%)	Fruit set (%)
250	15	333.8 a	13.34de	1.26e	18.0d	45.2c
	20	332.7a	12.97f	1.25e	16.2d	31.3d
	25	264.5h	11.83 g	1.20f	12.2e	14.4e
500	15	324.6bc	15.62a	1.60b	64.6a	56.8a
	20	319.0cd	14.07c	1.52cd	39.9b	51.2b
	25	281.5g	13.03ef	1.48d	24.3c	13.6e
750	15	315.2d	14.84b	1.67a	69.9a	52.7b
	20	304.0e	14.67b	1.58b	41.9b	54.6ab
	25	290.0f	12.91f	1.53cd	25.4c	14.0e
Chilling hour (CH)		*	*	*	*	*
Forcing temperature (FT)		*	*	*	*	*
CH×FT		*	ns	ns	ns	*

Values are indicated as means and analyzed by ANOVA, * or ns denotes the significance level at $p<0.05$ or non-significance
Different letters within the same column show a significant difference at $p<0.05$ by DMRT.

A previous study reported that the reproductive phase is one of the most sensitive stages to temperature stress (Hall, 1992). High temperature exposure before budburst accelerated anthesis but retarded reproductive organ development, resulting in premature flowering. Producing flowers with short styles and underdeveloped pollen. These flower abnormalities are possibly related to poor pollen germination and fruit set. Similarly, we found flowers that developed at an elevated temperature weighed less than those at the lower temperatures. These flowers showed less development of the pistil and ovary. Pollen germination and fruit set rate showed a decreasing trend with increasing forcing temperature. Temperature strongly influences flower development, pollen germination, and fruit set. A decrease in reproductive organ size and pollen germination because of elevated temperatures occurred in high-chilling peaches and sweet cherries (Beppu et al., 1997; Kozai et al., 2004). Excessively high temperatures significantly affect flowering, stigma receptivity, and ovule longevity, resulting in abnormal flower development and delayed or reduced fertilization (Kozlowski and Pallardy, 1997), leading to poor fruit set. Furthermore, the fruit set was reduced due to non-uniform flowering. The late bloom wave has a low chance for fruit set because of competition with stronger sinks leading to a reduced yield (Erez, 2000). However, the difference in the size of reproductive organs and fruit set among the different forcing temperatures diminished as the chilling accumulation increased.

CONCLUSIONS

Our results indicated that prolonged chilling exposure and higher forcing temperatures hastened bud burst and flowering, as well as increased the level and uniformity of bud break in the 'KU-PP2' peach cultivar. However, inadequate chilling exposure and excessive forcing temperatures negatively affected dormancy-breaking, flower development, anthesis, and fruit set in 'KU-PP2'. Budburst and flowering were significantly retarded by insufficient chilling and elevated forcing temperatures of 25-30°C used in this study. The minimum chilling requirement of the 'KU-PP2' peach cultivar was 500 CH and it required heat accumulation of 9,000 GDH for sufficient flowering. This study showed that trees exposed to excessive chilling accumulation (750 CH) required less forcing (4,000 GDH) to reach full bloom. Taken together, our findings indicate that prolonging chilling exposure and optimizing heating are crucial to accelerate bud burst and ensure normal flower development under forced cultivation, while also ensuring energy costs are reduced.

ACKNOWLEDGEMENTS

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Effects of intensity and duration of UV-B irradiation on growth and intumescence development in two tomato cultivars at the seedling stage

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Abstract

Intumescence is a physiological disorder in plants characterized by hypertrophy and, in some cases, hyperplasia. Ultraviolet B (UV-B) irradiation inhibits intumescence development; however, the degree of intumescence inhibition by UV-B irradiation may differ depending on the cultivar. In this study, we determined the effective range and duration of UV-B irradiation for intumescence inhibition in two cultivars of tomato seedlings in a closed plant production system. Seedlings of two cultivars were grown in a 128-cell tray until 21 days after sowing (DAS). Plants were exposed to either white LEDs with three levels UV-B irradiation (0, 0.5 and 1.0 W m^{-2}) using UV-B fluorescent lamps or white fluorescent lamps during the light period. In both cultivars, the intumescence area under LEDs alone treatment was the same, but those in 'Gohobi' were quite inhibited in all treatments with UV-B. In 'Rinka 409', the intumescence area was alleviated depending on the intensity of UV-B and was the smallest at 1.0 W m^{-2} among the treatments. In 'Rinka 409', total leaf area and dry weight of leaves in the LED alone treatment were the lowest among the treatments due to severe intumescence development, but no damage was observed on the leaves in all treatments in 'Gohobi'. To determine the effective duration of UV-B irradiation, half of the seedlings grown hydroponically under LEDs alone and half of those grown under UV-B (0.5 and 1.0 W m^{-2}) were exchanged between treatments on 14 DAS and grown for the next seven days under new conditions, whereas the remaining half of the seedlings were grown under the same light conditions until 21 DAS. Results showed that intumescence development was inhibited only during the UV-B irradiation period in both cultivars, and intumescences occurred on all leaves, regardless of leaf age, under light conditions without UV-B.

Keywords: closed plant production system, light emitting diodes (LEDs), edema, physiological disorder, *Solanum lycopersicum*

INTRODUCTION

Intumescence development is a physiological disorder characterized by hypertrophy and possibly hyperplasia of plant cells. Many plant species, including tomato, eggplant, potato and apple (La Rue, 1933; Craver, 2014) are susceptible to intumescence development on leaves and stems. When an intumescence occurs on leaves, the leaf area for photosynthesis decreases, leading to growth inhibition. Seedlings with small leaf areas often exhibit severe damage due to intumescence development; therefore, the development of methods for intumescence inhibition is required. However, the specific mechanisms or environmental factors that induce intumescence occurrence remain uncertain (Lang et al., 1983; Pinkard et al., 2006; Craver, 2014).

Many reports on intumescence development in tomato have aimed at elucidating the mechanisms involved and to develop prevention methods for this disorder (Atkinson, 1893; Sagi and Rylski, 1978; Lang et al., 1983; Morrow and Tibbitts, 1988). Previous reports have suggested that relative humidity, light quality (Rangarajan and Tibbitts, 1994; Eguchi et al., 2016a), light intensity, and air temperature during plant cultivation may be important factors

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in the development of this disorder. According to reports on tomato seedlings and other plants (Craver, 2014; Craver et al., 2014; Kubota et al., 2017), the intumescence disorder is severe under light emitting diodes (LEDs) compared to that under fluorescent lamps (FLs) because LEDs do not emit ultraviolet (UV) light, especially UV-B irradiation.

Recently, the light source for seedling production has been transitioned from FLs to LEDs, which have no UV-B light, increasing the problem of intumescence development in seedlings under artificial conditions (Williams et al., 2016). In addition, FLs emitting a low intensity of UV-B are being replaced with those without any UV-B. For these reasons, the problem of intumescence development is widespread in seedling production of many tomato cultivars under artificial conditions. Thus, UV-B is effective for inhibiting intumescence development (Eguchi et al., 2016b). However, the underlying mechanism and effective range of UV-B intensity and duration for prevention of intumescences in tolerant and susceptible cultivars has not been clarified.

In the present study, we aimed to identify the effective UV-B radiation intensity and irradiation duration for alleviating intumescence symptoms of tomato seedlings in two cultivars with different susceptibility to intumescence development.

MATERIALS AND METHODS

Plant material and treatment plots

All experiments were conducted in a closed plant production system. Seedlings of two tomato cultivars 'Gohobi' and 'Rinka 409' (Sakata Seed Corporation), which differ in their susceptibility to intumescence development ('Gohobi' is tolerant and 'Rinka 409' is susceptible) were cultivated using two methods, soil cultivation in a cell tray (experiment for UV-B radiation intensity) and hydroponic cultivation (experiment for UV-B irradiation period). In the cell-tray cultivation method, 64 seeds of each cultivar were sown in a 128-well cell tray filled with a commercial substrate (pH 6.0-6.5, N 50 mg L^{-1}, P_2O_5 500 mg L^{-1}, K_2O 100 mg L^{-1}) (Napura Soil Type S; Yanmar Agricultural Machinery Co., Ltd.). In hydroponic cultivation, seeds of both cultivars were sown in urethane sponges and transplanted into a container with half concentration of a standard nutrient solution (pH 6.0-6.5, EC 1.3 dS m^{-1}, N 9.3, P 2.6, K 4.3, Ca 4.1 and Mg 1.5 mg L^{-1}) (Otsuka House Fertilizer A prescription, OAT Agrio Co., Ltd.) after three days of germination at an air temperature of 26°C. All subsequent experiments were performed from three days after sowing (DAS) until 21 DAS.

In the experiment that investigated UV-B radiation intensity, tap water was used for irrigation for 0-6 DAS, and the same nutrient solution mentioned above was then irrigated from 7 to 21 DAS. One liter of the nutrient solution per tray was irrigated from the bottom of the cell tray once every two days. At 21 DAS, the seedlings reached shipment size at which stage they are commercially sold just at the point of mutual shading started.

In the experiment that investigated the UV-B irradiation period, the seedlings were grown in a hydroponic container filled with a half concentration of the standard nutrient solution, and the same solution was added once every three days. In both experiments, the environmental conditions, except for UV-B irradiation intensities among the treatments from 3 to 21 DAS, were as follows: air temperature was 25/20°C (light/dark), and the CO_2 concentration and relative humidity were 1000 μmol mol^{-1} and 70%, respectively.

Treatments

1. UV-B radiation intensity in soil culture.

In all treatments, the photosynthetic photon flux density (PPFD) was set to 225 μmol m^{-2} s^{-1} on the surface of the cell tray. White FLs (FL20SS/EX-N/18; Panasonic Corporation) and white LEDs (LDL40S/L/19/21; Panasonic Corporation) were used as the main light sources. Two levels of UV-B radiation intensity, 0.5 and 1.0 W m^{-2}, were applied along with LEDs using UV-B FLs (LEDs + 0.5 W and LEDs + 1.0 W; TL 20W/01 RS, 310 nm; Phillips Co.) (Figure 1). The UV-B radiation intensities were adjusted on the surface of cell trays. UV-B was irradiated during the light period (16 h). The growth and intumescence symptoms were

measured on three to five plants in each plot at 21 DAS, when the treatment was terminated. This experiment had three replicates.

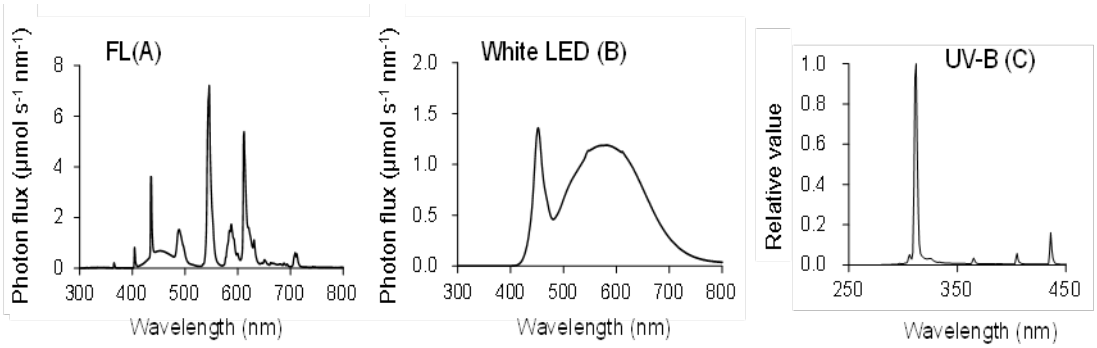

Figure 1. Photon flux distribution of white fluorescent light (FL) (A), white light emitting diode (LED) (B), and relative value of UV-B light (C). These parameters were measured using a spectroradiometer (USR-45DA, Ushio Inc.).

2. UV-B irradiation period in hydroponics.

To determine the effective duration of UV-B irradiation, the period of UV-B exposure was varied. The seedlings were grown hydroponically to ensure the same water content and leaf water potential in all plants. The intensity, including PPFD and UV-B light, was set on the surface of the cultivation panel. The same light sources and intensities of UV-B light as those used in the experiment for UV-B radiation intensity were used. Half of the seedlings were grown under LEDs alone and half of the seedlings were grown under UV-B (0.5 and 1.0 W). From within those treatment halves of the plants were interchanged with each other at 14 DAS, and grown for a further seven days until 21 DAS under the new conditions. The remaining half of the seedlings were continuously grown under the same light conditions until 21 DAS.

Data collection and intumescence area calculation

Stem length, number of leaves, total leaf area, leaf dry weight and leaf intumescence development were determined at 21 DAS. Total leaf area was calculated from a photograph of leaves using free imaging software (LIA 32 ver. 0.378). The leaf area of intumescence symptoms was scored based on the percentage of damaged leaf area on each leaf using the following scoring system: 0 – no symptoms; 1 – slight damage, several small spots; 2 – less than 25% of leaf area damaged; 3 – 25 to 50% of leaf area damaged; 4 – 50 to 75% of leaf area damaged; 5 – more than 75% of leaf area damaged (Figure 2). An integrated score was determined as intumescence area for the leaves from the cotyledon to the second leaf, and separately for those beyond the second leaf.

Statistical analysis

All data presented are mean values with standard errors. Treatment means were compared using the Tukey-Kramer test in Excel Statistical Analysis ver. 5.0 (ESUMI Co., Ltd.), and the level of significance was set at $p<0.05$.

RESULTS AND DISCUSSION

UV-B radiation intensity

1. Intumescence development in soil-cultivated seedlings.

In recent years, rapid intumescence development has been reported in various plants, including tomato, under light environments that do not include UV-B, such as lighting produced by LEDs (Craver et al., 2014; Williams et al., 2016). It is known that the degree of intumescence onset varies depending on the species or cultivar, even in the same light

environment (Lang et al., 1983). In the present study, both tomato cultivars had the same and highest intumescence score (intumescence area) in the treatment with LED alone at 21 DAS (Figure 3). However, the intumescence severity in the two cultivars differed. In 'Rinka 409', the onset of intumescence was observed earlier and the spread speed of intumescence area due to intumescence development was higher than that in 'Gohobi' (data not shown). This indicates that a scoring method in this study using only leaf area when symptoms occur is not discriminating enough to evaluate the degree of damage due to intumescence development.

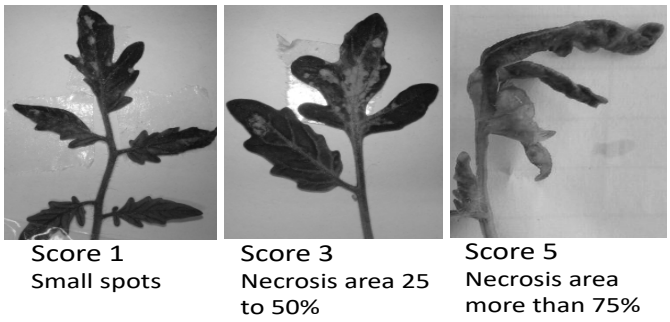

Figure 2. Typical intumescence symptoms on tomato leaves. The intumescence symptoms were scored based on the percentage of damaged leaf area on each leaf from 0 (no symptoms) to 5 (more than 75% of leaf area).

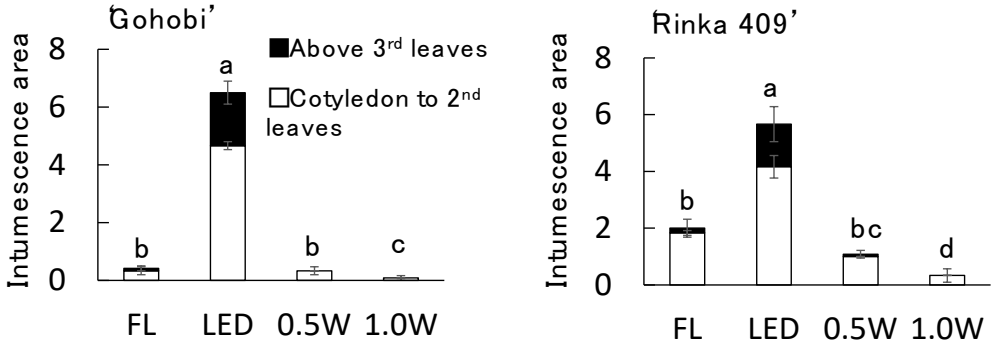

Figure 3. Effects of UV irradiation on intumescence area in tomato seedlings cultured in cell trays at 21 days after sowing. FL, fluorescent light; LED, light emitting diode; 0.5 and 1.0 W, UV-B radiation intensity of 0.5 and 1.0 W m^{-2}. The integrated score, based on the area of leaf damage as shown in Figure 2, was calculated for the leaves from the cotyledon to the second leaf, and separately for those beyond the second leaf. Vertical bars indicate standard error (n=4). Different letters indicate significance among treatments at $p<0.05$ by Tukey-Kramer's test.

In 'Gohobi', the intumescence area under FL, and under the two treatments with UV-B at either 0.5 or 1.0 W m^{-2} were much lower and equally inhibited compared with those in the LED alone treatment (Figure 3). Based on these results, it is suggested that intumescence onset and development in 'Gohobi' was inhibited under the existence or quite low intensity of UV-B, even obtained from the FL light source (where the total intensity of UV-A and UV-B was below 0.1 W m^{-2}). Our results showed, therefore, that a UV-B intensity of approximately 0.5 W m^{-2} or less was sufficient to inhibit intumescence development in the more tolerant cultivar 'Gohobi'.

Although 'Rinka 409' was more sensitive to intumescence development compared to 'Gohobi', the intumescence area in the FL treatment were also lower compared with that observed in the LED alone treatment. Moreover, UV-B at either 0.5 or 1.0 W m^{-2} showed a

significant inhibition of intumescence area compared to the FL treatment. Therefore, it is possible that the intumescence development was inhibited depending on the UV-B intensity, and UV-B intensity of approximately 1.0 W m^{-2} or more was recommended for the suppression of intumescence development in susceptible cultivars.

2. Growth at 21 DAS of soil-cultivated seedlings.

All replicates in each cultivar showed a similar trend on the growth parameters (data not shown). In both cultivars, an increase in stem length was observed in the LED alone treatment (Figure 4A, B), suggesting that UV-B irradiation slightly suppressed stem elongation. In 'Gohobi', there was no significant difference in total leaf area (Figure 4C), number of leaves (data not shown) and dry weight of leaves (Figure 4E) among the treatments, although a slight decrease in leaf area was observed under the LED alone treatment due to the intumescence development in that treatment.

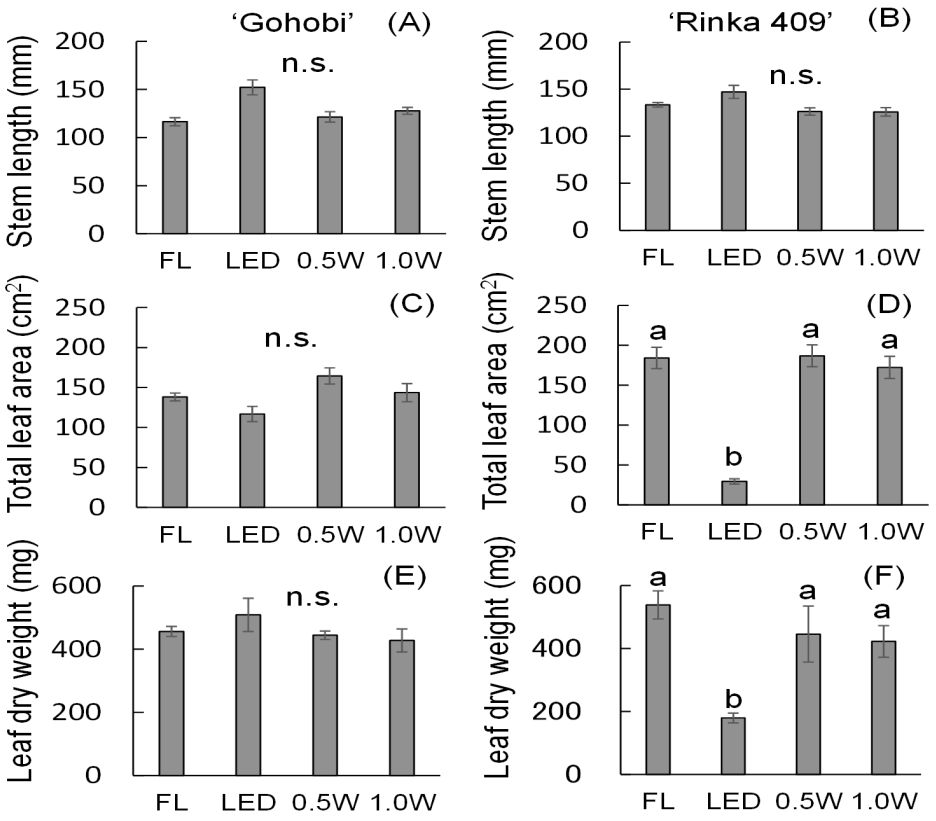

Figure 4. Effects of UV irradiation on growth of tomato seedlings grown in cell trays at 21 days after sowing. FL, fluorescent light; LED, light emitting diode; 0.5 and 1.0 W, UV-B radiation intensity of 0.5 and 1.0 W m^{-2}. Vertical bars indicate standard error (n=4). Different letters indicate significance among treatments at $p<0.05$ by Tukey-Kramer's test.

In 'Rinka 409', the total leaf area (Figure 4D), number of leaves (data not shown), and dry weight of leaves (Figure 4F) were negatively impacted in the LED alone treatment, but no significant difference was observed in stem length among the treatments. These results clearly indicate that 'Rinka 409', unlike 'Gohobi', was more susceptible to intumescence development. The marked inhibition in the growth of 'Rinka 409' under the LED alone treatment compared to that of 'Gohobi' could be due to the earlier onset of intumescence development and the subsequent severity of leaf damage that occurred (noting that the score

is related to the area of leaves affected and does not measure the severity of the impacted tissue).

Thus, the onset and development of intumescence in susceptible cultivars were early and severe, and the intumescence development inhibited photosynthesis due to the disruption of the affected leaf tissue. Therefore, we propose that UV-B irradiation of at least 0.5 W m^{-2} is required to maintain the growth rate of tomato seedlings, especially in cultivars that are susceptible to intumescence development. Based on these results, it was cleared that the treatments with UV-B irradiation in this experiment suppressed the intumescence development without any growth inhibition in both cultivars.

UV-B irradiation period

1. Intumescence development in hydroponic-cultivated seedlings.

The intumescence areas were lower in the seedlings grown hydroponically (Figure 5) than in those cultivated in cell trays filled with soil (Figure 3). Humidity conditions affect the degree of intumescence development (Atkinson, 1893; Sagi and Rylski, 1978; Lang et al., 1983), and differences in the internal water potential of the plants may explain the differences in susceptibility to intumescence development between the hydroponic and cell-tray cultures. In the hydroponic-cultivated seedlings, the effects of UV-B irradiation intensity on growth and intumescence development followed almost the same trend as that described in the experiment above which examined the effects of UV-B radiation intensity under soil culture; that is, UV-B inhibited the development of the intumescences (Figure 5). In addition, if the seedlings under light conditions which included UV-B (FL, 0.5 and 1.0 W) were moved to a LED alone treatment where the light source did not emit UV-B, the intumescences started to develop from 14 until 21 DAS in both cultivars, but especially in 'Rinka 409'.

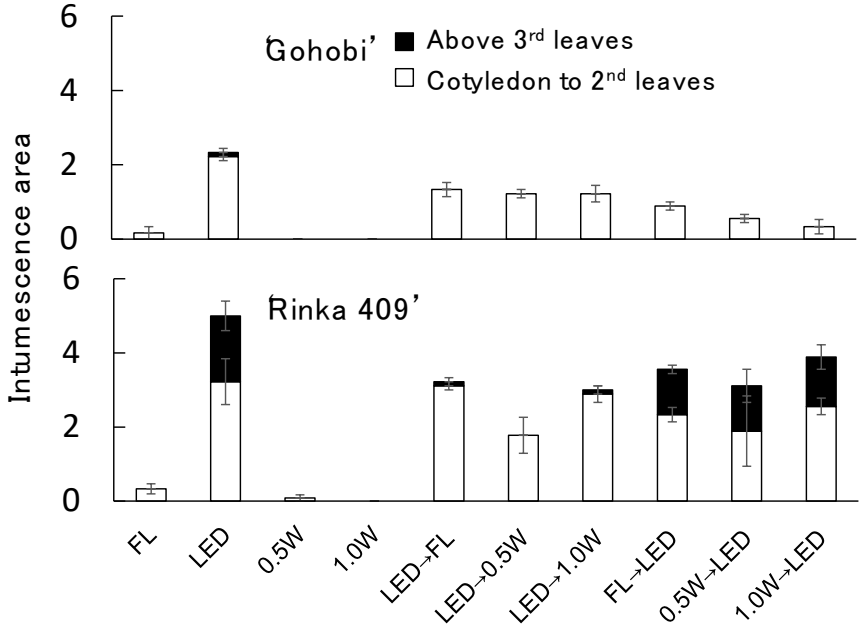

Figure 5. Effects of duration of UV-B irradiation on intumescence symptom development in tomato seedlings cultivated hydroponically at 21 days after sowing. Vertical bars indicate standard error (*n*=3-6). Half of the seedlings grown under light emitting diodes (LEDs) alone were interchanged with those grown under UV-B (0.5 and 1.0 W) at 14 days after sowing and grown for an additional seven days. The remaining half of the seedlings were continuously grown under the same light conditions until the end of the experiment.

Furthermore, growth inhibition of 'Rinka 409' was observed in the LED alone treatment and in treatments that included a period of LED irradiation (data not shown). In contrast, none of the growth parameters showed any differences among the treatments that incorporated a UV-B irradiation period in either cultivar (data not shown). These results were consistent with those obtained from the study of UV-B radiation intensity for plants under soil culture.

There was severe intumescence disorder above the third leaf in the LED alone treatment and in the treatments that exposed the plants to LED conditions from 14 until 21 DAS, in 'Rinka 409'. This suggests that the suppression of UV-B irradiation on intumescence development was lost when the UV-B irradiation was stopped, and conversely, that UV-B irradiation could suppress the spread of intumescence development even after its onset, although the existing intumescence symptoms could not be reversed. These results suggest that intumescence development was inhibited only during the UV-B irradiation period in both cultivars, and that intumescence development occurred in all leaves regardless of leaf age under the light conditions that lacked UV-B radiation.

CONCLUSIONS

The intumescence development was more severe in 'Rinka 409' as a susceptible cultivar than in 'Gohobi' as a tolerant one, but they were alleviated by UV-B irradiation. In 'Gohobi', intumescence development was inhibited under quite low intensity of UV-B, and that in 'Rinka 409' was inhibited depending on the intensity of UV-B and was the smallest at 1.0 W m^{-2} among the treatments in this study. Intumescence development was inhibited only during the UV-B irradiation period in both cultivars, and it occurred in all leaves regardless of their age under light lacking UV-B.

ACKNOWLEDGEMENTS

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The effect of different LED light on growth and anthocyanin of hydroponic red oak lettuce in a closed system

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Abstract

Light emitting diodes can potentially be a useful tool for indoor lettuce production. The aim of study is to test coloration, biomass and anthocyanin concentration in red oak lettuce grown under different LED lighting. This study will provide us with insights on how the LED light spectra can improve coloration and increase productivity. The experiment was conducted in a closed system, with light parameters, temperature, and humidity controls. Twelve lighting treatments were used: 1) green LEDs; 2) red:white LEDs (3:1 ratio); 3) red:blue LEDs (5:1 ratio); 4) red LEDs; 5) red:white LEDs (2:1 ratio); 6) red:white:blue LEDs (3:1:1 ratio); 7) blue LEDs; 8) red:white LEDs (1:1 ratio); 9) red:white:blue LEDs (1:1:1 ratio); 10) white LEDs; 11) red:white T8 type (1:1 ratio); and 12) red:blue LEDs (3:1 ratio). Significant differences were found between treatments for color ratings, growth, biomass, and anthocyanin concentration. Results showed the blue LEDs treatment provided a higher red pigment in the plants than any of the other artificial light treatments. The red LEDs treatment increased plant growth, biomass, photosynthesis efficiency and phenolic compound. The use of supplementary LEDs lighting technology could provide an alternative lighting source to improve the red oak lettuce growth in closed production systems.

Keywords: light intensity, closed system, and anthocyanin

INTRODUCTION

Vegetables are important for human health, as they provide various phytochemicals that function as antioxidants, phytoestrogens, and anti-inflammatory agents. Additionally, they are the main source of dietary fiber, which is associated with reduced incidence of cardiovascular disease, hypertension, and obesity. Lettuce is consumed worldwide because it is highly nutritious (Kim et al., 2004). Lettuce is low in fat, sodium, and calories, but high in fiber, folate, iron, vitamin C, and other bioactive compounds which are beneficial to human health (Kim et al., 2016; Eaknarin and Weeraya, 2018). In recent years, epidemiological studies show humans have an increased awareness of the benefits that phytochemicals provide for their health. Accordingly, it has been proposed that diets rich in vegetables and fruits can protect against some forms of cancer and cardiovascular diseases. Phytochemicals flavonoids and phenolic acids play an important role in acting as antioxidants. However, the antioxidant content of different plant species varies. In addition, it is also dependent on environmental stresses and their growing conditions (Pérez-López et al., 2018). Plants have developed a wide spectrum of antioxidant compounds that enhance their defense mechanisms to cope with biotic and abiotic stresses (Huang et al., 2017).

Different lights are widely used to study the effects of spectral quality on plant growth. It has been proven that enhancement of plant growth can be obtained by adjusting the spectral quality (Chen et al., 2014). The use of LED lighting is gradually increasing worldwide, due to their small size, long life span, selectable spectral composition, and cooler emitting temperatures compared with other light sources (Zhang et al., 2018). LED is a promising light

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source for use in plant physiology research in enclosed facilities. Various studies on photobiological research including chlorophyll formation, photosynthesis and morphogenesis have been done by applying LED to various plants in which fluorescent lamps are always used as the control. In addition, previous studies using LED lighting were performed without supplemental broad spectrum irradiation, focusing on the effects of red plus blue LED lighting compared to only red LED lighting or only blue LED light (Domurath et al., 2012; Jokhan et al., 2010; Chen et al., 2014).

It is well known that biosynthetic wavelengths produce plant pigments. These biosynthetic wavelengths are referred to as the action spectra, having the ability to extract maximum plant action in the red and blue ranges. Red light is important for the development of the photosynthetic apparatus, while blue light is important in the chloroplast development, chlorophyll synthesis, stomatal opening, and photo-morphogenesis. Combined red and blue LED have been proven to be highly effective for plant species production, including lettuce (Lee et al., 2007; Chen et al., 2014). In this study, the effects of light intensity and light quality on the growth and quality of hydroponically grown red oak lettuce is being studied. The effects of monochromic red or blue and mixed radiation of white LED on plant growth in a vertical plant factory of red oak lettuce hydroponically cultured is being investigated. This study is to determine the optimal light conditions for commercial lettuce production in a controlled environment with artificial lighting. Growth responses such as shoot growth, plant biomass and accumulations of anthocyanin and phenolic compound will also be investigated.

MATERIALS AND METHODS

Experimental set up and growth conditions

Seeds of red oak lettuce (*Lactuca sativa* var. *crispa* Lin.) were incubated at 4°C on moistened germination gauze for five days. The germinated seeds were sown into sponge cubes (2×2×2 cm) above the hydroponic boxes in an environmentally controlled growth room. Each hydroponic box (150×30×20 cm) contained seven plants spaced 15 cm apart. All plants were grown hydroponically using modified half-strength Hoagland's solution (Hoagland and Arnon, 1950). The nutrient solution was renewed weekly, with the electrical conductivity and pH of the nutrient solution controlled at 0.8-1.5 mS cm^{-1} and 6.5-7.5, respectively. Air temperature, CO_2 level, and relative humidity (RH), were maintained at 25/23°C (day/night), 450-500 ppm and 60%, respectively.

Light quality treatments

This experiment was conducted by using a completely randomized design with 3 replications. Treatments in the study consisted 12 light treatments: 1) white LEDs; 2) red LEDs; 3) blue LEDs; 4) green LEDs; 5) red:white, T8 type (1:1 ratio); 6) red:white LEDs (1:1 ratio); 7) red:white LEDs (2:1 ratio); 8) red:white LEDs (3:1 ratio); 9) red:blue LEDs (3:1 ratio); 10) red:blue LEDs (5:1 ratio); 11) red:white:blue LEDs (1:1:1 ratio); and 12) red:white:blue LEDs (3:1:1 ratio). All LEDs provided by the Lighting and Equipment Manufacturing Public Company Limited, Thailand.

Measurements of plant growth and morphology

Plant height was measured weekly while other bio-metric measurements; fresh weight (FW) and dry weight (DW) of shoots and roots, plant diameter, stem length and leaf number were collected at 42 DAS. Plant samples were oven-dried at 80°C for 48 h to determine dry weight.

Total anthocyanin contents

Total anthocyanin contents were determined according to the method described by Giusti and Wrolstad (2001). Diluted plant extract, 0.2 mL was prepared and placed into two small test tubes. In the first tube, 2.8 mL of KCl solution (pH=1.0) was added. In the second tube 2.8 mL sodium acetate solution (pH=4.5) was added. The solutions in each test tube were mixed by a vortex for 10 s. The samples were incubated in the dark at a temperature of 25°C

for 30 min. The light absorbance was measured by a spectrophotometer at the wavelength of 510 nm for the first solution (pH 1.0) and at 700 nm for the second one (pH 4.5). The calculation of total anthocyanin contents was calculated based on the equation as below (Mezadri et al., 2008) and expressed as mg 100 g^{-1} FW.

$$\text{Concentration anthocyanin} = A = (A510\text{-}A700)\ pH{=}1.0 - (A510\text{-}A700)\ pH{=}4.5$$

$$\text{Monomeric anthocyanin pigment} = (A \times MW \times DE \times 100)/(\varepsilon)$$

where MW = 499.2 mL of cyanidin-3-glucoside, DE = 1.5 dilution factors, ε = 26900 molar absorptivity.

Statistical analysis

The data analyzed using Statistix 10 program. Analysis of variance performed and mean comparision calculated using LSD (least square difference) method at a confidence level of 95%.

RESULTS AND DISCUSSION

Plant morphology

At harvest time (42 DAS), shoot growth under monochromic red light was fragile due to excessive elongation. Plant height was restrained under blue light which was the lowest for all the light sources (Table 1). Large-sized and compact seedlings with dark red leaves were detected under blue, red:blue and red:blue:white LED lights while seedlings with stem elongation and light green leaves were obtained under monochromic green light (Figure 1). Furthermore, seedlings under monochromatic green light looked weak and creeping, while those under monochromatic blue appeared dwarfed and small. Normal plant appearance was observed under W, R:W (1:1, 2:1 and 3:1), R:W (3:1 and 5:1), R:B:W (1:1:1 and 3:1:1) (Figure 1). These results were consistent with Lapjit et al. (2019) who reported that plants growing under supplemental white: red light appeared bloomy and vigorous. Supplemental LED lights induced morphological changes. For instance, plants under the white and red light were compact and vigorous while those under fluorescent light looked sparse.

Table 1. Influence of different light spectra combination on plant height, canopy diameter, leaf width, leaf length and number of leaves per plant at harvest maturity (42 DAS) of red oak lettuce grown under various light treatments.

Light treatments	Plant height (cm)	Canopy width (cm)	Leaf width (cm)	Leaf length (cm)	Leaf number
White (W)	12.58hi	22.04e	8.56d	9.80d	16.00ef
Red (R)	17.60ab	32.16a	13.60a	14.18b	24.20ab
Blue (B)	8.76j	15.25f	5.96e	7.02f	14.02fg
Green (G)	18.08a	31.10ab	9.28d	16.20a	12.60 g
R:W (1:1) (T8)	15.86cd	28.26c	8.20d	12.36c	13.40 g
R:W (1:1)	15.52de	28.36c	12.16b	12.02c	24.60a
R:W (2:1)	16.76bc	30.15bc	11.70bc	12.24c	23.00ab
R:W (3:1)	16.88bc	29.89bc	11.62bc	11.90c	24.20ab
R:B (3:1)	13.36 gh	24.04d	8.32d	9.44de	20.00cd
R:B (5:1)	14.52ef	25.13d	10.64c	11.74c	20.02bc
R:B:W (1:1:1)	12.04i	21.72e	8.00d	8.54e	17.80de
R:B:W (3:1:1)	13.94fg	25.36d	9.16d	10.38d	20.00cd
F-test	**	**	**	**	**
CV (%)	5.56	5.88	10.72	7.97	8.98

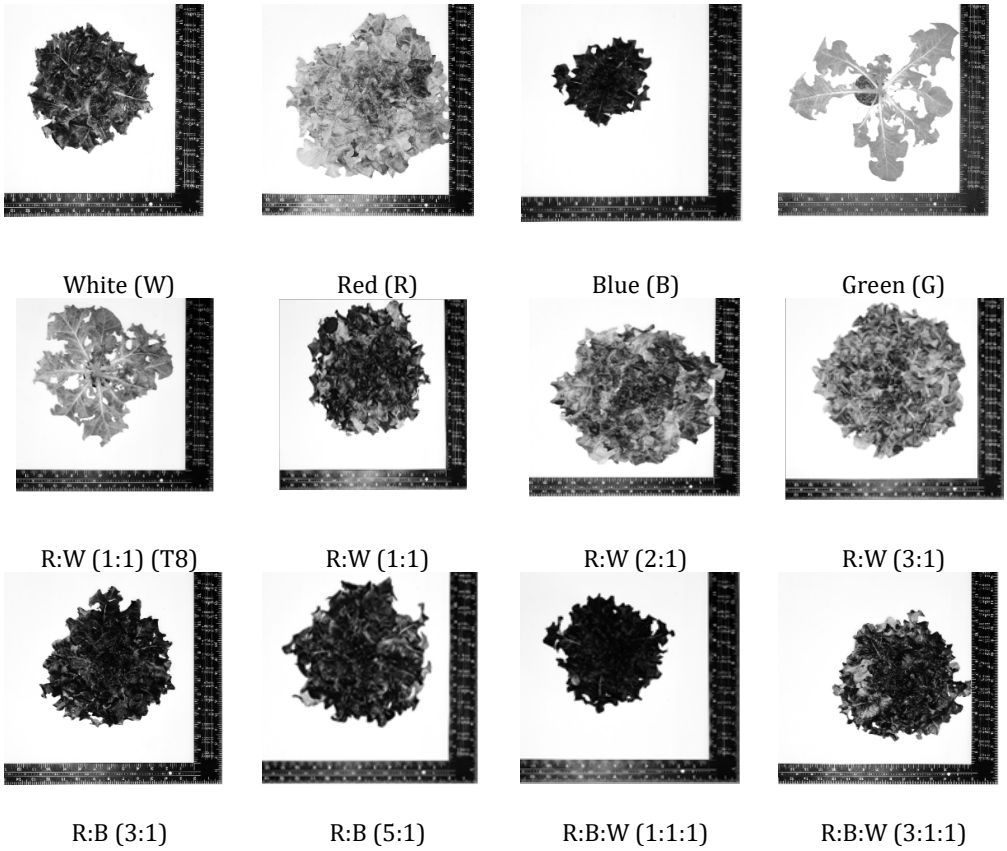

Figure 1. Morphology and growth under different light spectrum treatments of red oak lettuce harvested after 42 DAS.

The leaf is the most important photosynthetic plant organ, the greater plant height, plant diameter, leaf number in the red:white treatments reflected red light stimulated photosynthesis (Lapjit et al., 2019). It has been reported that red and blue light spectra strongly shape plant morphology and red light resulted in elongation of hypocotyls and leaf blades and increase of leaf number (Jokhan et al., 2010; Chen et al., 2014). Lettuce has very short stem, therefore the hypocotyl is generally considered as stem. In this study, plants under monochromatic red appeared to have long stems and tortuous leaves while lettuce leaves with radiation of monochromatic blue light looked upright and flat.

Plant fresh and dry weight

In this study, at harvest time (42 DAS), plants had a greater fresh weight and dry weight of shoot and root, 131.08, 14.56, 5.63 and 0.63 g, respectively in the red light than compared all the other lighting. However, no differences were found in fresh weight of root for R:W (1:1) and R:W (2:1) light treatments. In addition, no difference in dry weight of root from R:W (1:1), R:W (2:1) R:W (3:1) and R:B (5:1) light treatments (Table 2). These results support Lapjit et al. (2019) findings, who reported that supplemental red and white LEDs promoted biomass accumulation. Lin et al. (2013) showed that greater lettuce biomass was obtained under red:blue:white (1:1:1) light treatment than under fluorescent light. This suggests that red or blue light responses might also depend on the remaining spectral composition, and the light effect differed when other parts of the spectrum varied (Lapjit et al., 2019).

Photosynthetic efficiency (Fv/Fm)

There was no difference in photosynthetic rates for all light treatments (Table 2). In contrast, Frąszczak et al. (2014) found that basil grown under LEDs with a light intensity of

approximately 172 µmol m^{-2} s^{-1} had the highest rate of quantum yield.

Table 2. Fresh mass and dry mass harvested at 42 DAS and photosynthetic efficiency of red oak lettuce growing under various light spectrum treatments.

Light treatments	Fresh weight (g)		Dry weight (g)		Fv/Fm
	Shoot	Root	Shoot	Root	
White (W)	29.76e	5.42d	1.55ef	0.27d	0.828
Red (R)	131.08a	14.56a	5.63a	0.63a	0.825
Blue (B)	11.05e	3.20e	0.72 g	0.15e	0.837
Green (G)	12.36e	2.23e	0.68 g	0.12e	0.822
R:W (1:1) (T8)	16.02e	2.53e	0.79fg	0.14e	0.759
R:W (1:1)	82.64bc	12.53ab	4.11bc	0.60a	0.825
R:W (2:1)	98.88b	12.80ab	4.55b	0.57ab	0.833
R:W (3:1)	91.03b	11.27bc	4.35b	0.54a-c	0.827
R:B (3:1)	57.13d	9.62c	3.03d	0.50bc	0.821
R:B (5:1)	60.17d	11.58bc	3.33cd	0.54a-c	0.826
R:B:W (1:1:1)	29.63e	6.28d	1.78e	0.30d	0.833
R:B:W (3:1:1)	64.91cd	9.81c	3.45cd	0.46c	0.834
F-test	**	**	**	**	ns
CV (%)	20.36	14.96	17.17	13.85	3.63

Means followed by different letters within the same columns were significantly different tested by LSD's multiple comparison at p≤0.05.
ns = not significant, ** significant at p≤0.01.

Chlorophyll a and chlorophyll b

This study found the light treatments of R, G, R:W (1:1) and R:B (3:1) had no significant difference in the content of chlorophyll a and chlorophyll b for red oak lettuce (Figure 2). The results show the combined monochromatic R and G light only is not different with R:W (1:1) and R:B (3:1). However, its effectiveness was greater than the monochromatic white and blue light only. Even though the R light is more effective in improving photosynthesis compared than B or G light, the red light alone caused elongated hypocotyls and cotyledons (Meas et al., 2020). Meas et al. (2020) found blue light increased the ratio between chlorophyll a and b, which affected plant photomorphogenesis, promoting stomata opening in amaranths species namely; *Amaranthus cruentus* L. (red amaranth) and *Amaranthus gangeticus* L. (leafy vegetable amaranth). The combination of R and B LED lighting resulted in higher photosynthetic activity than monochromatic R or Blight light only. As a result, it produced an improved excitation of photoreceptors including phytochromes, cryptochromes and phototropins, which resulted in higher photosynthetic activity as reported by Sabzalian et al. (2014).

Phytochemical content

Results show light treatment impacted phytochemical content of different compounds (Figure 3). A significant increase of anthocyanin content was observed in red oak lettuce. The highest anthocyanin content was observed under R:B:W (3:1:1) followed by R:B (5:1), R:B (3:1), R:W (3:1) and R:W (1:1) light (Figure 3A). Under R:B:W (3:1:1) light, the red oak lettuce had significantly greater anthocyanin content than the other LED light treatments (Figure 2A). This result agrees with research conducted by Jokhan et al. (2010), who found that anthocyanin of 17-day lettuce seedlings illuminated under combined of red plus blue light spectrum was greater than that illuminated with monochromatic red, blue LED light and fluorescent light alone. The total phenolic content of lettuce was 190-198 (mg 100 g^{-1} FW) and highest values were found in the monochromatic red and green light only (Figure 3B). Studies on basil growth under four colors of light by Shiga et al. (2009), found that blue light and red light produced the highest phenolic content at 14 days This is different to this study's findings,

where red and green light produced the highest amounts of phenolic compounds. The amount of phenolics is minimal in plants grown under UV-B. White light generally stimulates a high accumulation of phenolics which is different to our findings. Overall, the effect of artificial lighting on antioxidants and growth for basil is consistent with the findings of Januthai et al. (2019) and this study. As recently found and with our study for lettuce, environmental stresses moderate the intensity and can induce accumulation of phenolic compounds as well as an enhancement of antioxidant capacity. These findings were consistent with previous studies that showed that mild light stress could induce the synthesis of phenolic compounds with antioxidant function in lettuce (Pérez-López et al., 2018).

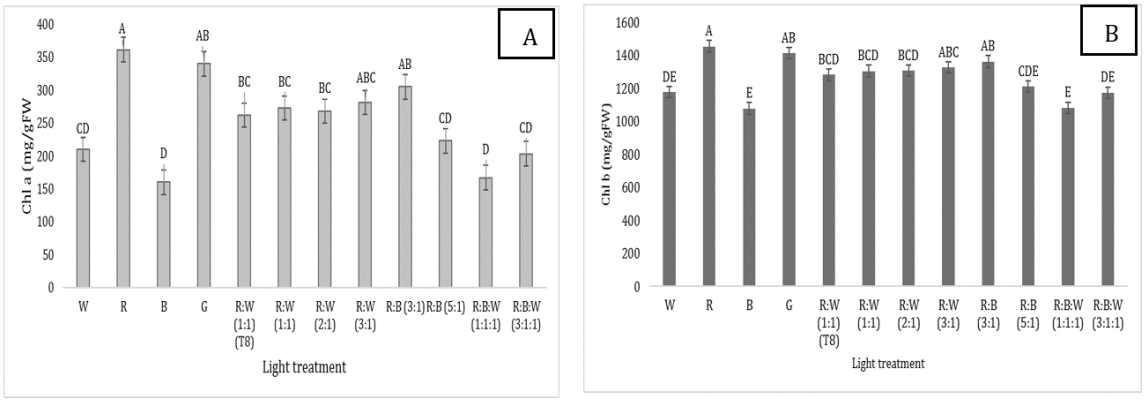

Figure 2. Chlorophyll a (A), and chlorophyll b (B)of red oak lettuce as affected by different light spectra.

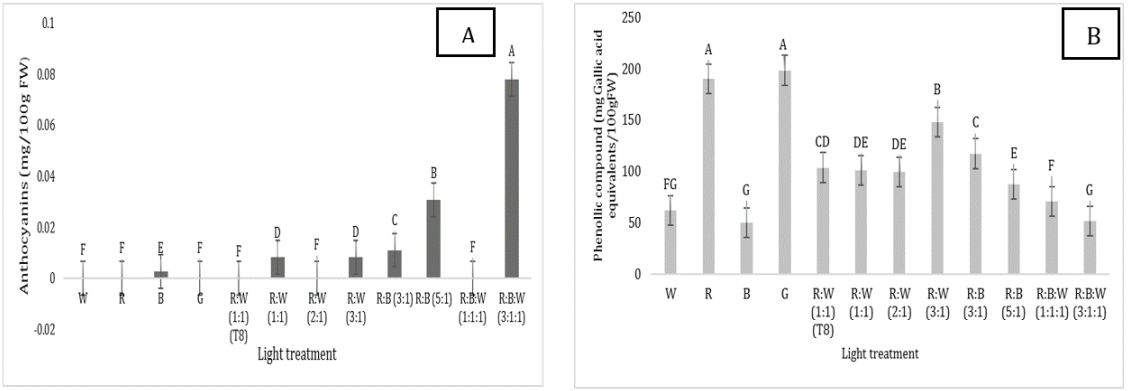

Figure 3. Anthocyanin (A) and phenolic compound (B) as affected by different light spectra.

CONCLUSIONS

The objective of this study was to investigate the response of red oak lettuce to different of light spectra. It can be concluded that the blue LED treatments provided a higher red pigment than the other artificial lighting. The lettuce plants, in the red LED treatments showed increased plant growth, biomass and phenolic compound. The supplementary LED lighting technology could provide to be an alternative lighting source, improving red oak lettuce growth in a closed system in a vertical plant factory.

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Effect of blue LED light irradiation on anthocyanin synthesis in the skin of detached apples

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Abstract

This study aimed to improve the marketability of poorly colored apples by increasing the anthocyanin concentration in the skin of fruit using blue LED light irradiation. Apple samples were collected at commercial harvest time from Morioka, a cold region in Japan. To determine the suitable temperatures for anthocyanin synthesis in the skin of apples, fruit were irradiated with blue LED lights in incubators at 11, 15 and 20°C for four days. For 'Tsugaru' and 'Shinano Sweet' apples, more anthocyanin accumulated under higher temperature conditions. Whereas for 'Jonagold' and 'Fuji' apples, anthocyanin levels were similar for all three temperature conditions. Therefore, in terms of improving apple pigmentation and maintaining firmness, irradiation at 15°C is preferred. The irradiation equipment used was adjusted to 15°C through a vent system when placed in the refrigerator. The 1-methylcyclopropene (1-MCP) treatment of 'Shinano Sweet', 'Jonagold' and 'Fuji' apples did not influence anthocyanin accumulation or fruit firmness. The skin of the apples after irradiation at 15°C for four days with blue LED light showed improved fruit skin pigmentation. However, it did not affect fruit firmness regardless of 1-MCP treatment. Blue LED light irradiation was effective for anthocyanin accumulation in 'Fuji' apples harvested from different production areas (cold and warm regions) in Japan.

Keywords: anthocyanin, apple, blue LED light, fruit firmness, 1-methylcyclopropene

INTRODUCTION

Anthocyanin is a flavonoid compound responsible for the red color in the skin of apples. The intensity of the red skin color is an important factor to enhance the commercial value of apple fruit in Japan. The redder the fruit, the higher the marketability. Anthocyanin synthesis is influenced, not only by genetic factors, but also by environmental factors including light and temperature (Honda and Moriya, 2018). High temperature conditions in the field at harvest time suppress anthocyanin accumulation in the skin of apples (Lin-Wang et al., 2011). Recently, poor red coloration has become a serious problem in the commercial apple industry owing to global warming (Iglesias et al., 2016).

In addition to red fruit coloration, fruit firmness is an important fruit attribute in terms of marketability. Fruit storage at lower temperatures extends the shelf life of apple fruit by maintaining fruit firmness. The application of 1-methylcyclopropene (1-MCP) to apple fruit is effective in maintaining fruit firmness during storage (Tatsuki et al., 2011). Therefore, the utilization of 1-MCP on apples by producers and distributors is expected to expand.

Previous studies have shown that irradiation with white and UV-B (emission peak of 320 nm) light is effective in increasing anthocyanin accumulation in the skin of detached apples (Arakawa 1991; Honda et al., 2014). Azuma et al. (2019) reported that for grape berries, white and UV-B light, or blue LED light (peak wavelength of 445 nm) irradiation after harvest promoted anthocyanin synthesis and the expression levels of anthocyanin synthesis-related genes. Whereas the effects of irradiation varied among grape cultivars. In the current study, investigation into the conditions required to promote anthocyanin synthesis under blue LED light irradiation in the skin of apples after harvest while trying to maintain fruit firmness, was

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undertaken.

MATERIALS AND METHODS

Plant materials

Apple (*Malus* × *domestica*) fruit samples with poor coloration were collected at commercial harvest times from mature trees at various locations across Japan (Figure 1A). Fruit of 'Tsugaru,' 'Shinano Sweet', 'Jonathan' and 'Fuji' apples were harvested at Morioka city (Iwate Prefecture, Apple Research Station, Institute of Fruit Tree and Tea Science, NARO, Japan). The 'Fuji' apples harvested at Morioka are of the original type. The 'Fuji' fruit harvested at Kuroishi city (Aomori Prefecture, Japan), Fukushima city (Fukushima Prefecture, Japan) and Yamaguchi city (Yamaguchi Prefecture, Japan) were purchased from apple producers. The effects of light irradiation on apples from different regions were examined in three separate experiments in 2017, 2018 and 2019. The first experiment consisted of four apple cultivars; early season-ripening cultivar ('Tsugaru'); mid-season-ripening cultivars ('Shinano Sweet' and 'Jonathan') and late season-ripening cultivar ('Fuji') harvested from Morioka in 2017. In 2018, the second experiment was carried out on two apple cultivars 'Shinano Sweet' and 'Fuji' harvested from Morioka. In 2019, the third experiment was carried out on the cultivar 'Fuji' harvested across the Japanese apple production regions of Kuroishi, Morioka and Fukushima, in northern Japan and Yamaguchi in southern Japan.

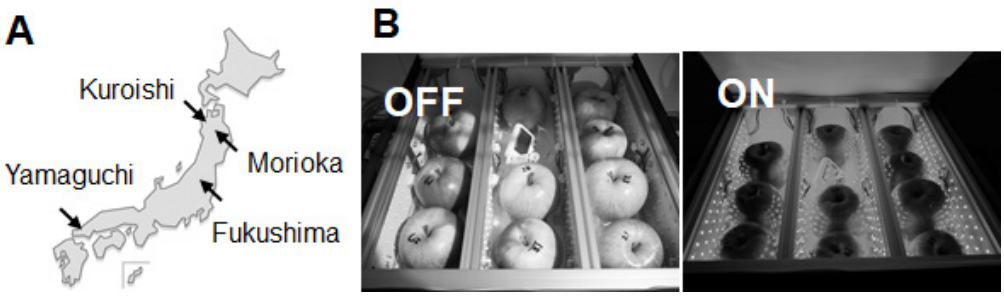

Figure 1. Location map of apple production in Japan (A) and blue LED light irradiation equipment (B). In B, 'Shinano Sweet' apples are placed in the irradiation equipment and the top is opened for the purpose of taking a photograph of the equipment.

Blue LED light irradiation

All fruit were stored at 1°C until the start of irradiation treatment. Control fruit samples were kept at 1°C in dark condition until assessment. Irradiation with blue LED light (peak wavelength, 445 nm; 20 W m^{-2}) started three days after harvest (DAH). In the experiment on temperature effect, the blue LED light was irradiated on one surface of the fruit where no anthocyanin had accumulated. The blue LED light sources were aligned in a grid pattern and electric power was supplied by a DC power source. Irradiation occurred in an incubator. Temperatures in the incubator were maintained at 11, 15 and 20°C for four days. After incubation, the anthocyanin concentration in the irradiated skin of the fruit was measured according to the method described by Ban et al. (2007).

To analyze the effect of 1-MCP application and compare the effect of irradiation on fruit from different production locations, fruit were irradiated with blue LED light (20 W m^{-2}) on the entire fruit surfaces using the equipment as shown in Figure 1B. The equipment was set up in a refrigerator (≈5°C) and the temperatures in the equipment were maintained at 15°C by adjusting the dimensions of the air holes. After 4-5 days of incubation, fruit were removed and placed in refrigerated storage at 1°C overnight until the fruit quality assessments were performed.

1-MCP application

Fruit harvested at Morioka in 2017 were treated with 1-MCP (Smart Fresh™, Agro Fresh Inc., Collegeville, PA) according to Honda et al. (2014). The timeline from harvest to fruit quality assessment is shown in Figure 2. Control (CK) fruit without irradiation were kept at 1°C until fruit quality assessment.

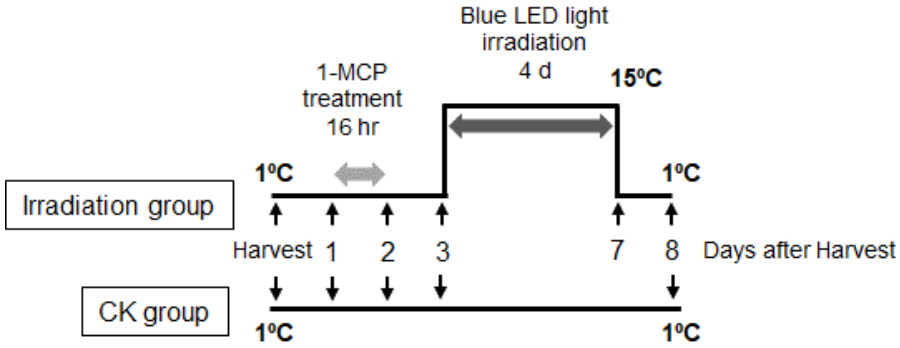

Figure 2. Timeline of 1-MCP application and blue LED light irradiation experiment conducted in 2017. The fruit were treated with 1-MCP at one day after harvest (DAH) and light irradiation started 3 DAH for four days.

Fruit quality assessment

The fruit quality attributes were assessed according to Honda et al. (2017). The starch index was estimated on a scale of 0-5 (0 = completely white, not stained with an iodine solution to 5 = completely black, fully stained). Titration acidity was expressed as g malic acid 100 mL^{-1} juice. The red color intensity index of the fruit was estimated on a scale of 0-5 (0 = no red coloration to 5 = intense red coloration). The color percentage on the surface of each fruit sampled was scored on a scale of 0-100% (0% = no red coloration to 100% = complete red coloration). The estimation was performed before and after irradiation.

Statistical analysis

The red coloration data (anthocyanin concentration) presented in Figure 3 and the fruit quality data in Table 1, were statistically analyzed using Tukey's test. The data on the red color intensity index and the color percentage of the total fruit surface area presented in Figures 4 and 5 were statistically analyzed using a t-test.

RESULTS AND DISCUSSION

Effect of temperature on apples under blue LED light irradiation

Lower temperatures during irradiation are preferred because storage at higher temperatures decreases fruit firmness. The blue LED light was irradiated on one surface of the fruit where no anthocyanin was accumulated. After light irradiation, anthocyanin was synthesized at all three temperature treatments in the four cultivars (Figure 3). The anthocyanin concentration was the highest in the fruit skin of 'Tsugaru' and 'Shinano Sweet' apples after irradiation at 20°C, whereas in 'Jonagold' and 'Fuji' apples it was not influenced by temperature. These results indicate that lower temperature conditions (11°C) did not necessarily induce anthocyanin accumulation in the skin of apple fruit. In 20°C treatment, fruit firmness substantially decreased (data not shown), although the red pigmentation improved in 'Tsugaru' and 'Shinano Sweet' apples. Therefore, the 15°C treatment was preferable in terms of improving skin pigmentation and maintaining fruit firmness.

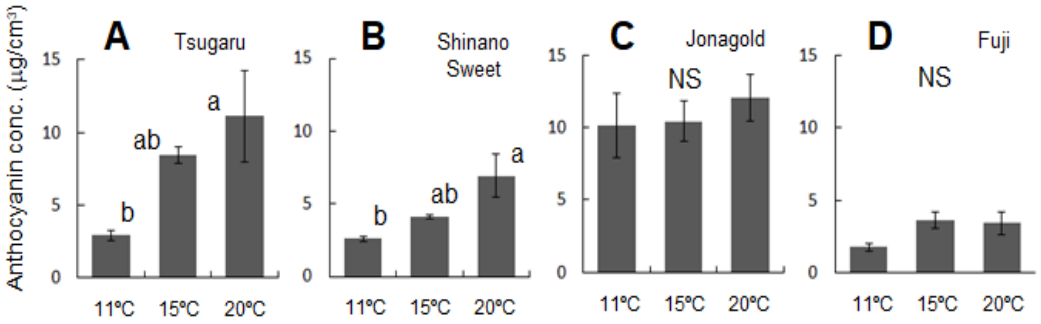

Figure 3. Effect of blue LED light irradiation in combination with low temperature treatment on anthocyanin concentrations in apple fruit skin. Fruit (n=3) were irradiated with blue LED light for four days. 'Tsugaru' (A), 'Shinano Sweet' (B), 'Jonagold' (C) and 'Fuji' (D). Apples were collected from the commercial harvest in 2017. NS letters indicate no significant difference while letters a and b indicate significant differences at $p<0.05$, respectively, as determined by Tukey's test.

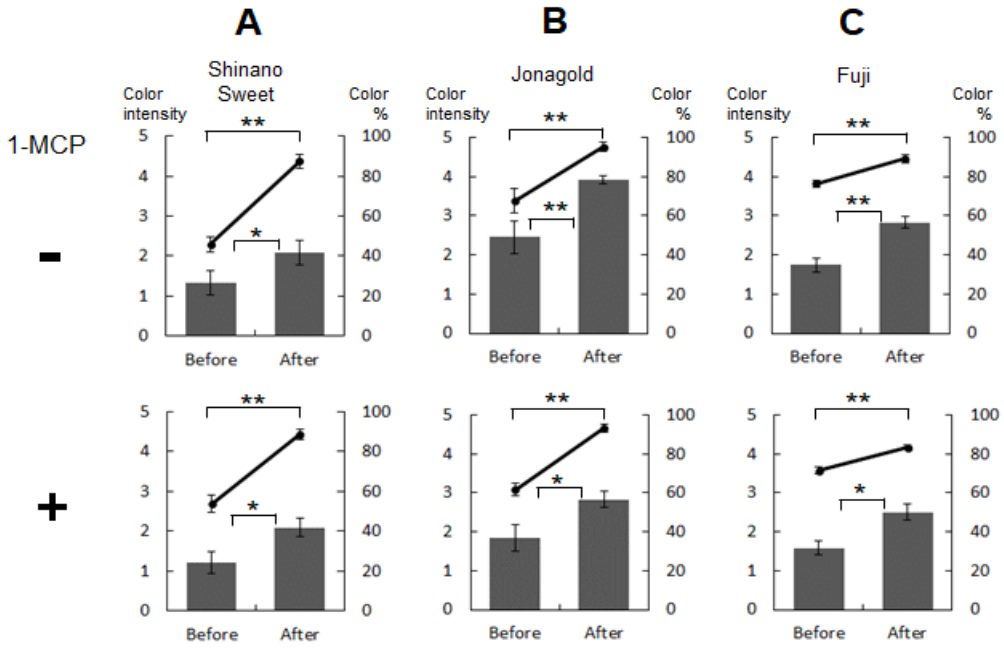

Figure 4. Effect of 1-MCP application on red pigmentation in the apple fruit skin. Fruit (n=6) were irradiated with blue LED light at 15°C for four days. 'Shinano Sweet' (A), 'Jonagold' (B) and 'Fuji' (C). Fruit were collected at the commercial harvest in 2017. The red color intensity index of the fruit is shown on the left axis (column) and the color percentage of fruit is shown on the right axis (line). * and ** indicate significant differences at $p<0.05$ and $p<0.01$, respectively, as determined by the t-test.

Effect of 1-MCP application on anthocyanin synthesis

The application of 1-MCP to harvested apples is effective for maintaining fruit firmness (Jung and Lee, 2009; Tatsuki et al., 2011). The response of anthocyanin synthesis in the skin of 1-MCP treated apples varied among some early ripening cultivars (Honda et al., 2014). As 'Tsugaru' is an early ripening cultivar and does not have a long shelf life, it was omitted from the 1-MCP application test.

Table 1. Effect of blue LED light irradiation on fruit quality at 15°C for four days.

Cultivar	Group	1-MCP treatment	Blue LED light irradiation	Fruit weight (g)	Fruit firmness (lbs)	Starch index	Soluble solid content (°Brix)	Titration acidity
Shinano Sweet	CK	-	-	279.1	11.9	2.8	14.8	0.414
	Irradiation	-	+	278.7	12.1	1.9	14.9	0.391
		+	+	294.7	11.3	2.1	14.8	0.391
Jonagold	CK	-	-	358.2	13.9	1.1	13.8	0.711a
	Irradiation	-	+	346.5	13.2	0.8	14.1	0.621b
		+	+	357.3	14.3	0.8	13.9	0.673ab
Fuji	CK	-	-	315.1	13.8	1.6	14.6	0.439a
	Irradiation	-	+	335.5	13.8	1.2	14.6	0.379b
		+	+	337.2	13.5	0.8	14.1	0.381b

Different letters indicate significant differences at $p<0.05$, as determined by Tukey's test.
CK = control fruit (not irradiated).

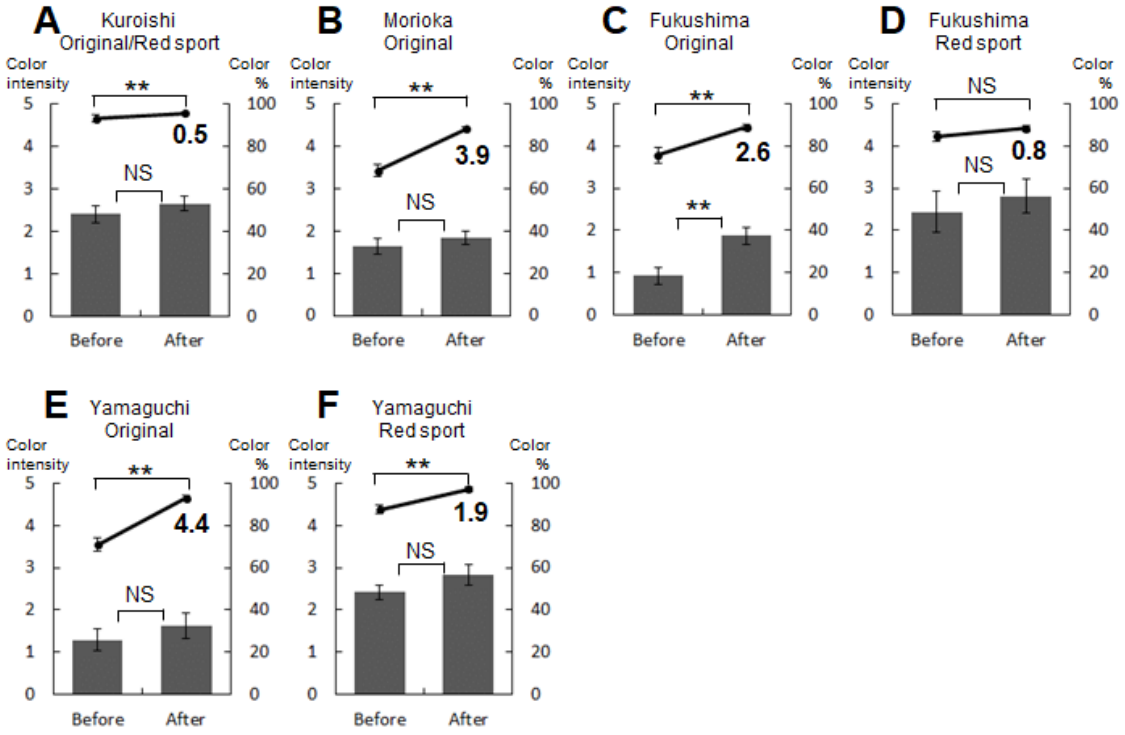

Figure 5. Comparing the effect of blue LED light irradiation on red pigmentation in 'Fuji' apples from different production locations. Fruit were irradiated with blue LED light at 15°C for five days in 2019. Kuroishi (A), Morioka (B), Fukushima (C and D) and Yamaguchi (E and F). The red color intensity index of the fruit is shown on the left axis (column) and the color percentage of the fruit is shown on the right axis (line). Bold numbers are the increments in the color percentage per day. NS, * and ** indicate no significant or significant differences at $p<0.05$ and $p<0.01$, respectively, as determined by the t-test.

After application of 1-MCP the effect of irradiation on anthocyanin synthesis in apples harvested at Morioka in 2017 showed an improvement in red pigmentation for red color

intensity index and percentage coloring (Figure 4). Both scores increased after blue LED light irradiation at 15°C for four days in the three apple cultivars ('Shinano Sweet', 'Jonathan' and 'Fuji') regardless of 1-MCP application. The fruit attributes were compared to those of the control (CK) fruit, which were not irradiated and stored at 1°C until the fruit quality assessments were undertaken (Table 1). In the three cultivars, the fruit firmness in the irradiated fruit, with or without 1-MCP treatment, was not different from that in the CK fruit. This indicates that irradiation with blue LED light at 15°C for four days improved fruit skin pigmentation but did not affect fruit firmness.

The studies found that irradiation did not influence fruit attributes except for titration acidity, which decreased in 'Jonathan' and 'Fuji' apples. It is considered that inhibition of ethylene signal transduction by 1-MCP treatment is less effective for maintaining titration acidity than fruit firmness in apple fruit. In 2018, it was confirmed that irradiation at 15°C for five days did not decrease fruit firmness in 'Shinano Sweet' and 'Fuji' apples with or without 1-MCP application (data not shown).

Comparison of the effect of light irradiation among apple production locations

Kuroishi and Morioka are the main apple production regions in northern Japan, whereas Fukushima is the main production region in southern Japan (Figure 1A). Yamaguchi has a small apple production area in the warm southwest region of Japan. 'Fuji' apple is the most popular cultivar in Japan and the effect of blue LED light irradiation on pigmentation was compared in 2019 using apples harvested at Kuroishi, Morioka, Fukushima and Yamaguchi. The harvest day and the start day of irradiation at each location are shown in Table 2.

Table 2. Apple harvest day and irradiation start day at each location in 2019.

Location	Harvest day	Start day of irradiation	Coloration type	Fruit number
Kuroishi	November 6	November 26	Original/red sport	10
Morioka	November 6	November 13	Original	6
Fukushima	November 21	December 4	Original	8
			Red sport	8
Yamaguchi	November 26/27	December 4	Original	12
			Red sport	12

The original 'Fuji' apple type is less red than the red sport type of 'Fuji' apple. In fact, the intensity scores of the 'Fuji' red sports were higher than those of the original 'Fuji' type harvested at Fukushima and Yamaguchi (Figure 5C, D, E, F). Apples harvested at Kuroishi were a mixture of the original type and red sports and the red intensity indices did not increase, while the color percentages increased to a small extent (Figure 5A). In the original type of 'Fuji' apple harvested at Morioka, the red intensity indices did not increase, whereas the color percentages increased significantly (Figure 5B). At Fukushima, both the red intensity indices and color percentages increased in the original type, whereas both indices did not increase in the red sport type (Figure 5C, D). At Yamaguchi, the red intensity indices did not increase, whereas the color percentages increased in both apple types (Figure 5E, F). Blue LED light irradiation was effective at increasing anthocyanin synthesis in the skin in 'Fuji' apples from all areas tested in this study.

The increments in the percentage coloring in the original type of 'Fuji' apples from Morioka, Fukushima and Yamaguchi were 3.9, 2.6 and 4.4, respectively (Figure 5). Whereas those in the red sports from Fukushima and Yamaguchi were 0.8 and 1.9, respectively (Figure 5). The increments in the original apples were larger than those in the red sports. This indicates the possibility that the effect on the improvement in the percentage coloring in the original type of 'Fuji' apples is more apparent than that in the red sports and irradiation is less effective for apple fruit of red sports types whose color intensity indices are more than 2 and color percentages are more than 80%.

CONCLUSIONS

Blue LED light irradiation promoted anthocyanin synthesis in the skin of apple fruit with poor coloration at harvest. Light irradiation treatment at 15°C for four days increased anthocyanin accumulation of the apple cultivars 'Shinano Sweet,' 'Jonathan,' and 'Fuji'. Light irradiation with or without 1-MCP treatment did not affect fruit firmness. The inductive effect of irradiation was further confirmed in 'Fuji' apples harvested from four different production locations in Japan, although the effects were of varying degrees.

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Effect of chitosan application on some secondary plant metabolites in chili

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Abstract

The use of the chitosan elicitor has alleviated biotic and abiotic stress. Chitosan is particularly effective in inducing phytochemical properties such as phenolic compounds and flavonoids. Chili is an excellent source of carotenoid and flavonoids. The aim of this study is to determine the effect of chitosan on some secondary plant metabolites in chili. Chitosan at 0, 60, 120 and 240 mg L^{-1} were sprayed once a week after transplanting until harvest. Mature fruits were harvest at 45 days after anthesis and analyzed for secondary plant metabolites, including total phenolic compounds, total flavonoid, salicylic acid, carotenoid content and DPPH radical scavenging. Results showed that chitosan application at 120 mg L^{-1} increased total phenolic, total flavonoid, salicylic acid, carotenoid content and DPPH radical scavenging by 2.37-4.90, 1.11, 1.19-1.41, 2- and 1.05-1.72 folds, respectively. However, chitosan at a concentration of 240 mg L^{-1} reduced all secondary plant metabolites. Results of this study supported the application of chitosan at 120 mg L^{-1} to produced good quality chili.

Keywords: phenolic compounds, total flavonoid content, salicylic acid, carotenoid, DPPH radical scavenging

INTRODUCTION

Chili (*Capsicum annum* L.) is an economically important crop in Thailand. Chili is known to be a rich source of antioxidant activity compounds, which can be used to develop functional ingredients for food and pharmaceuticals. *Capsicum* fruits are a rich source of carotenoids, flavonoids, and ascorbic acid (Campos et al., 2013). The amount and composition of these metabolites vary among genotypes and are affected by many factors such as fruit maturity, cultivation systems, and processing methods (Howard et al., 2000). These phytochemicals exhibit high antioxidant activity, and their consumption has been linked to a decreased risk of developing chronic and degenerative diseases (Biswas et al., 2011).

Chitosan is primarily produced from chitin, which is widely distributed in nature, mainly as the structural component of the exoskeletons of arthropods, in marine diatom, algae, and in some fungal cell wall (Radman et al., 2003). It has been reported that chitosan is effective in inducing various plant defence responses such as induction of phenylalnine ammonia lyase (PAL), phenolic compounds, and antioxidant activity (Kim et al., 2005). Therefore, chitosan based agronanochemicals can provide a sustainable alternative to conventional agrochemicals in crop disease management (Maluin and Hussein, 2020). Chitosan elicitor is a key regulator to the activity of PAL that produces many phenolic compounds in plants through the phenylpropanoid pathway without any stress (Notsu et al., 1994). Singh (2016) reported that chitosan application to spinach at 0.01 mg L^{-1} stimulates the production of phenolic compound and flavonoid. Also, chitosan at 60 mL L^{-1} treated in coffee increase carotenoids by 73.5% (Dzung et al., 2011). Chitosan application (0.0625%) in wheat improve antioxidant enzyme activity (Ma et al., 2014). However, the effect of chitosan on induction of secondary metabolites in chili has not been well investigated. In this study, the effect of chitosan on some physiochemical properties of chili was investigated.

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MATERIALS AND METHODS

Seeds of chili (*Capsicum annuum* var. Bird chili) from Chia Tai co. Ltd. were grown in a seedling tray. Seedlings at 30 days after sowing were transplanted into plastic pots, 12 inches in diameter. The experiment was conducted in completely randomized block design with four treatments and 12 replications. Six plants were grown in each replication. They were sprayed with chitosan solutions at 0, 60, 120 and 240 mg L^{-1} once a week for 13 weeks after transplanting. Chitosan powder (85% deacetylated) was purchased from Alfa Aesar (Germany). Chemical fertilizer formula 15-15-15 was applied at 250 kg ha^{-1}, which was divided into 10 times every two weeks after transplanting. Mature fruits at 45 days after anthesis (DAA) were harvested and separated into pericarp and seed. These were analysed for phytochemicals content.

Determination of total phenolic compound content

The total amount of phenolic compounds was determined by Folin-Ciocalteu's (FC) reagent according to the method of Singleton et al. (1999) with minor modifications. One gram of fresh pericarp or seed of chili was extracted with 10 mL of 20% methanol and the mixture was centrifuged at 5,000 rpm for 15 min. Total 50 µL of supernatant was mixed with 250 µL of FC reagent and kept at room temperature for 8 min. Thereafter, the mixture was added to 750 µL of 20% Na_2CO_3 and 950 µL of distilled water and incubated at 25°C for 30 min. After incubation, the absorbance was measured at 765 nm with the UV vis spectrophotometer (UV-1800, Shimadzu, Japan). Gallic acid was used as the standard, and the results were expressed as of mg of gallic acid equivalent (GAE) g^{-1} FW.

Determination of flavonoid content

A total flavonoid content assay was performed using the $AlCl_3$ colourimetric method (Tunna et al., 2015). One gram of fresh fruit pericarp or seed of chili was extracted with 80% metanol 10 mL. The supernatant (500 µL) was mixed with 2 mL of distilled water and 15 µL of 5% $NaNO_2$ and incubated at room temperature for 6 min. Subsequently, 150 µL of 10% $AlCl_3$, 2 mL of 2 M NaOH and 200 µL of distilled water were added. The solution was mixed and incubated at 25°C for 30 min. Then, the absorbance was measured at 415 nm using the UV-vis spectrophotometer. Quercetin was used as the standard, and the results were expressed as mg of quercetin equivalent (QE) g^{-1} FW.

Determination of salicylic acid content

Salicylic acid content assay was performed using the method of Hemsanit and Prathuangwong (2009) with minor modifications. Fresh fruit sample of 0.5 g was ground with liquid N_2 and extracted with 500 µL of 90% methanol. The mixture was centrifuged at 12,000 rpm for 15 min. The pellet was extracted with 500 µL of 100% methanol and centrifuged at 12,000 rpm for 15 min. The supernatant (500 µL) was mixed with 500 µL of ferric ammonium sulfate at 0.02 M and incubated at 30°C for 5 min. The absorbance was measured at 530 nm using the UV-vis spectrophotometer. Salicylic acid was used as the standard and the results were expressed as µg g^{-1} FW.

Determination of carotenoid content

The carotenoid content assay was determined as previously described with minor modifications (Porra, 2002). Fresh pericarp of 100 mg was extracted with 20 mL of 80% acetone for 24 h under dark condition. Then filtered and adjusted the volume to 30 mL by acetone. The solution was measured at 440, 645 and 663 nm using the UV-vis spectrophotometer. The carotenoid content was calculated using the following formula:

$$\text{Carotenoid content (mg g}^{-1}\text{ FW)} = 4.69\ (A_{440})\text{-}0.268[20.2\ (A_{645})\text{-}8.02(A_{663})]\times[V/1{,}000\times W]$$

where V = total volume of acetone, W = sample weight, A = absorbent.

DPPH free radical scavenging activity

The radical scavenging activity of the sample was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay (Brand-Williams et al., 1995) with some modifications. One gram of fresh sample was ground in liquid N_2 and extracted with 10 mL of absolute ethanol. The mixture was then centrifuged at 5,000 rpm for 10 min. The mixture filtered and adjusted to a volume of 100 mL by absolute ethanol. The different concentrate of extract solution at 0, 10, 100, 1,000 and 10,000 µL mL^{-1} were prepared. The sample solution (1.9 mL) was mixed with 100 µL of 1mM of freshly prepared DPPH. The reaction mixture was shaken and incubated in the dark at room temperature for 30 min. Absorbance was measured at 517 nm using the UV-vis spectrophotometer. Controls were prepared as for the tested group without the plant solution, which was replaced with the extraction solvent. The DPPH free radical scavenging activity was calculated using the following formula:

$$\text{DPPH scavenging activity (\%)} = [(A0–A1)/A0]\times100$$

where A0 is the absorbance of the control and A1 is the absorbance of the sample.

DPPH scavenging activity of different concentrate in extract solution at 0, 10, 100, 1,000, and 10,000 µL mL^{-1} were used to obtain antiradical curves for calculating the effective concentration (EC_{50}) values. Antiradical curves were plotted referring to concentration on the *x*-axis and their relative scavenging capacity on the *y*-axis. The EC_{50} value herein refers to the effective chitosan concentration where DPPH radical were scavenged by 50%. Ascorbic acid and trolox were used as a standard in all experiments.

Statistical analysis

All the data expressed as the mean of four replications. Data were subjected to analysis of variance followed by Duncan's multiple range tests at $p<0.05$ and 0.01 was considered statistically significant using the R project version 3.6.3 for statistical computing.

RESULTS AND DISCUSSION

Chitosan application increased the total phenolic compound and total flavonoid in mature chili fruit compared to the control treatment. The total phenolic compound was significantly increased by the application of 120 mg L^{-1} of chitosan in both pericarp and seed by 2.37- and 4.90-folds, respectively (Table 1). No further increase in phenolic content was observed in 240 mg L^{-1} chitosan treatment. In contrast, the flavonoid content increased with chitosan treatment only in the pericarp. Chitosan applied at 120 mg L^{-1}, produced the highest level of flavonoid content. Flavonoid content was 0.62 mg QE g^{-1} FW higher than the control of 0.56 mg QE g^{-1} FW. These results confirm previously reported results, that chitosan promoted isoflavone content in soybean seed by 16-96% (Al-Tawaha et al., 2005). Similarly, Kim et al. (2005) reported the phenolic compound content in sweet basil increased after chitosan treatment. Yin et al. (2012), who found that chitosan at 50 and 200 mL L^{-1}, increased the polyphenol content in Greek oregano. These results agree with a report showing that chitosan induced glucose-6-phosphate dehydrogenase in the pentose phosphate pathway that lead to phenolic synthesis (Sarkar et al., 2010). Chitosan also influences PAL activity in flavonoid metabolic pathway (Khan et al., 2002).

Salicylic acid contents were not significantly different between the chitosan treatment concentrations applied to the fruit (Table 2). Salicylic acid contents in seed significantly increased with chitosan treatment by 1.3- to 1.4-folds compared with the control. Jia et al. (2016) reported that chitosan induced the expression of the defence-related gene PR1, which is a marker of salicylic acid signaling pathway, increasing the content of salicylic acid in *Arabidopsis*.

Chitosan application significantly increased carotenoid content in the pericarp (Table 2). In plants sprayed with 120 mg L^{-1} of chitosan, carotenoid content increased 2.32-folds (0.10 mg g^{-1} FW) compared to the control treatment. These results agree with Farouk et al. (2013) who reported that chitosan sprays increased carotenoid content in the leaf tissue of cowpea.

Table 1. Effects of chitosan on total phenolic compounds and total flavonoid contents in the pericarp and seed of chili.

Chitosan (mg L^{-1})	Total phenolic compounds (mg GAE g^{-1} FW)		Total flavonoid content (mg QE g^{-1} FW)	
	Pericarp	Seed	Pericarp	Seed
0	39.05b	23.61c	0.56c	0.77b
60	46.05b	47.74ab	0.58b	0.79ab
120	92.68a	115.80a	0.62a	0.82a
240	51.55b	54.18ab	0.59b	0.81ab
F-test	**	**	**	*
CV (%)	19.02	22.75	1.56	2.57

Mean followed by the same letter in the same column are not statistically different according to LSD.
* significantly different at 0.05 probability, ** significantly different at 0.01 probability.

Table 2. Effects of chitosan on salicylic acid and carotenoid contents and DPPH radical scavenging in chili.

Chitosan (mg L^{-1})	Salicylic acid (µg g^{-1} FW)		Carotenoid (mg g^{-1} FW)	DPPH radical scavenging (%)	
	Pericarp	Seed	Pericarp	Pericarp	Seed
0	13.61b	10.18b	0.05c	14.88a	8.31c
60	14.64ab	14.04a	0.07bc	15.46a	11.07b
120	16.29a	14.42a	0.10a	15.64a	14.31a
240	14.97ab	13.37a	0.08ab	12.60b	15.65a
F-test	*	**	**	*	*
CV (%)	8.45	7.18	17.36	7.10	8.98

Mean followed by the same letter in the same column are not statistically different according to LSD.
* significantly different at 0.05 probability, ** significantly different at 0.01 probability.
EC_{50} Trolox = 0.0085 mg mL^{-1}, EC_{50} ascorbic acid = 0.0102 mg mL^{-1}, EC_{50} pericarp = 1.34 mg mL^{-1}, EC_{50} seed = 3.33 mg mL^{-1}.

The effect of chitosan on antioxidant activity was determined using DPPH radical scavenging. The radical scavenging of fruit in both pericarp and seed increased in response to increasing chitosan (Table 2). The highest activity in radical scavenging was observed in pericarp with 120 mg L^{-1} of applied chitosan and in seed with 240 mg L^{-1} of applied chitosan. The EC_{50} value of pericarp was 1.34 mg mL^{-1}, while of seed was 3.33 mg mL^{-1}.

The DPPH is a stable free radicle, which has been wildly accepted as a tool for estimating the free radical scavenging activity of antioxidants. The antioxidant activity in chili is contributed from phenolic compound, flavonoid, and carotenoid. The lower EC_{50} value indicated a strong ability of the extract to act as a DPPH scavenging reaction. The higher EC_{50} value indicated a lower scavenging activity of the scavenger, as more scavenger is required to achieve 50% scavenging reaction. Results of this study showed that the extract from chili had higher antioxidant activity at the 120 mg L^{-1} chitosan treatment (Table 2). From this result, antioxidant activity in chili is contributed to by phenolic compound, flavonoid, and carotenoid. Kim et al. (2005) found that antioxidant activity increased after chitosan application in sweet basil. Possibly, chitosan could regulate the activity of antioxidant enzyme and increase plant quality with health beneficial substances.

CONCLUSIONS

The chitosan application at 120 mg L^{-1} increased the phenolic compound (2.37- to 4.90-folds), total flavonoid (1.11-folds), carotenoid (2.0-folds), and DPPH radical scavenging (1.05- to 1.72-folds) greater than the control treatment. Based on this study, the application of chitosan can improve anti-oxidation compounds in chili. This may be helpful for consumers and processing chili products in the future.

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Effect of calcium silicate on number of trichomes, leaf thickness and chlorophyll in tomato

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Abstract

Silicon plays a role in alleviating biotic and abiotic stress in plants. A similar mechanism also acts against insects by inducing the formation of the trichomes and changing leaf structure. However, there are few studies on the role and effects of silicon on leaf structure. In previous studies, silicon protects plants from pathogens and insects and improves growth under stress conditions. This study looks at the effects of calcium silicate on salicylic acid content, chlorophyll content, the number of trichomes, and thickness of tomato leaves. The experimental design was a completely randomized design with five treatments and 10 replications. Calcium silicate was added to the planting media at 0, 62.5, 125, 187.5 and 250 kg ha^{-1}, seven days after transplanting (DAT). Leaves at position three to five from the shoot apex at 60 DAT were collected and analyzed for chlorophyll and salicylic acid content. The number of trichomes examined at the leaf margin, leaf blade, lower leaf vein, and upper leaf vein. Leaf thickness measured via cross-section with and without the vascular bundle. The concentration of calcium silicate at 250 kg ha^{-1} increased the number of trichome at the leaf margin, leaf blade, lower leaf vein, and upper leaf vein by 25, 40, 41 and 23%, respectively, compared to the control treatment. The lower leaf vein had a higher number of trichomes compared to the other leaf sectors examined. In addition, calcium silicate at 187.5 kg ha^{-1} enhanced leaf thickness by 43% and total chlorophyll content by 13% compared with the control treatment. Furthermore, calcium silicate did not result in a significant increase in leaf salicylic content compared to the control treatment.

Keywords: leaf blade, leaf margin, lower leaf vein, salicylic acid, silicon

INTRODUCTION

Silicon (Si) has been recognized as a beneficial element because it stimulates plant growth for some species. Si plays an important role in cell wall stability by bridging between polyuronides and stimulating lignin synthesis. Si can improve plant growth, leaf erectness, water use efficiency, photosynthetic ability, and protect plants from pests and diseases (Broadley et al., 2012; Xie et al., 2014). In addition, Si may act to alleviate salt stress in tomato by decreasing the permeability of plasma membranes and affects maintenance of cell form and structure due to the increase of antioxidative enzymes (Al-Aghabary et al., 2005). The use of Si improves the physical and chemical quality of tomato fruit by increasing soluble solids, vitamin C, lycopene, and firmness (Marodin et al., 2016). The application of Si to tomato via a nutrient solution produced healthier fruit as it lowered the incidence of blossom end rot (Costan et al., 2020). Samuels et al. (1991) reported Si in cucumber leaf suppresses the growth of powdery mildew fungus (*Sphaerotheca fuliginea*). *Cucurbitaceae* species show a high degree of intraspecific and interspecific variation, and the pattern of depositing Si in leaf trichomes. In leaves of cucumber, melon and watermelon, high accumulation of silicon was detected in cells surrounding the base of trichome hairs and the hairs as well (Abe, 2019). The application of silicate increases the number of trichomes in soybean (Souza et al., 2014) and in maize (Nitmee et al., 2015). The literature indicates Si alleviates biotic stress in plants. Examining the effects of Si fertilizer on tomato is important due to improvements in fruit

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quality. However, understanding the role of silicon on the number of trichomes at the leaf surface is limited. In this study, the effect of calcium silicate on the number of trichomes on the leaf surface, leaf thickness, chlorophyll content, and salicylic acid content in tomato was investigated.

MATERIALS AND METHODS

Seeds of 'Sida' tomato (*Lycopersicon esculentum*) were soaked with 8 g L^{-1} of calcium silicate for 4 h and sown into peat moss. Seedling at 21 days after sowing were transplanted to 12-inch plastic containers. The experiment design was a completely randomized design (CRD) with 10 replications and five treatments. Calcium silicate fertilizer applied to plant media at a rate of 0, 62.5, 125, 187.5 and 250 kg ha^{-1} at 7 DAT. Fresh green fully expanded leaves at third to the fifth leaf from the apex were sampled and data collected at 60 DAT.

Determination the number of trichome in different position

Fresh green leaves were collected and observed by stereomicroscope and compound microscope. Photographs obtained using a MSHOT digital microscope camera. Measurements were performed on the number of trichomes at the different leaf positions for the leaf margin, leaf blade, lower leaf vein (abaxial surface vein), and upper leaf vein (adaxial surface vein) (Figure 1). Leaf thickness was investigated by freehand cross-section at the position with vascular bundle and without the vascular bundle under the compound microscope.

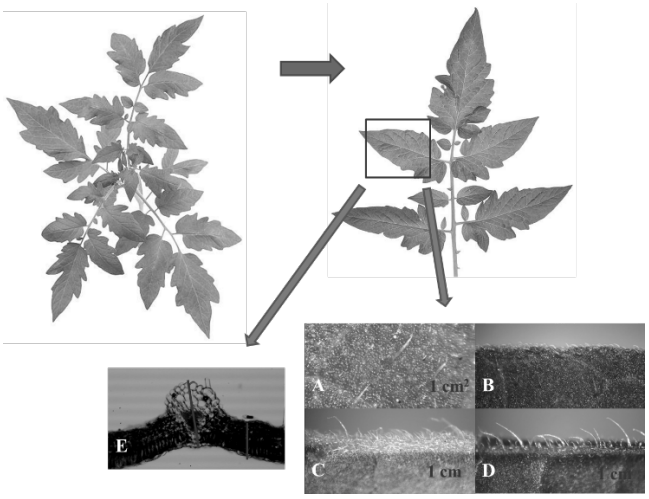

Figure 1. Sample position for observation the number of trichome (A = leaf blade, B-D = leaf margin, and E = leaf thickness).

Determination of chlorophyll content and chlorophyll relative content

Chlorophyll content in leaves was determined and calculated according to Arnon (1949). Fresh leaf samples of one g were soaked in 3 mL of dimethyl sulfoxide (DMSO) and incubated in the dark at room temperature for 48 h. Samples were filtered, and absorbance measured at 648 and 665 nm using the UV-vis spectrophotometer. The chlorophyll content results are expressed as a unit's milligram per gram fresh weight (mg g^{-1} FW). Chlorophyll a = $14.58A_{665}$-$5.14A_{648}$×V/1,000 W; Chlorophyll b = $25.48A_{648}$-$7.36A_{665}$×V/1,000 W; total chlorophyll = $7.49A_{665}$+$20.34A_{648}$×V/1,000 W; when V = final volume (mL) and W = sample weight (g).

In addition, the chlorophyll relative content was collected from four different leaf positions $plant^{-1}$ using a chlorophyll meter (Minolta, Model SPAD 502).

Determination of salicylic acid content

Salicylic acid content determined using ammonium ferric sulfate reagent, according to the method of Hemsanit and Prathuangwong (2009) with a minor modification. Leaf samples of 0.5 g were ground with liquid N_2 and extracted with 0.5 mL of 90% methanol and

centrifuged at 12,000 rpm for 15 min. Pellet was extracted with 0.5 mL of 100% methanol and centrifuged again. The supernatant of 0.5 mL was mixed with 0.5 mL of 0.02 M ammonium ferric sulfate and incubated at room temperature (30°C) for 5 min. After incubation the absorbance was measured at 530 nm with the UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan). Salicylic acid was used as the standard. The salicylic acid content results are expressed as the unit's microgram per gram fresh weight (μg g^{-1} FW).

Statistical analysis

All data are expressed as mean of four replications. Data were subjected to one-way analysis of variance followed by the least significant difference (LSD) test at $p<0.01$ being considered statistically significant different using the R project for statistical computing version 3.6.3.

RESULTS AND DISCUSSION

The density of trichomes increased slightly after treatment with calcium silicate compared to the control treatment. Calcium silicate at the concentration of 250 kg ha^{-1} increased the number of trichome at the leaf margin, leaf blade, lower leaf vein, and upper leaf veins by 25, 40, 41 and 23%, respectively, compared to the control treatment (Table 1). The lower leaf vein had a higher number of trichomes than the other positions. These results indicate that the application of 250 kg ha^{-1} of calcium silicate, induced the highest density of trichomes for the different leaf parts compared to the other calcium silicate treatments. These observations agreed with data obtained on the number of trichomes observed for soybean (Souza et al., 2014). For maize, the number of trichomes increased with the concentration of silicon (Nitmee et al., 2015). Furthermore, Si effects trichomes in *Artemisia annua* (Rostkowska et al., 2016) and rice. Trichomes identified by SEM and X-ray microanalysis, show the deposited of Si in cell walls of wheat and cell surrounding the base of trichome hair and hair itself for *Cucurbitaceae*. It has been reported that the location of silica acts as a physical barrier (Abe, 2019).

Table 1. Effect of calcium silicate on number of trichrome in tomato 'Sida' leaf.

Ca_2SiO_4 (kg ha^{-1})	No. of trichomes			
	Leaf margin	Leaf blade (mm^2)	Lower leaf vein	Upper leaf vein
0.0	7.50b	5.33b	16.50b	6.80b
62.5	8.50ab	5.67b	18.14b	7.78ab
125.0	8.75ab	6.50ab	18.67ab	8.09a
187.5	8.83ab	6.50ab	20.67ab	8.20a
250.0	9.42a	7.50a	23.33a	8.38a
F-test	**	**	**	**
CV (%)	18.8	9.04	15.92	9.29

Means followed by the same letters in the same column are not statistically different from each other according to LSD.
** significant difference at 0.01 probability.

Findings showed the application of calcium silicate also significantly increased leaves thickness. The treatment of calcium silicate at 187.5 kg ha^{-1} resulted in maximum leaf thickness, with the vascular bundle and without the vascular bundle increasing by 24 and 43%, respectively, compared with the control treatment (Table 2). The application of silicon enhanced the mechanisms for resistance to diseases and pests. It has been proposed that Si acts as a physical barrier by increased cell wall elasticity during extension growth. In addition, Si remains in the apoplast and is deposited after water evaporation as amorphous silica. These deposits are found at the termini of the transpiration stream, mainly on the outer walls of the epidermal cells on both surfaces of the leaves and trichomes (Ma and Takahashi, 2002; Broadley et al., 2012). As previously reported, the addition of silicate increased mesophyll, adaxial and abaxial epidermis thickness in orchid (Soares et al., 2012) and adaxial epidermis in passion fruit (Costa et al., 2018).

Table 2. Effect of calcium silicate on leaf thickness in tomato.

Ca_2SiO_4 (kg ha^{-1})	Leaf thickness (mm)	
	With vascular bundle	Without vascular bundle
0.0	0.49b	0.32b
62.5	0.53ab	0.32b
125.0	0.50b	0.26c
187.5	0.61a	0.46a
250.0	0.54ab	0.40a
F-test	**	**
CV (%)	13.65	13.60

Means followed by the same letters in the same column are not statistically different from each other according to LSD.
** significant difference at 0.01 probability.

Chlorophyll content in leaves was significantly higher in the calcium silicate treatments compared to the control treatment (Table 3). Calcium silicate at a rate of 187.5 kg ha^{-1} induced the maximum chlorophyll b, total chlorophyll, and chlorophyll relative content (SPAD index) of 0.073 and 0.175 mg g^{-1} FW, and 48.5, respectively. These findings showed a positive correlation between chlorophyll content and the SPAD index. This result was similar to the study conducted by Asmar et al. (2013) who found that chlorophyll content in banana increased after treatment with silicate. Therefore, the addition of silicate to the culture medium affected the photosynthesis and leaf anatomy of *A. andraeanum* 'Rubi'. 'Rubi' developed anatomical and physiological characteristics that contributed to its survival ex vitro (De et al., 2014). This study agreed with data presented by Al-Aghabary et al. (2005), Xie et al. (2014), Soundararajan et al. (2015), and Siddiqui et al. (2014) who found that silicon promoted chlorophyll content in tomato, maize, carnation, and squash.

Table 3. Effect of calcium silicate on chlorophyll contents in tomato leaf.

Ca_2SiO_4 (kg ha^{-1})	Chlorophyll a (mg g^{-1} FW)	Chlorophyll b (mg g^{-1} FW)	Total chlorophyll (mg g^{-1} FW)	Chlorophyll relative content (SPAD)
0.0	0.091	0.060e	0.154d	38.7c
62.5	0.099	0.062d	0.163c	46.6ab
125.0	0.102	0.069b	0.173ab	44.9ab
187.5	0.099	0.073a	0.175a	48.5a
250.0	0.102	0.067c	0.171b	43.3bc
F-test	ns	**	**	**
CV (%)	4.79	1.12	0.57	4.10

Means followed by the same letters in the same column are not statistically different from each other according to LSD.
ns = non-significant at 0.01 probability and ** significant at 0.01 probability.

Salicylic acid regulates a plants immunity network and defense responses (Clarke et al., 2000). The application of calcium silicate did not significantly affect the salicylic acid content in the tomato leaf compared with the control treatment (Figure 2). Similarly, Khan et al. (2019) reported that Si application at different pH levels to the substrate did not significantly affect the salicylic acid content in tomato seedlings. However, low levels of salicylic acid at high pH (9) considerably reduce the amount of stress in the seedling.

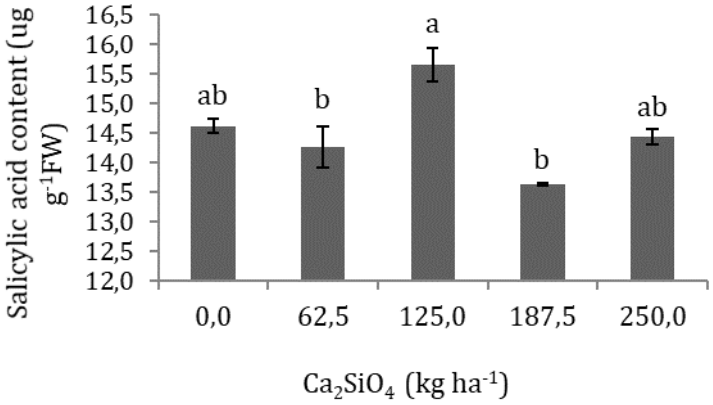

Figure 2. Effect of calcium silicate on salicylic acid content in tomato leaf. Bar represent means ± SE (n=4). Different letters mean significant differences between the treatments at 0.01 level.

CONCLUSIONS

Compared to the control treatment, the concentration of calcium silicate at 250 kg ha^{-1} increased the number of trichome at the leaf margin, leaf blade, lower leaf vein and upper leaf vein by 25, 40, 41 and 23%, respectively. In addition, calcium silicate at 187.5 kg ha^{-1} enhanced leaf thickness and total chlorophyll content, compared to the control treatment.

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Effects of CO_2 enrichment and 5-aminolevulinic acid-based fertilizer application on cut flower yield of *Alstroemeria* in low light conditions during winter

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Abstract

The effects of CO_2 enrichment and 5-aminolevulinic acid (ALA)-based fertilizer application on cut flower production of *Alstroemeria* 'Avalange' were investigated. Seedlings were potted in 45-cm diameter polyethylene pots in September 2017. Four treatments were applied: general liquid fertilizer applied regularly (control group); ALA-based fertilizer (ALA group); CO_2 enrichment (CO_2 group); and ALA-based fertilizer combined with CO_2 enrichment (ALA+CO_2 group). In the first year, the experiment was conducted in a greenhouse until April 2018 and the CO_2 concentration in the CO_2 and ALA+CO_2 groups was increased to a maximum of 800 µmol mol^{-1} for 5 h of the light period. The number of cut flowers and the SPAD value of leaves in the ALA+CO_2 group were significantly higher than those in the control group. There were no significant differences in cut flower length, cut flower weight, stem diameter, the number of peduncles and the number of florets among the treatments. In the second year, the experiment was conducted in a phytotron from November, 2018 to March, 2019 and the CO_2 concentration in the CO_2 and ALA+CO_2 groups was increased to 900 µmol mol^{-1} for 7 h. The number of cut flowers in the ALA+CO_2 group was significantly higher than those in the control and CO_2 groups. Stem diameter in the ALA, CO_2 and ALA+CO_2 groups was thicker than that of the control group. There were no significant differences in cut flower length, cut flower weight, the number of peduncles and the number of florets among the treatments. The number of shoots in the CO_2 group was significantly greater than those in the control and ALA groups. These findings demonstrate that ALA-based fertilizer application under CO_2 enrichment conditions increases *Alstroemeria* cut flower numbers in low light conditions during winter.

Keywords: chlorophyll, cultural practice, environmental control, greenhouse, perennial

INTRODUCTION

Alstroemeria spp. are perennial plants belonging to the *Alstroemeriaceae*. They are very popular as cut flowers and potted flowers because of a wide range of flower colors, flower patterns and long flower longevity. Flower bud differentiation is generally induced by chilling, which is different among cultivars. Although *Alstroemeria* cut flowers are produced all year round, growth is suppressed during winter which, in the greenhouse, includes low light intensity and low CO_2 concentration (Leonardos et al., 1994).

Enrichment with CO_2 has been known to promote plant growth and to improve the yield and quality of many horticultural crops (Mortensen, 1987). In cut flower production of *Alstroemeria* in a greenhouse, when the CO_2 concentration was increased to 900 µmol mol^{-1} the number of flower stalks increased 1.5 times compared to control cultivation in normal air (Van Labeke and Dambre, 1998). Leonardos et al. (1994) showed that CO_2 enrichment of 1,500 to 2,000 µmol mol^{-1} doubled whole-plant net photosynthesis in *Alstroemeria* 'Jacqueline'.

The compound, 5-aminolevulinic acid (ALA), is a natural amino acid that is ubiquitous in all organisms. It is a precursor in the biosynthesis of tetrapyrrole which is incorporated into such compounds as heme and chlorophyll. Exogenous low-level application of ALA to plants results in increased chlorophyll content and enhanced photosynthetic activity. In addition, the

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application of ALA increased the yield of various crops (Hotta et al., 1997; Hotta and Watanabe, 1999; Sasaki et al., 2002; Tanaka et al., 2005). Recently, ALA-based fertilizer was shown to promote the growth of date palm (*Phoenix dactylifera* L.) (Awad, 2008). It has also been reported that under low light conditions, ALA-based fertilizer under CO_2 enrichment promoted early growth of *Limonium sinuatum*, which is an ornamental plant belonging to the *Plumbaginaceae* (Mori and Chino, 2018).

In this study, to improve of the yield and quality of *Alstroemeria* cut flower during winter, the effects of CO_2 enrichment and application of ALA-based fertilizer on the growth of cut flower cultivar 'Avalange' in a greenhouse and in a phytotron were examined.

MATERIALS AND METHODS

The studies were performed in Ebetsu, Hokkaido, Japan (latitude 43°6'13"N; longitude 141°32'10"E). Figure 1 shows changes of solar radiation in Sapporo near Ebetsu, Japan (Japan Meteorological Agency). Seedings of *Alstroemeria* 'Avalange', which is an ever-flowering cultivar for cut flower production, were used in the study. The seedlings were potted in 45-cm diameter polyethylene pots on September 22, 2017. A CO_2 recorder (TR-76Ui; T&D Co., Matsumoto, Japan) was used to measure temperature and CO_2 concentration. Photosynthetic photon flux density (PPFD) was measured using a light analyzer (LA-105, Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan).

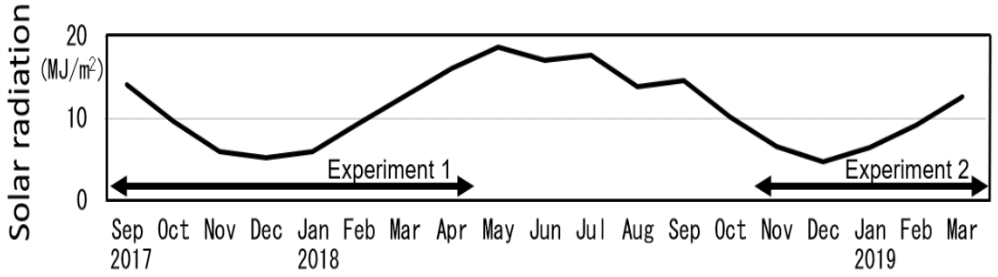

Figure 1. Changes of solar radiation during cultivation in Sapporo, Japan.

Experiment 1 (September 22, 2017-April 27, 2018)

Plants were cultivated in the greenhouse which was closed using thermal screens all day during the examination period with a minimum temperature of 10°C and a ventilation temperature of 23°C. The PPFD in the greenhouse was, for example, 80.5 μmol m^{-2} s^{-1} at 10 am on December 12, 2017 (cloudy conditions). Four treatments were applied to the potted plants: general liquid fertilizer applied regularly (control group), ALA-based fertilizer (ALA group), CO_2 enrichment (CO_2 group) and ALA-based fertilizer combined with CO_2 enrichment (ALA+CO_2 group). The CO_2 concentration in the CO_2 and ALA+CO_2 groups was increased to a maximum of 800 μmol mol^{-1} for 5 h of the light period using a CO_2 controller attached to a CO_2 cylinder. Common liquid fertilizer was applied to all plants one to four times a month.

The control group was cultivated in a non-CO_2 enriched greenhouse and only common liquid fertilizer was applied. The CO_2 group was cultivated in a CO_2 enriched greenhouse and only common liquid fertilizer was applied. The ALA group was cultivated in the non-CO_2 enriched greenhouse and the appropriate concentration of ALA-based fertilizer (described below) was applied in addition to the common liquid fertilizer that was applied at the supplement application. The ALA+CO_2 group was cultivated in the CO_2 enriched greenhouse and the appropriate concentration of ALA-based fertilizer was applied as well as the common liquid fertilizer. ALA-based fertilizer (Pentakeep V; SEIWA Co., Ltd., Shimotsuke, Japan) contains, in gravimetric percentage, 9.5% N (3.8% nitrate nitrogen and 5.7% ammonium nitrogen), 5.7% MgO, 0.14% B, 0.02% Cu, 0.6% Fe-DTPA, 0.23% Mn, 0.02% Mo and 0.16% Zn. However, the ALA concentration was not declared by the producer (Smolen and Sady, 2010). It was used at 5,000 times dilution.

The quality of the cut flowers was examined during the flowering period. Cut flower length of 65 cm or more, stem diameter of 4.5 mm or more, and three or more peduncles were

examined. Stem diameter was measured 10 cm above the cut base. The soil plant analysis development (SPAD) value of the involucral scales (three bract leaves) was measured using a chlorophyll meter (SPAD-502 Plus; KONICA MINOLTA JAPAN, Inc., Tokyo, Japan). Nine plants were used for each test group.

Experiment 2 (November 2, 2018-March 28, 2019)

All plants continued to be cultivated in the non-CO_2 enriched greenhouse following the completion of experiment 1. All shoots on these plants were cut back to a height of 10 cm on October 12, 2018 and these trimmed plants were used for experiment 2. The experiment was conducted in a phytotron set at 10°C night/15°C day and 70% relative humidity (RH), which had a non-CO_2 enriched room and a separate CO_2 enriched room, from November, 2018 to March, 2019. The PPFD in the phytotron was, for example, 444.6 µmol m^{-2} s^{-1} at 10 am on December 20, 2018 (sunny conditions). Four treatments (the same as those in experiment 1) were applied to the potted plants: control group, ALA group, CO_2 group and ALA+CO_2 group. The CO_2 concentration in the CO_2 and ALA+CO_2 groups was increased to 900 µmol mol^{-1} for 7 h during the day. Common liquid fertilizer was applied to all plants once a week. ALA-based fertilizer was applied in the ALA and ALA+CO_2 groups at the time of the supplemental fertilizer application.

Shoot thinning was performed on December 4, 2018 and January 31, 2019, leaving the thickest 10 or 20 shoots, respectively. The yield and quality of cut flowers were investigated during the flowering period, and at the end of the examination, the growth of the plants and the spots formed on the leaves were examined. Three plants were used for each test group.

Effects of CO_2 enrichment and ALA-based fertilizer were examined by analysis of variance and the Tukey-Kramer test (p=0.05) was calculated to compare treatment means.

RESULTS

Experiment 1 (September 22, 2017-April 27, 2018)

CO_2 concentration in the CO_2 enriched greenhouses was successfully controlled to the set-point (data not shown). The number of cut flowers (29% higher) and the SPAD value of leaves in the ALA+CO_2 group were significantly higher than those in the control group (Table 1). There were no significant differences in cut flower length, cut flower weight, stem diameter, the number of peduncles and the number of flowers among treatments (Table 1).

Table 1. Effects of CO_2 enrichment and ALA-based fertilizer on the yield and quality of cut flowers of *Alstroemeria* (September 22, 2017-April 27, 2018) under greenhouse conditions.

Treatments	No. of cut flowers pot^{-1}	Length of cut flower (cm)	Weight of cut flower (g)	Stem diameter (mm)	SPAD value	No. of peduncles $cut\ flower^{-1}$	No. of flowers per cut flower
Control	21.1b	90.0a	52.4a	7.6a	51.8b	5.2a	12.8a
ALA	25.9ab	92.8a	53.7a	7.6a	52.7ab	5.1a	12.8a
CO_2	21.9ab	89.3a	55.4a	7.8a	52.8ab	5.1a	13.0a
ALA+CO_2	27.1a	90.8a	58.3a	7.9a	54.1a	5.1a	13.2a

Values within the same column followed by different letters are significantly different at p<0.05 according to Tukey-Kramer's test.

Experiment 2 (November 2, 2018-March 28, 2019)

The CO_2 concentration in the CO_2 enriched phytotron greenhouse was successfully controlled to the set-point (data not shown). Because experiment 2 was performed in a small space which was different from that in the greenhouse used in experiment 1, the CO_2 concentration was reduced to 200 µmol mol^{-1} in the non-CO_2 enriched room but was immediately increased to the set value in the CO_2 enriched room.

The harvest of the cut flowers was performed from mid-February, 2019. The number of

cut flowers in the ALA+CO_2 group was significantly higher (by 2.4 times) than that in the control group (Table 2). Stem diameter in the ALA, CO_2 and ALA+CO_2 groups was significantly thicker than that in the control group. There were no significant differences in cut flower length, cut flower weight, the number of peduncles and the number of flowers among the treatments.

Table 2. Effects of CO_2 enrichment and ALA-based fertilizer on the yield and quality of cut flowers of *Alstroemeria* (November 2, 2018-March 28, 2019) in the phytotron greenhouses.

Treatments	No. of cut flowers pot^{-1}	Length of cut flower (cm)	Weight of cut flower (g)	Stem diameter (mm)	No. of peduncles per cut flower	No. of flowers per cut flower
Control	4.7b	86.1a	88a	8.5b	4.9a	13.8a
ALA	7.0ab	90.5a	95a	9.4a	4.8a	14.8a
CO_2	3.3b	88.0a	102a	9.6a	5.4a	18.4a
ALA+CO_2	11.3a	98.8a	112a	9.7a	5.1a	16.4a

Values within the same column followed by different letters are significantly different at $p<0.05$ according to Tukey-Kramer's test.

On March 28, 2019, the final day of the study, the number of shoots and dry weight of shoots in the CO_2 group were significantly greater than those in the control and ALA groups (Table 3). Unexpectedly, leaf spots occurred on some of the plants examined and these were rated according to a leaf spot index. The leaf spot indices in the CO_2 and ALA+CO_2 groups were significantly higher than those in the control and ALA groups (Figure 2).

Table 3. Effects of CO_2 enrichment and ALA-based fertilizer on growth of *Alstroemeria* (March 28, 2019).

Treatments	Plant height (cm)	No. of shoots pot^{-1}	Fresh weight of shoots (g)	Fresh weight shoot^{-1} (g)	Dry weight of shoots (g)
Control	120a	61.7b	2867a	46.5a	252b
ALA	119a	61.7b	2807a	45.7a	243b
CO_2	111a	99.7a	3953a	39.7a	341a
ALA+CO_2	130a	74.0ab	3260a	44.0a	254ab

Values within the same column followed by different letters are significantly different at $p<0.05$ according to Tukey-Kramer's test.

DISCUSSION

In a previous study, Van Labeke and Dambre (1998) reported that CO_2 enrichment increased the number of flower stems in some *Alstroemeria* cultivars and the effect and the optimal CO_2 concentration were cultivar dependent. Additionally, in *Alstroemeria* 'Jacqueline', CO_2 enrichment doubled whole-plant net photosynthesis at 1,200 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), but its positive effect was remarkedly limited at the lower irradiance level of 200 μmol m^{-2} s^{-1} PAR, which is similar to the level on a cloudy winter day (Leonardos et al., 1994). In this study, in experiment 2 that was performed during winter without supplementary lighting, CO_2 enrichment up to 900 μmol mol^{-1} alone did not improve the number of cut flowers although stem diameter, the number of shoots and the dry weight of shoots were all increased (Tables 2 and 3). These findings suggest that improving the yield and quality in *Alstroemeria* cut flower production during winter requires control of other factors in addition to CO_2 enrichment.

The application of ALA has been known to enhance photosynthetic activity and to promote the growth of some plants (Tanaka et al., 2005). However, in this study, performed during winter, application of ALA-based fertilizer alone to *Alstroemeria* only increased the thickness of the stem (Tables 1-3). However, application of both ALA-based fertilizer and CO_2 in combination improved the yield and quality of cut flowers that were cultivated under low solar radiation (Tables 1 and 2). Mori and Chino (2018) reported that the early growth of *L.*

sinuatum under low light conditions was promoted with the application of both ALA-based fertilizer and CO_2, which is consistent with the results presented in this current study. It is unclear, however, whether ALA-based fertilizer and CO_2 activated photosynthetic activity in *Alstroemeria*. ALA has been known to have various physiological activities, such as improvement of fertilizer use efficiency, salt tolerance, cold temperature tolerance or the coloration of the pericarp (Sasaki et al., 2002; Tanaka et al., 2005; Wu et al., 2019). *Alstroemeria* has two types of shoots: the flowering shoots with flower buds and the vegetative shoots without flower buds. Application of ALA-based fertilizer and CO_2 might have induced flower bud formation and, therefore, increased the number of cut flowers. In addition, ALA and CO_2 might have promoted flower bud development after flower bud differentiation and resulted in early flowering. Elucidation of the mechanisms that led to an increase in yield with application of both ALA-based fertilizer and CO_2 is needed.

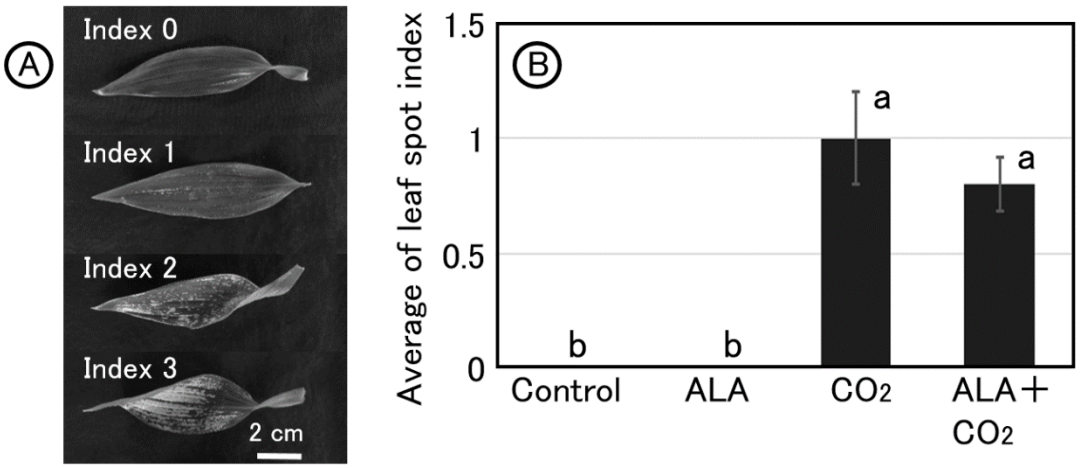

Figure 2. Leaf spot index; 0: no spots, 1: slight spots, 2: spots on less than half of the leaf, 3: spots on half or more than half of the leaf (A). A comparison of the leaf spot index for the leaves in the four treatments (B). Different letters show significant difference at $p<0.05$ according to Tukey-Kramer's test.

High CO_2 concentrations have been known to induce injury on leaves of some horticultural crops. For example, with chrysanthemum, chlorosis and necrosis were induced (van Berkel, 1984). In this study, leaf spots formed in *Alstroemeria* under CO_2 enrichment conditions (Figure 2). Because experiment 2 was performed in a phytotron with a small space, which is different from the greenhouse conditions used in experiment 1, the physiological injury observed might have been caused by other environmental factors in addition to the high CO_2 concentration. These causes remain to be clarified.

In conclusion, these findings demonstrate that ALA-based fertilizer application under CO_2 enrichment conditions increased *Alstroemeria* cut flower numbers in low light conditions during winter where supplementary lighting was not being used. In future, the optimal environmental conditions that do not cause of physiological injury will be examined and the positive effects from the use of ALA-based fertilizer and CO_2 enrichment will be verified using several cultivars.

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Growth and yield performance of tomato (*Solanum lycopersicum* Linn.) genotypes under protected and conventional cultivation systems

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Abstract

Protected cultivation is a production system that ensures a continuous vegetable industry in the Philippines. The University of Science and Technology of Southern Philippines (USTP) in Claveria, Misamis Oriental, Philippines conducted a study evaluating the performance of different fresh market tomato genotypes grown under different cropping environments. This study consisted of two cultivation systems (protected cultivation and conventional or open field cultivation) and seven genotypes ('AVTO 1173', 'AVTO 1004', 'Harabas', 'Athena', 'Sakata', 'Atlas', and 'Diamante'). The study was a factorial randomized complete block design (RCBD). Results show that tomato plants grown under a protected structure had the highest yield of 17.29 t ha^{-1} compared to those in the open field system, producing only 11.28 t ha^{-1}. This study found the fresh market tomato 'AVTO 1173', produced the highest yield of 18.67 t ha^{-1}. That cultivar is a potential substitute for presently used commercial hybrid genotypes. Plants under the protected cropping systems showed a significantly lower diseases infestation (32.62%) compared to the open field system (80.95%). This study provides scientific evidence for farmers to engage in advanced production systems. The protected cropping systems evaluated to enhance their ability to compete in today's modern agricultural marketing system.

Keywords: cultivation systems, tomato cultivars, greenhouse, hybrid genotypes, yield, pest and disease infestation

INTRODUCTION

The University of Science and Technology of Southern Philippines (USTP) in Claveria, Misamis Oriental, Philippines conducted a study on different fresh market tomato lines from the World Vegetable Centre (AVRDC). This study aimed to determine the various tomato genotype options which can compete with the local cultivar. Furthermore, this study evaluated protected structures, providing information for tomato growers in the area on their conventional production system. Presently, farmers are facing adverse climatic conditions and this information is vital for growers to make informed decisions.

Martínez-Blanco et al. (2011) reported protected structures not only lessens water and pesticide requirements but also enhances yield by controlled climatic and soil conditions, enabling vegetable crops to express their yield potentials. Furthermore, environmental stress is the primary cause of crop losses worldwide, reducing average yields by more than 50% (Boyer, 1982). Therefore, lessening environmental stress using protected structures, makes it possible to obtain a higher number of fruits compared to an open field situation. Protective cropping reduced periods of leaf wetness, creating less favourable conditions for diseases to infect. In addition, it protects fruit from direct contact with soil, by reducing splashing during heavy rains. Foliage diseases are easier to control under protected cropping structures (Capuno et al., 2015). Furthermore, vegetable production under protected structures reduces

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weed growth, moderates' soil and air temperatures, enables maximization of applied fertilizers, by reducing leaching of nutrients, therefore optimizing crop yield performance (Ghorbani et al., 2009).

In the Philippines, most of the tomato growers have little knowledge about this new type of cultivation system. Particularly in Claveria, Misamis Oriental farmers are afraid to take risks on this new advanced farming system. Farmers usually think greenhouses or protected structures are costly, which is often the reason why they do not try these systems.

This study was conducted to evaluate different tomato cultivars grown under conventional and protected cropping systems. Specifically, the study aimed to: 1) evaluate the growth performance of different tomato genotypes; 2) determine the yield and its component of these genotypes; and 3) assess the incidence and severity of disease for these different tomato genotypes under the different types of cultivation systems.

MATERIALS AND METHODS

Site details

This study was conducted at the Research Development and Extension Station of University of Science and Technology of Southern Philippines (USTP), located SE 8°36.667'N; 124°52.964'E and 590 m a.s.l., Claveria, Misamis Oriental, Philippines.

Treatments and experimental design

The experimental area was laid out using factorial in randomized complete block design (RCBD) with three replications. Two types of cultivation systems (open field condition or conventional and protected cropping) serves as the Factor A, and seven table tomato cultivars ('AVTO 1173', 'AVTO 1004', 'Harabas', 'Athena', 'Sakata', 'Atlas' and 'Diamante') as Factor B. Three existing university protective structures were used as replications. Each structure measures 6 m wide × 47 m long made of rigid pipes and is covered with heat resistant plastic film.

Cultural management practices

The land was ploughed and harrowed thoroughly twice, using a draught animal. In the open field, each plot had a planting distance of 1.5×0.4 m. Plants grown under the protected structure, three plots were established for each structure. These were then divided into the seven treatments plots with two rows per plot. There was a 1 m gap between plots. Plot dimensions were 1×5 m with 10 plants row^{-1}. A sterilized soil mixture of garden soil, vermicast (worm castings), lime and sand with the ratio, 4:5:½:1. Plants were transplanted upon reaching the five-leaf stage, about 4-5 weeks after sowing.

For both the open field and protected structure, basal applications were carried out with a commercial organic fertilizer, 20 g mixed with 10 g of complete fertilizer (14-14-14) per hill. A side dressing of 10 g of complete fertilizer (14-14-14) was applied per hill at 14 days after transplanting (DAT). Five grams of urea (46-0-0) and 5 g of potash (0-0-60) per hill was applied at 21 DAT. Manual weeding was practiced regularly in both the open field and protected cropping to keep the treatment plants free from weeds.

Insect sprays of Lannate TM and Karate® and fungicide sprays of Daconil™ carried out weekly. A rate of 2-3 teaspoons of insecticide per 16 L of water starting at 15 DAT was carried out at weekly intervals. Likewise, fungicide at four teaspoons per 16 L of water applied once a week after 15 DAT. The rate of application changed, as necessary.

Trellising was carried out to support the plants and lateral branches to stop lodging using 2 m bamboo sticks set at an interval of 3 m in each plot. Nylon climbing trellises were tied from the bamboo sticks to support the plant.

First harvest was carried out at 61 DAT. Three harvests were carried out on the open field plots while tomatoes under the protected structures required five harvests. Tomatoes were harvested at mature green stage when cream coloured streaks were present at the blossom end. Fruits were classified as marketable (fruits free from disease, insect damage and mechanical injuries) and non-marketable (fruits affected by diseased, small fruit and those

fruit with mechanical injuries).

Data collection and analysis

Growth parameters were recorded for plant height (cm) and dry matter yield (g). Dry matter yield was obtained by taking two average sized sample plants per treatment for every replication. These were placed in labelled paper bags. The samples were oven-dried at 70°C until a constant weight was attained for about 48 h. Collected yield parameters included: fruit number and weight (g) with all fruit classified as marketable and non-marketable, average fruit weight (g) and total fruit yield (t ha^{-1}). Total fruit yield is measured by adding the total weight of both marketable and non-marketable fruit per plot.

Disease incidence such as *Tomato yellow leaf curl virus* (TYLCV) and Fusarium wilt was evaluated and recorded. The incidence was calculated using the equation shown below.

$$\% \text{ Disease Incidence} = \frac{\text{No.of infested plants}}{\text{Total number of plants inspected}} \times 100$$

The severity of these diseases was monitored, and rated weekly using the following criteria. Rating scale: 1 – no infection (none of the total plant population is infected), 2 – mild infection (1-25% of the total plant population are infected), 3 – moderate infection (26-50% of the total plant population are infected), 4 – severe infection (51-75% of the total plant population are infected), and 5 – very severe infection (76-100% of the total plant population are infected).

RESULTS AND DISCUSSION

Plant height

The plant height and total dry matter production increased under the greenhouse conditions compared to the open field system. These findings support research by Rana et al. (2014). Our findings also support te study of Heuvelink and Buiskool (1995) that improved daily light interception increases the sink source for plants, thereby increasing plant height. Protected structures allow plants to increase their light interception and photosynthetic responses compared to field plants. This is due to the interception of more light and heat in filed grown plants that can exceed their photosynthetic capacity. Therefore, plants in productive structures can achieve optimum growth performance compared to open field grown plants. Research has shown that total dry matter production increases for greenhouse tomatoes, and follows an almost linear growth pattern, resulting in taller plants (Heuvelink and Buiskool., 1995; Russell et al., 1989). The growth pattern of protected structure tomato plants under constant climatic conditions produce uniform stands of vegetation, often accumulate dry matter at a rate closely related to the rate at which foliage intercepts radiant energy (Russell et al., 1989). Furthermore, Russell et al. (1989) showed that dry matter partitioning was strongly influenced by the number of fruits on the plant and dry matter content can affect yield by as much as 15%.

The plant height results showed a significant interaction between the type of cultivation used and the tomato genotypes (Table 1). Significant variations occurred between the cultivars at 60 days after transplanting. The cultivars 'Harabas' and 'Diamante' recorded the tallest plant height (117.33 cm) while 'AVTO 1173' was the shortest in plant height at 105.67 cm. Plants grown under the protective structure were significantly higher in dry matter yield, at all growth stages (30, 45 and 60 DTA). The cultivar 'Sakata', constantly had the highest significant dry matter yield at all growth stages, with a yield of 261.33 g at 90 DAT. Meanwhile, cultivars 'Atlas' and 'AVTO 1173' had the lowest dry matter yield, ranging from 174.50 to 183.17 g, respectively.

Number and weight of fruit $plant^{-1}$

Tomatoes planted in the greenhouse had significantly greater fruit weight $plant^{-1}$ compared to those grown in the open field (Table 2). Among the genotypes, 'AVTO 1173' had

significantly higher yields (18.67 t ha^{-1}). The lowest yield of 8.54 t ha^{-1} was attained by 'Athena'. This is almost half the harvest for 'AVTO 1173'. For 'AVTO 1173', this can be attributed to the larger number and higher total fruit weight plant^{-1}, possibly due to its genetic makeup. As reported in the literature, average fruit weight increased when under controlled conditions, such as in greenhouse or protected structures (Rana et al., 2014). The size of fruits may also have influenced the yield as 'Athena' produced significantly smaller fruit, possibly due to its genetics and the environmental growing conditions.

Table 1. Comparison of greenhouse and open filed plant height and dry matter yield of tomato genotypes at 30, 60 and 90 days after transplanting at Claveria, Misamis Oriental, Philippines.

Treatments	Plant height (cm)			Dry matter yield (g plant^{-1})		
	30 DAT	60 DAT	90 DAT	30 DAT	60 DAT	90 DAT
Types of cultivation (A)						
Open field	84.76	99.09b	111.05b	110.62b	142.19a	85.24b
Greenhouse	84.62	125.52a	132.52a	211.14a	303.14a	342.57a
	ns	**	**	**	**	**
Tomato genotypes (B)						
AVTO 1173	85.16	105.67b	117.67	126.83c	187.83cd	174.50d
AVTO 1004	85.16	109.17ab	117.67	156.17b	212.50bc	210.00c
Harabas	85.00	117.33a	122.67	198.33a	219.50b	221.33bc
Athena	81.66	108.50ab	121.37	162.67b	217.83b	208.33c
Sakata	85.66	115.00ab	123.33	203.83a	255.33a	261.33a
Atlas	87.50	113.50ab	122.17	104.67c	183.83d	183.17d
Diamante	82.66	117.00a	127.83	173.67b	281.83a	238.67b
	ns	*	ns	**	**	**
A×B	**	**	*	**	**	**

DAT = days after transplanting.
** Significant at a level of 1% of probability (p<.01); * Significant at a level of 5% of probability (p<.05); ns = not significant (p>0.05).

Table 2. Comparison of harvested fruit number, yield per plant, and fruit weight plant^{-1} for tomato genotypes when grown in open field and under protected structures at Claveria, Misamis Oriental, Philippines.

Treatments	Number of fruit		Weight of fruit (kg)		Average fruit weight (g)	Average yield (t ha^{-1})
	Marketable	Non-marketable	Marketable	Non-marketable		
Types of cultivation (A)						
Open field	54.32b	19.41a	0.56b	0.43a	10.51b	11.28b
Greenhouse	66.95a	9.31b	0.86a	0.35b	13.23a	17.29a
	**	**	**	**	**	**
Tomato genotypes (B)						
AVTO 1173	77.10a	13.81bc	0.93a	0.34cd	12.04bc	18.67a
AVTO 1004	65.38b	14.52b	0.78b	0.44b	11.80c	15.60b
Harabas	47.99d	8.71d	0.73bc	0.32d	15.21a	14.63bc
Athena	59.71bc	30.72a	0.43d	0.61a	6.99d	8.54d
Sakata	54.49cd	8.80d	0.69bc	0.32d	13.80ab	13.89bc
Atlas	55.16cd	14.50b	0.65c	0.42bc	11.58c	13.11c
Diamante	64.64b	9.48cd	0.78b	0.27d	11.67c	15.57b
	**	**	**	**	**	**
A×B	**	**	**	**	**	**

** Significant at a level of 1% of probability (p<0.01); ns = not significant (p>0.05).

The number of fruit plant^{-1} was influenced by two factors, growing conditions and genetic makeup. The greenhouse plants produced the most marketable fruit compared with those subjected to the open field conditions. In addition, the cultivar 'AVTO 1173' consistently outperformed the other genotypes under both types of cultivation systems. 'AVTO 1173' together with 'Diamante' significantly had a higher number of fruits than the rest of the genotypes when grown under a protected structure (Table 2).

Weight fruit^{-1}

Like the yield attribute parameters, the average fruit weight was significantly greater for tomatoes planted in the greenhouse compared to the open field system (Table 2). Significant variations were observed in the protected cropping systems for the tomato genotype lines.

The average fruit weight was significantly different between the two factors (Figure 1). For the tomato genotypes yield performance was optimized under the greenhouse structure compared to the open field system.

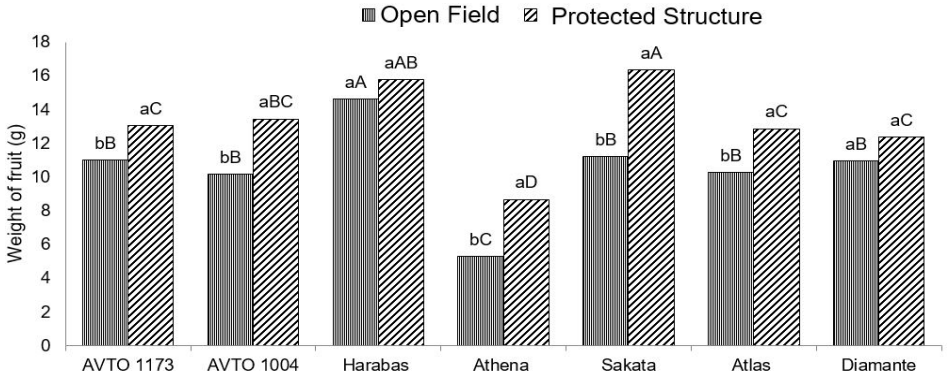

Figure 1. Average fruit weight for the tomato genotype cultivars trailed under greenhouse conditions at Claveria, Misamis Oriental, Philippines. Small case notations describe significant variations between type of cultivation whereas capital letters denote significant differences among tomato genotypes.

The increase in yield under the greenhouse systems and for genotypes was due to the number of fruits and the average weight of fruit plant^{-1}. Environmental stress is the primary cause of crop losses worldwide, reducing average yields for most major crops by more than 50% (Boyer, 1982). Reducing environmental stresses by using a greenhouse production system makes it possible to obtain higher yields in many locations.

Total yield

The tomato genotype cultivars grown under the protected structure were significantly different in terms of greater fruit yields plant^{-1} compared to the tomatoes genotypes planted in the open field system (Table 2). The greenhouse tomato genotype plants had an average yield of 17.29 t ha^{-1} while the open field tomato genotypes average yield was 11.28 t ha^{-1}.

Comparing the tomato genotypes, 'AVTO 1173' had a significantly higher yield (18.67 t ha^{-1}), while the 'Athena' tomato genotype cultivar had the lowest yield of only 8.54 t ha^{-1}. This is approximately half the harvested yield of 'AVTO 1173'. A significant interaction was observed between the treatments (Figure 2). Results showed the tomato genotype cultivar 'AVTO 1173' performed well under the greenhouse and open field systems. The tomato genotype cultivar 'Sakata', was the next best performer compared to the other tomato genotypes in the open field production system. The tomato genotype cultivar 'Athena' was the worst preforming cultivar under greenhouse and open field production systems.

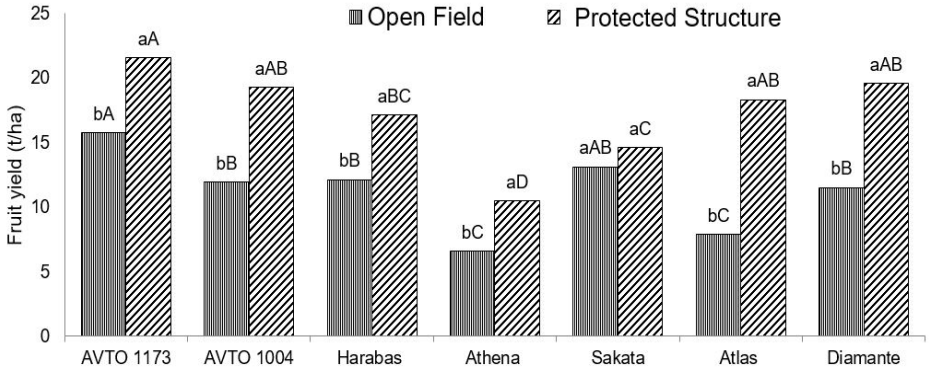

Figure 2. Comparison of the total fruit yield (t ha^{-1}) of the different tomato genotype cultivars grown under greenhouse conditions and the open field systems at Claveria, Misamis Oriental, Philippines. Small case notations describe significant variations between type of cultivation whereas capital letters denote significant differences among tomato genotypes.

TYLCV occurrence

The tomatoes genotype cultivars planted in the greenhouse production systems were significantly different in the incidence of TYLCV and Fusarium wilt. The average disease incidence (plant population infected) under the greenhouse system and open field production system was 32.62 and 80.95%, respectively (Table 3). Comparing the tomato genotypes, the cultivar 'Harabas', had the lowest incidence of TYLCV and Fusarium wilt. Furthermore, the tomato genotype cultivar 'Athena' had the highest incidence, 70.83% of the plant population infected with TYLCV and Fusarium wilt.

Table 3. Comparing the severity and incidence of TYLCV and Fusarium wilt in tomato genotypes in response to protected cropping and open field production systems at Claveria, Misamis Oriental, Philippines.

Treatments	Average disease severity	Average disease incidence (%)
Types of cultivation (A)		
Open field	1.55a	80.95a
Greenhouse	0.82b	32.62b
	**	**
Tomato genotypes (B)		
AVTO 1173	0.87b	54.17bc
AVTO 1004	1.20ab	58.33abc
Harabas	0.87b	45.83c
Athena	1.59a	70.83a
Sakata	1.42ab	68.00ab
Atlas	1.24ab	50.33c
Diamante	1.09ab	50.00c
	**	**
A×B	ns	ns

TYLCV and fusarium wilt severity rating: 0 – no infestation (none of the total plant population were infested), 1 – mild infestation (1-25% of the total plant population were infested), 2 – moderate infestation (26-50% of the total plant population were infested), 3 – severe infestation (51-75% of the total plant population were infested), 4 – very severe infestation (76-100% of the total plant population were infested).

** Significant at a level of 1% of probability ($p<0.01$); ns = not significant ($p>0.05$).

In terms of disease severity, greenhouse produced plants exhibited almost no infestation (0.82) while the open field had a mild infestation (1.55) (Table 3). The tomato genotype cultivar 'Athena' was most severely affected, with 'AVTO 1173' and 'Harabas' the least affected with TYLCV and Fusarium wilt in terms of severity.

The tomato genotype cultivars planted in the greenhouse had a lower disease incidence compared to the open field production system since foliage diseases are easier to control under protected cropping structures (Capuno et al., 2015). The moisture on the leaves in the open field production system, especially during heavy rains, is conducive to the motile bacterial wilt pathogens. Surface water run-off to other field areas also favours the dissemination of the water-borne inoculum to the area planted. However, inside protective structures, moisture extremes are regulated. Therefore, unfavourable to soil-borne pathogens incidence is lower (Ghorbani et al., 2009). In addition, reducing the periods of leaf wetness also creates less favourable conditions for diseases to infect fruit. Fruit are also protected from direct contact with soil, and weed growth is also reduced. In addition, rain splashing or flooding can help to disperse disease spores and infect plants (Rana et al., 2014). This is the reason why the TYLCV severity would be lessened under greenhouse production systems. The above factors support the results of the present study, that protected cropping creates a healthier environment for disease control for tomato plants compared to open field production system.

Reducing leaf wetness, creates less favourable conditions for diseases to infect. Under greenhouse conditions fruits are protected from direct contact with soil, have reduced weed growth, moderate soil and air temperatures, reduced leaching of nutrients from soils, optimize their yield performance (Ghorbani et al., 2009). In addition, clear plastic rain shelters prevent water logging and rain impact damage on developing fruits, improving tomato yields.

CONCLUSIONS

The potential of protected cropping compared to open field production systems revealed results that tomato genotypes cultivars grown under a structure have a higher productivity in terms of number and total fruit weight plant^{-1}. Results showed the highest fruit yield of 17.29 t ha^{-1} was achieved. The same tomato genotype cultivars grown in the open field production system yielded 11.28 t ha^{-1}. In addition the tomato genotype cultivars studied under protective cropping structures had statistically lower disease incidence (32.62%). The tomato genotype cultivars grown under the protective structures showed very minimal infestation of diseases (32.62%), less than a half compared to the open field production system (80.95%). Among the genotypes, fresh market tomato line 'AVTO 1173' proved to be a potential substitute, compared to other sources of planting material and the other commercial hybrids tested. 'AVTO 1173' consistently performed higher compared to the other genotypes, under both types of cultivation systems. It significantly produced the greatest number of marketable and highest total fruit weight plant^{-1}.

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Productivity of 'Robusta' coffee trees in response to different pruning systems in an acid upland soil

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Abstract

Pruning of coffee trees is one of the most important cultural practices vital to stimulating the production of new wood, intensifying the formation of flowers and fruit buds while keeping the tree at a manageable size to facilitate easy harvesting. The experiment aimed to evaluate yield increase and the profitability of rejuvenating 22-year-old 'Robusta' coffee trees in a plantation in an upland agroecosystem of Claveria, Misamis Oriental, Philippines. The study was laid out in a simple RCBD with five treatments which were replicated three times with eight trees per treatment per replication. Pruning was done by cutting all old stems at about 40 cm from ground level. The treatments were imposed as follows: PS0 AS – all water sprouts allowed to grow; PS1 2Sdb – two vigorous water sprouts allowed to grow and maintained at approximately 1.5 m height; PS2 4Sdb – four vigorous water sprouts were maintained at 1.5 m height; PS3 2S – two vigorous water sprouts were allowed to grow to their full height; and PS4 4S – four vigorous water sprouts were allowed to grow to their full height. Allowing all water sprouts to grow (PS0 AS) had significantly greater responses when compared with the other pruning treatments. Rejuvenation using the PS0 AS and PS3 2S pruning systems can be considered as a suitable mechanism for reviving and extending the productive life of old coffee trees. Return on investment (ROI) analysis after the fourth year of pruning showed that the PS0 AS treatment, followed by PS3 2S treatment, gave the highest values which were associated with greater profitability.

Keywords: rejuvenation, 'Robusta', ROI, water sprouts, Claveria, Misamis Oriental

INTRODUCTION

Coffee is a member of the *Rubiaceae* or Madder family. It is by far the most economically important plant species globally. After oil, coffee is the most valuable traded commodity worldwide, with global retail sales estimated to be US$ 90 billion. Coffee has been one of the most important plantation crops in the Philippines since its introduction in 1740 in Lipa, Batangas. As a food commodity, coffee is highly valued in local and foreign markets. The Philippines was one of the world's top ten coffee producers. However, since 1980, the world's coffee production has continued to fall behind demand for coffee and coffee products and the decrease in coffee production in the Philippines can be attributed to factors such as a lack of government support and poor crop management within the coffee farms.

In the Philippines, the bulk of coffee production is found in Mindanao. Among the cultivars produced are 'Arabica', 'Robusta', 'Excelsa' and 'Liberica'. At present, coffee production in the Philippines is 17,220 t and the SOCCSKSARGEN region was the highest coffee producer with 31.9% of total production. 'Robusta' coffee is currently the most produced, which accounted for 69.2% of total coffee production (Philippines Statistics Authority, 2020).

In Region X (Northern Mindanao), 'Robusta' coffee has been a traditional crop for over 70 years. It presently occupies an area of 11,600 ha with a total production of 5,695 t (Philippines Statistics Authority, 2018). Claveria, located in the southern upland part of Northern Mindanao, is noted for its relatively high production levels of vegetables, corn and specific permanent crops such as coffee.

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Pruning is one of the most important cultural practices for coffee trees. It is considered to be an important technique for stimulating the formation of flowers and fruit buds. The most effective pruning is done early in the season (March-December), when the buds begin to break and the soft tissue can be pinched off. The farmer must prune correctly for efficient annual production and the maintenance of healthy trees. Pruning is vital to stimulating the production of new wood and keep the tree to a manageable size. Appropriate mechanized pruning strategies vary by cultivar, location, age of trees, and cultural practice (Gautz et al., 2002).

The University of Science and Technology of Southern Philippines (USTP) - Claveria Campus, located in the upland of Claveria, has had an existing 1.5 ha area of Ivory Coast 'Robusta' coffee trees for 22 years, giving an average income of PhP 32,000 year^{-1} with less than 600 g tree^{-1} year^{-1}. The trees were rejuvenated 8 years ago and these trees are now typically 6-8 m in height, resulting in the coffee berries being difficult to harvest without the expensive use of ladders. The height of trees also results in a significant number of unharvested berries, which are then susceptible to coffee berry borer contributing to the low production overall. The University through its Research, Development and Extension Office, supported the development of technology on rejuvenating old coffee trees with the aim of increasing yield and profitability. The development of successful new technologies would help the development of the coffee industry in this region.

MATERIALS AND METHODS

Location and duration of study

The study was conducted in an existing 22-year-old 'Robusta' coffee plantation at the USTP. The experimental site was an area of 1.5 ha, where the last recorded rejuvenation was 8 years previously. The study was conducted over 4.5 years, where the first harvest commenced 18 months after imposition of the different pruning systems and continued for 54 months, in order to evaluate the productivity of the rejuvenated coffee trees in response to the treatments.

Site characteristics

The soil in Claveria, Misamis Oriental is classified under the Jasaan Series, fine mixed isohyperthermic, Ultic Haplorthox (Mercado, 2007). The site is acid upland (pH<5.0), located in a slightly sloping area. Parent materials are derived from residual andesite, basalts and pyroclastic materials (volcanic). Drainage is good to excessive (external) and internal is fair. Vegetation in the area consists of native grasses, broadleaved and sparse fruit trees.

Pruning technique employed

The various pruning systems were implemented on May 1, 2008. A hand-held chainsaw was used for pruning. All stems of the 22-year-old trees were pruned at about 30-50 cm from ground level. Immediately after pruning, a motor oil and paint mixture was used as a protectant against fungal infection on the cut surface. Water sprouts emerged on each cut stem within about two months after pruning.

Experimental design and treatments

The study was laid out in a simple randomized completely block design (RCBD), with five treatments each replicated three times with eight trees designated as sample trees per treatment per replication. Spacing between trees was 2×2 m on a square planting system or about 2,500 trees hill^{-1} ha^{-1}, the assumed population density. Pruning treatments imposed were as follows:

1. PS0 AS: control – all old stems/branches were cut and all waters sprouts were allowed to grow without removal of apical buds and without re-pruning from the start of rejuvenation;
2. PS1 2Sdb: modified cavite pruning system – all old stems/branches were cut; only two vigorous water sprouts were selected and allowed to grow and be maintained; apical

buds were de-budded at approximately 1.5 m stem height and repeatedly de-budded every other month at the same height;

3. PS2 4Sdb: all old stems/branches were cut; four vigorous water sprouts were selected and allowed to grow and be maintained; apical buds were de-budded at approximately 1.5 m stem height and repeatedly de-budded every other month at the same height;
4. PS3 2S: all old stems/branches were cut; two vigorous water sprouts were selected and allowed to grow throughout the study duration without removal of apical buds;
5. PS2 4S: all old stems/branches were cut; three or four vigorous water sprouts were selected and allowed to grow throughout the study duration without removal of apical buds.

where PS = pruning system, A = all stem, S = sprout, and Sdb = debudding.

Care and maintenance

Herbicide (glyphosate) was sprayed periodically to control weeds. Extra care was observed in spraying to avoid contacting the newly-emerged water sprouts with spray drift. In addition, pesticides were used if pest and disease presence was detected above the economic threshold level.

Lime and fertilizer application

Applications of lime, organic and inorganic fertilizers were applied usually at the onset and end of the rainy season. Ammonium phosphate (16-20-0) and muriate of potash (0-0-60) were applied per hill based on soil analysis. Fertilizer sources were applied to the upper portion of the hills. Similarly, coffee pulp and corn cobs were spread out on the upper portion of the hills while at the same time serving as a mulch.

Harvesting

At about 18 months after rejuvenation, harvesting of berries started and was repeated every yearly cycle for four years or for 54 months after the initial pruning (MAP). Only mature berries (drupes) were picked at harvest. Harvested berries were immersed per treatment in tap water. Sample of berries was immediately de-pulped, dried and placed in a bag per treatment. Classification of marketable and non-marketable green beans then followed. Sorting of beans into marketable and nonmarketable classes was done following the standard criteria with marketable beans being free from defects and insect damages, no black color, the required size and without any brokenness.

Measurements

Parameters gathered include proportions of marketable and non-marketable berries, fresh and dried weights, numbers of fruiting woods, numbers of internodes, tree height and length of fruiting wood. Return on investment (ROI), as an index of profitability, was also determined using actual field data on labor and input costs.

Statistical analysis

Statistical analyses were done using ASSISTAT (Version 7.7 Beta). Analysis of variance (ANOVA) and the least significance difference (LSD) test were used to compare the differences among treatment means.

RESULTS AND DISCUSSION

Marketable beans

The first harvesting was done 18 months after the different pruning systems had been imposed. At this time, the PS3 2S and PS4 4S treatments both had the highest marketable yields (Table 1). In contrast, marketable beans obtained from the PS0 AS treatment had the lowest yield. During the second year, 30 MAP, all treatments except PS3 2S had statistically similar yields. PS3 2S also had the lowest yield of marketable berries when yield was

computed on a per tree per hill basis. In the third (42 MAP) and fourth (54 MAP) years, the PS0 AS treatment produced the highest marketable yield which differed from the rest of the treatments. Morais et al. (2012) previously concluded that the economical operation of coffee plantations was intrinsically linked to the efficiency of the pruning system, which could improve yields and ensure the longevity of a plantation.

Table 1. Weight of dried marketable beans of 'Robusta' coffee following different pruning treatments in an acid upland soil.

Pruning treatments	Weight of marketable beans (kg ha^{-1})			
	18 MAP	30 MAP	42 MAP	54 MAP
PS0 AS	414.2bcB	183.8aD	305.9aC	885.5aA
PS1 2Sdb	478.5bA	181.3aB	164.6bB	227.5dB
PS2 4Sdb	363.3cA	150.1abB	179.0bB	325.0cA
PS3 2S	551.9aB	88.2bD	188.3bC	645.0bA
PS4 4S	569.7aA	169.7aC	170.5bC	345.0cB

In a column or a row, means having the same letter are not significantly different at 5% level of significance by LSD. Summary of mean difference for columns and rows were designated with lower case and uppercase letters, respectively.
MAP – months after pruning.

When yield was compared on a yearly basis, a high marketable yield tree^{-1} was obtained during the first year which can be attributed to more vigorous growth in that year. The periodic stresses which were incurred during the removal of water sprouts and other vegetative parts may have contributed to the variability that was shown in marketable yield. Also, the results obtained are generally consistent with the typical biennial bearing habit of the crop, wherein a typical light bearing year is commonly followed by a heavy crop in the succeeding year. However, the presence of coffee borer also impacted strongly on these results.

Beaumont and Fukunaga (1958) termed this variation in year-to-year fruiting behavior alternate bearing – the habit of the coffee tree to produce a heavy crop in one year followed by a light crop in the second year. Several physiological and morphological reasons have been cited in different studies for this trend. Because growing wood is being produced while the fruit is maturing, there is believed to be a limitation in the supply of carbohydrates and/or mineral nutrients to the growing wood and the developing fruit. As a result, when the tree has a high yield of fruit, little growing wood is produced (DaMatta et al., 2007). Hence, the following year's crop is produced on the current year's growing wood, resulting in a small crop the following year. In contrast, if the crop is small in the current year and the tree is able to produce adequate growing wood, the result will be a larger crop in the ensuing fruiting season.

As observed, during the third year (30 MAP) and fourth year (42 MAP) of the experiment, a heavy incidence of coffee borer affected the quality and quantity of the harvested berries.

Fresh berries

Weight of harvested fresh berries varied markedly from year-to-year in all treatments (Table 2). In the first harvest at 18MAP, the weight of fresh berries was highest in the PS3 2S and PS4 4S treatments. It was lowest in the PS2 4Sdb and PS0 AS treatments. The weight of fresh berries in the PS0 AS treatment consistently increased from year-to-year after rejuvenation. In contrast, the weight of fresh berries progressively declined in the PS3 2S treatment. Highest mean yields were obtained in the PS0 AS and PS4 4S treatments.

Yield

In the first year of harvest (18 MAP) treatments PS3 2S, PS4 4S and PS1 2Sdb had a greater weight of dried berries ha^{-1} compared to the PS2 4Sbd and PS0 AS treatments (Table 3). In the second (30 MAP) and third years (42 MAP) after pruning, all treatments except PS0

AS recorded a decrease in the weight of dried berries as compared to the first year following pruning. However, a significant increase in yield was recorded for treatments with modified pruning systems (PS1 2Sdb, PS2 4Sdb, PS4 4S, and PS3 2S) between the third and fourth year (54 MAP) of harvest, except for PS0 AS which had a decrease. Among the various pruning systems and harvesting years, PS0 AS and PS4 4S had the highest quantity of dried berries with the latter showing more consistency in yield.

Table 2. Weight of marketable and unmarketable fresh berries of 'Robusta' coffee following different pruning treatments in an acid upland soil.

Pruning treatments	Weight of fresh berries (kg ha^{-1})			
	18 MAP	30 MAP	42 MAP	54 MAP
PS0 AS	822.0cC	872.0cC	1382.0aB	1604.0bA
PS1 2Sdb	1231.8bA	1110.0abB	612.0dC	1272.0cA
PS2 4Sdb	825.6cC	1198.0aB	720.0bcC	1744.0aA
PS3 2S	1358.0aA	818.0cB	812.0bB	306.0cA
PS4 4S	1332.0abB	1042.0bC	650.0cdD	1700.0abA

In a column and rows, means having the same letter are not significantly different at 5% level of significance by LSD. Summary of mean difference for columns and rows were designated with lower case and uppercase letters, respectively.
MAP – months after pruning.

Table 3. Yield of dried 'Robusta' coffee berries following different pruning treatments in an acid upland soil. Marketable and unmarketable berries are included in these values.

Pruning treatments	Yield (kg ha^{-1})			
	18 MAP	30 MAP	42 MAP	54 MAP
PS0 AS	290.4bC	334.0bcC	642.0aA	556.0abB
PS1 2Sdb	458.2aA	416.0abA	232.0bB	436.0cA
PS2 4Sdb	303.6bC	400.0abB	274.0bC	596.0aA
PS3 2S	514.0aA	284.0cB	288.0bB	478.0bcA
PS4 4S	494.0aB	426.0aB	252.0bC	590.0aA

In a column and rows, means having the same letter are not significantly different at 5% level of significance by LSD. Summary of mean difference for columns and rows were designated with lower case and uppercase letters, respectively.
MAP – months after pruning.

Productive lateral branches and internodes

Across pruning treatments imposed, PS0 AS, PS1 2Sdb and PS3 2S had statistically similar lateral branch numbers (Table 4). The PS1 2Sdb and PS3 treatments produced the longest productive lateral branches. In contrast, trees in the PS0 AS treatment had the shortest productive lateral branches, which was a consequence of allowing all water sprouts to grow following pruning. Fruiting on coffee trees occurs on the lateral branches where flower bud development occurs. The length and numbers of laterals are, therefore, critical factors in determining the size of harvest. New growth on laterals, also known as growing wood, supports fruit clusters which appear at the nodes of this new growth which usually grows at approximately 18 MAP. Harvest size of the following year's crop depends on how much growing wood is produced during the current year, or, more exactly, on the number of new nodes on the lateral shoots (DaMatta et al., 2007).

The number of internodes was counted during the second and fourth year after imposition of the different pruning treatments (Table 4). The treatments used failed to influence the number of internodes during the first two years after pruning. However, after almost four years (54 MAP) PS1 2Sdb and PS0 AS had the greatest numbers of internodes while PS2 4Sdb had the lowest number. When two vigorous water sprouts were allowed to grow and then maintained at approximately 1.5 m in height (PS1 2Sdb), the number of nodes

per fruiting wood length was comparable to the treatment where all water sprouts were allowed to grow (PS0 AS) and then pruned to allow four vigorous water sprouts to grow at their maximum height. The greatest mean number of internodes occurred in treatment PS1 2Sdb which was comparable to PS4 4S.

Table 4. Number and length of productive lateral branches and number of internodes measured at the end of the first and fourth years after the imposition of various pruning treatments.

Pruning treatments	No. of productive lateral branches	Length of productive lateral branches (cm)		No. of internodes	
		18 MAP	54 MAP	18 MAP	54 MAP
PS0 AS	60.4a	87.4bB	143.6dA	13.0aB	26.2abA
PS1 2Sdb	57.6ab	101.6abB	184.0aA	15.6aB	29.4aA
PS2 4Sdb	50.6b	100.8abB	152.0cdA	14.4aB	21.2dA
PS3 2S	54.4ab	106.4aB	167.0bcA	16.0aB	22.8cdA
PS4 4S	52.6b	103.8aB	170.8abA	15.6aB	25.0bcA

In a column and rows, means having the same letter are not significantly different at 5% level of significance by LSD. Summary of mean difference for columns and rows were designated with lower case and uppercase letters, respectively.
MAP – months after pruning.

The number of internodes per length of fruiting wood appears to be a major morphological determinant or feature of a good species genotype. It also indicates the suitability of management practices applied in the plantation. In addition to the number of fruiting woods developed per tree, the number of internodes produced per length of fruiting wood was highly associated with yield tree^{-1} (DaMatta et al., 2007). The productivity of coffee trees could, therefore, be highly influenced by the number and length of fruiting wood which together determine the number of nodes at which flowers are produced and to the number of flowers and fruits produced at each node.

Tree height

During the first year, there were no significant differences in tree height among the different treatments (Table 5). The treatments showed that plants that had four and two vigorous water sprouts per plant, as imposed for PS4 4S and PS3 2S treatments, respectively, had similar heights at 24 MAP. Height is crucial in coffee, since taller cultivars or strains or management techniques that lead to taller plants, may impose shading within the tree and create a suitable microclimate for potential buildup of pests and diseases (Oberthur et al., 2012).

Table 5. Height of coffee trees taken at different intervals after imposition of different pruning treatments.

Pruning treatments	Tree height (cm)			
	6 MAP	12 MAP	18 MAP	24 MAP
PS0 AS	95.6aD	133.0aC	150.2aB	181.4aA
PS1 2Sdb	91.4aC	125.8aB	140.4aA	138.6bA
PS2 4Sdb	83.6aD	123.8aBC	141.0bCD	146.2bA
PS3 2S	89.0aC	125.4aB	147.8aA	143.4bA
PS4 4S	91.8aC	134.4aB	162.4aA	159.2abA

In a column and rows, means having the same letter are not significantly different at 5% level of significance by LSD. Summary of mean difference for columns and rows were designated with lower case and uppercase letters, respectively.
MAP – months after pruning.

Economic analysis

An economic analysis was carried out on the different treatments using actual data for prices of marketable beans and costs of labor and inputs at the trial site. Indices were computed across the pruning systems for the four-year experimental period (Table 6). Due to the apparent decline in the proportion of marketable beans during the second year (18 MAP), an erratic relationship for several of the profitability measures was noted. Negative net incomes were noted especially at the second (30 MAP) and third years (42 MAP) of the study duration which were mainly attributed to a major decline in the amount of marketable beans in those years. The ROI value is commonly regarded as a stable and good indicator of economic profitability. ROI values obtained in the fourth year (54 MAP) in particular showed that the PS0 AS treatment gave the highest return.

Table 6. Economic analysis of different pruning systems employed in 'Robusta' coffee in Claveria, Misamis Oriental.

Harvest period		Gross return (PhP)	Total cost (PhP)	Net income (PhP)	Return on investment (ROI)
18 MAP	PS0 AS	24055.50	16453.12	7602.38	0.46
	PS1 2Sdb	27750.10	21921.88	5828.22	0.27
	PS2 4Sdb	21068.50	21921.88	(853.38)	-
	PS3 2S	32013.10	21921.88	10091.22	-
	PS4 4S	33043.00	21921.88	11121.88	-
30 MAP	PS0 AS	12866.00	16453.12	(3587.12)	-
	PS1 2Sdb	12689.60	21921.88	(9232.28)	-
	PS2 4Sdb	10508.40	21921.88	(11413.48)	-
	PS3 2S	6170.50	21921.88	(15751.38)	-
	PS4 4S	11875.50	21921.88	(10046.38)	-
42 MAP	PS0 AS	25243.35	16453.12	8790.23	0.53
	PS1 2Sdb	13575.38	21921.88	(8346.50)	-
	PS2 4Sdb	14769.15	21921.88	(7152.73)	-
	PS3 2S	15547.12	21921.88	(6374.76)	-
	PS4 4S	14067.90	21921.88	(7853.98)	-
54 MAP	PS0 AS	67740.00	16453.12	51286.88	3.11
	PS1 2Sdb	17403.75	21921.88	(4518.13)	-
	PS2 4Sdb	24862.50	21921.88	2940.62	0.13
	PS3 2S	49342.50	21921.88	27420.62	1.25
	PS4 4S	26812.50	21921.88	4890.62	0.22

Actual prices of coffee beans in Philippines Pesos (PhP): Year 1 (PhP 58 kg^{-1}), Year 2 (PhP 70 kg^{-1}), Year 3 (PhP 82.5 kg^{-1}) and Year 4 (PhP 76.5 kg^{-1}), respectively.

Labour and input costs were based on the actual figures incurred in the study and were based in the prevailing rates at the study site.

CONCLUSIONS

Different pruning systems were employed on 22-year-old 'Robusta' trees, planted in an acid upland agroecosystem, over a 4.5-year period. Coffee trees which had been subjected to a pruning system where all branches were cut and then all water sprouts were allowed to grow without removal of apical buds and without repruning from the start of rejuvenation (PS0 AS), had the best agronomic responses when compared with all other pruning treatments. Rejuvenation can be considered as a mechanism to revive and lengthen the productive life of a coffee plantation. ROI values were highest using the control pruning system (PS0 AS). The pruning system where all old stems/branches were cut-off and where two shoots were allowed to grow (PS3 2S), also had a high ROI but only at 54 MAP.

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Yield and postharvest performance of green onion (*Allium fistulosum* L.) as influenced by planting distance and fertilizer application

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Abstract

Green onion (*Allium fistulosum* L.) is known throughout the world by its culinary and medicinal properties. Its production, therefore, must be maximized through appropriate cultural management practices, including the use of optimum population density per unit area and appropriate fertilizer management. This study was conducted to evaluate the performance of green onion in response to plant density, to determine suitable fertilizer material that would optimize growth and productivity per unit area, and also to assess its postharvest quality. The experiment was laid out in a split-plot design in RCBD with three replications. The main plot consisted of the type of fertilizer material (no application of fertilizer, organic fertilizer, vermicast, and inorganic fertilizer at recommended rate) and the sub-plots were four different planting distances using a single row planting of 25×15, 15×15, 10×15 and a double-row 10×15×15 cm planting as practiced by farmers. Results revealed that the population density that maximized onion grown was a 10×15 cm spacing. This had a significantly increased plant height, number of suckers per hill and total yield ha^{-1}. Application of vermicast resulted in a significant increase in, weight of bunch per hill and yield compared with other fertilizer materials. However, postharvest quality assessments showed that application of organic fertilizers significantly increased percent weight loss six days after harvest compared to plants applied with either inorganic fertilizer or with no fertilizer application, both of which had significantly lower percent weight loss. Highest return on investment (ROI) was found at the combined use of recommended dose of synthetic fertilizer and 15×15 cm planting distance to obtained an ROI of 2.59 pesos per 1-peso investment.

Keywords: *Allium fistulosum,* postharvest, shelf-life, population density

INTRODUCTION

Allium fistulosum L. (green onion, Welsh onion and Japanese bunching onion) belongs to the family *Liliaceae*; popularly known as *Mizo-purun* in Mizoram, India; and widely grown and consumed in East/Southeast Asian countries. It originated in Asia, probably in the region of Mongolia, Siberia or north-western China (Friesen, 1995). It is commonly called as *sibuyas dahon* in the Philippines.

Green onion is known for its fairly moderate smell of onion and leek. Throughout the ages it has been used to flavor soups, steamed-boils, fries, vegetables, salads, dals and other cooked products (Nencini et al., 2007). It is also reported to help in recovery from eyesight problems, common colds, headaches, heart problems, wounds and festering sores; to reduce fat accumulation and serum lipid concentrations; and root exudates in the soil root-zone have anti-termite, anti-fungal and anti-microbial activities (Singh and Ramakrishna, 2017; Chang et al., 2013).

Green onions are tolerant to abiotic stresses (excess moisture, drought and high humidity), biotic stresses (neck rot, leaf blight, pink root disease, smut, anthracnose, downy mildew, Fusarium basal rot, thrips, onion fly and *Onion yellow dwarf virus*); and are good

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sources of total soluble solids and folic acid (Singh and Ramakrishna, 2017). The antioxidant activity of *Allium* species is due to a variety of sulfur-containing compounds and their precursors, and the onions are high in polyphenols, dietary fiber and microelements (Nencini et al., 2007). Extracts of *A. fistulosum* have antimicrobial activity, being active against *Bacillus sublilis*, a common foodborne pathogen (Chang et al., 2013).

Cultivation of green onions has increased due to market demands and insufficient supply. Green onion grows best under acidic conditions (pH 5.5-6.5), in well drained heavy soils, under irrigated, humid, long-day conditions. It tends to bolt profusely in short-days which is ultimately detrimental to leaf growth, quality and aroma (Singh and Ramakrishna, 2017). Proper plant spacing increases air flow and reduces both blight and purple blotch. Current field spacings and related plant populations are those that maximize yield and quality without unduly increasing production costs. Bautista and Mabesa (1989) found that as plant population per unit area increased, the yield per unit area also increased until the spacing was so close that excessive competition between rows and in rows among plants occurred. With growers, a wider spacing is sometimes needed where the plant requirements for moistures and nutrients cannot be met at closer spacings, and where large vegetables are desirable or where the variety requires a wide spacing. Existing production practices recommended by the Department of Agriculture (in the Philippines) for green onion and those currently practiced by farmers vary greatly.

Organic fertilizers typically contain lower concentrations of major nutrients, but a wider variety of micronutrients. In contrast, inorganic fertilizers typically contain much higher concentrations of all of the nutrients, and so very precise application is needed in order to avoid damaging the crop.

Green onion is one of the commonly produced commodities in Claveria, Misamis Oriental, the Philippines, particularly in high elevation areas. Cultivation has increased due to market demand but there is still insufficient supply. The perishability of the commodity adds to its marketing problems. With these shortfalls, developing a package of technology for green onion production from the producers and through the postharvest chain to the market is needed in order to provide support to the local industry.

This study intends to enhance the knowledge of farmers relating to good growth and yield performance of green onion in response to different planting distances, and the application of suitable inorganic and organic fertilizers.

MATERIALS AND METHODS

The study was conducted at the Research Station of the USTP Claveria campus in Claveria, Misamis Oriental with an elevation of 650 m a.s.l. The soils in the upland areas of Claveria, which are derived from pyroclastic materials, are deep, well-drained and represent most of the acid uplands in Southeast Asia.

Experimental design and treatments

The experimental layout used was a split-plot design with three replications. The sub-plot was the four different planting distances (25×15 cm – 3 rows, 15×15 cm – 5 rows, 10×15 cm – 7 rows, and 10×15×15 cm – 6 rows). The main-plot was the four different fertilization treatments as follows: T1 = no application of fertilizer (or control); T2 = application of organic fertilizer at a rate of 15 t ha^{-1}; T3 = application of vermicast fertilizer at a rate of 15 t ha^{-1}; T4 = application of recommended doses of inorganic fertilizers (NPK).

Cultural management and practices

A land area for the 48 plots, measuring 1.0×2.5 m $plot^{-1}$, was prepared for the study. The experimental field was plowed and harrowed thoroughly until the soil was well pulverized. After plots were outlined, a basal application of vermi compost fertilizer (20 g $hill^{-1}$) was applied and thoroughly mixed into the soil. The plots were levelled and irrigated manually to achieve uniform moisture before planting.

Planting material (suckers) was sourced from Malagana, Claveria, Misamis Oriental, where a large production of green onions can be found. Suckers of good appearance and at

least 5 mm diameter were chosen. One green onion sucker was planted on each hill about 5 cm deep. The number of hills established in each plot depended on planting distance, as follows: B1 = 25×15 cm, 50 hills plot^{-1}; B2 = 15×15 cm, 83 hills plot^{-1}; B3 = 10×15 cm, 116 hills plot^{-1}; B4 = 10×15×15 cm, 100 hills plot^{-1}.

Treatments involving inorganic, single source fertilizer for NPK were applied as four split applications. The first application was applied 15 days after planting (DAP), the second at 30 DAP, the third at 45 DAP and the remaining application at 60 DAP. Applications of organic fertilizer were split into two portions, one-half was applied at 15 DAP and the remaining portion was applied at 30 DAP.

Water was applied immediately after planting and then as necessary during early morning or late afternoon, especially if there had been no rain.

Onions are weak-rooted and aggressive weeds can severely reduce yields. Removal of weeds was done in every two weeks by hand. Careful cultivation was used to avoid damage to the test crop. Prevention and control measures against insect pest and diseases were done by immediately removing any diseased leaves and spraying the plants with pesticides only when necessary.

Data gathered

Plant height (cm) was determined 75 days from planting. Ten sample plants were measured randomly from each plot from the base of the leaf sheath to the tip of the longest leaf.

Five sample plants per plot were selected for gathering the relative chlorophyll content using a chlorophyll meter (SPAD-502 Minolta, Japan). Average relative chlorophyll value per plant was determined by measuring the middle portion of each of the selected three fully expanded leaves per plant between 8:00 to 11:00 am at 30 and 60 days after planting (DAP). Moreover, three plants from each plot were collected randomly to determine dry matter accumulation. These samples were dried at 70°C in an oven for 24 h until a constant weight was attained.

A stem diameter greater than 5 mm was recognized as marketable yield. Ten sample plants per plot were measured to determine this parameter.

The freshly harvested green onions were stored in plastic crates under ambient conditions. Weight loss was measured daily until leaves exhibited a 50% weight loss. Weight loss was expressed as a percentage of the initial weight.

All inputs, including planting materials, fertilizers, pesticides, and labor among others were recorded, together with sales data in order to compute the return on investment. All data obtained were statistically analyzed using analysis of variance (ANOVA). The Tukey test was used to determine significant differences among treatments means at the 5% ($p<0.05$) level of significance.

RESULTS AND DISCUSSION

Plant height

Plots supplied with organic fertilizer resulted to tallest plant height. However, these were statistically comparable to plants applied with vermicast or inorganic fertilizer (Table 1) while the control (no fertilizer applied) plants were significantly shorter. Plant height was not affected by planting distance. These results are in contrast to those of Yassen and Khalid (2009) who showed that all organic fertilizer treatments improved vegetative growth characters, essential oil concentration, some of the main constituents of essential oil, and NPK contents. Biru (2015) showed the closer spacing resulted in competition for nutrients and light and thus resulted in plants that were short while the wider spaced plants had adequate space for their growth and development.

Relative chlorophyll content

There were no significant effects of fertilizer application or planting distance on relative chlorophyll content (SPAD) at 30 DAP. However, SPAD reading for plants applied with

vermicast and inorganic fertilizer at 60 DAP showed the highest content (Table 1). Application of organic fertilizer resulted in a comparable SPAD reading to those plants with no fertilizer at 60 DAP. Significant changes in crop chlorophyll content were previously positively related to increases in plant height, number of leaves and dry weight/plant in onion (Yaso et al., 2007; Ewais et al., 2010). As changes in chlorophyll content are regarded as a relatively late and independent mechanism of photosynthetic adaptation (Alonso et al., 2002), It appears that several biologically-active constituents of photosynthesis have been affected by the fertilizer treatments used in this study.

Table 1. Summary table of agronomic and physiological parameters of green onion as influenced by planting distance and fertilizer application.

Treatments	SPAD		Plant weight (g)		Plant height (cm)
	30 DAP	60 DAP	Fresh	Dry	75 DAP
Factor A (fertilizer application)					
Control	61.58	54.51b	74.81	6.90	45.40b
Organic fertilizer	69.98	62.97ab	107.67	8.33	52.29a
Vermicast	66.40	71.18a	113.25	8.64	52.03a
Inorganic Fertilizer (RR)	67.49	65.91a	100.17	7.39	51.98a
F-test	ns	**	ns	ns	**
Factor B (planting distance)					
25×15 cm	67.77	63.90	103.86	7.95	50.46
15×15 cm	67.48	62.89	103.47	8.31	50.12
10×15 cm	64.85	65.46	95.61	7.55	50.94
10×15×15 cm	65.34	62.32	92.94	7.44	50.18
F-test	ns	ns	ns	ns	ns
A×B					
F-test	ns	ns	ns	ns	ns
CV (%)					
A	14.92	10.98	37.78	21.41	3.96
B	12.49	6.55	19.09	21.08	3.13

** Significant at p=.01, * significant at p=.05, ns = not-significant.

Plant weight

Bautista and Mabesa (1989) explained that when the spacing of a number of vegetable crops is too close, excessive competition between rows and within rows occurs resulting to yield reductions. However, no significant differences in plant weight were observed among either the fertilizer or the spacing treatments treatment means in this study (Table 1). Sangoi (2001) suggested that the use of high populations increases interplant competition for light, water and nutrients which results to inhibited growth and productivity. However, competition may not be as severe where leaves are usually erect, such as with onion. Further, in onion large amount of dry matter as both leaf blade development and as storage root growth can continue almost indefinitely, providing continuously available sinks (Tei et al., 1996).

Number of suckers

The number of marketable suckers produced were not significantly affected by either fertilizer or planting distance treatments (Table 2). However, the number of non-marketable suckers produced was significantly affected. The control plots had the highest number of unmarketable suckers which were comparable to values from the organic fertilizer treatment (Table 2). The planting distance of 10×15×15 cm produced the lowest number of unmarketable suckers, which is consistent with that used in farmer's practices. These results are in contrast to those of Guzman (2011) who reported that the wider the spacing, the higher

the number of suckers produced.

Table 2. Yield parameters of green onion as influenced by planting distance and fertilizer application.

Treatments	No. of suckers per hill		Weight of bunch per hill (g)		Yield (t ha^{-1})
	Marketable	Non-marketable	Marketable	Non-marketable	
Factor A (fertilizer application)					
Control	5.76	5.16a	70.26c	15.00b	5.94b
Organic fertilizer	6.74	4.49a	106.85a	17.00a	7.81ab
Vermicast	6.79	2.12c	111.05a	8.50c	8.02a
Inorganic fertilizer (RR)	6.92	3.62b	91.21b	9.16c	6.85ab
F-test	ns	**	**	**	*
Factor B (planting distance)					
25×15 cm	6.39	4.20ab	102.95a	15.25a	4.41c
15×15 cm	7.10	4.65a	97.65a	11.00b	6.40b
10×15 cm	6.36	4.65a	90.75b	15.33a	9.66a
10×15×15 cm	6.35	1.90b	88.02b	8.08c	8.16a
F-test	ns	**	**	**	**
A×B					
F-test	ns	**	**	**	ns
CV (%)					
A	24.19	15.74	4.82	4.90	19.29
B	20.60	15.92	5.79	10.86	20.03

** Significant at p=.01, * significant at p=.05, ns = not-significant.

Weight of bunch per hill

The weight of a marketable bunch per hill was strongly affected by the fertilizers that had been applied (Table 2). Vermicast and organic fertilizer produced the heaviest marketable suckers per hill and organic fertilizer also produced the heaviest non-marketable weight per bunch. Plots with lowest plant population density recorded the heaviest marketable onions per hill, while those at 10×15×15 cm produced both the lowest marketable and the lowest non-marketable weights. This is similar to the findings of Walle et al. (2018) for the yield of onion bulbs (*Allium cepa*) planted in north western Ethiopia where the number and yield of marketable onion bulbs tended to decrease at higher plant densities.

Yield ha^{-1}

All fertilizer treatments produced similar yields except for the control plots which had the lowest yield (Table 2). In terms of yield per area, the highest populations had the highest yields per area as shown by the spacings at 10×15 and 10×15×15 cm. Some authors reported that most of the crops tend to increase yield per unit area as plant populations increase, but only up to a certain density after which yield starts to decline (Guzman, 2011).

Weight loss

Green onion leaves exhibited an average of 50% weight loss within a week of harvest. Application of organic fertilizer resulted to a highest weight loss of 61.02% at 6 DAH (Table 3). Findings of this study affirmed observations of green onion growers in the area that harvested plants applied with organic fertilizers hastened weight loss thus reducing shelf-life. This was similar to the findings of Shah et al. (2019) using combined organic fertilizer of onion that there were increase in fresh weight of allium bulb in early stage of growth and faster weight loss at later stage. Application of salicylic acid is recommended by Freddo et al. (2013)

as it decreases the weight loss as well as reducing the growth of rots on shoots and leaves. Planting distance showed no significant effect on the weight loss (Table 3).

Table 3. Postharvest evaluation of green onion as influenced by planting distance and fertilizer application.

Treatments	Postharvest weight loss (%)		
	2 DAH	4 DAH	6 DAH
Factor A (fertilizer application)			
Control	5.02c	37.35	43.23b
Organic fertilizer	15.96b	42.32	61.02a
Vermicast	13.98b	44.81	53.01ab
Recommended Rate (N-P-K)	19.37a	44.97	46.09b
F-test	**	ns	**
Factor B (planting distance)			
25×15 cm	16.83a	42.93	52.63
15×15 cm	10.62b	40.95	48.34
10×15 cm	14.50a	41.56	51.99
10×15×15 cm	14.04a	44.01	50.39
F-test	**	ns	ns
A×B			
F-test	**	ns	ns
CV (%)			
A	13.40	18.94	17.19
B	15.46	11.58	10.42

** Significant at p=.01, * significant at p=.05, ns = not-significant.

Return on investment

Return on per peso investment values for green onion production showed that the use of the recommended dose of synthetic fertilizer resulted in the highest ROI (Table 4). Returns from vermicast applied at a 10×15 cm spacing or at the farmer's practice of 10×15×15 cm produced returns of only 13% of that value. The combination of the recommended dose of synthetic fertilizer and 15×15 cm planting distance offers the highest return of investment of 2.59 per peso investment. Green onion applied with the commercial organic fertilizer at the 10×15×15 cm spacing gave a negative net income of 23,649 pesos. Application of organic fertilizers may have resulted to commendable marketable yield, however, these correspondingly resulted to highest non-marketable parts during harvest (Table 2) aside from obtaining significantly highest weight loss a week after harvest (Table 3) which will consequently increase postharvest losses eventually giving non-marketable products. The use of organic materials was found to be expensive considering that a bulk amount is required to compensate the recommended nutrient requirement of the plants. As a result, additional expenses on the labor upon application is incurred. These factors among others contributed to the negative ROI on the used of commercial organic fertilizer.

CONCLUSIONS

Application of vermicast showed a promising result in terms of increasing productivity of green onion and its marketability. Moreover, 10×15 cm spacing was considered to be the best planting distance for green onion for its positive effect on the growth and yield per unit area. Post-harvest quality of onion planted in 15×15 cm had a longer shelf life with respect to weight loss and all fertilizers had similar weight losses per unit time. In terms of return on per peso investment it was found that the use of recommended dose of synthetic fertilizer at a 15×15 cm planting distance obtained the highest return on investment with 2.59 pesos per 1 peso invested.

Table 4. Tabulated return on investment (ROI) per hectare of green onion as influenced by planting distance and fertilizer application.

Treatments	Production costs (Php)	Net income[a] (Php)	ROI
A_1B_1 (control, 25×15 cm)	78,149.00	21,151.00	0.27
A_1B_2 (control, 15×15 cm)	94,149.00	67,551.00	0.72
A_1B_3 (control, 10×15 cm)	144,149.00	9,751.00	0.07
A_1B_4 (control, 10×15×15 cm)	104,149.00	9,851.00	0.10
A_2B_1 (ComOrgFert, 25×15 cm)	172,549.00	- 5,749.00	-0.04
A_2B_2 (ComOrgFert, 15×15 cm)	188,549.00	39,451.00	0.21
A_2B_3 (ComOrgFert, 10×15 cm)	208,549.00	- 9,649.00	-0.05
A_2B_4 (ComOrgFert, 10×15×15 cm)	98,549.00	- 23,649.00	-0.12
A_3B_1 (Vermicast, 25×15 cm)	201,549.00	52,551.00	0.26
A_3B_2 (Vermicast, 15×15 cm)	218,549.00	67,651.00	0.31
A_3B_3 (Vermicast, 10×15 cm)	238,549.00	77,051.00	0.33
A_3B_4 (Vermicast, 10×15×15 cm)	228,549.00	74,751.00	0.33
A_4B_1 (RR, 25×15 cm)	72,949.00	72,949.00	1.64
A_4B_2 (RR, 15×15 cm)	72,949.00	188,651.00	2.59
A_4B_3 (RR, 10×15 cm)	130,549.00	164,051.00	1.26
A_4B_4 (RR, 10×15×15 cm)	120,549.00	109,851.00	0.92

[a] Php = Philippine peso.

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A new asparagus cultivation method for beginners: field tests on the whole harvest asparagus cultivation method of one-year-old plants

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Abstract

In Japan, open field cultivation of asparagus is based on seedlings planted in the spring of the first year. Spears of the same asparagus plant stocks are harvested in spring the following year for 10 to 20 years. However, this cultivation method has the following problems: the yield is small in the first year (the year after planting), the longer cultivation period, the higher risk of disease damage; and it is difficult to determine when to stop harvesting to grow mother fern (stocks). A study was conducted to examine the yields produced by asparagus cultivation beginners, involving 14-16 growers who had never cultivated asparagus before, using a new cultivation method called "Whole Harvest Asparagus Cultivation Method of One-year-old Plants". All spears grown from stocks cultivated in the first year are harvested in the following spring. In the present study, two-year field tests of asparagus were conducted. In the first year, the yields produced by approximately 70% of the beginners were equivalent to or larger than the mean yield produced in open field cultivation of asparagus in Japan. In the second year, although the yields decreased due to typhoons, in open field cultivation of asparagus in Japan. The mean yield of all growers was equivalent to or larger than the mean yield produced by the conventional open field cultivation method.

Keywords: *Asparagus officinalis* L., open field cultivation, soil volumetric water content, upland converted paddy field, yield

INTRODUCTION

In open field cultivation of asparagus, the conventional cropping type system in Japan, asparagus seedlings are planted in the spring of the first year and the spears are harvested in spring from the following year for 10 to 20 years. However, yields from the first harvest (in the year after planting) are small and stable yields are not obtained until three years after planting (Figure 1) (Motoki, 2003). Furthermore, the risk of the plants becoming infected with disease increases over time. In addition, it is difficult to determine when to stop harvesting and to grow the mother fern for the next harvesting (Motoki and Inoue, 2008).

To address these problems, Kabuno et al. (2018) developed a new cultivation method, whole harvest cultivation method of one-year-old plants (WHACM). Asparagus seedlings are planted in open fields in the first year, and in the following spring, all spears from the stocks are harvested without allowing the mother ferns to grow. In this new cultivation method, seedlings are planted earlier than in the conventional cultivation method to extend the stock cultivation period and increase yields (Figure 1). This earlier planting of asparagus seedlings requires a prevention method to stop frost damage. In the new cultivation method, plug seedlings are planted deeply in an earlier period, using a newly designed hole-maker (Figure 2) (Shimizu et al., 2016; Taguchi et al., 2020).

To compare growth and yield, Kabuno et al. (2018) and Taguchi et al. (2018) planted asparagus every month during the five months from February to June. As a result, the yield from the asparagus planted in February to April was equivalent to or larger than the mean

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yield from all the conventional cropping type systems in Japan. However, no studies have been conducted to examine the yield of planting asparagus earlier than February and its effects on growth and yield.

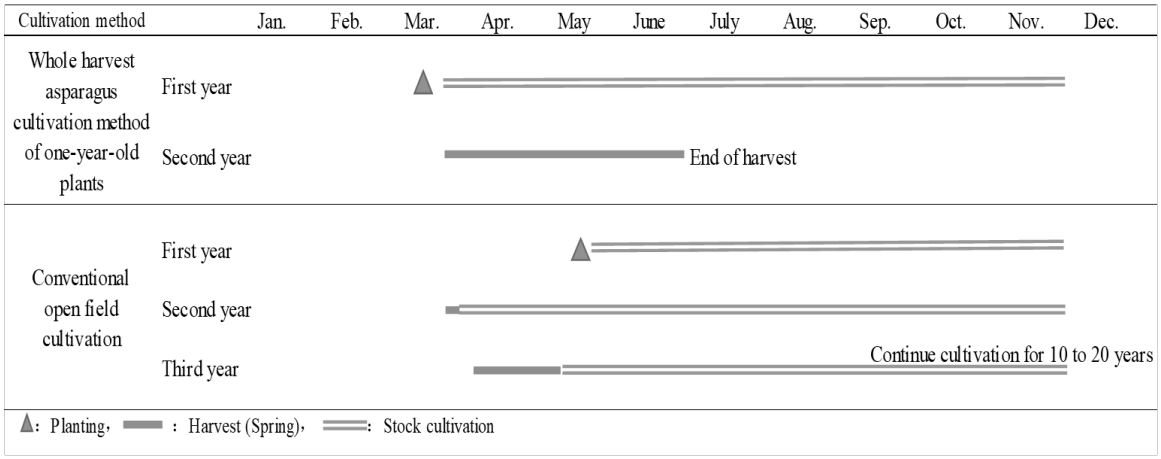

Figure 1. The cultivation method for whole harvest asparagus cultivation method of one-year-old plants and conventional open field cultivation in Japan.

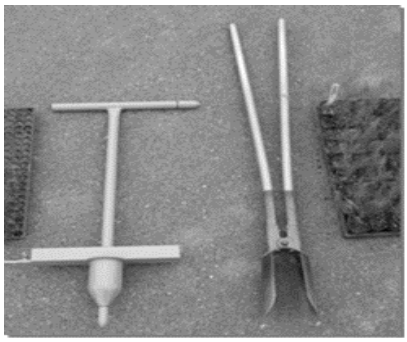

Figure 2. Photographs of two different hole makers. Left = new hole maker and right = conventional hole maker.

Compared to the conventional asparagus cultivation system, this new cultivation method does not require a plastic house and other facilities. These facilities are necessary for semi-forcing asparagus. This new system will be labor-saving and production costs will be lower than the conventional cultivation method. Therefore, the new cultivation method is profitable and can be initiated even by beginners who wish to cultivate asparagus (Kabuno et al., 2018; Taguchi et al., 2018). This new cultivation method will quickly become the preferred method of asparagus cultivation among beginners. However, no studies have been conducted to examine the yields produced by beginners of asparagus using this new cultivation method.

The present study was conducted to spread the new cultivation method: In Test 1, asparagus seedlings were planted every month during the five months from December to April to determine the appropriate timing of planting. In Test 2, there were 14-16 growers who had never cultivated asparagus before and were asked to cultivate asparagus for two years using the new cultivation method. The yields were compared with each grower's field and the experimental field.

MATERIALS AND METHODS

Test 1 – determine the appropriate timing of planting

The experiment was conducted in the test field at Meiji University (Kanagawa, 140°E; 36°N and 65 m a.s.l.). The field soil conditions were: low-humic andosol, pH 6.0 and EC 0.10 dS m^{-2}. The green asparagus cultivar was 'Taiho-wase'. Seeds were sown onto 128-hole cell trays each month between September 2016 and January 2017 (Table 1). Plug seedlings of 20 cm in plant height of three individual stems were planted using the new hole-maker (Figure 2) (Shimizu et al., 2016; Taguchi et al., 2020). Plantings were conducted on December 16, 2016, January 18, February 15, March 13 and April 14, 2017 (Table 1). The asparagus seedlings were planted in single row bed (width 1.4 m) at a distance of 0.4 m between plants at a density of 17,860 plants ha^{-1}. The crop was fertilized with 40 t ha^{-1} of fully fermented cattle manure pre-planting, and a controlled release fertilizer ($N:P_2O_5:K_2O$ = 150:150:150 kg ha^{-1}) applied to the crop. The beds were covered with a black plastic mulch. No spears were harvested during the year the seedlings were planted. No irrigation was applied or additional fertilizers were added during the stock cultivation. Other cultural practices were implemented in accordance with the WHACM for one-year-old plants (Kabuno et al., 2018; Motoki, 2019; Taguchi et al., 2018). Plant measurements were conducted on December 25, 2017. Average sized stocks were selected to measure plant height, maximum stem diameter, and number of effective stems. Stem diameter measurements were conducted on May 25, 2018. Sample size consisted of ten average sized asparagus stock plants. The diameter of the stems of each harvested stock plant measured using digital calipers. Yields and the classes of spear weight estimated using a yield estimation program (Motoki et al., 2007; Tsuda and Motoki, 2018).

Table 1. Seeding and planting date in different planting month.

Planting month	Seeding day	Planting day	Raising seedling day
December	September 21, 2016	December 16, 2016	86
January	October 18, 2016	January 18, 2017	92
February	November 16, 2016	February 15, 2017	91
March	December 14, 2016	March 13, 2017	89
April	January 13, 2017	April 14, 2017	91

Test 2 – field tests

Field tests were conducted in growers' fields Kawasaki City (Kanagawa Prefecture, 140°E, 36°N on 16 sites in 2017 and 14 sites in 2018, fields numbers 1-17). The soil conditions of the field were Haplic lowland paddy soils for field No. 7 and low-humic allophanic andosols for all other sites. For two years the control treatment sites were conducted in three fields cultivated by the authors. These consisted of an upland field, an upland converted paddy field at the field and experimental site Meiji University (Experimental fields). The soil conditions of these fields were low-humic allophanic andosols for upland field, and Haplic lowland paddy soils for the upland converted paddy field. The cultivars were 'Early California' in 2017 and 'Taiho-wase' in 2018. Planting was undertaken in middle March in both 2017 and 2018. The stem diameter measurements were conducted on June 15, 2018, and May 28, 2019, as per Test 1. The planting density, spacing, and other cultural practices were the same as Test 1. In 2017, the soil volumetric water content was measured. Soil at the depths of 10, 20, and 30 cm from the bed surface for the upland converted paddy field were measured using a SenSprout Pro sensor (SenSprout Inc.). Since the growers had never cultivated asparagus before, the authors visited the growers' fields once every two months and provided them with advice on asparagus cultural practices.

RESULTS AND DISCUSSION

Test 1 – determine the appropriate timing of planting

The effects of planting month on the growth of asparagus is shown in Table 2. Plant

height was significantly greater for asparagus planted in February than any other month. There was no significant difference in the number of effective stems and maximum stem diameter between planting months. The effect of planting month on the estimated yield of asparagus is shown in Table 3. The total and marketable yield was greater for asparagus planted in February than any other month. The number of thick spears (15 g or larger size) was greater for asparagus planted in March than any other month. The mean yield of asparagus from all cropping systems including cultivation in a plastic house, per unit of land in Japan, is 5,130 kg ha^{-1} (Ministry of Agriculture, 2019). According to the results of this study, the yield of asparagus planted in February and March is greater than the mean yield for all cropping systems in Japan. Other planting months were equivalent to the mean yield reported for Japan (5,130 kg ha^{-1}). The results of this study support the previous studies by Kabuno et al. (2018) and Taguchi et al. (2018) and are equivalent to the mean yield of asparagus from all cropping systems in Japan.

Table 2. Effect of planting month on growth of asparagus.

Planting month	Plant height (cm)	Number of effective stems[a]	Maximum stem diameter (mm)
December	215b	24.0a	12.1a
January	232b	26.4a	12.4a
February	252a	29.5a	14.1a
March	230b	28.5a	12.8a
April	219b	25.3a	12.8a

[a]Effective stem is a stem of more than 3 mm diameter.
Different letters in each planting month indicate significant difference between planting months at the 5% level by Tukey test (*n*=8-15).

Table 3. Effect of planting month on estimated yield of asparagus.

Planting month	Total yield (kg ha^{-1})	Marketable yield (kg ha^{-1})	Classes of weight (%)					Thick spears (>15 g) (%)
			>40 g	15-39 g	10-14 g	7-9 g	5-6 g	
December	5,619	5,135	3	51	29	11	6	54
January	5,895	5,544	2	45	27	14	12	47
February	9,280	8,955	9	57	21	7	6	66
March	7,663	7,474	5	69	18	6	2	74
April	5,383	5,071	6	61	18	9	7	67

This study was conducted in the southern Kanto region during the period of December to March which is mid-winter. Growers do not usually plant asparagus during this period. However, planting holes were made in the raised beds using the new hole-maker, enabling a plug-seedlings to be planted at a depth of approximately 15 cm (Figure 3). The temperature in the planting holes made by the new hole maker do not decline as rapidly in early spring, compared to seedlings planted into the soil as pot seedlings (Shimizu et al., 2016; Taguchi et al., 2020). Therefore, asparagus seedlings were successfully planted during the mid-winter period due to this new cultivation method.

The yields of asparagus planted in December and January were equivalent to yields reported for April. To increase the yield of asparagus, growers are advised to plant earlier, expanding the stock cultivation period. However, the results this study suggest timing of planting asparagus in the southern Kanto region should not be earlier than February. If asparagus is planted earlier than February, there is a significant delay in its growth, even when the new hole maker is used. This is due to markedly low temperatures. Therefore, the appropriate period for planting asparagus in the southern Kanto region using this new cultivation method is February to April.

Test 2 – field tests

The estimated yield from field tests of the WHACM for one-year-old plants is shown in Table 4. In both 2017 and 2018, the total yield and marketable yield showed large differences between the grower fields. The marketable yield in the field experiments ranged from 624 to 9,988 kg ha^{-1} in 2017 to 20-10,833 kg ha^{-1} in 2018. The mean value of the marketable yield in these field tests was 4,344 kg ha^{-1} in 2017 and 3,104 kg ha^{-1} in 2018. The mean yield produced in open field cultivation using a conventional cropping system, per unit of land in Japan was 3,200 kg ha^{-1} (Motoki, 2016). Therefore, in 2017 and 2018, the mean yield in the grower field test experiments was equivalent to or larger than the mean yield produced in open field cultivation of asparagus in Japan. Approximately 69 and 36% of the growers' field experiments were above the mean yield produced in open field cultivation of asparagus in Japan.

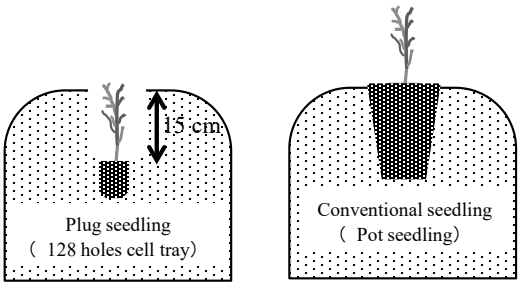

Figure 3. Shape of the planting hole and positioning of the seedling with the new hole maker compared to the conventional hole maker.

There are significant differences in the yield of asparagus between the different grower fields due to multiple number of reasons. One reason is the timing of chemical sprays. Cultural practices are very important because asparagus yields are significantly influenced by the timing of cultural practices e.g., chemical sprays (Motoki, 2003). In 2018, typhoons brought heavy rains and strong winds resulting in severe damage to the growers' fields. It is necessary to spray chemicals after a typhoon has passed. This is due to stem blight and other diseases that spread immediately after heavy rainfall and damage asparagus plants. Furthermore, the asparagus stocks cultivated in the growers' field were influenced by typhoons. Damage included, falls of asparagus stems increasing the risk of disease damage. Therefore, it is necessary to consider the typhoon influences and weather-related damage on the asparagus experiments using this new cultivation method.

As indicated above, in 2017, 69% of growers had yields of asparagus greater than the mean yield produced for open field cultivation of asparagus in Japan. Growers with no or little experience of cultivating asparagus, if they receive the correct advice on its cultivation, can produce a yield of asparagus equivalent to or larger than the mean yield produced form open field cultivation in Japan. The results of this study suggest that the new cultivation method is profitable and can be taken up by beginner asparagus growers.

For the two model farms (upland field and upland converted paddy field) the cultural practice experiments were conducted by the authors. The marketable yield varied depending on the year. In 2017, the yield of asparagus for the upland converted paddy field was greater than that the harvested from upland field. However, in 2018 the yield of asparagus in upland fields was larger than upland converted paddy field. These studies suggest the growth of asparagus in the upland converted paddy field is lower in general, due to these fields have a high water holding capacity and contain excessive amounts of water (Yanai et al., 2013). The soil volumetric water content at 30 cm below the surface for the upland converted paddy field in 2017 was between 20 to 50% (Figure 4). This soil moisture is considered not to be excessively moist. Therefore, the results of this study suggests that the yield of asparagus each year is influenced by climate conditions, including precipitation.

Table 4. Estimated yield of grower field experiments using the whole harvest asparagus cultivation method for one-year-old plants.

Field no. or name	Total yield (kg ha^{-1})		Marketable yield (kg ha^{-1})		Classes of spear weight (%)										Thick spears (>15 g) (%)	
					>40 g		15-39 g		10-14 g		7-9 g		5-6 g			
	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018
1	3,449	4,452	2,909	4,380	1	7	40	69	28	16	12	6	20	1	41	76
2	2.869	236	2,187	20	2	0	48	0	22	0	14	15	13	85	51	0
3	11,133	10,982	9,988	10,833	9	18	59	69	19	10	9	2	5	1	67	87
4	6,300	2,727	5,098	2,376	3	4	50	60	28	14	10	10	9	12	53	65
5	6,813	4,718	6,011	4,550	4	10	41	63	24	15	16	8	15	4	45	72
6	5,682	1,852	4,715	1,436	4	4	43	31	23	20	16	28	14	16	47	35
7	5,469	754	4,196	570	0	0	27	3	31	29	22	44	20	25	28	3
8	4,228	-[a]	3,677	-	2	-	45	-	26	-	16	-	11	-	47	-
9	2,580	-	2,302	-	1	-	42	-	28	-	18	-	11	-	43	-
10	4,255	2,285	3,946	1,954	1	0	45	32	32	27	14	21	8	20	46	32
11	5,146	1,938	4,826	1,403	0	3	37	33	33	21	18	20	13	24	37	36
12	7,146	5,506	6,584	5,079	3	2	51	46	20	25	16	15	10	12	54	48
13	1,766	1,907	1,265	1,323	0	4	31	36	24	30	25	15	21	15	31	39
14	6,463	-	5,928	-	3	-	60	-	19	-	11	-	7	-	63	-
15	5,728	5,233	5,254	4,595	3	6	46	56	26	19	16	13	10	7	49	61
16	768	2,844	624	2,580	0	0	19	41	54	35	17	16	9	8	19	41
17	-	2,616	-	2,362	-	3	-	35	-	27	-	23	-	13	-	38
Average	4,987	3,432	4,344	3,104	2	4	43	41	27	20	16	17	12	17	45	45
Upland field	4,603	5,729	4,132	5,218	3	12	45	57	25	18	16	8	11	5	48	69
Upland converted paddy field	8,537	3,917	7,801	3,487	5	1	50	35	24	19	13	23	8	22	56	36
Experimental field (Meiji Univ.)	6,572	7,432	6,161	6,868	5	2	59	37	18	26	9	21	8	14	64	39

[a]not tested.

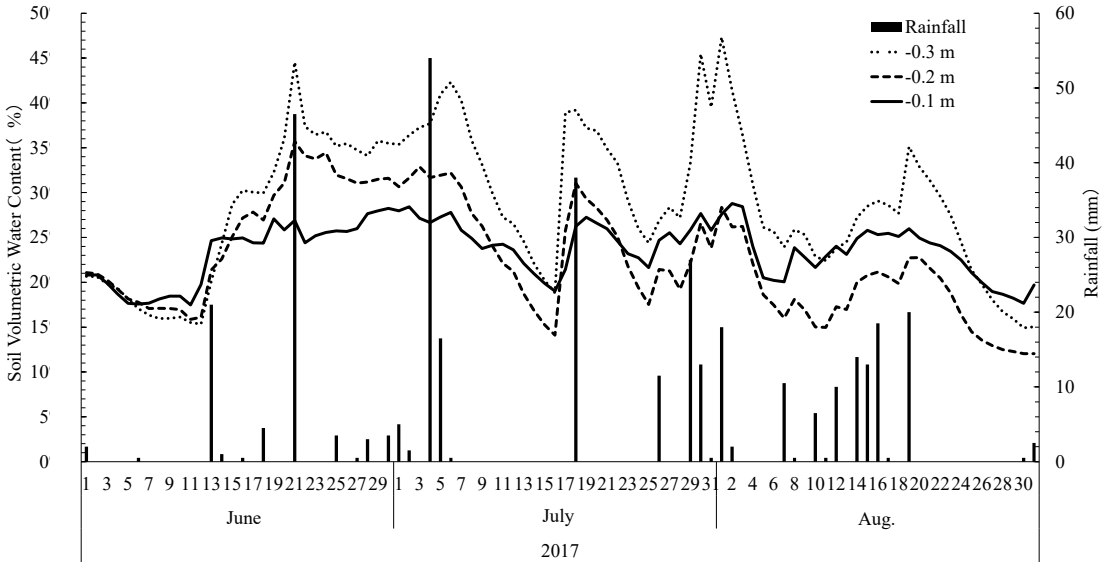

Figure 4. Trend of daily soil volumetric water content from June to August 2017 at three depths (-0.1, -0.2 and -0.3 m).

The marketable yield produced by grower three in 2017 and 2018 was approximately 10 t ha^{-1}. This was greater than the yields produced by other growers. Grower 3 irrigated the fields during the stock cultivation and harvest. This helped the soil retain an appropriate amount of moisture leading to increased yields. Therefore, it is necessary to examine the effects of irrigation on the yields of asparagus produced using this new cultivation method.

CONCLUSIONS

The results of this study suggest the new cultivation method is profitable and can be used by beginner growers for asparagus cultivation. However, the results of the study suggest factors that significantly influence the yields of asparagus using this new cultivation method need to be investigated. We plan to research methods to increase the yields based on this new cultivation method.

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Use of *Trichoderma harzianum* and *Daldinia eschscholtzii* to enhance the growth of 'Namwa' banana

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Abstract

The beneficial fungi *Trichoderma* and *Daldinia* are used as defenses against phytopathogenic fungi, as well as for the enhancement of plant growth. However, there are very few published studies on the effects of these fungi on plant growth and nutrient status, especially in banana. This research was conducted to evaluate the effects of *Trichoderma harzianum* GR03 and *Daldinia eschscholtzii* FL11 on growth and the macro-nutrient status of 'Namwa' banana. Banana sapling tissue cultures were transplanted into 15-L pots. The experiment was a completely randomized design with three treatments: the control; a hole dressing with *T. harzianum* GR03; and a hole dressing with *D. eschscholtzii* FL11. The rate of application was 10 g of beneficial fungi per pot. This work was conducted at the Fruit Tree Division, Department of Horticulture, Khon Kaen University from August to October, 2017. *T. harzianum* GR03 increased shoot height and pseudo-stem girth, while *D. eschscholtzii* FL11 had no effect on these attributes. The whole plant weight, shoot fresh weight, and shoot dry weight were also highest in the *T. harzianum* GR03 treatment but there were no significant differences in root fresh weight, root dry weight, root length, or leaf greenness. Neither *T. harzianum* GR03 nor *D. eschscholtzii* FL11 affected nitrogen, phosphorus or potassium contents in the shoots or roots. This research displayed the potential of *T. harzianum* GR03 to increase shoot growth.

Keywords: *Musa* 'Namwa', *Trichoderma*, *Daldinia*, shoot growth, root growth

INTRODUCTION

Plant growth can be enhanced by several factors, including mineral nutrient management, soil moisture regulation, and environmental control. The use of beneficial microorganisms also presents an alternative for sustaining high production with low ecological impact. Soil fungi can colonize plant roots and have positive effects on plant growth (Ousley et al., 1993; Masunaka et al., 2011; Hermosa et al., 2012). Several researchers confirmed that *Trichoderma* spp., in particular, increased plant growth in lettuce (Ousley et al., 1993; Pereira et al., 2019), lotus (Masunaka et al., 2011) and bitter gourd, loofah, and cucumber (Lo and Lin, 2002); while *Daldinia* spp. exhibited inhibition of plant-parasitic nematodes. Beneficial fungi have been reported to produce plant growth substances, such as auxin (Blanchard and Björkman, 1996), increase mineral nutrient uptake (Altomare et al., 1999) and suppress plant pathogens (Hermosa et al., 2012; Samuelian, 2016). These mechanisms have been shown to enhance plant growth and production but the effects of *Trichoderma* sp. and *Daldinia* sp. on growth in bananas have not been investigated. Our research, therefore, aimed to evaluate the potential of these two fungi on the growth and nutrient concentrations in banana shoots and roots in pot experiments.

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MATERIALS AND METHODS

Fungal preparation

Trichoderma harzianum GR03 and *Daldinia eschscholtzii* FL11 were cultured on PDA agar and incubated at 25±2°C for seven days. Agar plugs (5-mm diameter) with mycelial tips were transferred into polyethylene bags containing 200 g of autoclaved rice grain. The fungi were incubated at 25±2°C for 14 days and shaken every three days during incubation.

Plant material and pot planting

A total of 15 banana saplings were prepared from two-month-old saplings post-transplantation from tissue culture propagation. These saplings were planted in 15-L pots. The growing medium comprised soil:rice husk:burnt rice husk:manure, at 1:1:1:0.5. The experiment was arranged as a completely randomized design (CRD) with three treatments: 1) a hole dressing with 10 g sterilized rice grain (control), 2) a hole dressing with 10 g of rice grain containing *T. harzianum* GR03, and 3) a hole dressing with 10 g of rice grain containing *D. eschscholtzii* FL11. The experiment was conducted in the Fruit Tree Division, Department of Horticulture, Khon Kaen University, from August to October 2017.

Growth measurements

Weekly non-destructive growth measurements of shoot height and girth (5 cm above the soil) were conducted. Leaf greenness was measured using a dual-wavelength meter (Model SPAD-502, Minolta) on the 3rd leaf from the shoot tip, prior to the non-destructive measurements. At 60 days post-planting, the banana saplings were measured for shoot height, pseudo-stem girth, leaf number, and root length and number, as well as whole shoot, and root fresh weights. The shoot and root samples were then dried in a hot air oven at 65°C and the dry weights were measured.

Nutrients in shoot and root

The nitrogen (N), phosphorus (P), and potassium (K) concentrations in the shoots and roots were analyzed from the dried samples. Digestion used the wet acid method (Attanandana et al., 1989; Mills and Jones, 1996). Samples were digested with sulfuric acid with sodium sulfate and selenium. Nitrogen and phosphorus concentrations were analyzed colorimetrically using a spectrophotometer (Baethgen and Alley, 1989; Suwanwong, 2004), and the potassium concentration was analyzed using a flame photometer (Attanandana et al., 1989; Mills and Jones, 1996).

Data analysis

Data were analyzed using a one-way analysis of variance (SPSS 11.5, SPSS Inc., New York, USA). Comparisons of means were performed using Duncan's test.

RESULTS AND DISCUSSION

Changes in shoot height and girth

Weekly observations determined that the shoot heights in all treatments showed stable values in the first month after transplanting after which increases in shoot height occurred corresponding to the production of new leaves. The banana saplings treated with 10 g *T. harzianum* GR03 exhibited the greatest shoot height (Figure 1A), as well as the greatest increase in pseudo-stem girth (Figure 1B). Height (Figure 2A) and girth (Figure 2B) increments confirmed that *T. harzianum* enhanced growth in the banana saplings. *Trichoderma* sp. has been shown previously to have a positive effect on plant growth in other species (Ousley et al., 1993; Masunaka et al., 2011; Hermosa et al., 2012). However, this study did not demonstrate any significant effects on banana growth due to the *D. eschscholtzii* FL11 treatment. It is possible that these beneficial fungi may affect only plant-parasitic protection and that longer-term experimentation may be needed to demonstrate plant growth effects.

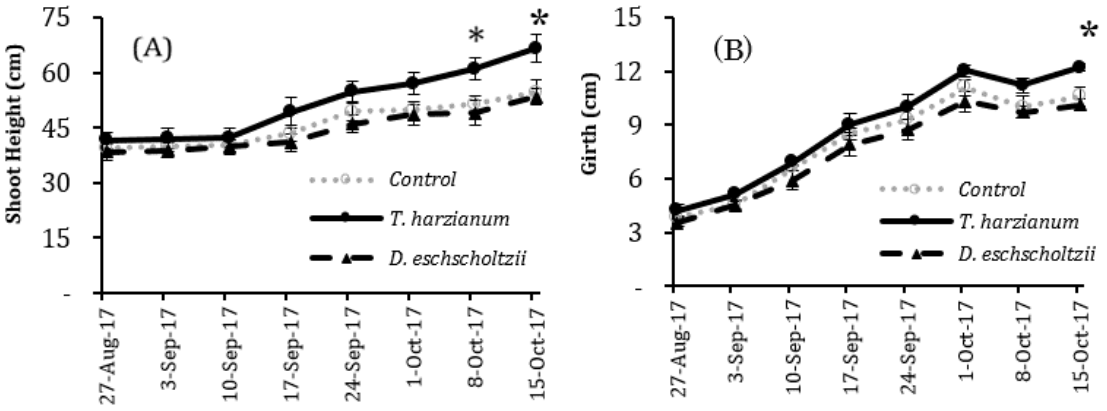

Figure 1. Effects of *T. harzianum* GR03 and *D. eschscholtzii* FL11 on shoot height (A) and pseudo-stem girth (B) of *Musa* 'Namwa'. * indicates significant difference at p≤0.05. Error bars represent standard errors.

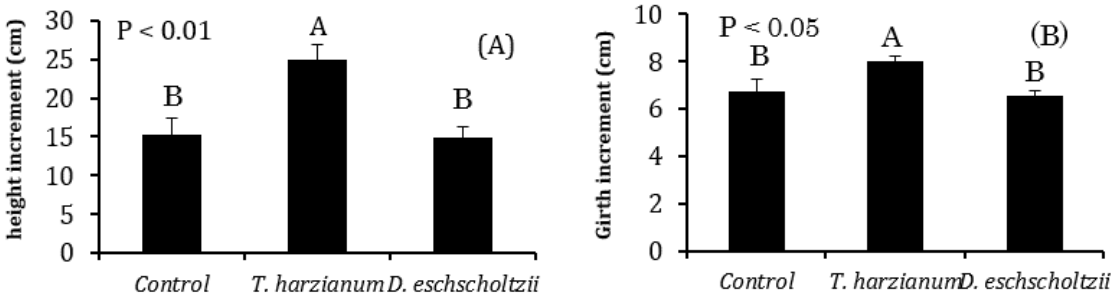

Figure 2. Effects of *T. harzianum* GR03 and *D. eschscholtzii* FL11 on shoot height increment (A) and pseudo-stem girth increment (B) of *Musa* 'Namwa' following 60 days of growth under each treatment. Different letters indicate a significant difference by DMRT. Error bars represent standard errors.

Destructive growth measurements

Two months after transplanting, while the banana saplings treated with *T. harzianum* GR03 were highest and had a greater pseudo-stem girth, there were no significant differences in number of leaves, leaf greenness, root length or root number among treatments (Table 1). *T. harzianum* GR03 treatment also resulted in increased whole fresh weight, shoot fresh weight, and shoot dry weight of the banana saplings, but did not affect root growth (Table 2). These results are in contrast with those of previous research in which *Trichoderma* spp. increased root growth in bitter gourd, loofah, and cucumber (Lo and Lin, 2002) and in *Arabidopsis* (Nieto-Jacobo et al., 2017). This difference in response may be due to the different root system in banana, as the formerly mentioned plants possess a taproot system, whereas the banana plant possesses a large rhizome. Masunaka et al. (2011) determined that *Trichoderma* spp. had no growth-promoting effects on *Lotus japonicas*.

Trichoderma spp. has been shown to typically increase the chlorophyll content in plant leaves (Lo and Lin, 2002; Nieto-Jacobo et al., 2017). However, in this study, there were no differences in leaf greenness, which is directly related to chlorophyll content (Table 1).

Shoot and root nutrients

Analysis of N, P and K in the shoots and roots, two months post-transplantation, found that banana saplings grown with the *D. eschscholtzii* FL11 supplement had the lowest K concentration, whereas the N and P concentrations were not significantly different (Table 3).

Table 1. Effects of *T. harzianum* GR03 and *D. eschscholtzii* FL11 on growth parameters of *Musa* 'Namwa'.

Treatment	Shoot height (cm)	Pseudo-stem girth (cm)	Leaf no.	Leaf greenness (SPAD unit)	Root length (cm)	Root no.
Control	56.60b	10.10b	7.60	33.81	176.60	21.00
T. harzianum	74.70a	12.80a	8.00	38.80	171.80	24.40
D. eschscholtzii	58.60b	11.80ab	7.60	34.76	155.70	19.60
F-test	**	*	ns	ns	ns	ns

ns indicates a non-significant difference. *, ** indicate significant differences at levels 0.05 and 0.01, respectively.
Means in the same column with the different letters are significantly different by DMRT.

Table 2. Effects of *T. harzianum* GR03 and *D. eschscholtzii* FL11 on fresh and dry weights of whole, shoot, and root parts of *Musa* 'Namwa'.

Treatments	Whole FW (g)	Shoot FW (g)	Root FW (g)	Whole DW (g)	Shoot DW (g)	Root DW (g)
Control	323.82b	154.66b	169.16	49.34	22.76b	26.58
T. harzianum	539.73a	286.96a	252.77	69.61	35.69a	33.92
D. eschscholtzii	349.23b	172.71b	176.52	52.19	24.96b	27.23
F-test	**	**	ns	ns	*	ns

ns indicates a non-significant difference. *, ** indicate significant differences at 0.05 and 0.01, respectively.
Means in the same column with the different letters are significantly different by DMRT.

Table 3. Effects of *T. harzianum* GR03 and *D. eschscholtzii* FL11 on various macronutrient concentrations in shoots and roots of *Musa* 'Namwa'.

Treatments	N (%)		P (%)		K (%)	
	Shoot	Root	Shoot	Root	Shoot	Root
Control	1.73	0.77	0.44	0.20	1.97a	1.40
T. harzianum	1.60	0.53	0.42	0.21	1.72ab	1.41
D. eschscholtzii	1.31	0.63	0.34	0.25	1.31b	1.28
F-test	ns	ns	ns	ns	*	ns

ns indicates a non-significant difference. * indicates significant differences at levels 0.05.
Means in the same column with the different letters are significantly different by DMRT

The nutrient contents per shoot and root were calculated and results showed that there was a non-significant difference among treatments for all nutrients for both shoots and roots (Table 4). However, the banana saplings treated with *T. harzianum* GR03 tended to have greater nutrient contents in both shoots and roots. Altomare et al. (1999) showed that *T. harzianum* promoted the solubilization of soil nutrients. In our research, the banana saplings treated with *T. harzianum* GR03 showed a greater uptake of nutrients than either that of the control or the *D. eschscholtzii* FL11 treatment, which resulted in the increased shoot growth (Figure 2).

CONCLUSIONS

A hole dressing with 10 g of *T. harzianum* GR03 increased the shoot height, pseudo-stem girth, whole fresh weight, shoot fresh weight, and shoot dry weight in pot-grown banana saplings. In contrast, *D. eschscholtzii* FL11 did not affect banana sapling growth. The use of *T. harzianum* GR03 and *D. eschscholtzii* FL11 had no clear effects on nitrogen, phosphorus, or potassium concentrations in the plant tissues. However, the responses of banana to the two fungi require further longer-term experimentation and evaluation under field conditions.

Table 4. Effects of *T. harzianum* GR03 and *D. eschscholtzii* FL11 on various macronutrient contents in shoots and roots of *Musa* 'Namwa'.

Treatments	N (mg part^{-1})		P (mg part^{-1})		K (mg part^{-1})	
	Shoot	Root	Shoot	Root	Shoot	Root
Control	398.07	199.11	98.61	50.94	475.37	371.18
T. harzianum	591.50	184.97	147.87	72.67	606.64	493.43
D. eschscholtzii	334.92	151.45	84.92	64.72	331.52	356.67
F-test	ns	ns	ns	ns	ns	ns

ns indicates a non-significant difference.

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Effect of foreign pollen on fruit set and quality of 'Monthong' durian

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Abstract

The effect of foreign pollen on fruit set and quality of 'Monthong' durian was studied in two experiments at the Chanthaburi Horticultural Research Center, Thailand. The first experiment investigated the in vitro germination of pollen from five commercial cultivars including 'Phuangmanee', 'Longlublae', 'Chanee', 'Kadum' and 'Monthong', five lesser-known cultivars namely 'Nokyhib', 'Kobmaethao', 'Chomphoosri', 'Kanyaosrinak', and 'Chaymafai', and one hybrid 'Chanthaburi 3' at different temperatures. 'Chanthaburi 3' was found to have the highest germination rates among cultivars across the 10 to 35°C temperature range. Consequently, 'Chanthaburi 3' pollen was chosen to study the effect on 'Monthong' fruit set under two weather conditions, in comparison with 'Monthong' pollen. The percentage of fruit set was not different between 18.5 and 31.6 and 23.3-34.2°C (min-max) temperature conditions. However, the number of fruit set tree^{-1} under the cooler condition was significantly higher, at 81 as compared with 65 fruit tree^{-1}. Similarly, pollen of 'Chanthaburi 3' did not result in higher fruit set percentage, but the number of fruits tree^{-1} was higher than with 'Monthong' pollen. 'Monthong' fruit which were pollinated with pollen from 'Chanthaburi 3' revealed no significant differences in pulp weight, seed weight, fruit weight, number of aborted seeds and total soluble solids from fruit which were pollinated with pollen from 'Monthong'. Nevertheless, analysis of volatile compounds from fruit pulp with a gas chromatograph-mass spectrometer, using headspace solid phase micro extraction with DVB/CAR/PDMS coated fiber, revealed a slightly different profile between fruit that had been pollinated with 'Chantaburi 3' compared with that of 'Monthong' pollen.

Keywords: durian, pollen germination, fruit set, climate changes

INTRODUCTION

Durian (*Durio zibethinus* Murray) is known as the king of tropical fruit and is widely cultivated in South-East Asia. In Thailand it is grown in the eastern, southern and northeastern regions. 'Monthong' is the most popular cultivar. The suitable growing temperature is 25-30°C, and 75-85% relative humidity. However, the weather at the present time often changes rapidly and may affect the productivity of durian. Kozai et al. (2014a) reported that too high or too low temperature during the flowering period affected the viability of 'Monthong' pollen, resulting in low fruit set. Kunjet et al. (2016) also reported that fruit set was reduced as the minimum temperature decreased from 22.4 to 12.9°C. Additionally, durian is a cross pollinated species. Consequently, durian flowers that were pollinated with pollen from other cultivars showed higher fruit set than those that were self-pollinated (Kozai et al., 2014b). This study was conducted, therefore, to find cultivar(s) of durian that show high pollen germination rates across a wide range of temperatures. Eleven cultivars of durian were tested. Besides pollen germination, the investigation also included and examination of the xenia effect. This is because the quality of durian pulp was reported to change according to pollen sources (Indriyani et al., 2012; Honsho et al., 2004; Honsho et al., 2007b). The information

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gained from this study would be beneficial to increasing durian fruit set across a wide range of temperatures.

MATERIAL AND METHODS

Experiment 1 – germination of durian pollen at different temperatures

The experiment was conducted from December 2016 to February 2017 during the durian flowering season. Eleven durian cultivars that were flowering at the same time were chosen from more than 200 durian cultivars grown at the Chanthaburi Horticulture Research Center. The cultivars included 'Nokyhib', 'Phuangmanee', 'Longlublae', 'Chanthaburi 3', 'Kobmaethao', 'Chomphoosri', 'Kanyaosrinak', 'Chanee', 'Chaymafai', 'Kadum' and 'Monthong'.

1. In vitro pollen germination.

Thirty flowers of each cultivar were collected at 17.00 pm before anther dehiscence, and delivered to the laboratory by 19.00 pm Pollen grains were placed on germination agar medium containing 100 mg L^{-1} H_3BO_3, 200 mg L^{-1} $MgSO_4.7H_2O$, 300 mg L^{-1} Ca $(NO_3)_2.4H_2O$, and 100 mg L^{-1} KNO_3 with 1% agar and 10% sucrose (Honsho et al., 2007a). The germination agar medium was placed on microscope slides in square petri dishes containing moist filter paper. After incubation at 10, 15, 20, 25, 30 or 35°C for 12 h, germination was observed under a microscope.

Experiment 2 – effect of the foreign pollen on the fruit quality of 'Monthong' durian

The experiment was performed from December 2017 to February 2018 during flowering time. Sixteen 8-year-old 'Monthong' trees were chosen for the study. Approximately 500 flowers $tree^{-1}$ were tagged and emasculated. A factorial 2×2 factor design was used. The trees are divided into four groups; groups 1 and 2 for pollination during relatively high ambient temperatures (min-max, 23.3-34.2°C) on February 23, 2018; groups 3 and 4 for pollination during low ambient temperatures (min-max, 18.5-31.6°C) on January 26, 2018. Anthers were emasculated at 16.00 pm, then pollination was done between 19.00 and 20.00 pm 'Monthong' pollen grains were pollinated on the stigma of 'Monthong' durian flowers in groups 1 and 3, while pollen of 'Chanthaburi 3', were pollinated on flowers of 'Monthong' in groups 2 and 4. Pollinated flowers $tree^{-1}$ were counted immediately after pollination. After seven days, the number of fruit set was recorded.

Forty fruit were harvested, 120 days after pollination, from the two treatments at the higher ambient temperatures. Fruit quality including fruit weight, peel weight, pulp to fruit weight ratio, seed weight, number of aborted seeds and total soluble solids (TSS) were determined two days after fruit stalk abscission. There were five fruit per replication and four replications. Moreover, five fruits which were pollinated with either 'Monthong' or 'Chanthaburi 3' were chosen to check for any xenia effect on the composition of volatile compounds. These were analyzed using headspace SPME coupled with GC-MS (Clarus 600, PerkonElmer, Inc., USA). The procedure was modified from the method of Chin et al. (2007). A 100-g fresh durian pulp sample was blended with 200 g distilled water in a Waring blender for 1 min to form a homogenate. A 15-g sample of durian homogenate was then transferred to a 30-mL vial together with a magnetic stirring bar and 5 g of NaCl, and then kept under constant vigorous stirring at 45°C for 30 min. A manual SPME (solid phase micro extraction) sampling unit, equipped with a 50/30 µm fiber with divinylbenzene/carboxen on polydimethylsiloxane (DVB/CAR/PDMS) coating (Supelco Co., Bellefonte, PA, USA), was inserted into the headspace of the vial for 30 min. After extraction, the SPME fiber was immediately introduced into the spitless GC injection port at 250°C and maintained for 5 min. The separation of volatiles was achieved with an Elite-5 capillary column (30 m × 0.25 mm I.D. × 0.25 µm film thickness) (PerkonElmer, Inc., USA) under an oven temperature program: 45°C initially, held for 5 min, then increased to 230°C at 7°C min^{-1}. Purified helium was used as the carrier gas at 1 mL min^{-1} constant flow rate. The mass spectrometer was operated in scan mode from m/z 33 to 500 at 2.05 scans s^{-1}, with 70 eV electron ionization at 230°C, quardrupole at 150°C. Data were analyzed using TotalChrom software (PerkonElmer, Inc.,

USA). Compounds were identified tentatively by comparison of the mass spectrum with the NIST library spectrum, with over 80% similarity being chosen.

RESULTS AND DISCUSSION

Experiment 1 – pollen germination of various durian cultivars at different temperatures

1. In vitro pollen germination.

The germination percentages of durian pollen in vitro at various temperatures were different among different cultivars. 'Chanthaburi 3' had a higher percentage of germination when compared with other cultivars. The rate was over 70% at relatively low temperatures (10-20°C) and at higher temperatures the rate was over 59%. 'Longlublae' and 'Phungmanee' had 70-80% germination rate at 15-25°C, but it was lower than 50% outside this temperature range. Other cultivars including 'Chanee', 'Chompoosri', 'Chaymafai', 'Kadum', 'Kanyaosrinak', 'Kobmaethao', and 'Nokyhib' had a germination rate below 50% at most temperatures. 'Monthong' pollen had the highest germination percentage of 57% at 15°C but this decreased to 18% at 3°C, and 32% at 10°C (Figure 1). Most cultivars, except 'Chanthaburi 3', exhibited low pollen germination at 10 and 35°C, consistent with the results of Kozai et al. (2014a). From these results, 'Chanthaburi 3' was chosen for further study.

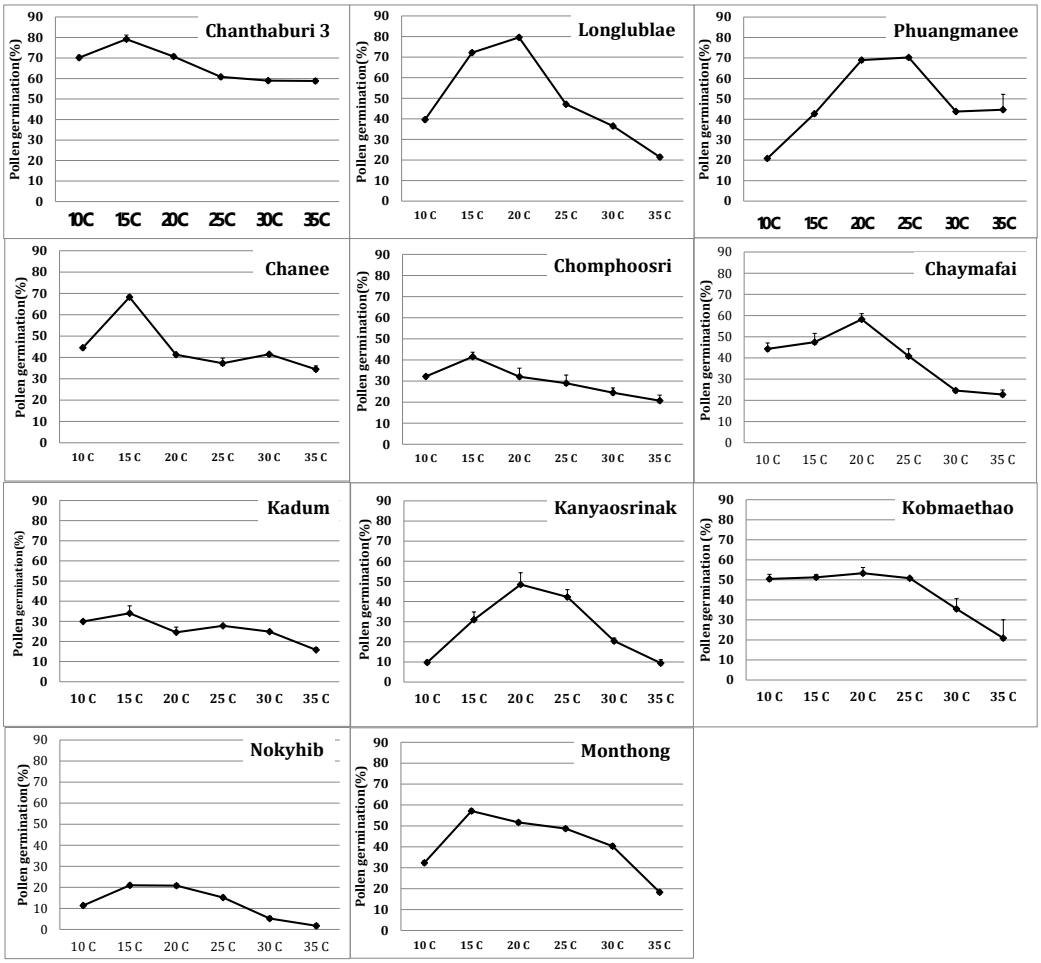

Figure 1. Percentage of in vitro durian pollen germination after incubated 12 h at 10-35°C.

Experiment 2 – effect of 'Chanthaburi 3' pollen on the fruit set and fruit quality of 'Monthong'

1. Fruit set.

The percentage fruit set was similar when either 'Monthong' or 'Chanthaburi 3' pollen were used to pollinate 'Monthong' flowers. However, the number of fruit set tree^{-1} was higher when 'Chanthaburi 3' pollen was used as compared with 'Monthong' pollen. No significant difference in the percentage of fruit set was detected under the two different temperature ranges. However, the number of fruit set tree^{-1} under the cooler temperature range was significantly higher than that under the warmer temperature range. No interaction was found between temperature and cultivar (Table 1).

Table 1. Number and percentage fruit set in 'Monthong' durian when either 'Chanthaburi 3' or 'Monthong' pollen grains were placed on the stigma of 'Monthong' at two temperature ranges.

	Number of fruit set tree^{-1}	Percentage fruit set
Temperature (A)		
23.3-34.2°C	65.4 B[a] (504.0)[b]	12.9
18.5-31.6°C	81.0 A (495.6)	16.3
Cultivars (B)		
Chanthaburi 3	83.5 a (515.3)	16.2
Monthong	62.9 b (484.4)	13.0
Temperature × cultivar (A×B)		
23.3-34.2°C		
Chanthaburi 3	75.8 (514.2)	14.7
Monthong	55.0 (493.8)	11.1
18.5-31.6°C		
Chanthaburi 3	91.2 (516.2)	17.7
Monthong	70.8 (475.0)	14.9
A	*	ns
B	*	ns
A×B	ns	ns

[a]Mean values followed by different letters in the same column are significantly different using DMRT at 95%.
[b]Total number of flower pollinated tree^{-1}.

From the results obtained, we cannot recommend the use of 'Chanthaburi 3' pollen to pollinate 'Monthong' flowers, since fruit set was not clearly improved when compared with the use of 'Monthong' pollen itself. However, hand pollination is recommended due to the fact that fruit set percentages were much higher than occurs with natural open pollination which resulted only 1.4% of fruit set (Honsho et al., 2004). With regard to the influence of temperature on fruit set, this study did not find that low temperature had an adverse effect. On the contrary, there was a tendency for fruit set to be higher during the cooler growing period. This result is contrary to reports of Kozai et al. (2014a) and Kunjet et al. (2016). Since the two temperature regimes were not carried out concurrently, it is possible that other factors such as humidity, wind or pollinators could affect fruit set rather than solely the ability of pollen to germinate at low temperature. Thus, the effect of temperature on fruit set should be further examined.

2. Fruit quality.

The comparison between pollination with either 'Chanthaburi 3' or 'Monthong' on fruit quality showed no significant differences (Table 2). However, there was a tendency for fruit weight and the number of aborted seed in fruit to be lower in fruit that were pollinated with pollen of 'Chanthaburi 3'.

Table 2. Fruit quality of 'Monthong' durian derived from flowers pollinated with pollen of 'Chanthaburi 3' and 'Monthong' at 23.3-34.2°C.

Cultivar of pollen	Fruit weight (g)	Pulp to fruit weight ratio	Seed weight (g)	Aborted seed (%)	TSS (%)
Chanthaburi 3	3582	0.36	140.5	72.4	6.8
Monthong	4100	0.37	137.5	93.8	6.8
t-test	ns	ns	ns	ns	ns

ns = not-significantly different.

The aroma components in the pulp of 'Monthong' fruit derived from flowers pollinated with either 'Monthong' or 'Chanthaburi 3' pollen were similar, except that ethanethiol, propyl propanoate and dithioacetic acid were not found in those pollinated with 'Chanthaburi 3'. In addition, the dominant compound in fruit derived from self-pollinated flowers was ethyl propanoate, while it was ethyl 2-methylbutanoate in fruit derived from cross pollination with 'Chanthaburi 3' (Figure 2).

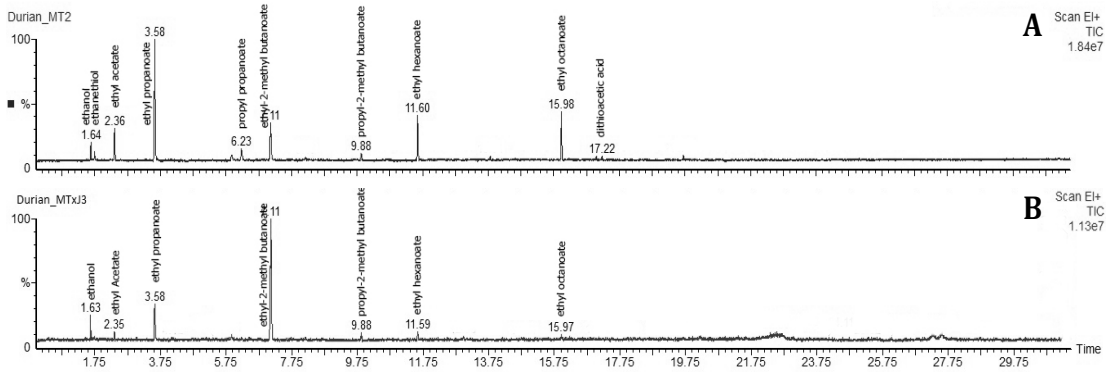

Figure 2. Total ion chromatograms of the volatile compounds of durian pulp from 'Monthong' self pollinated (A) and 'Monthong' pollinated with 'Chanthaburi 3' pollen (B), obtained by HS-SPME with DVB/CAR/PDMS fiber.

Furthermore, a preliminary sensory test, using 10 laboratory personnel, revealed no differences in taste and aroma in fruit of 'Monthong' that was self pollinated and 'Monthong' that was cross pollinated with 'Chanthaburi 3'. The results did not support the occurrence of a xenia effect as reported earlier by Indriyani et al. (2012). Although there was a tendency for foreign pollen ('Chanthaburi 3') to result in smaller fruit, a lower percentage of aborted seed, and less volatile sulfur compounds, further experiments are required before the use of foreign pollen can be recommended for the pollination of 'Monthong'.

CONCLUSIONS

Among 11 cultivars studied, 'Chanthaburi 3' pollen showed the highest percentage of germination in vitro at 10 to 35°C. In the natural condition, 'Chanthaburi 3' pollen gave the higher number of fruit setting tree^{-1} than 'Monthong' pollen, but not the fruit set percentage. Pollination at cooler weather condition gave no different in fruit set percentage, but the number of fruit set tree^{-1} was higher than at warmer weather. No difference was found in fruit quality, except for a slightly different in volatiles profile, when the two pollen were compared.

ACKNOWLEDGEMENTS

We would like to express our sincere thanks to Mr. Songsak Permpol, the owner of the 'Monthong' commercial orchard at Mueang District, Chanthaburi Province, Thailand, for allowing us to collect data and for his hospitality during the field work involved with this study.

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Yield trials of lotus (*Nelumbo nucifera* Gaertn.) grown for seed production

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Abstract

Lotus (*Nelumbo nucifera* Gaertn.) is an aquatic plant that is a popular cash crop in Thailand. A major problem with cultivated lotus is the poor yield of harvested seeds in tropical regions because of low seed set. The aim of this study was to evaluate seed yield and seed quality in eight lotus cultivars ('Nnu_A001', 'Nnu_A010', 'ChHy04', 'Khon Khen 6', 'Bang Phra 3/2', 'Prachuap Khiri Khan 29', 'Yasothon 1' and a local cultivar). The study was conducted at the Si Sa Ket Horticultural Research Center, from October 2017 to September 2018. The lotus rhizomes were grown in an aquatic pond, with dimensions of 6 m in length, 1 m in width and 0.5 m in depth. The design was a randomized complete block design with three replications. Treatments were eight cultivars of lotus. 'Nnu_A010', 'Prachuap Khiri Khan 29', 'Yasothon 1' and the local cultivar showed the shortest times to first flowering at 86, 77, 80 and 83 days after planting, respectively. The greatest pod size, seed size and seed fresh weight occurred in 'ChHy04'. The local lotus cultivar was found to have the highest number of seeds pod^{-1}. However, 'Yasothon 1' had the highest percentage of normal seed and the highest seed yield. 'ChHy04', 'Yasothon 1' and the local cultivar were selected for evaluation under field conditions for further varietal improvement.

Keywords: breeding program, torus, aquatic plant, wetland, sacred lotus

INTRODUCTION

Lotus (*Nelumbo nucifera* Gaertn.) is an aquatic herbaceous perennial plant within the family *Nelumbonaceae*. It has several common names, including Indian lotus, Chinese water lily and sacred lotus. Lotus seeds are recognized as a delicious and nutritious food. The seeds are rich in protein, amino acids, vitamins (B1, B2, B6, C and E) and phospholipids (Wu et al., 2007). Moreover, the alkaloid extracted from seeds is effective in the treatment of arrhythmia (Ling et al., 2005). Lotus is an important and popular cash crop in Asian countries. In Thailand, lotus is grown mostly for the production of flowers and seedpods (Theravanich et al., 2007). In 2019, returns of 4,700 baht rai^{-1} were achieved from lotus crop production (Department of Agricultural Extension, 2019). Low seed set of lotus limits the quality of seedpod production in tropical areas. Seed set is limited by pollen viability, stigma receptivity, self-incompatibility, and embryo development (Wang et al., 2012; Khatfan et al., 2014). There is very little available information on factors that influence the yields of economically important traits in lotus. In addition, there is a poor understanding of the impacts of cultivars on cultivar development in breeding programs that are designed for commercial outcomes. Therefore, the objective of this work was to evaluate seven named lotus cultivars and one local cultivar for yield and seed quality under open-field conditions in the next phase of a varietal improvement program.

MATERIALS AND METHODS

In this study, seven lotus cultivars were selected from a germplasm collection at the Si Sa Ket Horticultural Research Center ('Nnu_A001', 'Nnu_A010' and 'ChHy 04'), at the Khon Kaen Agricultural Production Science Research and Development Center ('Khon Khen 6' and 'Bang Phra 3/2') and at the Phichit Agricultural Research and Development Center ('Prachuap Khiri Khan 29' and 'Yasothon 1'), and one un-named local cultivar that was collected from a

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commercial lotus field in Si Sa Ket (Figure 1).

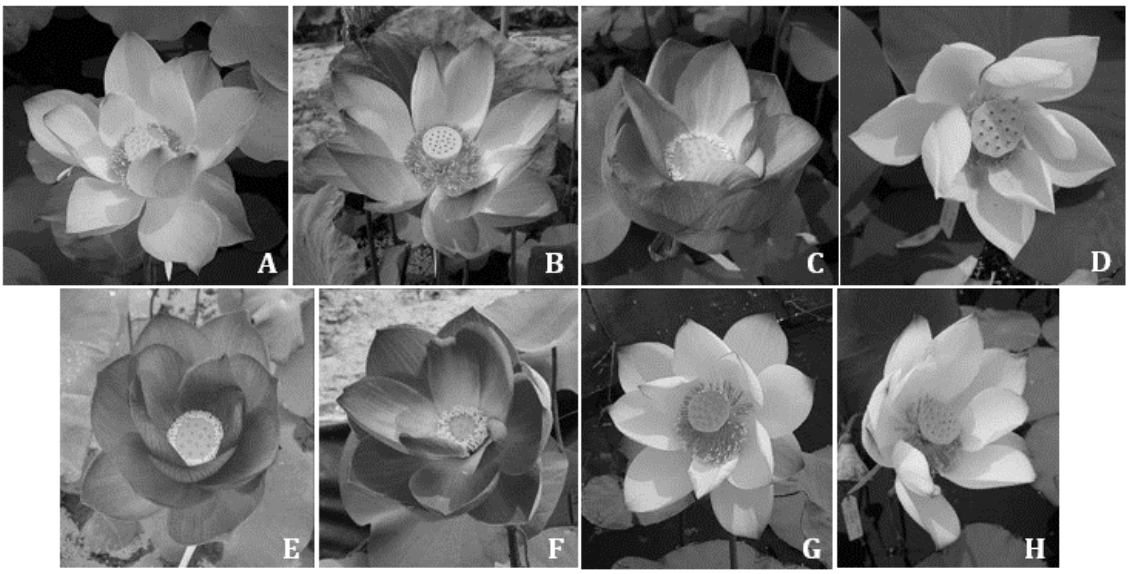

Figure 1. Flower characteristics of 'Nnu_A001' (A), 'Nnu_A010' (B), 'ChHy 04' (C), 'Khon Khen 6' (D), 'Bang Phra 3/2' (E), 'Prachuap Khiri Khan 29' (F), 'Yasothon 1' (G) and a local cultivar (H).

Primary rhizomes of 'Nnu_A001', 'Nnu_A010', 'ChHy04', 'Khon Khen 6', 'Bang Phra 3/2', 'Prachuap Khiri Khan 29', 'Yasothon 1' and the local cultivar were prepared in the form of a rhizome consisting of a shoot apex and the two adjacent nodes. The rhizomes were grown in an aquatic pond (6 m in length, 1 m in width and 0.5 m in depth) with a mixture of loam and dry compost (3:1 v/v) ≈20 cm in depth and immediately filled with water after planting. Chemical fertilization began when several floating leaves had developed. Lotus in each pond was feed 95 g of 15N-15P-15K at 15-days interval. The experiment was conducted at Si Sa Ket Horticultural Research Center, Si Sa Ket, Thailand from October 2017 to September 2018. A randomized complete block design with three replications per treatment was used. The treatments were the eight lotus cultivars and six rhizomes were planted in each replication.

The number of days after planting date until the first flower bloomed in each replication was used to define the speed to first flowering after the planting date. Pods and seeds were harvested at 18±1 day after the first day of flowering. Data of pod size, number of seedpod^{-1}, and the percentage of normal seedpod^{-1} were recorded and are presented as the average from 10 pods within each replication. Seed size and seed weight were recorded and expressed as means for 100 seeds in each replication. These traits were used as evaluation criteria. Data for each trait were statistically analyzed using one-way analysis of variance (ANOVA) and the means were separated using the Duncan's multiple range test (DMRT) at 0.05 level.

RESULTS AND DISCUSSION

The earliest first flowering occurred in 'Nnu_A010', 'Prachuap Khiri Khan 29', 'Yasothon 1' and the local cultivar (Table 1). 'Bang Phra 3/2' was the slowest to first flowering when compared with other treatments. Ishizuna and Tsutsumi (2014) observed that a flower bud of lotus is formed at every node. However, the development of flower buds to reach full flowering was limited by environmental factors. In this study, the speed of appearance of the first flowering of the eight lotus cultivars is likely to have shown a difference in response to environmental factors as well as differences due to genetic factors.

Table 1. The speed of appearance first flowering after planting of eight lotus cultivars.

Cultivars	Time to first flowering (days)
Nnu_A001	130cd
Nnu_A010	86a
ChHy 04	91b
Khon Khen 6	104c
Bang Phra 3/2	168d
Prachuap Khiri Khan 29	77a
Yasothon 1	80a
Local cultivar	83a
F-test	**
CV (%)	8.31

Means with the same letter within a column are not significantly different by DMRT ($p<0.05$).
** Significantly different.

'ChHy 04' had the highest of pod width, pod height, seed width, seed length and seed fresh weight. The smallest pod size, seed size and seed weight were in the cultivar 'Yasothon 1' (Table 2; Figure 2).

Table 2. Pod size, seed size and seed weight of eight lotus cultivars.

Cultivars	Pod width (cm)	Pod height (cm)	Seed width (cm)	Seed length (cm)	Seed fresh weight (g)	Seed dry weight (g)
Nnu_A001	10.13b	5.17a	1.43bc	2.04a	2.42ab	1.13
Nnu_A010	9.67bc	4.12b	1.45bc	1.87b	2.55a	1.43
ChHy 04	11.69a	5.13a	1.60a	1.93ab	2.76a	1.39
Khon Khen 6	9.19cde	4.25b	1.31c	1.63c	1.52c	0.90
Bang Phra 3/2	9.97b	4.02b	1.62a	1.85b	2.60a	1.13
Prachuap Khiri Khan 29	8.68ed	4.43b	1.45b	1.87b	2.20ab	1.15
Yasothon 1	8.46e	3.44c	1.41bc	1.65c	1.97bc	1.15
Local cultivar	9.51bcd	4.21b	1.43bc	1.88b	2.22ab	1.11
F-test	**	**	**	**	**	ns
CV (%)	4.56	6.28	4.20	3.68	13.01	19.46

Means with the same letter within a column are not significantly different by DMRT ($p<0.05$).
** Significantly different.

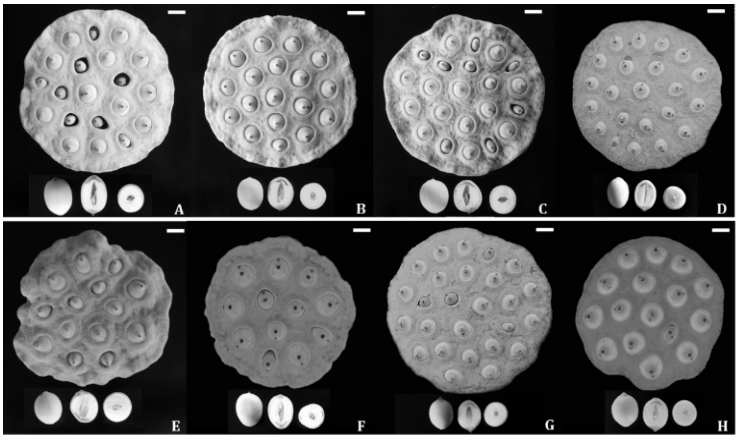

Figure 2. Pod and normal seed characteristics of 'Nnu_A001' (A), 'Nnu_A010' (B), 'ChHy04' (C), 'Khon Khen 6' (D), 'Bang Phra 3/2' (E), 'Prachuap Khiri Khan 29' (F), 'Yasothon 1' (G) and the local cultivar (H); Scale bar A-G = 1 cm.

The number of seedpod^{-1} was highest in the local cultivar and lowest in 'Bang Phra 3/2' (Table 3). The highest percentage of normal seeds and yield was obtained in 'Yasothon 1' (Table 3). Pollen viability and germination studies have shown that a low seed set rate is associated with self-incompatibility but that self-incompatibility does not exist in all lotus cultivars (Khatfan et al., 2014). Therefore, a lotus cultivar with a high percentage of normal seeds is usually one with low self-incompatibility.

Table 3. Number of seeds per pod, percentage of normal seeds and number of pods 6 m^{-2} of production area of eight lotus cultivars.

Cultivars	Number of seed pod^{-1}	Percentage of normal seed	Number of pods 6 m^{-2}
Nnu_A001	18bc	31.51d	20c
Nnu_A010	17dc	69.68ab	58bc
ChHy 04	19ab	60.79bc	85ab
Khon Khen 6	15dc	44.38dc	31c
Bang Phra 3/2	11e	37.70d	30c
Prachuap Khiri Khan 29	13e	70.07ab	63bc
Yasothon 1	17dc	80.56a	115a
Local cultivar	21a	67.05ab	80ab
F-test	**	**	**
CV (%)	7.01	17.22	41.76

Means with the same letter within a column are not significantly different by DMRT ($p<0.05$).
** Significantly different.

Based on the results found for 'ChHy 04' that had a high potential pod size, good seed size, high seed fresh weight (Table 2) and yield (Table 3) and for both 'Yasothon 1' and the local cultivar that had a positive response to environmental factors (Table 1), a low self-incompatibility and high yield (Table 3), these cultivars were selected for further evaluation under field conditions.

CONCLUSIONS

Basic knowledge gained from this study, selection of lotus cultivars could be made for their inclusion in the varietal improvement program. The results obtained showed that 'ChHy 04', 'Yasothon 1' and the local cultivar were suitable for further evaluation in field trials aimed at the further of improvement of cultivars.

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Impacts of cultivar and growing substrate on growth and yield of melon

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Abstract

The effects of substrates on melon production in a net house were studied. The experiment used a 2×3 factorial in a completely randomized design with four replications. Factor A was the melon cultivar; 'POT ORANGE T1957' and 'GREEN NET T778'. Factor B was the substrate type consisting of either sand mixed with coir dust, or sand mixed with rice husk charcoal, or sand mixed with peat (all with 1:1 v/v). 'POT ORANGE' had a higher vine length and fruit weight than 'GREEN NET'. Melons grown in sand mixed with peat gave the highest soluble solids of 17.4 °Brix, higher than those grown in sand mixed with coir dust (15.8 °Brix) or sand mixed with rice husk charcoal (15.7 °Brix). Yields were not significantly different among substrates. After harvest, sand mixed with peat and sand mixed with rice husk charcoal had an equal bulk density of 1.01 g cm^{-3}, and the mixes subsided by 2.95 and 4.08 cm, respectively. In contrast, sand mixed with coir dust had 0.9 g cm^{-3} bulk density and subsided by only 2.29 cm. Hence, sand mixed coir dust was considered to be the best substrate for melon culture in this study based on and overall consideration of plant responses and the physical properties of the growing medium.

Keywords: coir dust, rice husk charcoal, peat, sand, yield

INTRODUCTION

Thailand is a country rich in natural resources with an advantage of being located in the tropics where various types of agriculture, including fruit production, can be practiced throughout the year. Melon is one of the best options for production because it can quickly produce fruit and it grows well in hot weather with normal crop water requirements. Most importantly, melon is an expensive fruit, giving good returns to producers. Its sweetness and ease of preparation are attractive to many consumers. Due to wide range of favorable conditions in terms of climate and soils, various types of melons are currently cultivated in Thailand, mostly on soil. However, soil-borne pests can often occur during melon production that can require the use of pesticides. Such control measures are undesirable as they may contaminate the product and impact negatively on consumer health (Montri and Wattanapreechanon, 2007). An alternative approach is to use soilless culture where the crop can be grown in many different types of planting material. Plant nutrients can be added to the growing medium according to each particular crop's requirements. This approach can provide control over the consistency of production, including both product quality and yield. In addition, a smaller cultivated area and less manpower is usually required (Yu et al., 1997; Fukuda and Anami, 2002). Consequently, soilless culture is often applied to a number of different crops worldwide and has recently found application in Thailand. In Thailand, there are many common planting materials that are available that have different physical properties, including rice husk, rice husk charcoal, and coir dust. Since different growing media contain different properties that may influence growth and yield, this research aimed to investigate the effects of different types of planting material and crop cultivar on growth and yield of melon.

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MATERIALS AND METHODS

The effects of substrates on melon production were studied in a net house (32 mesh) at the Department of Soil Science, Faculty of Agriculture at the Kamphaeng Saen Campus of Kasetsart University, Thailand. The experiment used a 2×3 factorial in a completely randomized design with four replications. Factor A was the cultivar; 'POT ORANGE T1957' and 'GREEN NET T778'. Factor B was the substrate type consisting of either sand mixed with coir dust, or sand mixed with rice husk charcoal or sand mixed with peat (all with 1:1, v/v). The nutrient solution used was the Kae Te Chen's formula adjusted according to growth stage. Growth (vine length 30 and 60 days after planting), fruit quality (°Brix) and yield of melon were recorded. Some physical properties of each substrate were also determined after harvest. The data were analyzed using R-stat and the means compared by Duncan's new multiple range test (DMRT) at 95%.

RESULTS

Length of the vine

Differences in vine length between cultivars were significant at the first time of assessment (Table 1). Vines of 'POT ORANGE T1957' were significantly longer than those of 'GREEN NET T778'. Differences in vine length between substrates were significant at 30 days after planting. Vine length of melon grown in sand: peat was the longest, but not significantly different from that in sand: rice husk charcoal, while vine length in sand: coir dust (C) was shortest. When measured at the 25 mature leaf development stage, there was no effect of type of growing media on vine length. There was, therefore, an interaction between type of planting substrate and cultivar of melon but only at 30 days after planting The vines of 'POT ORANGE T1957' grown in sand:rice husk charcoal and in sand:peat were longer than those grown in sand: coir dust and the vines of 'GREEN NET T778' growing in any of the substrates.

Table 1. Effects of interaction between type of melon cultivar and planting materials on length of vine, fruit weight and sweetness.

Factors	Length of vine (cm)		Fruit weight	Sweetness
	30 DAT	60 DAT	(kg)	(°Brix)
Cultivar (A)				
Pot orange	64.8x	171.3x	1.73x	16.0
Green net	54.1y	158.1y	1.59y	16.6
F-test (A)	*	*	*	ns
Substrate (B)				
Sand:coir dust	54.2B	166.5	1.66	15.8B
Sand:rice husk charcoal	60.9AB	163.1	1.71	15.7B
Sand:peat	63.3A	164.4	1.60	17.4A
F-test (B)	*	ns	ns	*
Interaction				
Pot orange:coir dust	53.4b	172.2	1.64	15.3c
Pot orange:rice husk charcoal	70.8a	172.0	1.80	15.8bc
Pot orange:peat	70.3a	169.5	1.74	16.9a
Green net:coir dust	55.0b	160.8	1.68	16.3bc
Green net:rice husk charcoal	51.0b	154.3	1.62	15.5bc
Green net:peat	56.4b	159.3	1.47	17.8a
F-test (A×B)	*	ns	ns	*
CV (%)	17.03	20.04	10.30	7.35

Mean values followed by same small letters do not differ significantly according to DMRT test at 95%.
ns = non-significant at 0.05 probability, * significant at 0.05 probability.

Fruit weight

The fruit weight (FW) of 'POT ORANGE T1957' was significantly higher than that of 'GREEN NET T778' in most substrates (Table 1). However, there was no effect of type of substrate on fruit weight and no interaction between type of substrate and cultivar. Overall, the weight of melons was within a range of 1.47-1.80 kg.

Fruit sweetness

Sweetness of the melons was assessed using total soluble solids concentration (°Brix). In contrast to fruit weight, the type of planting substrate and not cultivar had an effect on sweetness (TSS). The highest TSS was found in melon that was planted in sand:peat (P). There was no significantly difference in TSS found between melon grown in sand:rice husk charcoal and that in sand:coir dust (Table 1).

Bulk density of substrate

The highest bulk density (g cm^{-3}) was found in the sand:rice husk charcoal and sand:peat media (Table 2).

Table 2. Effects of interaction between type of melon cultivar and planting materials on physical properties of the planting materials.

Factors	Bulk density (g cm^{-3})	Subsidence (cm)
Cultivar (A)		
Pot orange	0.99	3.10
Green net	0.96	3.11
F-test (A)	ns	ns
Substrate (B)		
Sand:coir dust	0.91B	2.29C
Sand:rice husk charcoal	1.01A	4.08A
Sand:peat	1.01A	2.95B
F-test (B)	*	*
Interaction		
Pot orange:coir dust	0.99ab	2.34c
Pot orange:rice husk charcoal	0.95b	4.06a
Pot orange:peat	1.02ab	2.89b
Green net:coir dust	0.82c	2.24 c
Green net:rice husk charcoal	1.07a	4.09 a
Green net:peat	1.00 ab	3.01 b
F-test (A×B)	*	*
CV (%)	9.27	4.95

Mean values followed by same small letters do not differ significantly according to DMRT test at 95%.
ns = non-significant at 0.05 probability, * significant at 0.05 probability.

Subsidence of substrate

Significant differences occurred with shrinkage of the growing substrate depending on the type of media (Table 2). The highest subsidence occurred with sand:rice husk charcoal, followed by sand:peat, and the lowest was with sand:coir dust. There was no impact of cultivar on the values obtained for subsidence.

DISCUSSION

The two cultivars of melon that were grown in the different planting substrates had different growth and fruit quality characteristics that are controlled genetically. Sweetness is an important characteristic in the determination of the quality of produce. Varieties of *C. melo*, such as *C. cantalupensis*, *C. reticulatus* and *C. inodorus*, all contain genotypes which accumulate

sucrose (Kyriacou et al., 2018). In additional, Nuñez-Palenius et al. (2008) reported that ripe melon fruits contain sucrose, glucose and fructose. The sucrose accumulated during fruit development while, glucose and fructose are used for the synthesis of sucrose during the development phase. Sucrose concentration affects the sweetness of the melon fruit. Chikh-Rouhou et al. (2019) reported that the quality attributes of melon, such as fruit weight and total soluble solids concentration, are genetically controlled but are influenced by the environment. Examination of the physical properties of the substrates used in this study showed that that sand:coir dust gave the lowest subsidence and had the lowest bulk density. Noguera et al. (2003) similarly reported that coir dust had a low bulk density that can result in enhanced root growth, together with having a high water holding capacity (WHC) and a high cation exchangeable capacity (CEC) leading plant nutrients to be retained in the substrate (Nguyen and Wang, 2017). Consequently, the coir dust is a desirable component in substrate mixtures and it can enhance plant production. The results in this study showed that sand combined with coir dust produced good vine growth, high fruit weights, low bulk density and the lowest amount of subsidence.

CONCLUSIONS

Sand combined with coir dust produced a high fresh fruit weight and had the best physical properties, such as the lowest bulk density and substrate subsidence.

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Influence of lime and phosphorus fertilizer on shallot growth and bulb yield in strongly acid soils in West Java, Indonesia

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Abstract

Shallot yield is lower in acidic soils and under low soil P status. Increasing shallot bulb yield can be achieved by improving the soil fertility of acidic soils. This study investigated the effect of lime and phosphorus (P) fertilizer on shallot growth and yield. Three field trials were set up in the early rainy season (2018-2019) in Kabupaten Bandung, West Java, Indonesia. They were: 1) Field 1, extremely acidic – low soil available-P (initial soil pH=4.23, Bray-P = 14 mg P kg^{-1}); 2) Field 2, very strongly acidic – medium soil available-P (initial soil pH=4.98, Bray-P = 27 mg P kg^{-1} and 3) Field 3, strongly acidic – low soil available-P (initial soil pH=5.15, Bray-P = 8 mg P kg^{-1}). Each trial site had 12 treatments; each replicated four times. The treatments were combinations of lime (0, 0.5 and 1.0 t ha^{-1}) and phosphorus fertilizer (0, 50, 100, 150 P kg ha^{-1}). Lime significantly increased soil pH, reduced Al^{3+} in all fields, but only increased dry weight at eight weeks after planting and shallot bulb yield in Field 2 (from 7.7 to 10.6 t ha^{-1}). Phosphorus fertilization increased soil available P in Field 1 and 3 but only improved dry weight at eight weeks after planting and shallot bulb yield in Field 3 (from 8.4 to 10.0 t ha^{-1}). However, neither lime nor phosphorus fertilizer improved shallot biomass nor bulb yield for Field 1. In conclusion, application of lime improved the shallot bulb yield in the strongly acidic soil by increasing soil pH, Ca^{2+} content and by reducing the exchangeable Al^{3+}. Phosphorus fertilization improved P-availability and improved the bulb yield in soil with low available-P status. A higher rate of lime (>1.0 t ha^{-1}) in extremely acid soil is required to increased soil pH, to adequately lower exchangeable Al^{3+} and to achieve a yield response. Bulb yield is a fresh bulb yield of shallot without leaves.

Keywords: limestone, acidic soils, P fertilizer, onion, soil amendment, aluminum toxicity

INTRODUCTION

Shallot (*Allium cepa* Aggregatum group) is an important vegetable crop in Indonesia; however, yields are highly variable, with lower yields occurring on more weathered acidic soils. Soil acidity is a common problem in agriculture systems, being a significant cause of reduced crop yield due to lower availability of essential plant nutrients (e.g., phosphorus and calcium) and increased availability of toxic elements (e.g., aluminum and manganese) (Li et al., 2019). Under acidic conditions, plant growth can be restricted by specific factors and the interactions between them, with often increasing levels of aluminum (Al) and manganese (Mn) resulting in toxicity, which restricts root development and decreases plant availability of P and basic cations (Haynes and Mokolobate, 2001; Marschner, 1991). To mitigate the harmful effects of acidic soils on plant growth, applications of liming materials is a standard method to increase soil pH levels (Kamprath and Foy, 1985; Kunhikrishnan et al., 2016). However, the effectiveness of liming varies and depends on lime source materials, the application rate and crop species (Li et al., 2019). There is limited information on shallot production under acid soil conditions and, consequently, there is a reliance on onion research to guide recommendations.

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A pot study found that onions failed to bulb when soil pH was <3 and applied agricultural lime, at a rate 12 t ha^{-1}, increased soil pH from 3.4 to 5.1 and increased onion bulb yield by 78% (van Lierop et al., 1979). Mathur and Levesque (1983) showed that onion bulb yield increased linearly with increasing soil pH from pH 3.0 to 5.8. In a field trial in less acidic soil conditions in Oregon USA, Hemphill (1987) reported that an application of 14.8 t ha^{-1} of agricultural limestone (95% $CaCO_3$ equivalent) increased soil pH from 5.5 to 6.6 and enhanced onion bulb yield from 4.0 to 30.4 t ha^{-1}. However, there have been limited detailed field studies that have investigated the effects of liming on shallot yield in very acid soil conditions (pH<5).

There have been some studies involving phosphorus fertilizer that have found that the optimum rate of P for onions varies depending on soil pH and P status. Chagas et al. (2016) reported that in acid soil (pH=5.5) with a deficient of soil available P (Olsen P = 3.2 mg P kg^{-1}), the optimum rate was very high at 149 kg P ha^{-1}, but was reduced to 77 kg P ha^{-1} when the initial Olsen was higher (Olsen P = 12 mg P kg^{-1}). In another study where the soil pH was near neutral (pH=6.5) and the Olsen P was 16 mg P kg^{-1}, the P fertilizer recommendation was 59 kg P ha^{-1} (Boyhan et al., 2007). However, there has been little quantitative analysis of optimum rates of phosphorus fertilizer on very acid soil conditions (pH<5) for either onion or shallots. One study reported that the application of P fertilizer at rates of 0-105 kg P ha^{-1} without liming did not increase shallot yield when soil pH was 4.3 and 4.8 and Olsen P was 10.2 and 5.1 mg P kg^{-1}, respectively (Sumarni et al., 2012).

One of the constraints to lime use by smallholder farmers in West Java, Indonesia, is the difficulty in transporting the vast quantities of lime often required, to remote farm sites with limited road access. Because this has constrained the use of lime by some farmers, it is useful to assess the benefits of lower rates of lime.

This study aimed to investigate the effect of lime and P fertilizer on shallot growth and yield in strongly acid soils with varying P status in West Java.

MATERIALS AND METHODS

Experimental sites and plant materials

The experiment was set up in the rainy season on three farmer's fields of Kecamatan Pacet, Kabupaten Bandung, West Java (6°41'-7°19'S; 107°22'-108°50'E) at an altitude 1000-1200 m a.s.l. Climatic data were collected from BMKG (Badan Meteorologi and Geofisika Jawa Barat/Meteorology and Geophysics Agency of West Java). During the crop growing period (November 15, 2018-February 12, 2019), the amount of rainfall was 840 mm, with a range of 123-440 mm $month^{-1}$. Mean temperatures ranged from 20.6 to 23.8°C with the minimum temperature 19°C and the maximum temperature 33°C. Average relative humidity was 80%, with the minimum being 66% and the maximum being 94%. The trial sites were on sloping areas of 10-30°. The soil type of the experimental area was an Inceptisol and the soil has a clay texture (61% clay, 25% silt and 14% sand). The trial site details and soil chemical properties are provided in Table 1. This field trial was a continued study from survey activity that was done in 2017 and 2018. The survey research indicated that Pacet, one of the shallot productions centers in West Java, had acidic soils and a lower bulb yield compared to other regions that have higher soil pH. Therefore, the research focused on how to manage the soil acidity and improve shallot production in this area. Shallots (*Allium cepa* Aggregatum group, 'Sumenep') were planted at 0.2×0.2 m spacing in bed size 1×3 m. The height of the seedbeds was approximately 30 cm, with the gap between beds being 60 cm.

Experimental design and treatments

The experiment was conducted to compare the effects of lime and phosphorus fertilizer on acid soil properties and shallot growth and yield. The effects of three different rates of lime: 0 (0 L), 0.5 (0.5 L) and 1.0 t ha^{-1} (1.0 L) and four different rates of phosphorus fertiliser: 0 (0 P), 50 (50 P), 100 (100 P) and 150 kg P ha^{-1} (150 P) were studied. Lime and phosphorus fertilizer was incorporated at a single application after manual ploughing on November 14, 2018, a day before planting.

Table 1. Characteristics of the field trial site.

	Field 1	Field 2	Field 3
Location	7°12'54"S;107°06'87"E	7°12'73"S;107°71'88"E	7°12'75"S;107°71'94"E
Elevation (m a.s.l.)	1060	1147	1172
pH	4.23 (extremely acid)	4.98 (very strongly acid)	5.15 (strongly acid)
Al^{3+} (cmol (+) kg^{-1})	1.60 (very high)	1.13 (high)	0.32 (low)
Ca^{2+} (cmol (+) kg^{-1})	4.60	6.79	8.03
Al^{3+}/Ca^{2+}	0.35 (high)	0.17 (medium)	0.04 (low)
Bray-P (mg P kg^{-1})	14.2 (low)	26.9 (medium)	7.7 (low)
P-retention (%)	61	52	48

Lime (CaO = 27%, $CaCO_3$ equivalent = 106%) and triple superphosphate fertiliser (0-20-0-0) were used as treatments. Ammonium sulfate (AS) (21-0-0-24) and urea (46-0-0-0) were used as the N sources. Potassium chloride (KCl) (0-50-0-0) was used as a potassium source. Ammonium sulfate was applied at a rate of 317 kg ha^{-1} at 21 days after planting. Urea was applied twice, at 35 and 49 days after planting, at a rate of 145 kg ha^{-1} per application. Potassium chloride was applied three times, at 21, 35 and 49 days after planting, at a rate of 107 kg ha^{-1} per application. The N and K fertilizers were applied at the same rate to all treatment plots.

Measurements and analysis

Eight weeks after planting, five shallot plants from each treatment were sampled, washed with de-ionized water, blotted dry with tissue paper and cut into separate components of roots and shoots. The plant materials were dried in an oven 65°C (Memmert type UN 450) and were measured on an electrical balance (Precisa type XB 620C) to get the root and biomass dry weight (g $plant^{-1}$). The dry weight of the bulb yield (g $plant^{-1}$) was measured by the same method.

Soil samples (0-20 cm soil depth; 6 cores per sample) for analyses were taken near the plants at eight weeks after planting. The soil pH (soil to water ratio of 1:2.5) was measured from soil suspensions using a Hanna pH meter (HI 2550). The plant-available phosphorus in the soil was determined using the Bray-1 method (Bray and Kurtz, 1945; Van Reeuwijk, 1993). The basic cations, Ca^{2+}, Mg^{2+}, K^{+} and Na^{+}, were measured by ammonium acetate (1 M $CH_3CO_3NH_4$, pH 7) extraction and analyzed using atomic absorption spectroscopy (AAS) (Hajek et al., 1972; Van Reeuwijk, 1993). Exchangeable Al and H were determined by 1 N KCl extraction (Van Reeuwijk, 1993).

Statistical analysis

In order to assess the effect of treatments on measured parameters, repeated-measures analyses of variance (ANOVA) were used using SAS (9.4 version). An ANOVA was observed separately for each field (three lime levels × four phosphorus fertilizer levels). Means were grouped according to the LSD test. In the tables, means followed by the same letter are not significantly different at $p<0.05$. To measure the correlation (r^2) between observations average of data were used and combined across the three fields.

RESULTS AND DISCUSSION

The results from each field were analyzed separately. However, for effective presentation, the results were compiled into one table. A bold field title was provided to highlight the field.

Effect of lime and phosphorus fertilizer on soil properties

The lime treatments raised the soil pH at all three-field sites (Table 2). The 0.5 t ha^{-1} lime rate significantly ($p\leq0.05$) increased the soil pH, compared to the no lime control, at both Field 2 and 3. Whereas the 1.0 t ha^{-1} lime rate significantly increased the soil pH, compared to the control, at all three field trial sites, but the effect was only significantly higher compared

to the 0.5 t ha^{-1} lime rate at Field 2. On average, pH increased by about 0.30 pH units when lime was applied at 1.0 t ha^{-1}. The highest increase in soil pH was achieved at Field 3, increasing by 0.39 pH units. However, the pH values were well below the optimum pH soil for onion growing of 6.1 (Reid and Morton, 2019).

Table 2. The effect of lime and phosphorus fertilizer on soil pH, available P, Al^{3+} and Ca^{2+} in each field (0-20 cm).

	pH	Bray-1 (mg P kg^{-1})	Al^{3+} (cmol (+) kg^{-1})	Ca^{2+} (cmol (+) kg^{-1})	Al^{3+}/Ca^{2+}
Field 1					
0 t ha^{-1} (0L)	3.94b	25.2ns	1.60a	3.33b	0.51a
0.5 t ha^{-1} (0.5L)	4.04b	28.4	1.59a	3.80ab	0.45a
1.0 t ha^{-1} (1.0L)	4.17a	27.8	1.16b	4.27a	0.29b
0 kg P ha^{-1} (0P)	4.00ns	21.6b	1.43ns	3.41b	0.44ns
50 kg P ha^{-1} (50P)	4.01	26.6ab	1.46	3.77ab	0.41
100 kg P ha^{-1} (100P)	4.08	25.4ab	1.56	3.96ab	0.46
150 kg P ha^{-1} (150P)	4.09	35.0a	1.36	4.09a	0.36
CV (%)	3.8	18.0	9.6	18.7	7.5
Field 2					
0 t ha^{-1} (0L)	4.10c	31.3ns	1.13b	4.26b	0.28a
0.5 t ha^{-1} (0.5L)	4.25b	34.4	1.00b	4.57b	0.24a
1.0 t ha^{-1} (1.0L)	4.39a	29.7	0.65a	5.57a	0.13b
0 kg P ha^{-1} (0P)	4.16b	28.3b*	1.01ns	4.74ab	0.24ns
50 kg P ha^{-1} (50P)	4.20b	31.3ab	1.07	4.41b	0.27
100 kg P ha^{-1} (100P)	4.26ab	35.9a	0.81	4.73ab	0.18
150 kg P ha^{-1} (150P)	4.36a	31.7ab	0.83	5.31a	0.17
CV (%)	4.0	15.5	14.7	20.2	8.3
Field 3					
0 t ha^{-1} (0L)	4.26b	26.1ns	0.32b	5.52b	0.08a
0.5 t ha^{-1} (0.5L)	4.59a	25.6	0.27b	6.28b	0.07ab
1.0 t ha^{-1} (1.0L)	4.65a	28.1	0.04a	8.29a	0.01b
0 kg P ha^{-1} (0P)	4.47ns	11.9c	0.23ns	6.58ab	0.05ns
50 kg P ha^{-1} (50P)	4.49	24.1b	0.23	6.34ab	0.07
100 kg P ha^{-1} (100P)	4.43	27.7ab	0.22	6.16b	0.06
150 kg P ha^{-1} (150P)	4.59	41.3a	0.16	7.71a	0.03
CV (%)	8.9	23.4	13.7	28.0	6.8

Tukey's method, for means comparison, means presenting the same letters are not significantly different. ns = not significantly different at α=5%. * significantly different at α=10%.

The lime application also reduced aluminum toxicity. On average, the lime application of 1.0 t ha^{-1} decreased exchangeable Al by 0.40 cmol (+) kg^{-1} across three sites. Lime applied at 1.0 t ha^{-1} decreased exchangeable Al from 1.60 to 1.16 cmol (+) kg^{-1} in Field 1, from 1.13 to 0.65 cmol (+) kg^{-1} in Field 2 and from 0.32 to 0.04 cmol (+) kg^{-1} in Field 3. Also, liming increased soil exchangeable calcium (Ca^{2+}). On average, the lime application of 1.0 t ha^{-1} raised exchangeable Ca^{2+} by 1.67 cmol (+) kg^{-1} across all fields.

Phosphorus fertilizer treatments significantly increased available soil P only at Field 1 and 3. At Field 1, only the 150 kg P ha^{-1} rate resulted in a Bray-P value significantly higher than the control treatment. At Field 3, all three P fertilizer rates significantly increased Bray-P, compared to the control treatment, with the 100 and 150 kg P ha^{-1} rates increasing Bray-P significantly higher than the 50 kg P ha^{-1} rate. At this site, adding 150 kg P ha^{-1} increased Bray-P from low to medium soil available-P. At Field 2, a P fertilizer rate of 100 kg P ha^{-1} slightly increased the P-Bray. However, this increase was not significant and it was assumed that the

variation within the field was high. This high variation increased the mean and the standard error and the CV(%) in this field were high. Furthermore, if the alpha (α) value was 10%, the result would have been significantly different.

The optimum or the target Olsen-P for onion was depended on the potential yield of the dry bulb. For a dry bulb matter of 6 t ha^{-1}, the target Olsen-P is 35 mg P kg^{-1} (Reid and Morton, 2019), which is equivalent to a Bray-P value of approximately 72 mg P kg^{-1} (the high range for Bray-P is 40-100 mg P kg^{-1}) (Khokhar, 2019; Mallarino, 1995). In the current study, the highest Bray P-value achieved was 41; therefore, at all sites available soil P status may have still been a limiting factor. However, given the very low soil pH, even after liming, available P will not be the only limiting factor at all field sites.

Effect of lime and phosphorus fertilizer application on root dry weight and shallot bulb yield

There was a varied response of the effect of the lime and phosphorus fertilizer treatments on root dry matter (Table 3). The effect of lime on biomass was only significant at Field 2 where root biomass was increased. The application of 1.0 t ha^{-1} lime increased root biomass about 31% compared to control, without lime. The use of phosphorus fertilizer significantly increased root dry weight at Field 3. The application of phosphorus fertilizer at rates of 50 kg P ha^{-1} enhanced the root biomass by about 32% compared with 0 kg P ha^{-1}. However, there was no significant difference between the rates from 50 to 150 kg P ha^{-1}.

Table 3. The effect of lime and phosphorus fertilizer on root dry weight (g $plant^{-1}$) at eight weeks after planting in each field.

Treatments	Root dry weight (g $plant^{-1}$)			Bulb yield (t ha^{-1})		
	Field 1	Field 2	Field 3	Field 1	Field 2	Field 3
0 t ha^{-1} (0 L)	0.14ns	0.26b	0.39ns	6.56ns	7.69b	9.85ns
0.5 t ha^{-1} (0.5 L)	0.19	0.31ab	0.35	6.43	8.96ab	9.43
1.0 t ha^{-1} (1.0 L)	0.17	0.34a	0.40	6.08	10.61a	9.63
0 kg P ha^{-1} (0 P)	0.16ns	0.31ns	0.31b	6.14ns	9.16ns	8.39b
50 kg P ha^{-1} (50 P)	0.15	0.30	0.41a	6.03	8.13	10.08a
100 kg P ha^{-1} (100 P)	0.18	0.30	0.40a	6.84	9.36	10.05a
150 kg P ha^{-1} (150 P)	0.17	0.31	0.41a	6.40	9.69	10.04a
CV (%)	5.2	28.6	25.0	18.3	25.6	24.2

Tukey's method for means comparison, the same letters are not significantly different. Ns = not significantly different at α=5%.

In general, Field 3 had the highest root dry weight compared to Fields 1 and 2. Field 3 had the highest pH value and the lowest Al^{3+} compared to Field 1 and 2. This study showed that the shallot was sensitive to the soil pH and to the concentration of exchangeable Al^{3+}. Furthermore, since Al^{3+} has an antagonistic correlation with Ca^{2+} (r=-0.77), the ratio of Al^{3+} and Ca^{2+} could be more useful on describing the effect of aluminum toxicity on plant growth. In this study, it was found that Al^{3+}/Ca^{2+} had a moderately negative correlation with root dry weight (g $plant^{-1}$) (r=-0.54). Root dry weight had a strong positive correlation in total biomass (r=0.72), and total biomass had a strong positive correlation with bulb yield (g $plant^{-1}$) (r=0.65). Further, shallot bulb yield (g $plant^{-1}$) had a strong positive correlation with fresh bulb yield per ha (r=0.70).

Table 3 shows the effect of lime and P-fertiliser rates on fresh bulb yield. There was no interaction between lime and phosphorus fertilizer on bulb yield in all fields. Lime significantly increased the fresh bulb yield in Field 2 but not the phosphorus fertilizer. Lime doses 0.5 and 1.0 t ha^{-1} improved the fresh bulb yield ha^{-1} by 17 and 38% over the control, respectively, in Field 2.

Phosphorus fertilizer increased the fresh bulb yield ha^{-1} at Field 3. The lowest fresh bulb yield was 8.4 t ha^{-1} at control treatment, without phosphorus fertilization. The highest fresh bulb yield was 10.1 t ha^{-1} at 50 kg P ha^{-1} that did not have a significant difference with other

doses.

CONCLUSIONS

Lime was effective in increasing soil pH and reducing exchangeable Al at all three sites but a lime rate of 1.0 t ha^{-1} only increased bulb yield at Field 2, with medium Al toxicity and high soil available-P (initial soil pH=4.23, Al^{3+}/Ca^{2+}=0.35, Bray-P = 27 mg P kg^{-1}). Phosphorus fertilization increased soil available-P at two sites (Fields 1 and 3) but only improved the bulb yield at Field 3, with low Al toxicity and low soil available-P (initial soil pH=5.15, Al^{3+}/Ca^{2+}=0.04, Bray-P = 8 mg kg^{-1}). Neither lime nor P fertilizer, improved shallot bulb yield at Field 1, with high Al toxicity and low available-P (initial soil pH=4.23, Al^{3+}/Ca^{2+}ratio=0.35, Bray-P = 14 mg P kg^{-1}). The low rate of lime (lime 1.0 t ha^{-1}) may not have been sufficient to increase the soil pH and Ca^{2+} and alleviate Al toxicity in this Field. Calcium deficiency and aluminum toxicity may have impeded the bulb yield in Field 1. Further Research is needed to understand the role of each nutrient and the interaction among them on the growth and development of shallot plants in strongly acidic soil.

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Physico-chemical evaluation of red-fleshed dragon fruit (*Hylocereus polyrhizus* Britton and Rose) as influenced by potassium fertilization

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Abstract

Potassium (K) is an important, essential nutrient because of its effects on quality factors and the fact that it plays an important regulatory role in various key physiological processes (i.e., translocation of sugars and formation of carbohydrates, protein synthesis and regulation of particular enzymes). Red-fleshed dragon fruit (*Hylocereus polyrhizus*) has high functional potential and is in demand in the market. This study aimed to evaluate the physico-chemical properties of freshly picked fruit at 38 days after anthesis (DAA) as influenced by five different potassium levels imposed as fertilizer treatments: T1) $N_{60}P_{120}K_{60}$; T2) $N_{60}P_{120}K_{120}$; T3) $N_{60}P_{120}K_{180}$; T4) $N_{60}P_{120}K_{240}$; and T5) $N_{60}P_{120}K_{300}$. Significant differences were observed in percent pulp and peel, equatorial diameter, number of fins and fin length and base width. Positive differences in pulp total soluble solids concentration (°Brix) and pulp acidity (pH) were also recorded. Application of 60-120 kg K produced the highest % pulp, while fruits applied with 300 kg K yielded the highest percent peel relative to other treatments. Treatment 1 (60 kg K) produced the longest fin length (42.33 mm) and greatest fin base width (14.78 mm), while T3 gave the highest equatorial length (92.22 mm). Application of 180 kg K ha^{-1} (T3) gave the highest total soluble solids concentration (13.40 °Brix) and pulp acidity (pH 5.23). The results indicate that most of the physical and physico-chemical parameters were influenced positively by the application of 180 kg K relative to the other rates that were applied.

Keywords: potassium, physico-chemical evaluation, postharvest, dragon fruit

INTRODUCTION

Dragon fruit has a pleasant taste and an exotic appearance and it has nutritional and functional properties, which makes it promising for cultivation. Among the known dragon fruit species, the red-fleshed dragon fruit (*Hylocereus polyrhizus*) is prominent due to its claimed functional properties, attracting the interest of consumers and producers (Magalhães et al., 2019). It has a bright red peel with overlapping green fins or bracts that cover the fruit, a characteristic that has gained popularity in different countries around the world (Jaafar et al., 2009).

Potassium is not a constituent of any plant structures or compounds, but it plays a part in many important regulatory roles, i.e., osmo-regulation processes, regulation of plant stomata and water use, translocation of sugars and formation of carbohydrates, energy status of the plant, the regulation of enzyme activities, protein synthesis and many other processes needed to sustain plant growth and reproduction (Hsiao and Läuchli, 1986). It is a highly mobile element and is characterized as having luxury consumption. Additionally, it plays an important role in the plant's tolerance of biotic and abiotic stresses. Importantly, K is known as a quality nutrient because of its important effects on quality factors (Imas and Bansal, 1999). With the exception of nitrogen, potassium is required by plants in much greater amounts than all the other soil-supplied nutrients (Tisdale et al., 1985).

The red-fleshed dragon fruit has been commercially grown in Claveria, Misamis Oriental

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for at least five years. Recently, its cultivation has been expanding as recorded both in the local agroecosystem and in northern Mindanao. Hence, marketing of the fruit has also increased, as consumers are looking for high quality fruits. Although research on dragon fruit (pitaya) has increased, there is still much to study (Ortiz-Hernandez and Carrillo-Salazar, 2012), especially in relation to the factors that influence growth and production. Among such factors, fertilization can be highlighted, since it is critical for achieving success in the cultivation of fruits (Pegoraro et al., 2014). Knowledge about the amount of nutrient uptake in the plant can aid in informing a balanced fertilizer recommendation for the crop. Adequate nutrition contributes to the ultimate expression of the crop's potential (Augostinho et al., 2008). While dragon fruit has a huge market potential both locally and abroad, its high perishability limits its export. Hence, postharvest studies are important and, through postharvest technologies, opportunities to extend fruit shelf-life might be available to widen the scope for exports.

This study aimed to evaluate the physico-chemical properties of red-fleshed dragon fruit as influenced by potassium fertilization. Physico-chemical evaluation of the freshly harvested dragon fruit was focused on assessing fruit weight, the proportions of peel and pulp, polar and equatorial diameters, peel thickness, the number of fins and their length and base width, pulp pH, total soluble solids concentration (TSS) and firmness of the fruit in one year of harvest.

MATERIALS AND METHODS

Field fertilization treatments were applied to plants in a 5-year-old red-fleshed dragon fruit plantation at the University of Science and Technology of Southern Philippines - Claveria (8°36'36.9"N; 124°52'59.9"E). The study focused particularly on varying the levels of potassium that were applied. Application of the nutrients imposed as treatments was made on the 15th day of each month. Single applications of urea (46-0-0), Solophos (0-18-0) and muriate of potash (0-0-60) were applied annually with the five NPK treatments.

Fruits were harvested 38 days after anthesis (DAA), followed by postharvest evaluation. The experimental was laid out as a completely randomized design with five treatments of the following rates of fertilizer: T1) $N_{60}P_{120}K_{60}$; T2) $N_{60}P_{120}K_{120}$; T3) $N_{60}P_{120}K_{180}$; T4) $N_{60}P_{120}K_{240}$; and T5) $N_{60}P_{120}K_{300}$. Each treatment was replicated three times. After harvest, the following physico-chemical parameters were measured: weight of the whole fruit, weight of the peel (exocarp + mesocarp) and pulp (endocarp), % pulp, polar and equatorial diameters, peel thickness, number, length and base width of fins, pulp pH, total soluble solids concentration (TSS) and fruit firmness.

The weight of fruits and peel were determined using a digital weighing scale. Pulp weight was obtained from the difference between total fruit weight and skin weight. Pulp yield was calculated by the formula: pulp weight × 100/weight of the whole fruit, expressed as percentage. Polar and equatorial diameter, as well as peel thickness, were measured using a digital caliper (RoHS, China) and expressed in mm. Pulp firmness was determined using a semi-manual penetrometer, with an 8-mm tip, with results expressed in kg. The fruit was broken in the middle and a firmness reading was taken perpendicular to the surface of the pulp. The sample was mashed and pH of the pulp was measured using a digital pH meter. Soluble solids concentration was measured on a pure juice sample from the pulp using a hand-held refractometer (Atago, Japan) and expressed in °Brix.

All results were expressed as means ± standard deviation of the five treatments with three replications. Data were interpreted by one-way analysis of variance (ANOVA) with Tukey HSD Test using Statistica 8.0 software (StatSoft Inc., Tulsa, OK74104, USA). The statistical significance was evaluated at $p<0.05$ level.

RESULTS AND DISCUSSION

There was no significant effect of potassium application on total fruit weight. This is in contrast to the results of Chakma et al. (2014) who showed highest fruit weight at $N_{400}P_{230}K_{185}$. However, the application rate of potassium produced a significant effect on the partitioning between pulp and peel (Table 1). At 300 kg ha^{-1} of potassium (T5) there was the lowest proportion of pulp (73%) and, consequently, the highest percentage of peel (27%). In contrast,

the lowest rate of applied potassium (T1) had the highest pulp (81%) and lowest proportion of peel (19%). Hence, when there was an increase in the rate of potassium application there was a decrease in the proportion of pulp and a significant increase in the proportion of peel in a fruit.

Table 1. Summary of the physical mass and proportion of pulp and flesh of red-fleshed dragon fruit as influenced by potassium fertilizer rate.

Treatments	Fertilizer NPK (kg ha^{-1})	Fresh wt. (g)	Pulp (%)	Peel (%)
1	60-120-60	477.43	81.07a	18.93c
2	60-120-120	430.78	80.57a	19.39c
3	60-120-180	510.46	78.58bc	21.27ab
4	60-120-240	442.14	77.22b	22.67b
5	60-120-300	469.44	72.91d	27.21a
p-value		0.09	0.00	0.00
Error between MS		2.102	10.2	9.81
F-test		ns	**	**

Mean values per column followed by the same letter are statistically comparable at 5% level of significance in a Tukey test. ns = not significant, ** significant at α=0.01, * significant at α=0.05.

Fruit dimensions, such as fruit weight, did not consistently differ among treatments (Table 2). Differences in polar length were not significantly different among potassium treatments and while equatorial length was significantly different among the potassium rates, there was no consistent trend in the data in spite of the widely varying rates of potassium that were applied. In the study of Centurion Yah et al. (2008), dragon fruit reached 82 mm diameter and an equatorial diameter of 79 mm was observed by Ortiz et al. (2015), both of which were significantly lower than that recorded in the present study (Table 2). Similarly, Martínez-Chávez (2011), working with different *Hylocereus* spp. genotypes, reported equatorial diameters between 52 and 78 mm. The values ranging from 87 to 93 mm in this study were considerably larger than those reported previously.

Table 2. Summary of fruit size and physical characteristics of the pericarp as influenced by potassium fertilizer rate.

Treatment	Fertilizer NPK (kg ha^{-1})	Polar length (mm)	Equatorial length (mm)	No. of fins	Fin length (mm)	Fin base width (mm)	Peel thickness (cm)
1	60-120-60	98.58	90.18ab	31a	42.33a	14.78a	1.97
2	60-120-120	98.13	86.64a	32a	25.13b	12.24ab	1.42
3	60-120-180	102.60	93.22b	27b	18.88b	5.47c	1.59
4	60-120-240	98.59	87.71ab	24b	41.64a	6.20c	0.90
5	60-120-300	99.91	90.56ab	34a	29.12ab	8.57bc	1.07
p-value		0.665	0.021	0.00	0.00	0.00	0.054
Error between MS		83.5	83.5	11.61	6.7681	9.6074	1.3677
F-test		ns	*	**	**	**	ns

Mean values per column followed by the same letter are statistically comparable at 5% level of significance in a Tukey test. ns = not significant, ** significant at α=0.01, * significant at α=0.05.

Potassium significantly affected on the development of fins (bracts) on dragon fruit. Treatment 1 (K_{60}) had the longest fin length and widest base; in contrast T3 (K_{180}) had the shortest fin length and lowest width. Hence, the lower the rate of potassium application, the wider the fin base. Kammapana et al. (2013) reported that the fin of dragon fruit at all stages of fruit development showed a significantly higher density of stomata than that of the fruit

peel, by about 3- to 4-folds. As a result, water transpired faster via the stomata located on the fin than from those on the peel. Thus, the fins of dragon fruit showed a rapid loss of visual quality and became visually unacceptable. This observation implies that where there is a greater number of fins (such as in T5) the fruit will degrade faster. Firmness is a characteristic of great importance in determining fruit quality and is directly related to palatability. Fruits soften as they mature and, consequently, it is an important parameter for determining product acceptability (Chitarra and Chitarra, 2005). Neither peel thickness or fruit firmness were significantly different among the potassium application rates (Table 3).

Table 3. Summary of physico-chemical properties of red-fleshed dragon fruit as influenced by potassium fertilizer rate.

Treatment	Fertilizer NPK (kg ha^{-1})	Firmness (kg)	Pulp TSS (°Brix)	Pulp pH
1	60-120-60	7.83	12.20bc	5.17bc
2	60-120-120	6.40	10.60d	5.06d
3	60-120-180	5.61	13.40a	5.23a
4	60-120-240	6.83	11.60b	5.15b
5	60-120-300	8.16	12.60c	5.22cd
p-value		0.089	0.00	0.000
Error between MS		7.765	0.41	0.003
F-test		ns	**	**

Mean values per column followed by the same letter are statistically comparable at 5% level of significance in a Tukey test.
ns = not significant, ** significant at α=0.01.

There were significant differences among treatments for TSS concentration in the pulp of harvested dragon fruit (Table 3). The highest mean TSS was recorded in T3 (13.4 °Brix) which was significantly different from other treatments and T2 (10.6°Brix) had the lowest TSS. The result from T3 was similar to the findings of Chakma et al. (2014) where the $N_{400}P_{230}K_{185}$ treatment had a TSS of 13.8 °Brix. The TSS decreased when there was an increase in potassium above the $N_{60}P_{120}K_{180}$ rate resulting in a relatively similar TSS to that in the lowest rate of potassium ($N_{60}P_{120}K_{60}$). The pH of fruit harvest at 38 DAA was significantly highest in T3 (pH 5.23). This pH is different to the results of Ortiz and Takahashi (2015) who found an average pH 4.6 in *H. undatus* fruit harvested at 28 DAA and from other research that reported pH in the range between 4.3 and 4.7. The results reported here indicate that potassium at a rate of 180 kg ha^{-1}, in combination with N_{60}, is considered optimal as Wrona (2004) previously reported that there was a decrease in total soluble solids or sucrose production with high application rates of nitrogen.

CONCLUSIONS

The results of the physico-chemical evaluations carried out at 38DAA in red-fleshed dragon fruit indicated that application of potassium at a rate of $N_{60}P_{120}K_{180}$ kg ha^{-1} (T3) would be optimal for producing the highest fruit yield (510.46 g), with proportions of 79% for the pulp and 21% for the peel, and for producing the highest TSS of 13.4 °Brix with a low acidity of pH 5.23. This rate of potassium also produced a lower number of smaller fins which would reduce the rate of post-harvest degradation.

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Growth, yield and postharvest quality of tomato (*Solanum lycopersicum* Mill.) as influenced by different rates of nitrogen fertilizer

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Abstract

There is no available information on optimal nitrogen (N) fertiliser rates to maximise tomato production in the Philippines hence excessive N application is a problem. This research was conducted to determine the optimum rate of N to enhance production of different tomato cultivars. The experiment was a randomized complete block factorial design evaluating a range of N rates (72.8, 122.8, 172.8, 222.8, 272.8 and 322.8 kg N ha^{-1}) in combination with two cultivars ('Harabas Rescuer 746' and 'AVTO1173') and replicated four times. On the 2nd crop, an N rate of 222.8 kg ha^{-1} produced the highest total plant dry matter yield (176 g plant^{-1}), the largest and heaviest fruits (mean 49.6 g) and the lowest weight loss. The highest fruit yields were also recorded at an N rate of 222.8 kg ha^{-1} for the first (14.4 t ha^{-1}) and second (28.1 t ha^{-1}) crops. 'AVTO1173' had the earliest flowering, a 47% higher dry matter value and produced the largest diameter fruit (5.33 cm in first crop and 4.9 cm in the second crop). 'AVTO1173' was less susceptible to diseases compared with 'Harabas Rescuer 746' and had less shrivelling in the 1st cropping cycle. It is recommended that nitrogen fertilization at 222.8 kg N ha^{-1} should be used for producing high yields and good tomato fruit quality.

Keywords: 'AVTO1173', fruit quality, nitrogen fertilization rate, shrivelling point, tomato productivity

INTRODUCTION

Tomato is generally considered to have high fertilizer requirements (Singh et al., 2010) with rate of uptake depending on crop growth stage. Fertilization is an important factor that affects yield and quality of tomatoes and many studies have been conducted to understand which rate and nutrient forms are most effective in maximizing production (Caralampides, 2012; Li et al., 2017).

Over-application of nitrogen (N) fertilizer is a serious problem that leads to large N losses through leaching and denitrification and potential environmental pollution (Zhang et al., 2010). Caralampides (2012) observed that despite the negative effects associated with excessive fertilizer application, growth and production of tomato continued to increase with increasing N rate. Excessive application of nitrogen fertilizer also contributes to negative economic returns for tomato producers (Yilmaz et al., 2010) particularly in the Philippines where fertilizer costs represent a substantial proportion of crop production costs.

Many Philippine tomato farmers, particularly in Claveria, northern Mindanao apply excessive N under the belief that the greater the amount of fertilizer applied, the more nutrient the plant will take. This research was therefore conducted to evaluate the response of two cultivars of tomato to a range of N rates. This study aimed to assess the growth and yield performance of two cultivars over a range of N application rates. Furthermore, the study evaluated the effects of N rates on disease incidence and postharvest quality.

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MATERIALS AND METHODS

The study was conducted for two cropping seasons from July to September 2016 (1st cropping) and January to April 2017 (2nd cropping) at the Agricultural Experimental Station of the University of Science and Technology of Southern Philippines-Claveria (80°36'37.0"N; 124°52'59.9"E) at an elevation of 615 m a.s.l.

Experimental design

In each cropping season a randomized complete block factorial design was used consisting of four replications of the two factors. The factors included N rates (72.8, 122.8, 172.8, 222.8, 272.8 and 322.8 kg N ha^{-1}) and cultivars ('Harabas Rescuer 746' as check cultivar and 'AVTO1173'). Nitrogen treatments were imposed using urea (Table 1). 'Harabas Rescuer 746' is a commercial cultivar planted by growers and is moderately resistant to *Tomato yellow leaf curl virus* (TYLCV), while 'AVTO1173' is a selection with a semi-determinate growth habit accessed from the World Vegetable Center (AVRDC, Taiwan).

Table 1. Application of fertilizer (g $plant^{-1}$) in trials conducted at Claveria Northern Mindanao, Philippines.

Treatments	Urea	Solophos	Muriate of potash
72.8 kg N ha^{-1}	0	15.5	4.5
122.8 kg N ha^{-1}	3	15.5	4.5
172.8 kg N ha^{-1}	6	15.5	4.5
222.8 kg N ha^{-1}	9	15.5	4.5
272.8 kg N ha^{-1}	12	15.5	4.5
322.8 kg N ha^{-1}	15	15.5	4.5

Cultural management practices

Each treatment plot had an area of 1×11 m. The trials were planted using seedlings that were raised in an enclosed nursery in a sterilized soil mixture comprising garden soil, vermicast, lime and sand (4:5:0.5:1 ratio). The seedlings were transplanted at about four to five weeks after sowing with a planting distance of 0.5×0.5 m in a double row. The plant population was approximately 40,000 plant ha^{-1}.

Preventive spraying of Gnatrol WDG® biological insecticide and Aztron® was done interchangeably twice weekly. Basal fertilizer application consisted of 20 g $plant^{-1}$ Well Grow (2.1N:0.32P:1.0K with 5.78% Ca) and trace elements of 0.87% (Fe, 0.019% Cu, 8.312 ppm Mg, 927 ppm Mn, and 339 ppm Zn) and 10 g of a complete fertilizer (14-6-11).

For each of the two cropping seasons, different rates of fertilizer were divided into 4-split applications at transplanting, 3, 5 and 7 weeks after transplanting. Urea (46-0-0), Solophos® (0-7.8-0) and muriate of potash (0-0-50) were used as sources of nitrogen, phosphorus and potassium (Table 1).

Data collection

The following factors were measured: days to flowering, plant establishment, and dry matter yield. Yield parameters included: number and total weight of marketable and non-marketable fruits, individual fruit weight, fruit size (polar and equatorial) and yield ha^{-1}. The first harvest was carried out 55 days after transplanting and six harvests were made in total. Tomatoes were harvested at the mature green stage and were classified as being either marketable or non-marketable.

Postharvest shelf-life was determined by measuring shrivelling under ambient conditions. The weight of fruit at initial stage and at 3-day intervals were determined and recorded. Weight loss was expressed as a percentage of the initial weight minus the recorded final fruit weight after 12 days observation of the shelf-life. Shrivelling was visually evaluated on a four-point scale as: 1) no shrivelling, 2) slight shrivelling (1-25% surface affected), 3) moderate shrivelling (25-50% surface affected), and 4) severe shrivelling (more than 50%

surface affected). Disease infestation rating was evaluated using the following criteria: 1) no infestation, 2) mild infestation (1-25% of the plant population), 3) moderate infestation (25-50% of the plant population), 4) severe infestation (50-75% of the plant population), and 5) very severe infestation (75-100% of the plant population).

Statistical analysis

Analysis of variance (ANOVA) was used to test significance among treatment means using ASSISTAT (version 7.0 beta) software. Tukey's test was used to compare significant differences among means.

RESULTS AND DISCUSSION

Growth parameters

'AVTO1173' had slightly earlier flowering than 'Harabas Rescuer 746' but only in the first cropping season (Table 2). Time to flowering was not affected by N rate in either cultivar. Variations in temperature could influence the development of the first inflorescence in tomatoes and genetic variation has been shown to affect time to first flowering (Marcelis et al., 2004).

Table 2. Effect of the varying nitrogen rates (72.8-322.8 kg ha^{-1}) on time to flowering of tomatoes in two cropping seasons in trials conducted at Claveria Northern Mindanao, Philippines.

Treatments	Days to flowering	
	1st cropping	2nd cropping
Rate of nitrogen (A)		
72.8 kg N ha^{-1}	29.2	35.6
122.8 kg N ha^{-1}	28.1	35.5
172.8 kg N ha^{-1}	28.5	35.4
222.8 kg N ha^{-1}	28.5	35.2
272.8 kg N ha^{-1}	28.1	34.1
322.8 kg N ha^{-1}	28.8	35.5
F-test	ns	ns
Cultivars (B)		
Harabas Rescuer 746	29.1a	35.4
AVTO1173	28.0b	35.1
F-test	*	ns
A×B		
F-test	ns	ns
CV (%)		
A	3.9	4.2
B	5.0	4.9

* significant at α=0.05, ns = not significant.

The highest count of surviving plants was recorded in the 322.8 kg N ha^{-1} treatment had in the period from 60 until 90 days after transplanting based on the average data for two cropping seasons (Table 3). There was an overall trend for plant number to be higher at the higher rates of N. It is well documented that N fertilization promotes vegetative growth and fruit yield of tomato and later application during plant development improves fruit development. This effect on tomato growth and development is marked in soils with limited N supply (Hokam et al., 2011; Fatimah et al., 2019). A greater plant population was maintained for' Harabas Rescuer 746' compared with 'AVTO1173'.

A nitrogen rate of 222.8 kg N ha^{-1} produced the highest mean dry matter yield (DMY) over the two cropping cycles (Table 3). 'AVTO1173' had 47% higher dry matter than 'Harabas

Rescuer 746'. Caralampides (2012) reported that N application is associated with more prolific vegetative growth and biomass accumulation but these factors may also depend on soil texture, initial soil fertility, weather conditions, and management practices (Sainju et al., 2000). DMY in tomato is associated with net photosynthetic gain, expressed as net carbon accumulation, hence appropriate nutrient levels (i.e., N and P) are critical. In addition, dry matter partitioning is based on the sink strengths of different plant organs, expressed by potential growth rate (Heuvelink, 1996).

Table 3. Plant stand and dry matter yield of tomatoes at different growth stages as affected by N rate expressed as the mean of two cropping seasons in trials conducted at Claveria Northern Mindanao, Philippines.

Treatments	Plant stand (%)					Dry matter yield (g plant^{-1})
	30 DAT	45 DAT	60 DAT	75 DAT	90 DAT	
Rate of nitrogen (A)						
72.8 kg N ha^{-1}	99.4	89.8	77.8b	59.0b	50.2b	142.4cd
122.8 kg N ha^{-1}	99.4	89.8	79.0ab	60.2b	51.3ab	153.5bc
172.8 kg N ha^{-1}	100.0	92.6	82.4ab	61.9ab	52.8ab	162.1b
222.8 kg N ha^{-1}	97.1	89.8	79.0ab	61.9ab	52.8ab	176.0a
272.8 kg N ha^{-1}	99.4	93.7	83.5ab	63.1ab	54.0ab	125.1e
322.8 kg N ha^{-1}	98.3	93.8	84.6a	66.47a	56.96a	132.6de
F-test	ns	ns	*	*	*	**
Cultivars (B)						
Harabas Rescuer 746	98.7	93.2a	84.6a	65.9a	56.6a	102.8b
AVTO1173	99.2	89.3b	77.46b	58.5b	49.3b	194.3a
F-test	ns	**	**	**	**	**
A×B						
F-test	ns	ns	ns	ns	ns	**
CV (%)						
A	2.1	4.1	4.9	5.7	6.6	4.7
B	2.1	3.3	5.9	6.3	5.8	7.9

** highly significant at α=0.01, * significant at α=0.05, ns = not significant.

Yield and yield components

The 222.8 kg N ha^{-1} rate produced heavier fruits during the second but not the first cropping season. The individual fruit were bigger, in both polar and equatorial dimensions, in both cropping periods in this treatment (Table 4). Irrespective of N treatment AVTO1173 had larger (polar and equatorial dimensions) fruit in both cropping periods. These results are similar to the findings of Warner et al. (2004) who found that application of 150 to 200 kg N ha^{-1} was sufficient to maximize marketable yield in the cultivars tested ('CC337', 'H9492' and 'H9553'); yield increased linearly with N fertilizer rate up to a maximum application of 200 kg N ha^{-1}.

In this study, a rate of 222.8 kg N ha^{-1} produced the greatest number and weight of marketable fruits, consequently, resulting in the highest yield ha^{-1} in both cropping cycles (Table 5). This result is different from that of Warner et al. (2004) who found that fruit size was not influenced by the rate of nitrogen fertilization. A rate of 322.8 kg N ha^{-1} produced the most non-marketable tomato, suggesting that application of more than 222.8 kg N ha^{-1} is not likely to increase yield and that the additional fertilizer is surplus to uptake and would increase the risk of N leaching (Sainju et al., 2000) as any soil mineral N not taken up immediately has a much higher chance of being lost through leaching and denitrification (Caralampides, 2012).

Table 4. Fruit weight and size of tomatoes treated with different rates of nitrogen for two cropping seasons in trials conducted at Claveria Northern Mindanao, Philippines.

Treatments	1st cropping			2nd cropping		
	Weight fruit^{-1} (g)	Fruit size (cm)		Weight fruit^{-1} (g)	Fruit size (cm)	
		Polar	Equatorial		Polar	Equatorial
Rate of nitrogen (A)						
72.8 kg N ha^{-1}	34.4b	4.5c	3.5c	43.4b	4.6b	4.0b
122.8 kg N ha^{-1}	36.5b	4.6bc	3.6bc	43.2b	4.7b	4.1ab
172.8 kg N ha^{-1}	36.2b	4.9b	3.8b	47.5ab	4.7b	4.2a
222.8 kg N ha^{-1}	36.5ab	5.3a	4.2a	49.6a	5.4a	4.2a
272.8 kg N ha^{-1}	39.1a	4.8b	3.8b	47.0ab	4.4b	4.1ab
322.8 kg N ha^{-1}	35.9b	4.5c	3.5c	47.1ab	4.6b	4.0ab
F-test	**	**	**	**	**	*
Cultivars (B)						
Harabas Rescuer 746	35.6b	4.2b	3.3b	49.3a	4.6b	4.2a
AVTO1173	37.2a	5.3a	4.2a	43.3b	4.9a	3.9b
F-test	*	**	**	**	*	**
A×B						
F-test	**	ns	ns	ns	*	ns
CV (%)						
A	4.5	3.9	4.0	6.4	7.2	2.4
B	6.4	4.8	4.8	6.4	7.1	3.3

** highly significant at α=0.01, * significant at α=0.05, ns = not significant.

Table 5. Yield performance of tomatoes as influenced by nitrogen rates in two cropping seasons in trials conducted at Claveria Northern Mindanao, Philippines.

Treatments	1st cropping					2nd cropping				
	No. fruit plant^{-1}		Yield plant^{-1} (kg)		Yield (t ha^{-1})	No. fruit plant^{-1}		Yield plant^{-1} (kg)		Yield (t ha^{-1})
	M	X	M	X		M	X	M	X	
Rate of nitrogen (A)										
72.8 kg N ha^{-1}	7.7c	6.6c	0.3c	0.1d	9.8c	13.6c	6.1d	0.6d	0.2abc	21.2d
122.8 kg N ha^{-1}	9.7b	8.8b	0.4b	0.2c	13.0b	16.4ab	7.2bc	0.8b	0.2bc	26.4b
172.8 kg N ha^{-1}	10.6a	9.0ab	0.4ab	0.2bc	13.4ab	17.2ab	7.1bc	0.8b	0.2cd	26.2b
222.8 kg N ha^{-1}	10.7a	9.4ab	0.4a	0.2ab	14.4a	18.2a	6.6cd	0.8a	0.2d	28.1a
272.8 kg N ha^{-1}	10.4a	9.2ab	0.4ab	0.2ab	13.7ab	17.0ab	7.5ab	0.8b	0.3ab	26.7b
322.8 kg N ha^{-1}	10.6a	9.7a	0.4ab	0.2a	13.9ab	15.7b	8.1a	0.7c	0.3a	23.6c
F-test	**	**	**	**	**	**	**	**	**	**
Cultivars (B)										
Harabas	10.0	10.2a	0.4b	0.2a	12.7b	17.2a	6.7b	0.8a	0.2a	29.6a
AVTO1173	9.9	7.3b	0.4a	0.2b	13.4a	15.5b	7.5a	0.6b	0.2b	21.1b
F-test	ns	**	**	**	**	**	**	**	**	**
A×B										
F-test	**	**	**	**	**	**	**	**	**	**
CV (%)										
A	2.6	5.6	5.6	9.3	5.6	7.1	5.8	3.0	7.3	3.1
B	3.3	6.2	4.6	8.5	4.6	5.3	4.4	1.6	6.5	1.6

** highly significant at α=0.01; ns = not significant.

M = marketable, X = non-marketable.

Disease incidence

There was no significant difference in disease incidence across the applied N rates in either of the cropping seasons (Table 6). Plots without N application resulted in the least disease incidence at most assessment times in both cropping seasons. There was no indication, therefore, that N application influenced the expression of diseases in the crops in this study (Table 6). Parisi et al. (2006) found that excess nitrogen supply lead to a greater incidence of viral diseases. Disease susceptibility may also be pathogen-specific, probably dependent on resources or sensitivity to plant resistance reactions (Hoffland et al., 2000). 'AVTO1173' was slightly less susceptible to diseases than 'Harabas Rescuer 746' throughout the growth stages in both cropping seasons.

Table 6. Incidence of diseases as influenced by different rates of nitrogen in two cropping seasons in trials conducted at Claveria Northern Mindanao, Philippines.

Treatments	1st cropping					2nd cropping				
	Disease incidence					Disease incidence				
	30 DAT	45 DAT	60 DAT	75 DAT	90 DAT	30 DAT	45 DAT	60 DAT	75 DAT	90 DAT
Rate of nitrogen (A)										
72.8 kg N ha^{-1}	1.5b	2.0b	3.0b	3.4	4.4	2.0	2.1b	2.9	3.0b	4.0b
122.8 kg N ha^{-1}	2.0a	2.6a	3.1ab	3.8	4.5	2.0	2.6a	3.0	3.6a	4.8a
172.8 kg N ha^{-1}	2.0a	2.9a	3.4a	4.0	4.9	2.0	2.9a	3.2	3.9a	4.8a
222.8 kg N ha^{-1}	2.0a	2.9a	3.4a	4.0	4.9	2.0	2.9a	3.2	3.9a	4.8a
272.8 kg N ha^{-1}	2.0a	2.8a	3.4a	4.0	4.8	2.0	2.5ab	3.0	3.9a	4.8a
322.8 kg N ha^{-1}	2.2a	2.5a	3.1ab	3.9	4.5	2.0	2.6a	3.0	3.9a	4.8a
F-test	**	**	**	ns	ns	ns	**	ns	**	**
Cultivars (B)										
Harabas Rescuer 746	2.0	2.5	3.3a	3.9	4.6	2.0	2.6	3.2a	3.7	4.7
AVTO1173	2.0	2.7	3.1b	3.8	4.7	2.0	2.6	3.0b	3.5	4.6
F-test	ns	ns	**	ns	ns	ns	ns	**	ns	ns
A×B										
F-test	ns	ns	ns	ns	ns	*	**	ns	ns	ns
CV (%)										
A	7.7	9.2	4.8	10.1	10.5	10.1	10.0	10.0	8.7	7.1
B	9.1	10.1	4.1	8.1	9.0	8.3	9.3	8.1	9.8	8.6

** highly significant at α=0.01, * significant at α=0.05, ns = not significant.

Postharvest quality

The 222.8 kg N ha^{-1} rate resulted in lower weight loss in tomato fruit in the 2nd cropping (Table 7). Weston and Barth (1997) reported that heavy N fertilization could affect nutritional quality, with a relationship between nitrogen and carbon. Other factors affecting postharvest ripening and storage can include maturity at harvest, and the interrelationships between nutrient transport, water relations and fruit growth (Ferguson et al., 1999). 'AVTO1173' had slightly less shrivelling than 'Harabas Rescuer 746' during the 1st cropping but there was no significant difference in the 2nd cropping.

CONCLUSIONS

Plots with an application rate of 222.8 kg N ha^{-1} produced the highest dry matter yield, heavier and bigger fruits (polar and equatorial size), highest yield and lowest weight loss after harvesting. 'AVTO1173' had earlier flowering, 47% higher dry matter yield, the largest polar fruit size, lower disease incidence and less shriveling at the 1st cropping compared with 'Harabas Rescuer 746'. These results indicate that a nitrogen fertilizer rate of 222.8 kg ha^{-1} can be recommended for achieving high yields and good fruit quality under the conditions at the test location in the Philippines. There is a need for further research to include additional cultivars.

Table 7. Postharvest responses of tomatoes subjected to different rates of nitrogen for two cropping seasons in trials conducted at Claveria Northern Mindanao, Philippines.

Treatment	1st cropping		2nd cropping	
	Weight loss (%)	Shriveling score	Weight loss (%)	Shriveling score
Rate of nitrogen (A)				
72.8 kg N ha^{-1}	55.3a	3.0a	46.3a	2.4
122.8 kg N ha^{-1}	51.7b	2.5b	42.4b	2.4
172.8 kg N ha^{-1}	52.2b	2.0c	41.4b	2.5
222.8 kg N ha^{-1}	51.6b	2.0c	34.43	2.4
272.8 kg N ha^{-1}	52.0b	2.0c	36.9c	2.3
322.8 kg N ha^{-1}	51.8b	2.1c	36.9c	2.6
F-test	**	**	**	ns
Cultivars (B)				
Harabas Rescuer 746	52.6	2.3a	38.8b	2.4
AVTO1173	52.2	2.2b	40.7a	2.4
F-test	ns	**	**	ns
A×B				
F-test	**	*	**	ns
CV (%)				
A	1.7	8.3	3.2	8.0
B	1.9	9.4	2.8	5.9

** highly significant at α=0.01, * significant at α=0.05, ns = not significant.

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A field study of cover crops to improve soil biochemical properties in bulk and rhizosphere soils of lettuce (*Lactuca sativa* L.)

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Abstract

Hairy vetch (*Vicia villosa* Roth; HV) and rye (*Secale cereale* L.) cover crops have been reported to increase soil nitrogen and microbial biomass and diversity. However, quantitative analyses based on field experiments have been limited. A field trial with HV and rye cover crop plots was compared with control plots that received 2.5 g N m^{-2} synthetic fertilizer. Lettuce (*Lactuca sativa* L.) was rotated after the cover crops were incorporated and harvested at 33 days, when bulk soil samples were collected and tested for nitrate, ammonium, and microbial biomass nitrogen (MBN). DNA from soils that attach lettuce roots (i.e., rhizosphere soil) was targeted for bacterial (*16S* rRNA) community determination through the next generation sequencing (NGS) approach. Lettuce yields in HV (0.20 kg m^{-2}) and control (0.21 kg m^{-2}) treatments were similar, whereas that in rye (0.15 kg m^{-2}) was reduced because of lower nitrate and ammonium concentrations in rye (7.56 and 8.06 mg kg^{-1} soil) compared with HV (16.8 mg kg^{-1} soil) and control (29.9 mg kg^{-1} soil). MBN in HV and rye treatments was 2.5 and 3 times lower than that in control in bulk soil, indicating smaller microbial biomass under the cover crop treatments. Bacterial diversity, determined as the Shannon index at family level, was enhanced in HV (3.98), compared with that in control (3.93) and rye (3.80) in lettuce rhizosphere soil. Specifically, bacteria in the families *Bacillaceae*, *Bacillales incertae sedis*, and *Sphingomonadaceae*, which include plant growth promotors, were relatively more abundant in HV (3.16, 0.38 and 4.46%, respectively) than in either control (1.79, 0.12 and 3.91%, respectively) or rye (2.24, 0.12 and 2.11%, respectively). Moreover, those families were positively correlated with the ability of lettuce to take up N under HV amendments, based on the results of a redundancy analysis (RDA). In summary, HV cover crops improved chemical and reduced biological properties in bulk soil but increased microbial properties in rhizosphere soil, which corresponded to lettuce yields.

Keywords: hairy vetch, rye, microbial biomass, inorganic nitrogen, bacterial diversity, influenced bacteria

INTRODUCTION

Cover crops, such as hairy vetch (*Vicia villosa* R.; HV) and rye (*Secale cereale* L.), can be applied in the field prior to vegetable cultivation with the aim of increasing and scavenging soil inorganic nitrogen (N) concentration, thereby enhancing productivity of the vegetable crop (Chahal and Van Eerd, 2019). HV residue is rich in organic N, supplying an adequate concentration of inorganic N to the soil. Conversely, rye is poor in organic N. In some current practice, cover crops are applied as an alternative to the usage of synthetic N fertilizers with the aim of suppressing N pollution (Komatsuzaki et al., 2020). It is, therefore, necessary to study the effects of HV and rye cover crops and synthetic N fertilizers on vegetables production, such as lettuce (*Lactuca sativa* L.) to further understand the processes involved in nutrient cycling.

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Inorganic N in nitrate and ammonium forms is mineralized from cover crop residues in bulk soils by soil microbes. The contribution of soil microbes to N mineralization can be evaluated as microbial biomass nitrogen (MBN; Sainju et al., 2001, 2003; Sugihara et al., 2010). Fields with HV and rye cover crops input can similarly increased MBN to that from synthetic N fertilizers (Sainju et al., 2001, 2003). In the process of N mineralization, a decline in MBN may indicate that inorganic N is released from microbial bodies to the soil (Sugihara et al., 2010). There remain limited field studies into the status of MBN when the inorganic N supply from cover crops is synchronized with vegetable N demands. The evaluation of biochemical indicators in bulk soils (i.e., soil nitrate and ammonium concentration, and MBN) at the same time as the vegetable harvest would be highly valuable in terms of providing a greater understanding of the processes associated with N mobilization in the bulk soil.

The readily absorbable inorganic N is taken up by vegetable plants via the plant rhizosphere, where diverse beneficial bacteria inhabit soil surrounding the rhizosphere zone and assist with N absorption (Brahmaprakash et al., 2017). Soil DNA-based molecular approaches have recently been used to identify and quantify the existence of beneficial bacteria. For example, these methods allowed the quantification of the composition of rhizosphere bacteria in soils with a rotation of barley-tomato, HV-tomato, and HV-zucchini. However, it was difficult to identify the specific bacteria involved in the cover crops that impacted on growth performance of these vegetable crops (Manici et al., 2018).

This study aimed, therefore, to compare the effects of cover crops and synthetic N fertilizer on 1) the biochemical properties in bulk soil, and 2) the diversity and contribution of specific rhizosphere bacteria on lettuce production. We hypothesized that HV and synthetic N fertilizer similarly supply inorganic N and improve lettuce productivity, but that HV promotes existence of beneficial bacteria in the lettuce rhizosphere soil.

MATERIALS AND METHODS

Rotation of cover crops lettuce

The research was conducted in an experimental field at Hokkaido University, Sapporo, Japan, as described by Chinta et al. (2020). HV and rye seeds were sown at 5 and 10 g m^{-2}, respectively, in 3×4 m plots at late autumn in 2016. HV and rye were grown until reaching 572 and 612 g m^{-2} dry weight (DW) biomass, each. They were terminated, chopped, and incorporated into soils during one day at early summer in 2017, when the organic nitrogen (N) inputs of HV and rye were 22.2 and 7.70 g m^{-2}, respectively. Additionally, the carbon (C) input and the C:N ratio were 238 g m^{-2} and 10.7, respectively, for HV and 260 g m^{-2} and 37.8, respectively, for rye. For comparison, a plot with 2.5 g N m^{-2} as ammonium sulfate [$(NH_4)_2SO_4$] fertilizer was prepared. The plots were designed in a randomized block layout with three replicates. Five days after incorporation, one month old lettuce seedlings were transplanted per plot. The plants were harvested 33 days later, oven-dried for five days and measured for DW. The lettuce mass was ground; and the N content was measured, using the dry combustion technique, to indicate the ability of plants to absorb inorganic N from soil.

Measurement of bulk soil biochemical properties

Bulk soils (Calcaric, Eutric Fluvisol light clay) were collected at 0-10 cm depth at the lettuce harvest time (i.e., 38 days after incorporation) and sieved through a 2-mm mesh. The concentration of nitrate and ammonium was measured from 2 g of air-dried soil in 10 mL of 2 M KCl solution using a Flow Injection Analyzer (FIA). Microbial biomass nitrogen (MBN) was evaluated based on the fumigation extraction method (Brookes et al., 1985). Total soluble N was converted into nitrate using a persulfate reagent mixture; and the nitrate as MBN was measured using FIA.

Assessment of bacterial community in the lettuce rhizosphere soil

Rhizosphere soil from the root zone of lettuce plants was detached; and the DNA was extracted using a PowerSoil DNA extraction kit. The technique of Oka and Uchida (2018) was followed to assess the bacterial community. In the first polymerase chain reaction (PCR), DNA

was amplified in AmpliTaq Gold 360 Master Mix and sets of primers (515F and 806R) targeting bacteria (*16S*) rRNA. The amplicon product was purified using AMPure XP reagent. To prepare bacterial libraries, a second PCR was carried out with the same primer sets attached with an adaptor and a barcode. The product was purified, diluted in 50 pM concentration, and subjected to the rRNA sequencing system using the Ion Torrent Personal Genome Machine (PGM) for the next generation sequencing (NGS). Bacterial sequences as operational taxonomic units (OTUs) were obtained from the Ion Reporter system, and then analyzed online for bacterial relative abundance using Torrent Suite Software V5.0 as performed by Toda and Uchida (2017) at family level. The Shannon index at family level was calculated based on the OTUs number and relative abundance to show bacterial diversity.

Statistical analyses

Analyses were done in R v.3.6.1 (R Core Team). All data were analyzed using a one-way ANOVA, followed by Tukey HSD at $p<0.10$ or $p<0.05$. Bacteria for which the relative abundance was statistically significant within the treatments were selected as influenced bacteria. To compare the effects of treatments on the whole of bacterial community structure, permutational ANOVA (perMANOVA) at $p<0.05$ was done based on Bray-Curtis distances using the "vegan" analysis package. A redundancy analysis (RDA) was performed by joining data for relative abundance of the influenced bacteria as community variables with data of lettuce yield and N content as the environment variables, using the "vegan" analysis package. A square root-conversion of the data was used to select significant variables.

RESULTS AND DISCUSSION

HV treatment and control similarly resulted in high lettuce production

HV treatment and control comprising synthetic fertilizer application similarly increased lettuce yield per area but had contrasting lettuce N content (Figure 1). HV with high organic N, therefore, provided high inorganic N demanded by lettuce plants and produced high lettuce biomass. The inorganic N supplied from HV residue was significantly taken up by lettuce plants (Figure 1B). Bottoms et al. (2012) previously concluded that N input is positively correlated with N content in lettuce cultivation. We assume that a biological factor, such as rhizosphere bacteria, differently influenced the N absorption between HV treatment and the control. In contrast to HV, rye treatment significantly reduced both the yield and N content of lettuce plants (Figure 1). This was likely due to the fact that there was 65.3% lower organic N input from rye than from HV, which may not be fully transformed as inorganic N. Lettuce yield promotion by HV and yield suppression by rye was shown in an earlier study with tomato and zucchini (Manici et al., 2018).

HV increased nitrate concentration and decreased MBN in bulk soil

Although soil nitrate concentration is not correlated with lettuce N content (Bottoms et al., 2012), the soil nitrate concentration in HV treatment was remained in much higher than that in the control (Figure 2A), suggesting a sufficiency of N supply during lettuce cultivation period. Ammonium sulfate as the N source in the control resulted in a high residual ammonium concentration (Figure 2B). Conversely, low N input from rye resulted in lower soil nitrate and ammonium concentrations compared with HV and the control, respectively. With regard to the lettuce DW production, lettuce N content, and soil nitrate status in HV treatment and noting that it has been previously shown that inorganic N is mineralized from cover crop residues incorporated before lettuce planting and during lettuce growing period (Sorensen and Thorup-Kristensen, 2003), we conclude that HV can provide an alternative source of N to synthetic N fertilizer for lettuce cultivation.

Both HV and rye had significantly reduced MBN compared with the control (Figure 2C). The result is in contrast with Sainju et al. (2001, 2003), who demonstrated similar MBN values under HV, rye, and 0.9 and 1.8 g N m^{-2} fertilizer treatments. In this study, the lower MBN in HV (Figure 2C) may indicate greater N mineralization as shown by Sugihara et al. (2010). Such mineralization would lead to a high concentration of residual soil nitrate, as was shown

(Figure 2A). In contrast, the low N input from rye would have resulted in a small size of soil microbes, which likely caused the low concentrations of residual nitrate and ammonium in bulk soil samples (Figures 2A, B). Thus, the lower MBN in rye treatment (Figure 2C) may not relate directly to N mineralization; but may show limited availability of rye residue for soil microbes.

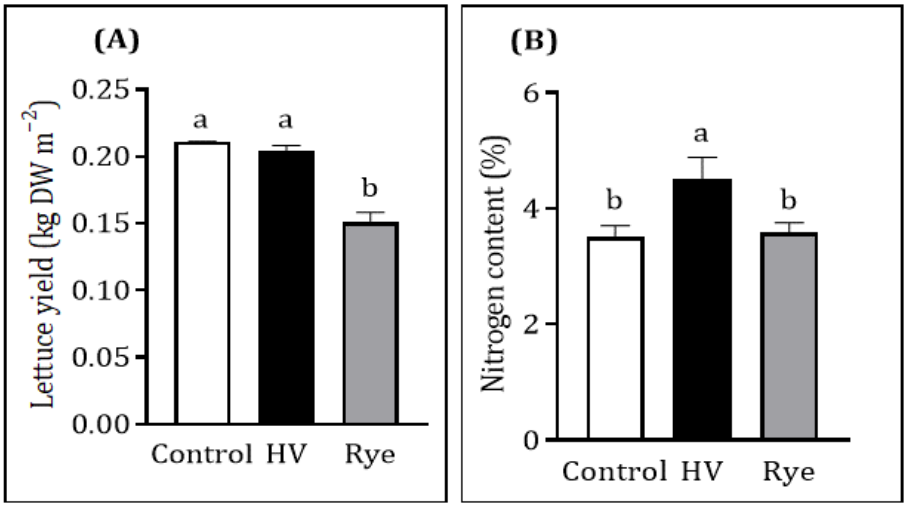


Figure 1. Lettuce yield based on dry weight (A) and lettuce nitrogen content (B) at 33 days after planting. Treatments are synthetic nitrogen fertilizer (control), hairy vetch (HV), and rye. Different letters denote significant difference according to Tukey HSD at p<0.10 (*n*=3). Bars show standard errors.

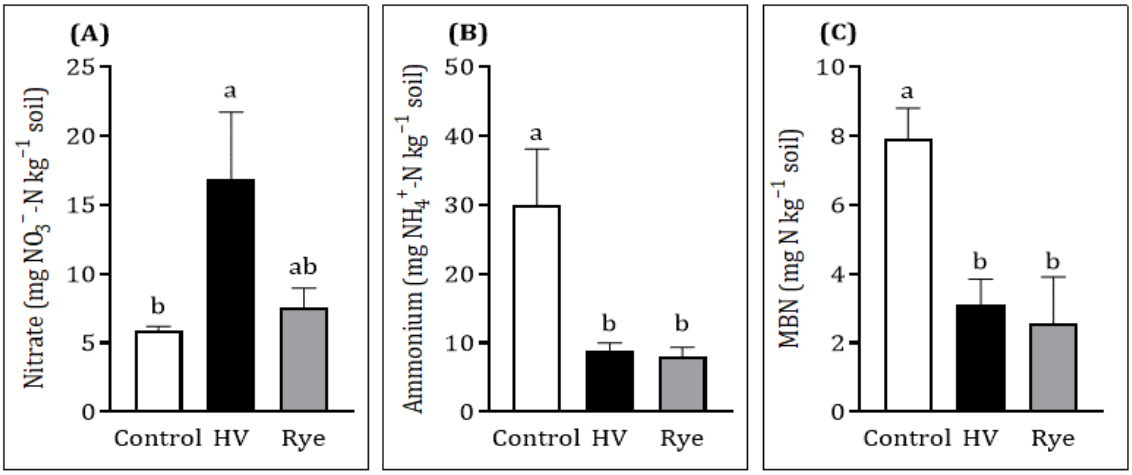
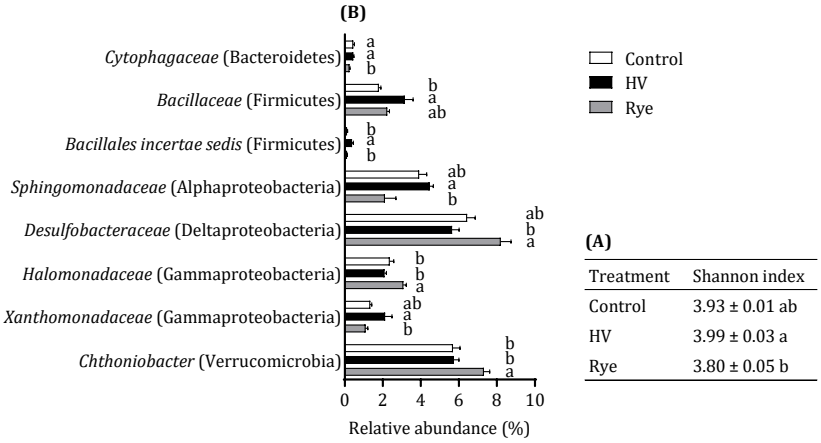


Figure 2. Concentration of nitrate (A) and ammonium (B) and microbial biomass nitrogen (MBN) (C) in bulk soils treated with synthetic nitrogen fertilizer (control), hairy vetch (HV) and rye inputs at 38 days after incorporation. Different letters denote significant difference according to Tukey HSD at p<0.10 (*n*=3). Bars show standard errors.

Plant growth-promoting bacteria existed in lettuce rhizosphere soil and was associated with lettuce N content and yield

Vigorous growth of lettuce in both HV and control treatments was associated with increased bacterial diversity in lettuce rhizosphere soil. In contrast, rhizosphere bacteria in rye treatment was less diverse (Figure 3A). These results are different to those of Manici et al.

(2018), who demonstrated that barley and HV incorporation into soils prior to tomato and zucchini cultivation did not vary rhizosphere bacterial diversity. Therefore, we assume that rhizosphere bacteria appeared to be selective to the species of plant (i.e., lettuce). The identified bacterial richness (i.e., the number) and evenness (i.e., the average of relative abundance in the community) were similar in HV (115 and 0.31), control (112 and 0.30), and rye (108 and 0.29) treatments. Nonetheless, the bacterial community composition differed depending on the treatment as assessed by the perMANOVA (P=0.034) analysis. This implies that increasing or decreasing the relative abundance in each specific bacterial family may account for the evenness that was determined.



(A)

Treatment	Shannon index
Control	3.93 ± 0.01 ab
HV	3.99 ± 0.03 a
Rye	3.80 ± 0.05 b

Figure 3. Shannon index of bacteria at the family level (A) and relative abundance of bacterial families (B) in the lettuce rhizosphere soil as influenced by synthetic nitrogen fertilizer (control), hairy vetch (HV) and rye treatments 33 days after planting. Different letters denote a significant difference according to Tukey HSD at p<0.05 (n=3). Bars show standard errors.

The relative abundance of some plant growth-promoting bacteria in the lettuce rhizosphere soil was positively influenced by HV cover crops and significantly associated with the lettuce DW yield. Phyla Firmicutes and Proteobacteria inhabiting plant rhizospheres include plant growth-promoting bacteria (Brahmaprakash et al., 2017), such as those shown in Figure 3B. HV treatment resulted in a significantly increased relative abundance of family *Bacillaceae* and *Bacillales incertae sedis*, over the control (Figure 3B). These families produce indole-3-acetic acid (IAA) hormone and antibiotics to promote plant growth and protect plant roots from soil-borne pathogens, respectively (Brahmaprakash et al., 2017). The RDA 1 axis indicates that bacteria in the family *Bacillaceae* are significantly associated with lettuce N content in HV treatment (Figure 4). The IAA may stimulate plants to absorb soil inorganic N. Family *Bacillales incertae sedis* in HV treatment, nonetheless, was also not significantly functioning in N absorption by lettuce plant. Further, HV treatment significantly increased relative abundance of families *Sphingomonadaceae* and *Xanthomonadaceae*, over those in rye treatment (but not the control; Figure 3B). Subrahmanyam et al. (2020) have shown that members of family *Sphingomonadaceae* (e.g., *Sphingomonas* sp.) produce gibberellic acid (GA_3). An increase in the relative abundance of family *Sphingomonadaceae* may, therefore, increase the amount of GA_3 leading to a promotion of lettuce leaf production in HV treatment (Figure 4). Families *Bacillaceae* and *Xanthomonadaceae* have been known to release antibiotics that suppress or kill plant pathogens (Brahmaprakash et al., 2017). Thus, HV incorporation into bulk soils could enhance the existence of biological control agents (BCAs) in lettuce rhizosphere. However, rye input did not lead to the similar enhancements in bacterial composition. Although the control using synthetic N fertilizer showed similarly high lettuce growth as HV (Figure 1A), control treatment did not result in significant enhancements

in relative abundance of some of the specific bacteria and, consequently, their association with the lettuce growth parameters shown in Figure 4. This result indicates a lower dependence of plant-microbe interactions with lettuce plants treated with synthetic N fertilizer.

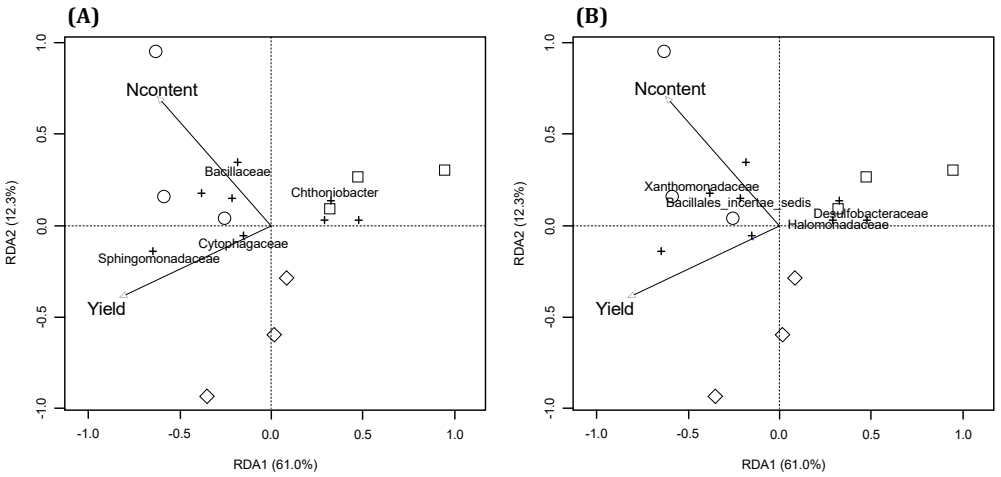


Figure 4. Redundancy analysis (RDA) showing significant (A) and non-significant (B) relationships between the influenced bacteria families in the lettuce soil rhizosphere and lettuce responses to synthetic nitrogen fertilizer (◇), hairy vetch (○), and rye (□) treatments. The variables are lettuce yield (Yield) and nitrogen content (N content).

Rye cover crops influenced C cycling-related bacteria in the lettuce rhizosphere. Rye promoted a relative abundance of bacterial families *Halomonadaceae*, *Chthoniobacter* and *Desulfobacteraceae*, but significantly decreased relative abundance of family *Cytophagaceae*, over that in the control and HV treatment (Figure 3B). These four bacteria families are involved in C cycling by degrading C substrates (Vreeland, 1992; McBride et al., 2014; Janssen, 2015; Kuever, 2014). Some plant-microbe interactions in rhizosphere soil involve the exchange of C between microbes and plant roots (Brahmaprakash et al., 2017). Lettuce roots in HV treatment and in the control may therefore grow vigorously and excrete C-rich substrate that supported the existence of bacterial family *Cytophagaceae*. This relationship between family *Cytophagaceae* and lettuce plants was identified in the RDA1 axis of Figure 4. Conversely, the poorer growth of lettuce in rye treatment may have limited the amount of C substrate that was released by the plant roots. Family *Halomonadaceae*, *Chthoniobacter*, and *Desulfobacteraceae* have been shown to be survive under a sole nutrient stress (Vreeland, 1992). Thus, those three rhizosphere bacteria families occurred in the same area of the RDA plots under rye treatment, where family *Chthoniobacter* was the most prolific (Figure 4).

CONCLUSIONS

Incorporation of hairy vetch (HV) cover crop into bulk soils resulted in a reduction of microbial biomass nitrogen compared with the application of synthetic nitrogen (N) fertilizer. This decrease may be related to the high amount of residual soil nitrate, which improved the N content and yield of the lettuce plants. Consequently, there were similar lettuce yields in HV and control treatments, indicating that HV could be an alternative to the application of synthetic N fertilizer. We also demonstrated that absorption of inorganic N by lettuce cultivated in HV treatment was associated with bacteria inhabiting the rhizosphere soil as HV increased bacterial diversity and relative abundance of plant growth-promoting bacteria (i.e., families *Bacillaceae*, *Sphingomonadaceae*, and *Bacillales incertae sedis*), compared with that occurring in rye treatment. Those bacteria families may be key to directly and indirectly contributing to lettuce N content and yield under HV treatment.

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Effects of girdling, chemicals and plant growth regulators on production and fruit quality of 'Hong Huay' lychee

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Abstract

Effect of girdling, chemicals and plant growth regulators for improving production and fruit quality in 'Hong Huay' lychee were conducted in Chiang Mai, Chiang Rai and Pha Yao Provinces, Thailand during 2016 and 2018. The experiments consisted of: 1) application of ring cincturing, foliar fertilizers and plant growth regulators for flower induction; 2) application of spiral cincturing, chemicals and plant growth regulators to increase fruit set; and 3) application of spiral cincturing and plant growth regulators to increase fruit size. There were six orchard × year combinations out of 15 tested had significantly different in average flowering percentages. Soil application of 300 g tree^{-1} paclobutrazol (10% WP) followed with 200 g tree^{-1} potassium chlorate (99% WP) had higher average flowering percentages than the control treatment but were significantly different in only three orchard×year combinations. Ring cincturing of limb followed with spraying with 10 g L^{-1} 0-52-34 (1%) fertilizer mixed with 800 mg L^{-1} ethephon (48%) had higher average flowering percentages than control treatment but were significantly different at only at two orchard×year combinations. Spiral cincturing of blooming branches tended to have the highest average fruit set which was higher than the control treatment but significantly different only at three orchard × year combinations out of seven tested. Spraying with 400 mg L^{-1} uniconazole also-increased average fruit set more than the control treatment but was significantly different only at one orchard. Spraying with 5 mg L^{-1} brassinolide tended to increase average fruit weight more than that in the control treatment but the effect was significant only at four orchard×year combinations out of eight tested. Further testing is required before recommendations can be made to lychee growers.

Keywords: flowering, fruit set, fruit weight, spiral cincturing

INTRODUCTION

Lychee (*Litchi chinensis* Sonn.) is a subtropical fruit tree that fruits well under full sun. Its production constraints are an alternate bearing habit and low productivity (Das et al., 2002; Malhotra et al., 2018). It is a commercially important fruit that is grown mainly in the upper north region of Thailand. There are many cultivars grown but 'Hong Huay' is the most widely produced (70%) commercial cultivar. However, the plantation area has decreased gradually due to an unfavorable climate, especially increasing temperatures and long drying periods, which has caused irregular flowering, poor fruit set and poor fruit quality. To overcome these problems, several studies have been carried out to enhance flower induction, increase fruit set and to improve fruit size. Various plant growth regulators and cincturing methods were able to induce flowering of lychee (Chen and Huang, 2001; Chen et al., 2013; Menzel and Simpson, 1987, 1990; Mitra and Sanyal, 2001). Many studies have shown that fruit set and fruit size in lychee and longan can be also induced by the application of some plant growth regulators (Yuan and Huang, 1991; Nie et al., 2001; Mandal et al., 2014; Stern et al., 2001). In addition, spiral cincturing has been reported to increase fruit set and fruit quality of lychee (Li and Xiao, 2001; Chen and Huang, 2001). However, previous results have not produced

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consistent results due to differences in locations, environments and cultivars.

This study was carried out to examine the effects of plant growth regulators, selected chemicals and girdling methods on flower induction, fruit set and fruit weight of 'Hong Huay' lychee under conditions in Thailand.

MATERIALS AND METHODS

Three experiments were conducted to improve fruit quantity and quality of 15- to 20-year-old 'Hong Huay' lychee in 15 commercial orchards at Chiang Mai, Chiang Rai and Pha Yao Provinces, Thailand during 2016 and 2018. Experiments were laid out in a randomized complete block design (RCBD). Data were analyzed for statistical differences for each treatment by an analysis of variance, means comparison with the DMRT ($p \leq 0.01$(**) and $p \leq 0.05$(*)). Three experiments were consisted of:

1) Application of ring cincturing, foliar fertilizers and plant growth regulators for flower induction comprising five replications and four treatments. Treatments were: T1) control treatment, T2) ring cincturing when the 2nd flush had matured, T3) soil application of 300 g tree^{-1} paclobutrazol ($C_{15}H_{20}CIN_3O$, 10% WP) when 2nd flush had fully matured and then sprayed with 200 g tree^{-1} potassium chlorate ($KClO_3$, 99%WP) 15 days later, and T4) ring cincturing when the 2nd flush had matured and then sprayed with 0-52-34 (1%) fertilizer mixed with 800 mg L^{-1} ethephon (48%) at 15, 20 and 25 days after cincturing. Ring cincturing was done on the limb with a pruning saw. The wound was 0.2-0.3 cm wide and 0.4 cm deep. Spiral cincturing was done on the limb with a special knife. The wound was 0.2 cm wide, 0.4 cm deep with 6-8 cm between two rings. The percentage of flowering was recorded by evaluating the whole canopy at fully bloom;
2) Application of spiral cincturing, chemicals and plant growth regulators to increase fruit set, comprising four replications and five treatments. Treatments were: T1) control treatment, T2) pollen grains in 5% sugar solution was sprayed two days at the female-bloom stage, T3) 10 mL L^{-1} 1-naphthaleneacetic acid (NAA) was sprayed twice at seven day-intervals at the full-bloom stage, T4) spiral cincturing was conducted at the full-bloom stage, and T5) 400 mL L^{-1} uniconazole was sprayed twice, once at inflorescence emerged and once at the full-bloom stage. Pollen grains were collected from dehisced male panicles kept overnight at room temperature before application. The number of fruits on each of ten panicles in each tree were counted at initial set and at harvest;
3) Application of spiral cincturing and plant growth regulators to increase fruit weight comprising four replications and five treatments. Treatments were: T1) control treatment, T2) sprayed twice at 7-day intervals with 5 mg L^{-1} brassinolide (BS), T3) sprayed once with 100 mg L^{-1} gibberellic acid (GA_3), T4) sprayed once with 200 mg L^{-1} 1-naphthaleneacetic acid (NAA), and T5) sprayed three times at seven day-intervals with 60 mg L^{-1} forchlorfenuron (CPPU). Trees were sprayed with plant growth regulators when fruit had browned seed. At harvest, ten fruits tree^{-1} were sampled and fruit weight and aril weight were recorded.

RESULTS AND DISCUSSION

Application of ring cincturing, foliar fertilizers and plant growth regulators for flower induction

The effects of treatments were inconsistent and varied among locations and years. This may have been due to differences in the management of each orchard, including pruning, fertilization or irrigation practices, which caused uneven leaf flushing and differences in leaf age prior to flower induction and after treatment. There was a significant difference in the percentage of flowering only in 6 of the 15 year × orchard combinations (Table 1). The control trees had a high variation in average percentage of flowering 23.8 (1.0-81.0)%. Poor flowering of trees in some years and orchards might have been because it rained in December and induced a new flush of leaf emergence. Stern and Mitra (2017) reported that young immature

terminal shoots in inductive temperatures have no bloom or poor flowering. Furthermore, dry winters are considered favorable for flowering in lychee, via the induction of vegetative dormancy (Menzel and Simpson, 1994).

Table 1. Effect of ring cincturing, foliar fertilizers and plant growth regulators on flowering percentage during 2016-2018.

Treatments	Lychee orchards × years							
	1[a]	2[a]	3[a]	4[b]	5[b]	6[b]	7[b]	8[b]
T1 = control	1.2	6.7	3.5	30.0a	7.0	12.5b	24.5b	14.1b
T2 = ring cincturing	5.9	7.2	6.5	20.0b	10.1	12.9b	12.1c	16.9b
T3 = 300 g tree^{-1} paclobutazol + 200 g tree^{-1} $KClO_3$	-	-	-	51.5a	4.8	24.5a	36.0a	9.9b
T4 = ring cincturing + 10 g L^{-1} 0-52-34 + 800 mg L^{-1} ethephon	4.8	0.5	14.5	27.8b	1.7	15.6b	13.2c	33.8a
F-test	ns	ns	ns	*	ns	*	**	*
CV (%)	137.4	133.8	124.6	45.4	116.9	39.1	28.5	57.6
Treatments	**Lychee orchards × years**							
	9[c]	**10[c]**	**11[c]**	**12[c]**	**13[c]**	**14[c]**	**15[c]**	**Mean**
T1 = control	81.0	33.0	22.5	14.5	1.0b	18.0	64.0a	23.75
T2 = ring cincturing	73.2	34.9	24.9	7.7	1.9b	12.3	37.0b	20.11
T3 = 300 g tree^{-1} paclobutazol + 200 g tree^{-1} $KClO_3$	83.0	48.0	16.5	13.0	11.8a	15.5	79.0a	34.70
T4 = ring cincturing +10 g L^{-1} 0-52-34 + 800 mg L^{-1} ethephon	71.6	20.2	22.8	10.2	10.3a	32.2	74.5a	24.53
F-test	ns	ns	ns	ns	*	ns	*	
CV (%)	12.20	54.7	43.0	49.3	92.6	150.0	18.6	

Means in each column followed by the same letters are not significantly different by DMRT at p≤0.01 (**) and p≤0.05 (*).
ns = not significantly different; - = treatment not available.
[a]Year 2016; [b]Year 2017; [c]Year 2018.

Application of pollen grains, spiral cincturing and plant growth regulators to increase fruit set

The number of fruit per panicle at initial set and at harvest varied among the treatments and orchards (Tables 2 and 3). There was significantly different number of fruit per panicle at both stages among the treatments in five of the orchard × year combinations. The response to treatment, however, was not consistent, as reviewed by Menzel (1983). In addition, the control tree had 19.48 (7.71-27.60) and 5.40 (2.22-10.6) fruit per panicle at the initial set and at harvest, respectively.

As pollination and fertilization are both required for sufficient initial fruit set, it is recommended that insects (e.g., honey bees) are introduced to the orchard during blooming (Huang and Chen, 2014). Insects are required to transport pollen grain from anthers to stigmas for fruit set. In this study, sprayed pollen grains had no effect on fruit set and resulted in fewer fruit per panicle than that in the control treatment. This result may have been due to the time to spraying which may not have matched the ideal time of receptivity of the female flower and resulted in poor fertilization. The average number of fruit per panicle after application of NAA was 12.52 (7.12-21.02) (Table 2). Spiral cincturing significantly increased number of fruit per panicle to 27.34 (13.63-38.54). Similar results have been reported in lychee by Chen and Huang (2001) and Li and Xiao (2001). In this study, application of uniconazole increased number of fruit per panicle at initial set but decreased it at harvest. Nie et al. (2001) previously reported that uniconazole increased photosynthesis, yield and total sugar content, but reduced fruit weight of 'Shixia' longan.

Table 2. Effect of pollen grains, plant growth regulators and spiral cincturing on number of fruit per panicle at initial set during 2016-2018.

Treatments	Lychee orchards × years							
	1[a]	2[b]	3[b]	4[c]	5[c]	6[c]	7[c]	Mean
T1 = Control	7.71	21.77a	19.35a	25.52b	27.60	16.24bc	18.16b	19.48
T2 = Pollen grains in 5% sugar	5.77	19.85b	11.67c	19.04bc	25.60	15.08c	20.34b	16.76
T3 = 10 mL L^{-1} NAA	8.79	12.53a	9.53bc	13.16c	21.02	7.12d	15.60bc	12.52
T4 = Spiral cincturing	-	14.98b	13.63b	33.12a	27.65	36.10a	38.54a	27.34
T5 = 400 mL L^{-1} Uniconazole	-	-	-	39.34a	27.60	22.56b	9.76c	24.82
F-test	ns	**	**	**	ns	**	**	
CV (%)	29.21	63.87	60.55	73.04	65.22	84.48	87.69	

Means in each column followed by the same letters are not significantly different by DMRT at p≤0.01 (**).
ns =not significantly different.
[a]Data of 2016; [b]Data of 2017; [c]Data of 2018.

After fruit set, most fruit drop occurred within 2 weeks with more than 50% of fruitlet loss of the initial fruit set (Figure 1). This pattern of fruit drop is similar to the pattern mentioned by Yuan and Huang (1993). Excessive fruit drop in lychee has been a serious problem, causing yield losses in some cultivars. The results presented in Table 3 indicate that fruit retention on the panicle was variable depending on year and location. At harvest, the average fruit number per panicle in the control treatment was 5.4 (2.22-10.60). In contrast, the average fruit number per panicle of all of the other treatments was in the range from 4.32 to 6.93. Studies in South Africa and Australia reported that cincturing soon after fruit set increased fruit retention and yield of lychee (Roe et al., 1997) which is similar to the overall results presented in this study.

Table 3. Effect of pollen grains, plant growth regulators and spiral cincturing on number of fruit per panicle at harvest during 2016-2018.

Treatments	Lychee orchards × years							
	1[a]	2[b]	3[b]	4[c]	5[c]	6[c]	7[c]	Mean
T1 = Control	5.24	6.30a	10.60a	2.22c	7.02	3.68bc	2.76bc	5.40
T2 = Pollen grains in 5% sugar	5.34	3.95b	5.75b	2.52c	7.55	3.92b	4.60b	4.80
T3 = 10 mL L^{-1} NAA	3.84	5.50b	7.17b	1.22c	7.40	2.42c	4.22b	4.54
T4 = Spiral cincturing	-	4.25b	7.28b	5.78a	8.02	7.26a	8.96a	6.93
T5 = 400 mL L^{-1} Uniconazole	-	-	-	4.30b	8.25	2.96bc	1.78c	4.32
F-test	ns	*	**	**	ns	**	**	
CV (%)	38.52	87.24	72.05	110.93	91.41	90.92	92.13	

Means in each column followed by the same letters are not significantly different by DMRT at p≤0.01 (**) and p≤0.05 (*).
ns = not significantly different
[a]Data of 2016; [b]Data of 2017; [c]Data of 2018.

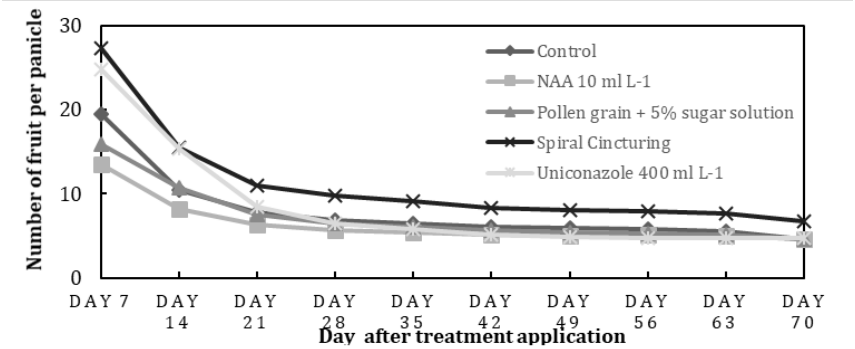

Figure 1. Number of fruit per panicle after application of plant growth regulators during 2016 and 2018.

Application of spiral cincturing and plant growth regulators to increase fruit size

The effects of plant growth regulators on fruit weight and aril weight are shown in Tables 4 and 5. The effectiveness was variable depending on the year and the orchard. Fruit weight was significantly different between treatments during 2016-2018 (Table 4). Average fruit weight of the control was 17.71 (14.79-20.78) g. Application of BS gave the greatest fruit weight in six of the orchard × year combinations with an average of 18.99 (13.82-25.20) g. Application of NAA gave the maximum fruit weight in 2 orchards with average 17.58 (14.48-21.48) g. Application of GA_3 produced an average fruit weight of 17.10 (13.89-21.36) g while with application of CPPU fruit weight was 17.29 (14.72-20.62) g. During the fruit development period, there were high temperatures (>38°C), low relative humidity (<30%) and low soil water content. This might have caused more fruit drop or low fruit quality. Menzel and Waite (2005) reported that water deficits before fruit set can reduce the number of fruit per panicle. In addition, water deficits after fruit set reduce fruit weight mainly due to the production of smaller arils, which account for 65-75% of final fresh weight.

Table 4. Effect of plant growth regulators on fruit weight (g) at harvest during 2016-2018.

Treatments	Lychee orchards × years								
	1[a]	2[a]	3[b]	4[b]	5[b]	6[c]	7[c]	8[c]	Mean
T1 = control	14.79bc	16.81bc	17.67a	15.55ab	17.28ab	20.78a	20.61b	18.22d	17.71
T2 = 5 mg L^{-1} BS	13.82c	17.81a	17.92a	16.21a	18.33a	20.33ab	25.20a	22.28a	18.99
T3 = 100 mg L^{-1} GA_3	14.17bc	16.15c	15.21b	13.89c	16.63b	19.23c	21.36b	20.12c	17.10
T4 = 200 mg L^{-1} NAA	17.11a	17.46ab	16.67ab	14.60bc	14.48c	19.26c	19.60b	21.48ab	17.58
T5 = 60 mg L^{-1} CPPU	14.77b	17.35ab	17.46a	14.96b	14.72c	19.40bc	19.03b	20.62bc	17.29
F-test	**	*	*	**	**	*	*	**	
CV (%)	19.60	19.54	37.15	20.83	23.78	17.05	52.45	18.15	

Means in each column followed by the same letters are not significantly different by DMRT at p≤0.01 (**) and p≤0.05 (*).
[a]Data of 2016; [b]Data of 2017; [c]Data of 2018.

Table 5. Effect of plant growth regulators on aril weight (g) at harvest during 2016-2018.

Treatments	Lychee orchards × years								
	1[a]	2[a]	3[b]	4[b]	5[b]	6[c]	7[c]	8[c]	Mean
T1 = control	8.33bc	10.17ab	11.06	9.49ab	9.39b	12.83a	12.95b	11.27c	10.69
T2 = 5 mg L^{-1} BS	7.66c	10.85a	11.23	10.20a	11.31a	11.19c	17.92a	14.82a	11.90
T3 = 100 mg L^{-1} GA_3	8.27bc	9.56b	9.67	8.37c	9.52b	11.63bc	14.03b	13.70b	10.59
T4 = 200 mg L^{-1} NAA	10.22a	10.45a	10.60	8.87bc	7.58c	12.21ab	11.95b	14.36a	10.78
T5 = 60 mg L^{-1} CPPU	8.50b	10.56a	10.90	9.50ab	8.92b	12.39ab	11.94b	12.81b	10.69
F-test	**	*	ns	**	**	*	*	**	
CV (%)	27.91	26.51	41.56	28.65	34.27	23.60	78.99	24.43	

Means in each column followed by the same letters are not significantly different by DMRT at p≤0.01 (**) and p≤0.05 (*).
ns = not significantly different
[a]Data of 2016; [b]Data of 2017; [c]Data of 2018.

Application of plant growth regulators significantly affected to aril weight in all orchards except in one orchard × year combination. The control had an average aril weight of 10.69 (8.33-12.95) g. Similar to fruit weight, application of BS produced the highest aril weight with an average of 11.90 (7.66-17.92) g. Application of NAA also induced a high aril weight with an average of 10.78 (7.58-14.36) g.

CONCLUSIONS

On the basis of these results, it can be concluded that, for flower induction, soil application of 300 g $tree^{-1}$ of paclobutrazol once the 2nd flush was fully matured followed 15 days later with a spray of 200 g $tree^{-1}$ of potassium chlorate was the best treatment. To promote fruit setting, spiral cincturing on branches of 5-10 cm diameter should be done at full-bloom. Application of 5 mg L^{-1} BS twice at seven days interval or 200 mg L^{-1} NAA once at

the brown-seed stage could be used to increase fruit weight and aril weight. In addition, tree management before flowering, such as pruning after harvesting, application of fertilizers or irrigation had positive effects on tree vigor, flowering and fruit setting. However, large scale field testing in 'Hong Huay' lychee is need before any commercial recommendations are made to lychee growers.

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Productivity enhancement of yard long bean (*Vigna unguiculata* ssp. *sesquipedalis*) using various detopping intervals

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Abstract

Detopping refers to the removal of terminal portion from the uppermost node to improve yield of vegetables in the Philippines. Yard long bean (*Vigna unguiculata* ssp. *sesquipedalis*) is an edible legume, mostly grown in home gardens or under partially shaded areas as a companion crop. A study was conducted at the Research Development Extension Unit of University of Science and Technology of Southern Philippines to enhance the productivity of yard long bean, through various detopping intervals. The study was laid out using split plot design with three replications. The main plot was cultivars (V1-'Montenegro' and V2-'Galante'), and the sub-plot was detopping intervals/days after planting (DAP) (DI1-14 DAP, DI2-21 DAP, DI3-28 DAP and DI4-35 DAP). Results revealed that detopping at 21 DAP, significantly increased pod yield to 23.52 t ha^{-1}, increased the number of stems, produced heavier fresh pods and attained the highest marketable pod yield compared to other treatments. The cultivar 'Galante' showed significant results compared to its counterpart 'Montenegro' on several parameters, like length of pods, pod fresh weight, marketable pod yield and dry matter yield.

Keywords: detopping, yard long bean, *Vigna unguiculata*, yield

INTRODUCTION

Detopping refers to nipping or the removal of terminal portion from the uppermost node to improve yield. This improves the function of remaining leaves by arresting unnecessary growth, decreasing mutual shading of leaves, enhancing light interception, and increasing nutrient uptake (Bhargavi et al., 2017). In addition, it is an effective way to reduce shedding of fruiting bodies and maintain the appropriate balance of vegetative and reproductive growth. Studies revealed that it can increase production. According to Firoz et al. (2010), detopping is a part of management practices which initiates new shoot and branches that can help increase yield.

Yard long bean (*Vigna unguiculata* ssp. *sesquipedalis*) is an edible legume well adapted to the lowland tropics at a temperature range of 20 to 35°C. It grows best under full sunlight but can tolerate partial shading. Adequate water supply and friable fertile soil can promote healthy growth and production of quality pods. Constant supply of moisture throughout the growing period is necessary. Adequate irrigation during the reproductive period can increase flowering and pod setting. Yard long bean has trifoliate leaves. The flowers are in pairs and borne on the axil of the leaf which vary in color depending on the cultivar. It is a vital annual crop, producing 30-60 cm long pods that hang in pairs with many seeds. Pods are either green, dark green, light green or purple (Bureau of Plant Industry, 2013).

In the Philippines, yard long bean is a vegetable available throughout the year and in some Filipino dishes it is one of the most important ingredients. It is grown in home gardens and on dikes around paddy fields and under partially shaded areas as a companion crop or commercial crop (Bureau of Plant Industry, 2013). The succulent young pods of yard long bean are eaten as whole pods and only need very light cooking. It can also be a good supplement to infant food whether cooked singly or mixed with other vegetables.

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Traditional farming practices among farmers' in the Philippines specifically Claveria does not involved innovative management practices such is detopping and relies heavily on a conventional approach when it comes to vegetable production.

The study was conducted under Claveria conditions to assess the response of different cultivars toward detopping intervals. This was carried out to enhance yard long bean productivity and validate such an innovative management method, compared to conventional practices. Specifically, the study aimed to: 1) assess the growth performance of yard long bean as influence by the different detopping intervals, and 2) determine the pod yield of yard long bean, respectively.

MATERIALS AND METHODS

Location of the study

This study was conducted from October 2018 to June 2019 at the Research Development and Extension Station of the University of Science and Technology of Southern Philippines (USTP). The research site is located 8°36.667'S;124°52.964'E and 590 m a.s.l. in Claveria, Misamis Oriental, Philippines.

Treatments and experimental design

The study design was a split plot with three replications. Cultivars were the main plot and detopping intervals were the sub-plots. The main plot (cultivar) was V1-'Montenegro' and V2-'Galante' and the sub-plot (detopping intervals (DI)) was DI0-no detopping, DI1-14 DAP, DI2-21 DAP, DI3-28 DAP and DI4-35 DAP.

Cultural management

Yard long bean seeds were directly seeded into the experimental plots. The experimental area was ploughed and harrowed before planting. The planting distance between hills was 30 and 75 cm between rows. Manual watering carried out when deemed necessary and seedling plants fertilized uniformly. A bamboo trellis was installed using twine when plants started to climb.

Data collection

1. Horticultural parameters.

The horticultural parameters, number of stems and pod length were determined through manual counting of stems and measurement of the length of the pods in cm. Collection of biomasses was carried out through random sampling of representatives per treatment per replication. Samples weighted in kg and means determined from the collected samples for analysis.

2. Yield parameters.

The pod yield plot^{-1} (kg ha^{-1}) recorded by weighing the harvest per replication per treatment per plot per plant, and converted to yield in t ha^{-1} by using the following formula:

$$\text{Yield (t ha}^{-1}\text{)} = \frac{\text{Plot yield (kg)}}{\text{Plot area (m}^2\text{)}} \times \frac{10{,}000\ (\text{m}^2)}{1\ \text{ha}} \times \frac{1\ \text{t}}{1{,}000\ \text{kg}} \quad (1)$$

Marketable pods are tender, straight, long, and unblemished. Non-marketable pods are short, curved, damaged by insects or diseases, and past the picking stage. However, they are still utilized as vegetable. The dry matter yield (non-consumable part stem, leaves, and roots of the plant) was determined by oven drying at 70°C. Harvest index was determined by dividing the weight of marketable yield by the combined weight of dry matter yield, marketable yield, and non-marketable yield and multiplying it by 100. The following formula was used to determine the harvest index:

$$\text{Harvest index} = \frac{\text{Marketable yield}}{\text{Marketable yield + non-marketable yield + dry matter yield}} \times 100 \quad (2)$$

Statistical analysis

Collected data was subjected to analysis of variance (ANOVA) to determine the level of significance. The Tukey's test or honest significant differences (HSD) was used to compare the significant differences between treatment means.

RESULTS AND DISCUSSION

Number of stems

Regardless of the cultivar, detopping of yard long bean at different intervals showed a significant effect on the number of stems produced. Results showed a significant difference in the number of stems promoted due to detopping compared to the un-topped yard long bean plants (Table 1). Detopping at 21 days after planting attained the highest number of stems (3.66). Upon detopping, a decapitated plant produces more axillary buds causing the increase of stems as per the concept proposed by Cline (1997). According to Mathan et al. (2016), the correct timing of detopping of yard long bean is important to increase yield, weight and fruit number. Therefore, the number of stems is related to pod fresh weight, which is related to flower number.

Table 1. Stem number per plant of yard long bean in response to different detopping interval using 'Montenegro' and 'Galante' cultivars.

Treatments	Number of stems
Cultivar (MP)	
Montenegro	2.69
Galante	2.64
F-test	ns
Detopping intervals (SP)	
No detopping	1.95c
14 DAP	2.72b
21 DAP	3.67a
28 DAP	2.44bc
35 DAP	2.56bc
F-test	**
MP×SP	
F-test	ns
CV (%)	
A	15.98
B	13.55

** significant at a level of 1% of probability ($p<0.01$).
ns = not significant ($p>0.05$).

Length of pods

The length of pods of yard long bean cultivars is shown in Table 2. Results showed there was a significant effect due to cultivar. 'Galante' pods averaged 60.58 cm in length, significantly longer than 'Montenegro'. This supports the 'Galante' description, stating that it has long pods with an average length of 58.5 cm. Furthermore, there were no significant effects with regards to detopping intervals. It has also been reported that removal of apical bud of yard long bean, regulated and increased shoot growth and fruit bearing capacity. This supports the study of Mollah et al. (2015) that detopping enhances pod yield.

Table 2. Fresh pod weight plant^{-1} and length of pods of yard long bean in response to different detopping intervals for the cultivars 'Montenegro' and 'Galante'.

Treatments	Fresh pod weight (g plant^{-1})	Length of pods (cm)
Cultivar (MP)		
Montenegro	391.38b	48.03b
Galante	515.61a	60.58a
F-test	**	**
Detopping intervals (SP)		
No detopping	451.94b	54.2
14 DAP	426.01cd	53.6
21 DAP	529.19a	55.24
28 DAP	416.16d	53.53
35 DAP	444.16bc	54.94
F-test	**	ns
MP×SP		
Montenegro × No detopping	353.89c	46.56b
Montenegro × 14 DAP	376.72bc	48.03ab
Montenegro × 21 DAP	461.49a	48.09ab
Montenegro × 28 DAP	368.72c	47.65ab
Montenegro × 35 DAP	396.07b	49.81a
Galante × No detopping	549.99bc	61.84ab
Galante × 14 DAP	475.29c	59.18b
Galante × 21 DAP	596.89a	62.39a
Galante × 28 DAP	463.61c	59.41b
Galante × 35 DAP	492.25c	60.07ab
F-test	**	**
CV (%)		
A	4.92	8.28
B	3.03	2.10

** significant at a level of 1% of probability ($p<0.01$); ns = not significant ($p>0.05$).

Fresh pod weight

Detopping had a significant effect on pod fresh weight as shown in Table 2. A 529.19 g pod fresh weight was the highest fresh weight recorded due to detopping of yard long bean at 21 DAP. Agro-climatic factors such as elevation and temperature often increase the fruit growth rate, but it has a greater affect in hastening maturity. As a result, the final mean weight of fruit is reduced (Mollah et al., 2015).

Non-marketable yield

Results showed that non-marketable fruits of yard long bean was influenced by different detopping intervals. Data are presented in Table 3. Significantly higher weight of non-marketable fruit (204.44 g) was observed for detopping at 28 DAP. There is also a significant effect of cultivar. 'Galante' is a relatively larger cultivar compared to its counterpart, 'Montenegro'. This gave the advantage to 'Galante' in terms of yield parameters compare to 'Montenegro'. The non-marketable yield parameter is associated with the effects of pest and diseases, physical damages, postharvest handling and field operations, or it is small in size and had an abnormal maturity, making them unmarketable.

Marketable yield

The marketable fruit in yard long bean was influenced by different detopping intervals. Table 3 shows the results found by this study. The highest marketable pod yield was 520.99 g. This was obtained by detopping treatment at 21 DAP. There is also a significant effect by

cultivar. 'Galante' was a relatively larger cultivar compared to its counterpart, 'Montenegro'. This gives an advantage to 'Galante', in the yield parameter compared to 'Montenegro'.

Table 3. Yield parameters and harvest index of yard long bean and response to different detopping intervals for the cultivars 'Montenegro' and 'Galante'.

Treatments	Marketable (g)	Non-marketable (g)	Dry matter yield (g)	Harvest index
Cultivar (MP)				
Montenegro	391.59b	127.46b	80.66b	65.65a
Galante	503.93a	194.13a	83.68a	64.17b
F-test	**	**	*	*
Detopping intervals (SP)				
No detopping	452.85ab	122.33d	81.42	69.33a
14 DAP	424.87b	146.40c	82.24	65.93a
21 DAP	520.99a	175.05b	82.74	66.86a
28 DAP	401.42b	204.44a	81.17	58.19b
35 DAP	438.66ab	155.75c	83.27	64.27a
F-test	**	**	ns	**
MP×SP				
F-test	ns	ns	ns	ns
CV (%)				
A	6.79	3.88	2.25	1.28
B	11.13	6.36	3.43	4.48

** significant at a level of 1% of probability ($p<0.01$); * significant at a level of 5% of probability ($0.01=<p<0.05$); ns = not significant ($p>0.05$).

Detopping allows increased air circulation to the plants. The openness also helps to subdue possibility of mildew and mold. The plant response is increased total fruit yield and the pruning of side shoots also reduces the number of marketable fruits carried by each cluster (Santos, 2008). According to Cline (1997), by detopping, the marketable yield is increased. Detopping at 21 days after resulted in the highest marketable yield for this trail.

Dry matter yield

This study found dry matter yield in yard long bean was not influenced by different detopping intervals. No significant results were found by this study (Table 3). On the other hand, 'Galante' showed significant results in terms of dry matter yield compared to its counterpart, 'Montenegro'. 'Galante' is a relatively larger cultivar compared to its counterpart, 'Montenegro'. Therefore, 'Galante' has an advantage for yield parameters, compared to 'Montenegro'.

Harvest index

Harvest index in yard long bean was influenced by different detopping intervals (Table 3). Comparing 'Galante' and 'Montenegro', a significant difference was found between the two cultivars as shown in Table 3. Detopping on 28 DAP showed the lowest return of investment (ROI) compared to all detopping intervals.

Furthermore, even if 'Galante' showed a significant result for several parameters, length of pods, pod fresh weight, marketable pod yield and dry matter yield compared to 'Montenegro'. However, the harvest index result showed 'Montenegro' was significantly better for economic yield compared to 'Galante'. These implies the efficiency of 'Montenegro' is economically viable for market. In addition, 'Montenegro', even with a lesser yield still has a higher harvest index compared to other high yielding cultivars.

Pod yield

The pod yield (t ha^{-1}) for yard long bean was not influenced by different detopping intervals. The results are shown in Table 4. Furthermore, 'Galante' was significantly different for pod yield (t ha^{-1}) compared to its counterpart, 'Montenegro'. As indicated previously, 'Galante' is relatively large cultivar compared to 'Montenegro'. Therefore, 'Galante' has an advantage in terms of yield compared to 'Montenegro'.

Table 4. Pod yield of yard long bean in response to different detopping intervals under the environmental conditions at Claveria, Misamis Oriental, Philippines.

Treatments	Pod yield (t ha^{-1})
Cultivar (MP)	
Montenegro	17.39b
Galante	22.25a
F-test	**
Detopping intervals (SP)	
No detopping	20.08ab
14 DAP	17.27b
21 DAP	23.52a
28 DAP	18.49bc
35 DAP	19.74bcs
F-test	**
MP×SP	
F-test	ns
CV (%)	
A	9.88
B	15.51

** significant at a level of 1% of probability ($p<0.01$).
ns = not significant ($p>0.05$).

CONCLUSIONS

Results show that detopping at 21 days after planting (DAP) has a significant result for pod yield. A pod yield of 23.52 t ha^{-1} was achieved compared to the other treatments, no detopping (20.08), 14 DAP (17.27), 28 DAP (18.49) and35 DAP (19.74), respectively. This is supported by findings which showed a greater number of stems plant^{-1}, more pods, a greater marketable yield, and pod weight compared to the other treatments. Detopping at 21 DAP, was found to be a suitable time for yield maximization compared to all other treatments. Detopping at different intervals showed no significant result in pod length, diameter, weight, and number of days to flowering and harvest.

'Galante' showed a significant difference for several parameters, length of pods, pod fresh weight, marketable pod yield and dry matter yield compared to 'Montenegro'. In contrast a significant result in harvest index showed that 'Montenegro' gave a higher harvest index compared to 'Galante'.

Therefore, detopping yard long bean cultivar 'Galante', at 21 days after planting enhanced productivity by increasing the number of lateral branches and stems plant^{-1}. These may serve as potential sources for photosynthates, thus helping the plants increased pod yield through increased pod length, resulting in increase in pod weight. However, the same treatment combination also resulted in the highest ROI. It is recommended that further studies be conducted on the practice of pruning through detopping to verify and strengthen these results.

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Influence of different mulching materials on growth and yield of tomato subjected to drip irrigation

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Abstract

Locally sourced plant material has potential to be used as a mulching option instead of being discarded and treated as a waste product. Furthermore, the introduction of drip irrigation, which promotes better water usage efficiency, could be used in conjunction with mulching to enhance tomato production in the field. This study aimed to determine the best mulching system that would optimize growth, yield, and resistance to diseases when used with drip irrigation. The study was laid out in randomized complete block design (RCBD) with different mulch materials as treatments (replicated three times). The different treatments were no mulch without drip (control), no mulch with drip and rice straw or corn stalk or black plastic mulch or silver plastic mulch with drip. Plots mulched with corn stalk had increased plant height. Black plastic mulch produced a consistent increase in number and weight of marketable fruits which, consequently, resulted in the highest yields of 39.55 and 37.50 t ha^{-1} for the first and second crops, respectively. Corn stalk mulch produced the lowest incidence of disease from 30 until 75 days after transplanting (DAT) during the first cropping, followed by silver plastic mulch. Results indicate that black-colored plastic or corn stalk mulch along with drip irrigation are the best mulching materials to use in order to significantly enhance height and yield and to reduce disease incidence in tomato crops under production conditions in the Philippines.

Keywords: black plastic mulch, corn stalk mulch, drip irrigation, enhanced yield, plant waste mulching material

INTRODUCTION

The use of plastic mulch has long been recognized throughout the world as a means of enhancing the production of a number of different crops. Plastic mulch increases soil temperature, reduces weed populations, improves moisture conservation, reduces the incidence of certain insect pests, produces higher crop yields, and leads to a more efficient use of soil nutrients. Mulching covers the soil in the crop zone and forms a physical barrier to limit soil water evaporation, to control weeds, maintain a good soil structure, and to protect crops from contamination with water-splashed soil (Kasirajan and Ngouajio, 2012). Cover crops and organic mulches (OMs) can be used reduce inputs such as synthetic fertilizers and to increase soil quality (Wang et al., 2009). Organic mulches serve as a substrate for many microorganisms in the soil which are necessary for maintaining and promoting soil structure. Mulches can also help to keep the soil temperature favorable for the activity of microorganisms.

The color of a mulch can determine the retention of energy and can, therefore, influence the plant microclimate. The color of the mulch determines the surface temperature of the mulch and the underlying soil temperature. Different colors of mulching materials reflect different radiation patterns into the plant canopy affecting photosynthesis and, in some cases, plant morphogenesis, and may advance crop maturity. Color also can affect the behavior of some insects (Lament, 1993).

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Plastic mulch provides producers with economic advantages. However, disposal of used plastic mulch materials is a major issue, particularly in developing countries because limited facilities exist for recycling agricultural plastics. Consequently, most plastic mulch waste is disposed of in landfills, buried on farms or illegally burned. Polyethylene is persistent in the soil, spanning several human lifetimes, and the toxic by-products of combustion present environmental risks (Cowan, 2013).

The use of plastic mulches in the Philippines is still in the developmental stage. Farmers in the highlands of Misamis Oriental have never used this practice as mulching is expensive. Considerable effort is still required to convince growers, particularly for large scale tomato production, that there are benefits from using mulching materials. As part of a number of research initiatives that are aimed at resolving problems due to weeds, soil moisture retention and pest and disease incidence, this evaluation trial involving different types of mulch materials was conducted. The objective was to compare bare soil or no mulch with different mulching materials that would address the problems associated with a change in cultural practices and with incurring a new financial outlay. The study was conducted to evaluate the productivity of tomato as influenced by different mulching materials for two cropping systems. Specifically, it aimed: 1) to evaluate the growth performance of tomato in response to mulching, 2) to determine the yield and its components under the different methods of cultivation, and 3) to evaluate the occurrence of various pests and diseases.

MATERIALS AND METHODS

Site details

The study was conducted at the Agriculture Experiment Station of the University of Science and Technology at the Southern Philippines - Claveria Campus, Claveria, Misamis Oriental from January to April 2016 (1st cropping) and from May to August 2016 (2nd cropping). The tomato production area was specifically located at 8°36.6390'N, 124°52.8670'E with an elevation of 615 m a.s.l. Figure 1 shows the average agroclimatic data during the conduct of the experiment.

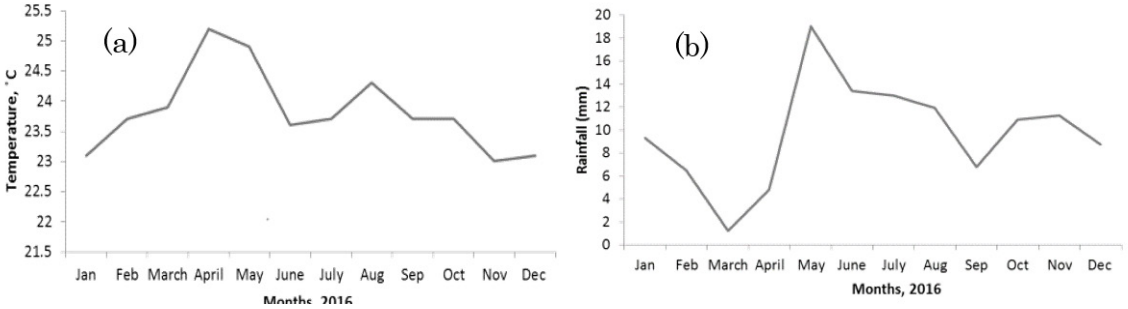

Figure 1. Average monthly temperature (a) and rainfall (b) at the USTP Claveria experimental area.

Cultural management practices

The experimental area had a total land area of 280 m^2. It was ploughed and harrowed twice then divided into experimental plots measuring 1×10 m each with an alley way of 1 m between plots. Installation of the different mulching materials followed the land preparation. Colored plastic mulch (black or silver) (1.5 m width) was cut and spread on top of the raised beds based on the length of the plot and soil was used to cover the sides of the plastic in order to keep it in place. Holes were made into the mulches based on the planting distance of crops. In the case of organic mulching, rice straw and corn stalks were collected from a harvested rice and corn field in the locality. Dried rice straw and corn stalks weighing about 12 and 15 kg, respectively, with an approximate thickness of 12.7 cm were used.

A sterilized soil mixture comprising garden soil, vermicast, lime and sand (4:5:0.5:1 v/v

ratio) in a seedling tray served as the planting medium for the tomato 'AVTO 1173' seedlings which were raised in an enclosed site protected with nylon nets. Seedlings were transplanted at the five-leaf stage about 4-5 weeks after sowing at a planting distance of 50×50 cm arranged in a double row.

Installation of the drip irrigation followed transplanting. One drip hose for every row was established in all experimental plots. Following transplanting, the drip was run for 2 h every morning and when plants were fully established, 15 days after transplanting (DAT), for 3 h every morning. Drip irrigation was not used when it was raining.

A basal application of a commercial organic fertilizer at a rate of 20 g per hill was applied together with 10 g of N-P-K fertilizer (14-14-14). A side dressing of 10 g of fertilizer (14-14-14) was applied per hill at 15 DAT and 10 g urea (46-0-0) and 10 g of muriate of potash (0-0-60) were applied per hill at 21 DAT. Fertigation was used at 40 DAT and then weekly until harvest, with the mixture of 8 kg urea (46-0-0), 8 kg muriate of potash (0-0-60), and 16 kg of Well Grow, diluted in 200 L of water.

Trellising was done at 30-35 DAT using bamboo sticks measuring 2 m length placed at an interval of 3 m in each plot. Spraying of insecticides (Lannate™ and Karate®) and fungicides (Daconil™) was done weekly at a rate of 10-15 mL 16 L^{-1} of water at 15 DAT and with the fungicide at a rate of 15-25 g 16 L^{-1} of water. The rate of application was changed as necessary. The first harvest was done at 55 DAT and was followed by seven subsequent harvests. After harvesting, the fruit were classified as marketable and non-marketable.

Data collection

Plant height (cm) was measured at 30, 45, 60, 75 and 90 DAT and at harvest the following parameters were determined: number and weight (g) of fruits classified into marketable and non-marketable, total fruit yield (t ha^{-1}) and fruit size (polar and equatorial diameter (cm)). Total fruit yield was measured by adding the total weight of both marketable and non-marketable fruits per plot in the harvested area as in Equation 1. Disease assessment was carried out at 30, 45, 60, 75 and 90 DAT.

$$\text{Yield (t ha}^{-1}\text{)} = \frac{\text{Plot yield (kg)}}{\text{Plot area (m}^2\text{)}} \times \frac{10{,}000\ (\text{m}^2)}{1\ \text{ha}} \times \frac{1\ \text{t}}{1{,}000\ \text{kg}} \quad (1)$$

Experimental and statistical design

The experiment consisted of two cropping systems which were laid out using a randomized complete block design (RCBD) with three replicates. In both cropping cycles, six plots were established with the different mulch materials as treatments (Table 1).

Table 1. Treatments applied for the growth and yield performance of tomato.

Treatments	Types of mulch
1	Control (no mulch, no drip)
2	No mulch with drip
3	Rice straw mulch with drip
4	Corn stalk mulch with drip
5	Black plastic mulch with drip
6	Silver plastic mulch with drip

During the first cropping, drip irrigation was established to continually supply water to the plants as, during those periods, extreme heat was experienced which was not favorable for tomato growth. The second cropping cycle was managed under protected cultivation. The greenhouse measured 6 m wide × 47 m long and was made up of heat resistant plastic film supported on rigid pipes.

RESULTS AND DISCUSSION

Plant height

Taller plants were observed where tomatoes were mulched with corn stalk in both cropping cycles and at all growth stages. The control plants were the shortest throughout both trials. There were statistically significant differences ($p<0.05$) among the mulches (Table 2) and plant height increased as the plants aged. The results were comparable to those of Olubanjo and Alade (2018). The respective mulches can, therefore, be recommended because the increased plant height (more flowering nodes) produced increased yields. Black plastic mulch was shown to enhance growth and yield of wheat plants in arid conditions, to increase soil porosity and to reduce soil compaction (Głąb and Kulig, 2008).

Mulching has been shown in other studies to decrease the fluctuations in temperature in the first 20-30 cm depth in soils and to promote root development, reduce weed populations, conserve moisture, reduce the incidence of certain insect pests, produce higher crop yields, and result in more efficient use of essential soil nutrients (Kasirajan and Ngouajio, 2012; Głąb and Kulig, 2008; Ham et al., 1993). Mulched drip irrigation is considered to be the most efficient irrigation method because it distributes water uniformly in the soil and minimizes unproductive evaporation (Zhang et al., 2020). Drip irrigation can be managed to maintain a more uniform and stable distribution of water throughout the cropping cycle in accordance with crop water requirements (Karlberg et al., 2007).

Size and weight of fruit

During first cropping cycle, plants grown at bare soil (no mulch) with drip irrigation showed comparable fruit size to that from the mulching treatments (Table 3). There were no significant differences ($p<0.05$) in the size or weight of fruits due to the different mulch types. In contrast, tomatoes with no mulch and no drip irrigation had the lowest fruit weight. Mulching has been shown to help to keep vegetables cleaner since there is no soil being splashed onto the plants or the fruits (Ham et al., 1993).

Yield plant^{-1} and yield ha^{-1}

Black plastic mulch enhanced the yield of tomato due to an increase in both the number and weight of marketable fruits in both cropping cycles (Table 4). Lament (1993) previously showed that plastic mulches aid in increasing yields but that the results obtained depended on location, soil type and mulch used. Certain colors of plastic mulch also influence the microclimate around the plants, and determine the surface temperature and the underlying soil temperature. Black mulch optimizes the transfer of heat from the mulch to the soil and the raised soil temperature can promote rapid crop development (Lament, 1993). Silver plastic mulch lessened the number of non-marketable fruit compared with the other treatments. It had the lowest number and lowest weight of non-marketable fruit during the first cropping cycle (Table 4). One attributing factor could have been due to aphids, which are an important vector of plant pathogenic viruses, as the color of a mulch can influence insect species (Farias-Larios and Orozco-Santos, 1997). An aluminum or silver surface color can repel certain aphids and reduce the incidence of aphid-borne viruses (Lament, 1993). This reduction in the incidence of pests has been attributed to a modification of the light environment around the plant (Diaz-Perez et al., 2007).

In contrast to the other mulch treatments, rice straw had the lowest number of marketable fruit, the lowest weight fruit^{-1} and the lowest yield plant^{-1}. This result is in agreement with Greer and Dole (2003) who found that rice straw as an organic mulch was not effective in increasing yields or in controlling insects and their damage. However, Olubanjo and Alade (2018) find that the proximate and mineral composition of fruit were higher in the crop mulched with rice husk. Overall, organic mulches usually cool soils which may lead to reduced yields.

Table 2. Plant height (cm) of tomato at each growth stage subjected to different mulching materials during the 1st and 2nd cropping cycles.

Treatments	1st cropping cycle					2nd cropping cycle					Mean
	30 DAT	45 DAT	60 DAT	75 DAT	90 DAT	30 DAT	45 DAT	60 DAT	75 DAT	90 DAT	90 DAT
1	50.60d	58.60e	67.80d	76.87d	65.67d	8.22e	6.33e	107.00d	116.00d	120.00e	92.84
2	56.33bc	64.73cd	74.46bc	86.86b	76.80b	99.13b	99.00d	109.30cd	118.67cd	122.67d	99.74
3	55.86bc	63.33d	71.53c	90.33a	81.87a	93.40c	101.66c	112.00c	121.00c	125.00c	103.44
4	58.20a	70.00a	81.86a	91.33a	80.87a	105.06a	113.33a	123.66a	132.67a	137.00a	108.94
5	55.46c	66.06bc	74.66bc	83.86a	73.13c	94.40c	102.33c	113.00c	122.00c	126.00c	99.57
6	57.33ab	67.26b	77.33 b	90.80a	82.87a	0.86d	107.33b	117.66b	126.67b	131.00b	106.94
F-test	**	**	**	**	**	**	**	**	**	**	
CV (%)	1.61	1.38	2.43	1.73	0.82	1.42	1.33	1.19	1.14	1.12	

DAT = days after transplanting.
** highly significant at α=0.01.

Table 3. Size and weight fruit^{-1} of tomato during the 1st and 2nd cropping cycles.

Treatments	1st cropping cycle			2nd cropping cycle		
	Fruit size (cm)		Weight fruit^{-1}	Fruit size (cm)		Weight fruit^{-1}
	Polar	Equatorial	(g)	Polar	Equatorial	(g)
1	3.58b	2.80b	19.66b	4.78	3.51b	28.64b
2	5.22a	3.88a	46.33a	5.36	3.99a	32.59ab
3	5.29a	3.81a	42.66a	4.97	3.78ab	45.72a
4	5.03a	3.80a	39.16a	5.32	4.01a	40.03a
5	5.12a	3.73a	41.00a	4.96	4.02a	39.77a
6	5.06a	3.88a	43.50a	5.17	4.08a	40.25a
F-test	**	**	**	ns	*	*
CV (%)	0.11	0.20	2.36	6.77	5.95	2.36

** highly significant at α=0.01, * significant at α=0.05, ns = not significant.

Table 4. Yield performance of tomato in response to mulching during the 1st and 2nd cropping.

Treatments	No. of fruits plant-1				Wt. of fruit plant-1 (kg)				Yield (t ha-1)	
	Marketable		Non-marketable		Marketable		Non-marketable			
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
1	33.46f	52.47ab	13.40a	13.40	0.70e	1.61b	0.23a	0.29	9.78d	22.63b
2	88.53c	63.40ab	8.73c	10.93	2.75b	2.19ab	0.18ab	0.21	38.55ab	30.70ab
3	74.00e	42.73b	8.33c	12.13	2.27d	1.60b	0.16ab	0.25	31.78c	22.53b
4	82.86d	69.80a	10.06b	10.87	2.64c	2.68a	0.18ab	0.32	36.97b	37.56a
5	92.26a	70.33a	5.93d	9.73	2.81a	2.68a	0.12bc	0.24	39.55a	37.50a
6	91.26b	57.60ab	4.46e	12.06	2.73b	2.14ab	0.08c	0.30	38.23ab	30.00ab
F-test	**	*	**	ns	**	*	**	ns	**	*
CV (%)	0.68	10.65	4.92	9.54	0.40	10.08	8.51	8.86	1.87	9.07

** highly significant at α=0.01, * significant at α=0.05, ns = not significant.

Disease incidence

Corn stalk mulch tended to suppress the incidence of diseases from 30 until 75 DAT during the first cropping cycle but differences among the different mulches were small or non-significant during both cropping cycles (Table 5). Previous reports have claimed that incorporation of crop residues has led to a reduction in the numbers of plant pathogenic nematodes (Litterick et al., 2004). Moreover, functional soil microbial and faunal communities have been shown to be higher in organic than in conventional fields and are associated with a reduction in the occurrence of large-scale disease epidemics (Litterick et al., 2004). In our study, the control treatment consistently had the highest disease incidence at the first cropping cycle.

Table 5. Influence of different mulching materials on the incidence of diseases in tomato during the 1st and 2nd cropping cycles.

Treatments	1st cropping cycle					2nd cropping cycle				
	30 DAT	45 DAT	60 DAT	75 DAT	90 DAT	30 DAT	45 DAT	60 DAT	75 DAT	90 DAT
1	1.66a	2.66a	3.66a	4.03a	4.06	1.67a	2.00	2.67	3.00	4.00
2	1.46b	2.46b	3.46ab	4.03a	4.03	1.00b	2.00	2.33	2.33	3.33
3	1.40bc	2.40bc	3.33ab	3.86ab	4.03	1.00b	1.67	2.67	2.67	3.33
4	1.26c	2.66c	3.00d	3.73b	4.03	1.00b	1.67	2.33	2.67	3.67
5	1.33bc	2.26c	3.13cd	3.86ab	4.03	1.00b	2.00	2.67	3.00	4.00
6	1.40bc	2.26c	3.26bc	3.73b	4.03	1.00b	1.33	2.00	2.33	3.33
F-test	**	**	**	**	ns	**	ns	ns	ns	ns
CV (%)	6.11	4.23	4.22	2.81	1.90	11.00	11.87	10.30	5.30	9.68

DAT = days after transplanting.

Disease incidence rating: 0 = none of the total population,1 = 1-25% of the population, 2 = 26-50% of the population, 3 = 51-75% of the population and 4 = 76-100% of the population.

** highly significant at α=0.01; ns = not significant.

CONCLUSIONS

Corn stalk, black, and silver plastic mulch consistently produced fruits with greater equatorial size (4.01-4.08 cm) and heaviest weight per fruit (39.77-40.25 g). Black plastic mulch produced an increased number and weight of marketable fruit in both cropping cycles which, consequently, resulted into the highest yield per hectare of 37.50-39.55 t ha-1. Crops grown with corn stalk mulch had the lowest disease incidence from 30 to 75 DAT during first cropping cycle. Overall, these results indicate that both black-colored plastic mulch and corn stalk mulch, combined with drip irrigation, were the best mulching materials for significantly

enhancing plant height and yield and for reducing disease incidence in tomato fruit under production conditions in the Philippines.

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Effect of hot wind on insects in longan (*Dimocarpus longan* Lour.) orchard during off-season production in the Chao Phraya Delta

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Abstract

Off-season longan is an important commercial frui crop grown in the alluvial plains of Chao Phraya Delta, Thailand. Hot winds during the off-season production period has been reported to significantly reduce shoot growth and cause flower buds to dry out and fall off. In addition, the effect of these hot winds on the bio-ecological environmental is still unknown. This study monitored the all year round (May 2018-April 2019) change in insect populations surrounding longan orchards. Five different insect trap sheets, four coloured (white, yellow, green and blue) and a transparant trap were located on the four compass directions (north, south, east and west) in Ban Phaeo District, Samut Sakhon Province. Apical shoots were induced in May (May pot) to initiated early flower buds to avoid the hot winds and compared with the flower buds initiated in June (June plot) that were exposed to the hot winds. The trap sheets were replaced and evaluated for insect population every month. The results showed, regardless of growth pattern differences between the two plots, insect populations in both plots clearly increased at the same time during June to November, when the annual hot winds blew at Ban Phaeo. Interestingly, during this period, insect populations increased in conjunction with situations of high-water vapour pressure deficit which is related to high temperature and low relative humidity. The insect category focused on, was those pests related to longan productivity, natural enemies, and water quality indicators. Trap location from the four compass directions showed the same trend. Major insect pests and enemies were found on yellow colour trap sheets. These results will benefit selective integrated pest management programs for controlling insect populations in longan orchards.

Keywords: integrated pest management, annual growth, cultural practice, climate change

INTRODUCTION

Longan (*Dimocarpus longan* Lour) is an important economic fruit tree crop in Southeast Asia. Subhadrabandhu (1990) reported Thailand is a major world exporter of fresh longan fruit. In 2017, about 20,995 million baht of fresh longan and about 2.35 million baht of frozen and 11,118 million baht of dried longan was exported (Office of Agriculture Economics, Thailand, 2017). The major longan growing areas and on-season production occurs in the northern regions of Thailand. Off-season production occurs in the eastern and central regions of Thailand. The use of potassium chlorate replaces the need for suitable climatic conditions for off-season production (Sukhvibul, 2015). All orchards near the capital Bangkok obtain a higher price for off-season longans. 'Phuang Thong' longan, growing in the Samut Sakhon Province in the Chao Phraya Delta, generally bloom in August and are harvested in February.

The issue with off-season longan production comes from the climate, namely hot winds, subsequently causing significantly low yield. This specific climatic effect includes a rapid increase in temperature to over 40°C which has a negative effect on yield (Pichakum et al., 2020). Recent climate inconstancy, has been reported to affect several important tropical fruit tree crops such as durian and mango in Thailand, causing uncertain flowering, harvesting and

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low fruit quality. Occurrences of these climate changes in Thailand is caused by an extreme increase in climatic event. Both seasonal and long-term changes affect various insect pests, their natural enemies and disease population dynamics and diversity. These climate factors may cause new pest and disease incursions which can influence world food security, especially in underdeveloped countries with rapid population increases. Abiotic parameters have direct effect on insect population dynamics through modulation of developmental rates, survival, fecundity, voltinism and dispersal (Karuppaiah and Sujayanad, 2012; Sharma, 2014). Epidemics of diseases and insects are associated with climate components such as ambient temperature, relative humidity, wind speed and direction. Climate variability can impose large fitness costs on insects, showing diapause and other life cycle responses and threatening population persistence. In response to stressful climatic conditions, insects also undergo ontogenetic changes including hardening and acclimation (Sgrò et al., 2016). For rice, temperature was reported as an important factor among the climatic components. The decline in the survival rate of brown plant hopper and rice leaf folder at higher temperatures, indicates the effects of rising temperatures may make on the changes in pest population dynamics of the rice ecosystem (Karuppaiah and Sujayanad, 2012). During heat waves, water-deficit stress effects, influences phloem-feeding insects. Moderate water-deficit stress during high day-night temperatures reduces aphid survival but has little effect on aphid population growth parameters especially for those aphids that have reached maturity. Drought and heat waves frequently occur together, and their combined effect on plant is complex. In addition insect herbivore interactions are also complex in the context of climate change (Beetge and Krüger, 2019). Evidence suggests that diverse environmental factors have a negative effect on organism. The annual hot wind phenomena in Chao Phraya Delta has affected longan production. This influence on other organisms in the biosphere should be investigated. This study aims to observe the insect dynamics surrounding longan orchards during hot wind exposure in the Chao Phraya Delta and the information was used to develop new pest management strategies.

MATERIALS AND METHODS

The observational experiment was carried out during off-season production of 'Phuang Thong' longan (*Dimocarpus longan* Lour) at farmer's orchards (13.592768E; 100.069948N) located in Ban Phaeo District, Samut Sakhon Province, Thailand in 2018-2019. Similar 19-year-old trees with a 3-m canopy diameter spaced at 6×6 m were selected. Two representative plots (approximately 1.12 ha each) located in the same orchard with industry standard cultural practices comprising of annual pruning, fertilizer application and crop protection measures were undertaken. In 2018, potassium chlorate ($KClO_3$) was applied to the two plots at different times to induce flowering. The first induction time was early May (May plot) and the second induction time was in late June (June plot). The $KClO_3$ was applied as a soil drenched at the rate of 100 g m^{-1} of canopy diameter. Seven days later a foliar spray at 1,500 ppm was applied to the same trees. The two plots were at different annual growth stages when encountering the hot winds. The May plot was fruiting, and the June plot was flowering (full bloom).

Two weather micro-stations consisting of a data logger and sensors (Hobo, Onset Corp., USA) were installed at the centre of each orchard. Weather data were recorded every 10 min throughout the seasonal year 2018-2019. Air temperature (Temp) and relative humidity (RH) sensors were installed at top-canopy level on the four sides (north, south, east and west) of the longan tree. Data were collected and analysed using the Hoboware pro software (version 3.7.12, Onset computer corporation, USA). Microclimates were identified regarding hot wind situations. In addition, the vapour pressure deficit (VPDs) was calculated. Based on the hot wind logged events, a VPDs of 2.71 kPa was the threshold causing dry-off symptoms in longan flower (Pichakum et al., 2020).

Insect traps consisted of four colour (white, yellow, green and blue) were designed and applied to boards of polypropylene sheeting coated with a sticky insect glue (Greenplana Co., Ltd., Thailand). A transparent sheet used as the control treatment. The sheets were set on four cardinal directions of north, south, east and west for each plot. Insect traps were 26×43 cm in

size and two traps were placed vertically at 140 cm height above ground level. Every month the trap sheets were replaced and the insect population identified and counted. Insect identification was carried out with reference to Triplehorn and Johnson (2005). Identified insects were classified into pests, natural enemies and those insects used as a water quality indicator (WQI).

RESULTS AND DISCUSSION

Environmental conditions

Annual fluctuations of Temp and RH showed considerable variation. Temperature ranged from 16 to 47°C and RH from 39% to nearly 100%. Interestingly, the high maximum Temp would suddenly increase to 47°C. This occurred in June and August to October. At the same time of these sudden increases in Temp, the minimum RH trended lower. These environmental factors in 2018 and 2019 of high Temp and low RH resulted in high VPDs (Figure 1).

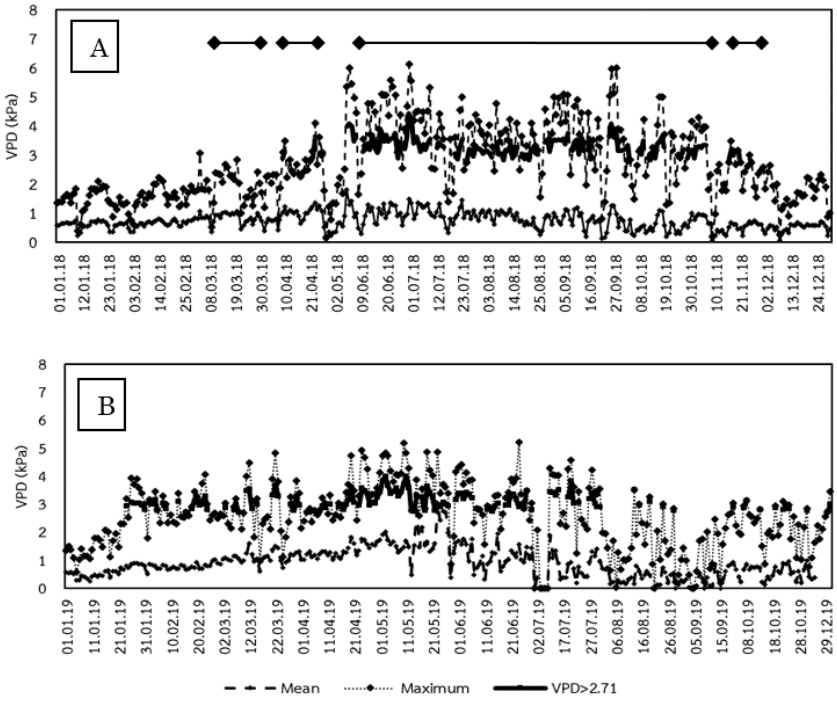

Figure 1. Daily vapour pressure deficit (VPDs; kPa), maximum and average (mean) readings above the threshold of 2.71 kPa located at the longan orchard in Ban Phaeo District, Samut Sakhon Province in 2018 (A) and 2019 (B). Solid line are the means of the hot wind events.

From these microclimatic data, results showed the VPDs affecting the longan flowers with dry-off symptoms. This experiment found the critical VPDs of 2.71 kPa as reported by Pichakum et al. (2020) was reached at this trial site due to hot winds. Therefore, the environmental conditions for longan flower dry-off (high VPDs) was caused by the hot wind phenomena at this trial site. This occurred on many occasions during 2018: 1) early and late March, 2) early and late April, 3) middle June to early November, and 4) late November. The highest VPDs occurred during June to November, reaching a high of 5 to 6 kPa. Regardless of the frequency of rain showers in 2019, VPDs peaks were found during the same periods as in 2018. Even though the critical VPDs levels were lower at 4-5 kPa, but still well above the threshold of 2.71 kPa. High VPDs played an important role in rapid loss of water loss from longan raceme tissue. Current year shoots have shown a low elongation rate as a negative

response to the hot winds. Shoot length and the number of compound leaves were low and incomplete flushing has appeared as reported by Traisuwan et al. (2020).

Pest population categorisation

The annual population dynamics of insect in the categories of pest, natural enemy and WQI in the longan orchard is showed in Figure 2. For the sampling time-period (2018-2019 season), the number for three categories of insect types varied with changes in VPDs. An increase in the numbers and peaks of pest and WQI was recorded twice, July and November, respectively. The results showed those peaks occurred at the same time as the elivated VPDs (June-November) and are the associated with the occurrence of higher VPDs values that are well above the normal daily VPDs for June to November due to hot winds (Figure 1A). However, the natural enemy category increased rapidly (March to April) after the longan off season harvest (November to February) (Figure 2).

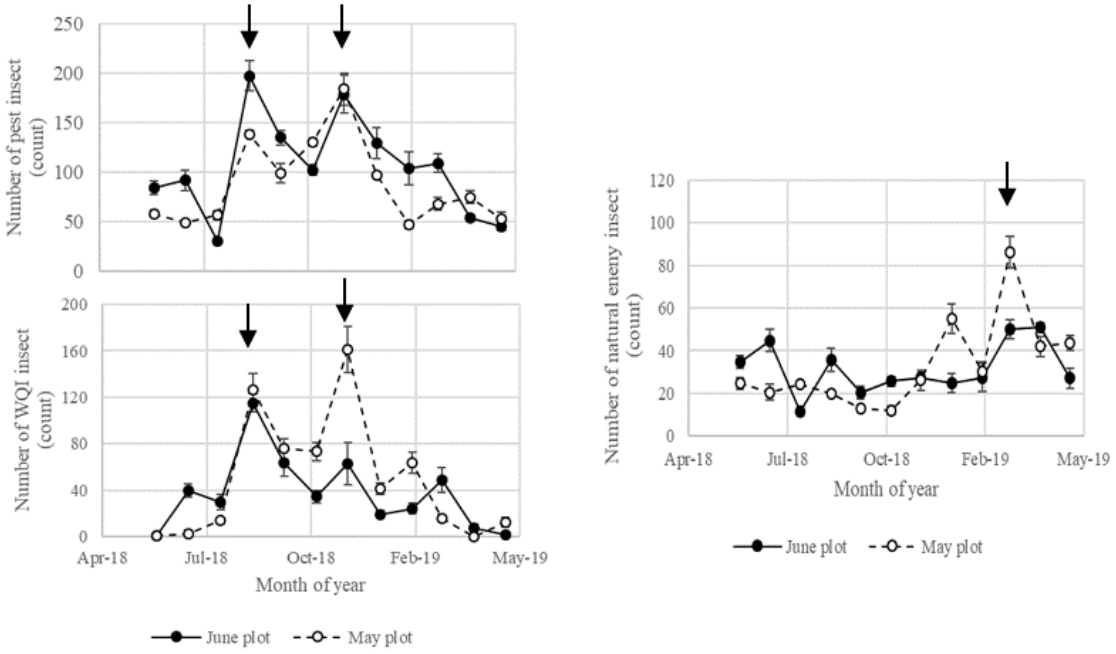

Figure 2. Annual population dynamics of insects (count) in each category (pest: top-left, water quality indicator (WQI), below-left, and natural enemy, right) observed in June Plot and May Plot in longan orchard located in Ban Phaeo District, Samut Sakhon Province in June 2018-May 2019. Arrows indicate hot wind events.

Although the experimental plots were different in annual growth stages, the insect population dynamics were similar. Consequently, the change in number of insects surrounding the longan orchard may be influenced by changing environmental conditions rather than host (longan) growth stages. This implies that climate change would be primary factor affecting changes in insect population dynamics in this region. Furthermore, various abiotic factors, such as increasing temperatures play a pivotal role, as a driving force influencing insect population dynamics. Temp has a direct effect on survival, growth, development, voltinism and dispersal of insects (Karuppaiah and Sujayanad, 2012). Increasing Temp can drive changes in the distribution and abundance of insects. Ward et al. (2019) indicated the time of year when warming occurs, spring and stable climatic conditions autumn, may disparately influence the phenology of herbivorous insects and their host plants in North America. Identification of observed insects, showed that the pests of longan orchards are whiteflies, leafhoppers, froghoppers, cicadas, treehoppers, stink bugs, red bugs, leaf bugs, gall midges, fruit flies, leaf beetles, scarab beetles, weevils, long-horned beetles, oil beetles,

noctuid moths, tent caterpillars, long-horned grasshoppers, locusts, pygmy grasshoppers, crickets and thrips. The natural enemy were braconids, ichnumonids, sphecid wasps, ensign wasps, green lacewings, earwigs, long-legged flies, ladybird beetles, big eye bugs, backswimmers, mantids, water striders, dragonflies and spiders. Whitefly and leafhopper were the important pest. Lastly fireflies and mayflies were identified as WQI insects. Annual insect pest numbers from cardinal directions (north: N, south: S, east: E, and west: W) is shown in Figure 3. Based on the data collected on the four directions, whenever the hot winds appeared, pests attack longan orchards from every direction.

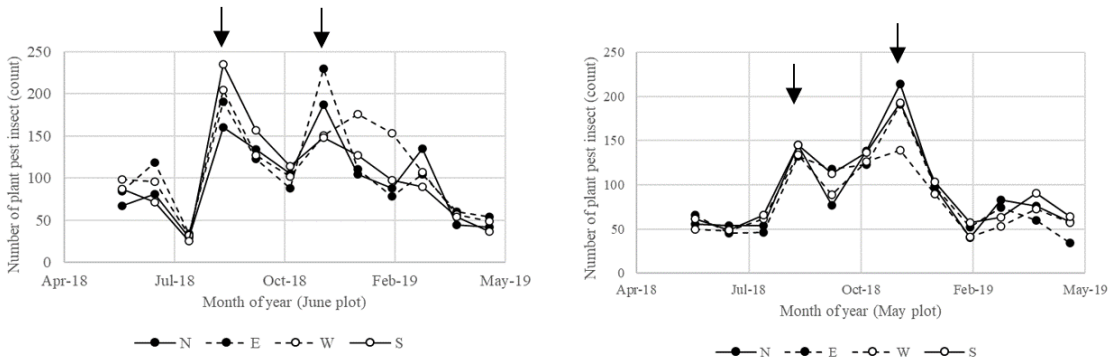

Figure 3. Annual population dynamics of insect pests observed on the insect traps located on the northern (N), southern (S), eastern (E) and western (W) cardinal directions for the June plot and May plot for the longan orchard located in Ban Phaeo District, Samut Sakhon Province in June 2018-May 2019. Arrows indicate hot wind events.

Insect pests and their natural enemies were found on all coloured traps (Table 1). Many insect pests (79-93 counts) were found on the four colours; white, yellow, green and blue, while lower counts were recorded on the transparent traps (control). High trapped numbers, for pests, natural enemies and WQI insects in longan orchard occurred on the four colours (average amount of 42-60 counts). Colour preference for pest and natural enemy (129 counts) were recorded on the yellow traps. This result is similarly to Ranamukhaarachchi and Wickramarachchim (2007) who recorded a high correlation between the cumulative numbers of trips trapped on colour cards and the percentage of infested tomato leaves in central Thailand. Thrips were found on yellow, blue, white, light green, dark green, orange, and transparent traps.

Table 1. Average amount of insect (number per trap sheet month^{-1}) at longan orchard located in the Ban Phaeo District, Samut Sakhon Province in June 2018-May 2019 (sheet size was 26×43 cm).

Type of insect	Colour of trap sheet				
	Transparent	White	Yellow	Green	Blue
Pest insect	61	84	84	79	93
Natural enemy	19	25	45	26	15
Water quality indicator	16	50	13	21	73
Average	32	53	47	42	60

CONCLUSIONS

The events of high Temp and low RH (representing high VPDs), occurring at Ban Phaeo District, Samut Sakhon Province in Chao Phraya Delta, Thailand for the year 2018, start in March and continue until November. The results clearly showed that high VPDs not only affected longan annual growth but changes in insect pests, natural enemies and the water

quality indicators insect populations surrounding the longan orchard. The cardinal insect direction traps clearly identify unaffected insect populations during hot windy conditions. The study agreed with previous studies that report the preferred colour for trapping insects in longan orchards is yellow.

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Effects of rhizome storage and handling on growth and development of *Curcuma* hybrid 'Great Reign'

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Abstract

Generally, the rhizome of Krajeaw (Subgenus *Eucurcuma*) has attached long storage roots. The attached roots are trimmed at harvest before the rhizome is kept in a cold room. Water loss generally occurs during storage and the dry rhizome limits its germination. This study is to determine the effects of rhizome storage and handling on plant growth and flowering. The rhizomes of *Curcuma* hybrid 'Great Reign' were used as plant material. The experimental design was a factorial in completely randomized design, consisting of 3 factors: 1) four levels of rhizome sizes (>3, 3.0-2.5, 2.5-1.5 and <1.5 cm diameter), 2) four levels of root trimming (0, 1, 3 and 5 cm trimmed root), and 3) two types of storage media (presence or absent of storage media). Rhizomes were kept at 15°C, 60% RH for two months before planting. At flowering, the results showed the larger rhizome size with 5 cm roots produced the tallest plants. Furthermore, the smallest rhizome gave the lowest photosynthetic rate of 4.83 µmol m^{-2} s^{-1} during the flowering stage. From this study, the appropriate postharvest handling of *Curcuma* hybrid 'Great Reign' rhizome to achieve the best plant growth and flowering was, using a combination of>3 cm rhizome size with 5 cm trimmed root length and stored in coconut dust.

Keywords: postharvest, *Eucurcuma*, flowering, root trimming, *Zingiberaceae*

INTRODUCTION

The genus *Curcuma* belongs to the family *Zingiberaceae*, which is distributed in tropical Asia, Papua New Guinea and Northern Australia (Ruamrungsri, 2015a). About 38 species are found in Thailand (Larsen and Larsen, 2006). There are two subgenera based on the presence or absence of anther spurs and true flower color: *Paracurcuma* and *Eucurcuma*. The purple flower is mainly found in *Paracurcuma,* whereas the yellowish true flowers are found in *Eucurcuma* (Sasikumar, 2005; Ruamrungsri, 2015b). The best well-known species in *Paracurcuma* is *C. alismatifolia* or Patumma which was introduced to the world market in previous decades (Kamenetsky and Okubo, 2013). Unlike *Paracurcuma*, *Eucurcuma* species mostly have yellowish flowers and a cone-shaped spike that emerges from the top or side of the pseudostem. Plant height averages 60-90 cm, with long and wide leaves. The underground stem is a stubbed rhizome with contractile roots and fibrous roots (Apavatjrut et al., 1999; Sasikumar, 2005). They are commonly used in landscape designs due to their vivid inflorescences and broad leaves. The world market demand for exotic flowers is increasing, especially *Curcuma*. Thailand exports approximately 2-3 million rhizomes to the world market, including Japan, USA and the Netherlands. For *Paracurcuma*, the standard size of a rhizome for European countries and the USA is up to 2 cm in diameter with four storage roots (Ministry of Agriculture and Cooperatives, 2011). The storage and handling of *Paracurcuma* or Patumma rhizomes has been intensively studied. Ruamrungsri (2015b) reported that ambient temperatures higher than 15°C caused water loss in Patumma rhizomes. In addition, *Eucurcuma* rhizomes should store at 15°C and covered with coconut coir dust. However, the investigation on *Eucurcuma* rhizome handling has been rarely reported (Ruamrungsri, 2015b). Therefore, this study aimed to elucidate the effect of rhizome size, roots trimming and

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presence of storage medium on growth and development of the Krajeaw 'Great Reign' *Curcuma*.

MATERIALS AND METHODS

Curcuma hybrid 'Great Reign' rhizomes were prepared. The experimental design was 4×4×2 factorial in CRD with 10 replications per treatment. The three factors were: 1) four levels of rhizome sizes (>3, 3.0-2.5, 2.5-1.5 and <1.5 cm of diameter), 2) four levels of root trimming (0, 1, 3 and 5 cm of root length trimming on rhizome), and 3) two levels of storage media (with or without coconut dust) (Figure 1). Rhizomes were kept at 15°C, 60% RH for two months before planting. Rhizomes were planted in a plastic bag (14.0×11.5×25.0 cm) with growing media consisting of soil:coconut dust:rice husk charcoal in a 1:1:1 ratio (w/w). Plants were watered daily and 15-15-15 NPK fertilizer was supplied when the plants started to sprout. Plant height (cm) and number of leaves were measured every two weeks. At 90 days after planting (DAP), leaf greenness of the first mature leaf was measured using chlorophyll meter (Konica Minolta®) and plant photosynthesis rate was recorded using the LCpro-SD Analyzer (ADC BioScientific). During the flowering stage, flower length, flower width and inflorescence length were recorded.

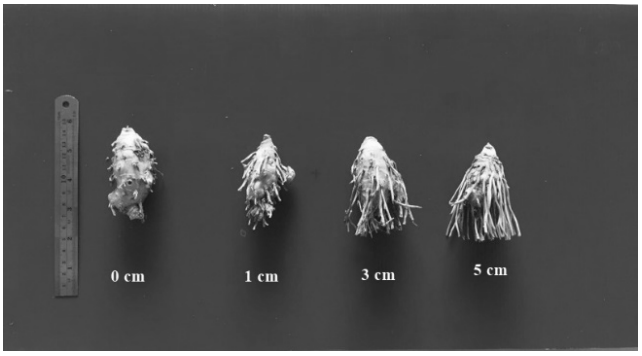

Figure 1. *Curcuma* 'Great Reign' rhizomes with various root-trimming lengths.

RESULTS AND DISCUSSION

Plant growth

Ninety days after planting, regardless of root trimming length and storage medium, rhizome diameters >3 cm produced the tallest plants, on average 90.6 cm in height (Table 1). However, these were not significantly different from rhizome diameters of 2.5-1.5 cm. Similar results were found in *Curcuma longa,* where larger rhizomes as a propagule, resulted in greater plant growth (Hossain et al., 2005). The larger rhizome clearly has more reserved nutrients, which enhanced plant growth and development (Ruamrungsri, 2015b). Longer trimmed roots produced taller plants, whereas total root removal, resulted in the shortest plants at 72.4 cm (Table 1). Pubuopiend (1992) reported that the number of storage roots of *C. sparganifolia* did not affect days to sprouting, but plants with three storage roots produced better growth than plants with 0, 1 and 2 storage roots. The shortest plants were obtained from the root sheared treatment, indicating that storage food in root affected plant growth. When roots were trimmed closer to the rhizome surface, a lesion may cause moisture loss in rhizomes. In ginger rhizomes, more moisture was lost from cut surfaces rather than from the intact surfaces (Akamine, 1962). Weight loss of *Zantedeschia* tuber occurred when its surface was cut and subsequently, white layers of dead tissue appeared on the cut surface (Hertogh and Nard, 1993). The combination of rhizome diameter >3 and 5 cm root trimming combined with coconut dust as the storage medium produced the tallest plants with an average height of 108.4 cm (data not shown). The darkest green leaf was obtained from the 5 cm root trimming treatment but not difference was found with 3 and 1 cm roots trimming treatments. Furthermore, the lightest, were those plants without roots (Table 1). Interestingly, a

combination of rhizome diameter 3.0-2.5 cm with 3 cm trimmed roots produced the greenest leaf, A SPAD reading of about 51.1 units (data not shown). There was no difference in the photosynthetic rate for all root-trimming treatments. However, the highest photosynthetic rate was observed in the combination treatment of rhizome diameter 3.0-2.5 cm with 5 cm trimmed roots and storage in coconut dust. The average photosynthetic rate was 7.26 µmol m^{-2} s^{-1} (data not shown).

Table 1. Effect of storage handling on the growth of *Curcuma* 'Great Reign'.

	Plant height (cm, 90 DAP)	Leaf greenness (SPAD unit)	Photosynthesis rate (µmol m^{-2} s^{-1})
Rhizome size			
>3 cm	90. 6a	47.9a	5.15ab
3.0-2.5 cm	82.5b	49.1a	5.76a
2.5-1.5 cm	88.2a	45.4b	5.22ab
<1.5 cm	64.2c	49.1a	4.83b
Root trimming			
0 cm	72.4d*	46.4b*	5.08a^{ns}
1 cm	78.3c	47.6ab	4.91a
3 cm	84.9b	48.2ab	5.38a
5 cm	90.0a	49.3a	5.59a
Storage material			
With coconut dust	81.0a^{ns}	47.9a^{ns}	5.29a^{ns}
Without coconut dust	81.8a	47.8a	5.19a
Interaction			
Rhizome size × root trimming	*	*	*
Rhizome size × storage material	*	*	*
Root trimming × storage material	*	*	*
Rhizome size × root trimming × storage material	*	*	*

The different letters following the means show the significant difference between treatments within the same column (LSD test at p≤0.05, ^{ns} = not significant).

Flowering parameters

The smallest rhizome diameter resulted in the shortest inflorescence (Table 2). Similar, to *Curcuma sparganifolia*, the small rhizomes produced the lowest spike quality (Pubuopiend, 1992). However, the largest rhizome produced the shorter inflorescence length, compared to rhizome diameters of 3.0-2.5 and 2.5-1.5 cm, regardless of root-trimming and storage medium. Chomtee (2012) reported that rhizome size did not affect flower length and width of *Globba winitii*. Roots trimmed to 1 cm in length produced the shortest inflorescence length compared to the other treatments (Table 2; Figure 2). The longest flowers were observed in the combination of 3.0-2.5 cm rhizome diameter and root-trimming to 5 cm in length without a storage medium (data not shown). On the other hand, the largest rhizomes with 5 cm trimmed roots and stored in coconut dust produced the highest values for spike width and inflorescence length (data not shown). Ruamrungsri (2015b) reported that after rhizome harvest, growers usually kept rhizome plants at room temperature (25±2°C, 60% RH) with good ventilation. When storage temperature increased, sucrose, fructose, and glucose concentrations in rhizomes decreased (Paz, 2003). In Patumma, the rhizome size and the number of storage roots was significant for plant quality. This is due to contained plant nutrition, especially carbohydrates, which are stored in the roots compared to the rhizomes. Primarily, the storage roots stored carbohydrates for use during the next growing season (Ruamrungsri, 2015a). Roots trimmed at 0 cm and stored without coconut dust resulted in a lower flowering percentage (data not shown). The minimum weight loss was obtained by completely covering the cut ends of the rhizomes with paraffin (Akamine, 1962). Although a higher flowering percentage obtained by covering the rhizome with medium, the rhizome stored without medium resulted in wider spike and longer inflorescence. This may indicate that some environmental conditions and conversions of carbohydrate in rhizome stored at

15°C, 60% RH for two months without medium enhanced flowering responses compared to rhizome stored in the medium.

Table 2. Effect of rhizome size on flowering of *Curcuma* 'Great Reign'.

	Spike length (cm)	Spike width (cm)	Inflorescence length (cm)
Rhizome size			
>3 cm	17.4b	9.9b	45.5b
3.0-2.5 cm	20.1a	10.8a	49.3a
2.5-1.5 cm	19.8a	10.3ab	49.2a
<1.5 cm	19.3a	10.1b	39.1c
Root trimming			
0 cm	19.3a	10.5a	44.2b
1 cm	16.3b	8.8b	40.2c
3 cm	20.6a	10.9a	48.8a
5 cm	20.5a	10.9a	49.9a
Storage material			
With coconut dust	18.7a^{ns}	9.8b	44.3b
Without coconut dust	19.7a	10.7a	47.2a
Interaction			
Rhizome size × root trimming	*	*	*
Rhizome size × storage material	*	*	*
Root trimming × storage material	*	*	*
Rhizome size × root trimming × storage material	*	*	*

The different letters follow means show the significant difference between treatments within the same column (LSD test at $p \leq 0.05$. ^{ns} = not significant).

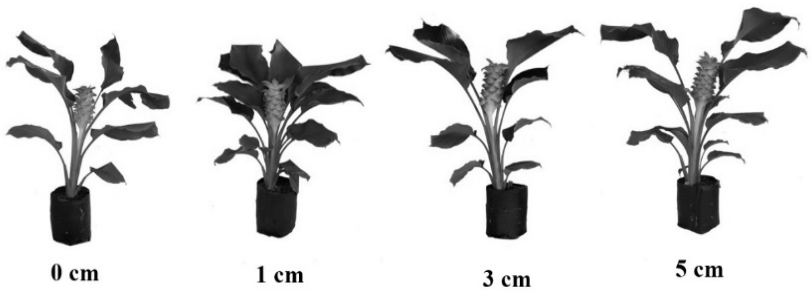

Figure 2. Effect of difference rhizome root length trimming on plant growth and flowering of *Curcuma* 'Great Reign' at flowering stage.

CONCLUSIONS

For *Curcuma* 'Great Reign', a rhizome diameter smaller than 1.5 cm produced the shortest plant height and inflorescence length. Rhizome diameter 1.5-3.0 cm and root trimming length of 5.0 cm are optimum for flower quality and plant height.

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Mother plant and pre-basic seed potato production in Thailand

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Abstract

Potato is an important economic crop in northern and northeastern Thailand. However, seed potatoes are expensive, with high production costs and they are often infected with diseases. The objective of this study was to develop and improve the method of seed potato production in Thailand to increase the yield and quality of the seed potatoes that are produced. The study evaluated mother plant production in soil compared with a hydroponic system with three replications. The height of mother plants in the soil medium in the rainy and in the cool season (33 and 31.1 cm, respectively) was significantly higher than that in the hydroponic system. Moreover, the number of apical cuttings 36 m^{-2} area in the rainy and in the cool season (9,084 and 6,165 shoots, respectively) and the number of times of harvest of the apical cuttings (8 and 5 times, respectively) were significantly higher than that in the hydroponic treatment. The soil medium system in the rainy season gave a unit cost of 4 baht $shoot^{-1}$ while it was 5 baht $shoot^{-1}$ in the cool season. In addition, a comparison of seed production using stem node cuttings in an aeroponic system was carried out with five treatments and four replications. In the cool season, two node cuttings (one node above and one under a foam layer) gave 415 tubers 2.4 m^{-2} area. The yields between the two and three node cutting methods in the 2.4 m^2 area were not different in the cool season (3.00-3.64 kg) or in the rainy season (4.75-5.75 kg). In summary, the soil medium system was shown to be an appropriate technique for mother plant production in both the cool and rainy seasons. Both two node cuttings and three node cuttings for pre-basic seed (G0) production were shown to increase tuber number, and were easy, rapid, and flexible to use under aeroponic conditions. Furthermore, these methods provided more security for control of phytosanitary quality, and reduced unit cost.

Keywords: mother plant, pre-basic seed (G0), production, cultivar, potato

INTRODUCTION

Potato (*Solanum tuberosum*) is an important economic crop in the northern part of Thailand. Farmers' income from planting potato per crop ha^{-1} is approximately 2,679-4,464 USD (25,000-15,000 Baht rai^{-1}) (Wongmetha, 2019). In 2016, the total area planted in potatoes was more than 7,011 ha (43,818 rai). Total production was estimated at 142,303 t with an average yield of 19,712.6 t ha^{-1} (3,154 kg rai^{-1}) (Office of Agricultural Economics, 2016). Normally, mother plants are planted in early-October and top shoot cuttings are transplanted in mid-November. For pre-basic seed (G0) production, seed potatoes are grown in mid-November and harvested in February (Wongmetha, 2019). Farmers and potato processing companies in Thailand sought to import 5,209 t of seed potatoes in 2020 for the production of potato chips (Kittipadakul et al., 2016; Office of Agricultural Economics, 2019). However, the imported seeds are expensive, the supply of high-quality seed is unreliable and an inadequate supply of imported seed limits production. It is also difficult to manage the period of time that imported seed is available in relation to the growing season. Farmers usually retain small tubers that they are unable to sell as seed (Kittipadakul et al., 2016) for planting in the next season as the seed is often infected with several diseases that can cause a reduction in total yield and quality (Wongmetha, 2019).

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Currently, a range of potato propagation methods are being used worldwide to multiply seed potatoes, including conventional seed potato production, micropropagation, and production in hydroponics and aeroponics systems (Tshisola, 2014). A raised soil bed method is used for potato mother plant production in soil media in Thailand (Wongmetha, 2019). However, hydroponic systems with or without substrates have been shown to be a good system for the production and multiplication of pre-basic potato seed (Corrêa et al., 2008). About 20 to 60 apical stem cuttings can be produced from each mother plant (Bryan et al., 1981). In addition, the conventional method of producing quality pre-basic potato seed is to plant mini tubers (1.5-2 cm) in a sterile soil medium or other suitable substrate under net house conditions where 5-10 mini tubers can be produced plant^{-1} (Otazú, 2010; Wongmetha, 2019). To increase pre-basic seed production, an aeroponic system, which is a soilless culture system where roots are kept in a dark environment saturated with an aerosol of nutrient solution, can produce high quality tubers efficiently (Tsoka et al., 2012; Rykaczewska, 2016). Moreover, it has been shown that as the number of stem nodes on cuttings was increased, the number of tubers plant^{-1} in sweet potato (3, 5 and 7 node cuttings) (Amoah, 1997), potato (2 and 3 node cuttings) (Kim, 2014) and sweet potato (9 node numbers) (Beyene et al., 2015) were increased.

The objective of this study was to investigate the multiplication rate of apical cuttings of mother plant production in a soil-based medium growing system and in a hydroponic system. In addition, the effect of node number on pre-basic seed production in an aeroponic system was evaluated.

MATERIALS AND METHODS

Plant material

In the first experiment, tissue-cultured plantlets of potato 'Chiangmai 1', after having been subcultured for 30 days, were transplanted into either a raised net bed in a sterilized (steam at 100°C for one hour) soil-based medium (soil:sand:coconut peat:black rice husk:husk in the ratio 0.5:1:1:1:1) or into a hydroponic container for mother plant production under net-house conditions at the Chiangmai Royal Agricultural Research Center (CMRARC) in 2016-2017. The net tray size in the soil media system was 1×12 m for each replication and a 4,500-L container (1 m wide, 18 m long and 0.25 m high) was used in the hydroponic system. The row and plant spacing was 10×10 cm. Stem cuttings of plantlets were subsequently transplanted into an aeroponic system 30-45 days after planting.

In the second experiment, a number of stem cutting sizes were examined: a two node cutting (one node above and one under the foam sheet), a three node cutting (either with one node above and two under the foam sheet or with two nodes above and one under the foam), a four node cutting (with two nodes above and two under the foam) and a five node cutting (with three nodes above and two under the foam). Plantlets of potato 'Chiangmai 1' were inserted into holes in the foam plastic sheet and connected with an aeroponic system in a net-house at CMRARC in 2015-2016, as described by Wongmetha (2019). The aeroponics system consisted of concrete chambers 0.8 m deep, 0.6 m wide and 18 m long. Plant density was 84 plants m^{-2} or 200 plants 2.4 m^{-2} in each replication. During plant growth, nutrient solutions (A, B and C, dependent on the period of growth) with controlled pH (5.5-6.5) and an EC of 1.1-2.0 mS cm^{-1} was applied. The application time of the nutrient solution was initially every 3 min for 2 min and then every 40 min for 2 min after 1 month. The seed tubers were harvested 90 days after planting (DAP) or when the plant's foliage or vines had died back. After collection, the tubers were graded for uniformity, size and shape.

Plant growth, yield and yield components, and quality attributes

Plant height (cm) was determined in both experiments. Root length (cm), stem diameter (mm), number of stem nodes, number of shoots plant^{-1}, number of shoots 36 m^{-2}, number of times of harvest of the cuttings, and production costs were recorded in the first experiment. Total yield (kg rai^{-1}), number of tubers plant^{-1}, number of tubers 2.4 m^{-2}, and number of tubers in size grade 1 (less than 2.5 cm diameter), and the number of tubers of a size suitable for

commercial marketing including grade 2 (2.5-3.5 cm), grade 3 (3.5-4.5 cm) and grade 4 (more than 4.5-6.5 cm) were evaluated in the second experiment. Total starch content (TSC) was measured on samples (5.00-5.05 kg each) using a digital starch content analyzer (Genix GX3000_ECOF) and expressed as percentage of starch. Total soluble solids (TSS) was measured using a digital hand refractometer (Atago Pocket refractometer PAL-1) with results expressed in °Brix. Measurements were taken in three areas at the shoulder, middle and base of tuber slices.

Statistical analysis

The first experiment was subjected to analysis using the two-sample t-test ($p \leq 0.05$) with two treatments and three replications. The second experiment was laid out using a randomized completely block design (RCBD) with five treatments and four replications. The data were analyzed using an analysis of variance (ANOVA). Where possible, mean comparisons were made using the Duncan's multiple range test (DMRT) at $p \leq 0.05$. Statistical analyses were carried out using the SAS program.

RESULTS AND DISCUSSION

Experiment 1 – soil-based growing medium and hydroponic production system

1. Plant growth and the production cost (variable cost).

The mother plants that were planted in the soil-based growing medium had significantly greater plant height, longer root length, a higher number of stem nodes, greater stem diametre, a higher number of shoots plant^{-1} and shoots 36 m^{-2}, and a greater number of cutting harvests than the hydroponic system in both the cool and rainy seasons (Table 1). Plant growth in the cool season was lower than that in the rainy season because the temperature of water in the hydroponics system during the cool season was lower than 1°C in the early morning and at night, while the temperatures in the soil-based medium were consistently above 7°C (Teagasc, 2013). Potato sprouts grow faster at temperatures above 12°C and up to about 24°C (Department of Environment and Primary Industries, 2010). Bryan et al. (1981) previously reported that soil media could be used to produce stem cuttings in a rapid multiplication technique.

Table 1. The average plant growth, number of shoots and number of times of harvest of the cuttings from potato mother plant production under a soil-based growing medium or under a hydroponic system at CMRARC in 2016 and 2017 during either the cool or the rainy season.

Systems	Plant height (cm)		Root length (cm)		Number of nodes		Stem diameter (mm)	
	Cool	Rainy	Cool	Rainy	Cool	Rainy	Cool	Rainy
Soil-based medium	31.1	33.0	22.0	19.8	7	7	4.2	4.1
Hydroponics	12.5	13.0	15.8	16.9	2	5	1.5	1.3
P-value	0.006*	0.019*	0.014*	0.35	0.02*	0.15	0.003*	0.003*
	Number of shoots plant^{-1}		Number of shoots 12 m^{-2}		Number of shoots 36 m^{-2}		Number of times of harvest of the cuttings	
	Cool	Rainy	Cool	Rainy	Cool	Rainy	Cool	Rainy
Soil-based medium	3	4	2,069	3,037	6,165	9,084	5	8
Hydroponics	2	2	182	1,495	528	4,484	2	3
P-value	0.024*	0.033*	0.0002*	0.028*	0.0001*	0.027*	0.037*	0.019*

*Indicates that variable is significant at 5% ($p<0.05$) level with the two-sample t-test.

The total cost of mother plant production under the soil-based medium growing system in both the cool and the rainy season was lower than in the hydroponics system (Table 2).

However, the number of shoots produced in the soil-based medium was very high and, therefore, the shoot price in both seasons was lower than that in the hydroponics system. Stem cuttings in a soil-based medium have been shown previously to give the highest net profit and to be a suitable method for growing potato (Ezzat, 2016).

Table 2. The production costs (variable costs) of potato mother plant production under either a soil-based medium or a hydroponic system at CMRARC in 2016 and 2017 during either the cool or the rainy season.

Item	Mother plant production (baht 36 m^{-2})			
	Cool season		Rainy season	
	Hydroponics	Soil-based medium	Hydroponics	Soil-based medium
1. Planting and harvesting costs	13,800	10,800	13,800	10,800
2. Laboratory equipment and test-kits	1,680	1,760	1,680	1,760
3. Agricultural equipment and supplies	19,469	10,318	19,089	10,878
- agricultural equipment	-	500	-	1,060
- tissue plantlets (2,130 plantlets at 4 baht $plantlet^{-1}$)	8,520	8,520	8,520	8,520
- chemical and manure fertilizers	10,342	760	10,342	760
- herbicides and pesticides	227	538	227	538
- maintenance and repairs to the net-house	8,900	-	8,900	6,000
4. Electricity costs	1,000	-	1,000	-
5. Other: gas for soil steaming and insect traps	-	5,540	-	5,540
Total costs of production (baht)	35,949	28,418	44,469	34,978
Number of times of cutting harvests	2	5	3	8
Number of shoots (plantlets)	528	6,165	4,484	9,084
Shoot price (baht $shoot^{-1}$)	68	5	10	4

1 net house = 6×15 m (90 m^2) and planting area = 36 m^2.
1 US dollar = 32 Thai baht.

Experiment 2 – impacts of stem node number

1. Plant growth, the total yield and yield components and the quality attribute.

Plant height of all stem cuttings was in the 30.4-32.1 cm range in cool conditions and 68.6-77.9 cm in the rainy season (Table 3). These results are similar to those of Safeer et al. (2013) who revealed that shoot tip cuttings with 1-6 nodes (~15 cm length) showed optimum growth performance and yield.

There were 3-4 tubers $plant^{-1}$ from all treatments in both the cool and rainy seasons (Table 3). However, the number of potato minitubers produced in an aeroponic system was 32.5-36.0 $plant^{-1}$ (Rykaczewska, 2016) and this higher number was probably because the mother plant had developed compound leaves and was physiologically older. Stem cuttings originating from mother plants with fully developed compound leaves generally yield 2-3 tubers $stem^{-1}$ (Parker, 2019).

The weight of tubers $plant^{-1}$ of the three node cuttings (two above and one under the foam sheet) in cool season was significantly higher than that from the five node cuttings (three above and two under the foam sheet) but not significantly different from the other treatments (Table 3).

The number of tubers 2.4 m^{-2} was highest in the two node cuttings (one above and one under the foam sheet) (Table 3). Tuber number of grade 1 and 3 sizes in both cool conditions and in the rainy season were also significantly greater from the two node cuttings than from the other shoot cuttings (Table 3). The number of tubers of grade 1 size was lower in the cool than in the rainy season while the number of grade 2, 3 and 4 in the cool season was greater than that in the rainy season. When stem node number increased, the total tuber number of tubers increased in sweet potato (Beyene et al., 2015).

Table 3. The average plant growth, total yield, yield components and quality attribute of pre-basic seed potato production under aeroponic system at CMRARC in 2015 and 2016.

Shoot cutting size	Plant height (cm)		Number of tubers plant^{-1}		Weight of tubers plant^{-1} (g)		Starch (%)		TSS (°Brix)	
	Cool	Rainy	Cool	Rainy	Cool	Rainy	Cool	Rainy	Cool	Rainy
2 SNC (1/1)	32.1	73.1	3	3	38.38ab	39.05	18.2	17.5	8.1	5.6d
3 SNC (1/2)	31.5	77.9	3	3	38.30ab	44.98	17.5	17.5	8.5	6.7a
3 SNC (2/1)	30.8	75.0	4	3	42.55a	44.48	18.0	17.5	8.8	6.2c
4 SNC (2/2)	31.9	74.7	3	3	40.23ab	47.90	18.0	17.1	8.5	6.4b
5 SNC (3/2)	30.4	68.6	3	4	31.93b	39.35	18.5	16.7	8.6	6.4b
F-test	ns	ns	ns	ns	*	ns	ns	ns	ns	*
CV (%)	8.7	8.3	20.2	19.7	13.9	23.1	4.24	3.65	11.47	1.94

	Number of tubers 2.4 m^{-2}											
Shoot cutting size	Total no. of tubers		No. of Grade 1		No. of Grade 2		No. of Grade 3		No. of Grade 4		Total yield (kg 2.4 m^{-2})	
	Cool	Rainy	Cool	Rainy	Cool	Rainy	Cool	Rainy	Cool	Rainy	Cool	Rainy
2 SNC (1/1)	415a	373	224a	240	88	62	59a	37a	44	35	3.64	4.75ab
3 SNC (1/2)	356b	319	184ab	213	78	55	48ab	22b	46	29	3.04	4.85ab
3 SNC (2/1)	330b	326	174ab	220	80	46	43b	30ab	33	30	3.00	5.75a
4 SNC (2/2)	332b	337	177ab	238	75	47	37b	26b	44	26	3.43	4.45b
5 SNC (3/2)	320b	294	160b	195	79	43	41b	24b	40	32	3.17	4.70ab
F-test	*	ns	*	ns	ns	ns	*	*	ns	ns	ns	*
CV (%)	8.0	18.5	16.9	24.5	10.7	32.2	18.8	22.9	21.0	36.8	14.2	14.6

Means followed by the same letter in a column are not significantly different but means followed by different letter in column are significantly different at the 95% ($p \leq 0.05$) using the DMRT.
2 SNC (1/1) = two node cutting (one above and one under the foam sheet), 3 SNC (1/2) = three node cutting (one above and two under the foam sheet), 3 SNC (2/1) = three node cutting (two above and one under the foam sheet), 4 SNC (2/2) = four node cutting (two above and two under the foam sheet) and 5 SNC (3/2) = five node cutting (three above and two under the foam sheet).
Tuber size grade 1 (least than 2.5 cm diam.), tuber size for commercial marketing such as grade 2 (2.5-3.5 cm), grade 3 (3.5-4.5 cm) and grade 4 (more than 4.5-6.5 cm).

Total yield 2.4 m^{-2} of tubers under rainy conditions was higher than that in cool weather and yield from the three node cuttings (two above and one under the foam sheet) was also highest under rainy season conditions (Table 3). These results are similar to those of Safeer et al. (2013) but different to those reported by Amoah (1997) who showed that 5 and 7 node cuttings gave significantly higher tuber yield than 3 node cuttings in sweet potato.

The starch content of each treatment in the cool season was higher than that in the rainy season (Table 3). The total soluble solids concentration (TSS) of all stem node cuttings in the cool season was in the range 8.1-8.6 °Brix (Table 3). However, in rainy season, the TSS in the three node cuttings (two above and one under the foam sheet) was significantly higher than that in all treatments within the rainy season but lower than that in the cool season.

CONCLUSIONS

The multiplication of apical cuttings from mother plant production in a soil-based growing medium was shown to be an appropriate method for increasing plant growth and for decreasing the variable costs of production for producing apical stem cuttings in both the cool and rainy seasons. Two node stem cuttings produced the highest number of tubers for G0 in the cool season and the three node stem cuttings also produced a high total number of tubers in the rainy season.

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Using liquid organic boron foliar sprays for increasing tomato growth and yield in a hydroponic system

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Abstract

Boron foliar sprays have been shown to increase marketable yield and fruit quality of tomato. Furthermore, the demand for organic produce is increasing due to consumer requirements for a healthy and safe product to eat. This greenhouse trial aimed to determine the effects of application methods and doses of liquid organic boron fertilizer. This fertilizer is made from green manure (*Leucaena leucocephala* leaves: *Pennisetum pururеum* leaves: *Musa acuminate* stem: *Allium cepa* group Aggregatum bulb 2:2:1:0.1/v:v:v:v).The effects liquid organic boron fertilizer on growth and yield of tomatoes grown hydroponically were studied. The study used a split-plot experimental design. The main plot was the application method of foliar and basal sprays. The subplot was the concentration of liquid boron organic fertilizer (4.10%B) was applied at 0, 25, 50, 75, 100, 125 and 150 mL L^{-1}. The results showed no interactions between the method and the concentration of liquid organic boron fertilizer (LOBF) on growth and yield of tomato plants. However, the concentration of liquid organic fertilizer significantly affected tomato growth and the yield. An application rate of 25 mL L^{-1} of liquid organic boron fertilizer increased the number of flower clusters by 22%. It also increased fruit yield $plant^{-1}$ by 18% and increased the number of tomato fruit $plant^{-1}$ by 14% compared to the control treatment (no liquid organic boron fertilizer). Results showed that foliar spray more efficient than basal spray.

Keywords: *Lycopersicum esculentum*, fruit yield, organic fertilizer, green manure

INTRODUCTION

One of the trusted agriculture systems that can produce high quality products is hydroponics. The controlling of environmental variables and optimum of nutrient management results in yield of ideal quality product. However, chemical fertilizers and fertigation may lead to environmental pollution, especially water pollution. Several studies using different sources of organic fertilizer have shown a reduction in chemical fertilizer.

The method for growing plants by organic hydroponics (using organic fertilizer) was developed in Europe (van Os, 2017). In the search for sustainable development, this method (organic hydroponics) can switch from the conventional hydroponic system using chemical fertilizers to a more sustainable agriculture system (Hsieh et al., 2018). However, this method faces some problems, especially fulfilling nutrient demand to support crop growth. The yield of crops reduced to 15% when fertilized with organic materials.

Tomato (*Lycopersicum esculentum* L.) is one of the most important vegetables in the world. Improving the quality of tomato will increase price and return. Hydroponic is one system that can provide a good quality product. Jones (2016) states the advantages of hydroponics, including the possibility to obtain maximum yield, eradication of soil-borne plant diseases and complete control of the environment to support the plant growth. Furthermore, organic fertilization improved the quality of the tomato fruit (Oliveira et al., 2013). Boron is a micronutrient that has a significant role to play in sugar translocation and metabolism of carbohydrate that influences tomato fruit quality (Bolan et al., 2003). Foliar boron application in a hydroponic system could increase potassium and calcium

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concentrations in the shoot and the dry matter of tomato fruit. Meanwhile, in field-system application of foliar boron improved plant growth, fruit yield and fruit quality of tomato (Davis et al., 2003).

In a hydroponic system, the nutrient solution is applied to fulfil the plant nutrient requirements. Other materials can be applied to increase plant responses. Yu et al. (1993) reported that the addition of activated charcoal to the nutrient solution increased the dry matter and yield of tomato in a hydroponic system. Organic materials can also be applied to support tomato growth. Application of organic N improved the quality of tomato fruits (Heeb et al., 2005).

Furthermore, Huang and Snapp (2009) reported the application of chemical boron fertilizer through leaves increased the tomato fruit yield and quality, and decreased the physiology disease incidence. However, there was still a limited supply of organic boron through the hydroponic system. This experiment aims to find the best concentration of liquid organic boron fertilizer made from green manure to increase the fruit yield of hydroponic tomato.

MATERIALS AND METHODS

Experimental site

A greenhouse trial was conducted in IVEGRI (Indonesian Vegetable Research Institute) located in 6°48'22"S; 107°38'42"E, 1200 m a.s.l. in 2016. The tomato cultivar used for this study was 'CLN 4046 Lot 4046' from UPBS IVEGRI. The tomatoes were grown on rice husk charcoal media provided by a local agricultural store in Lembang, West Java, Indonesia.

Liquid organic boron fertilizer

The liquid organic boron fertilizer was made from extracts of *Leucaena leucocephala* leaves *Pennisetum purureum* leaves; *Musa acuminata* shoot; *Allium cepa* group Aggregatum bulb with composition 2:2:1:0.1 (v/v/v/v). The liquid organic fertilizer contents high dose of boron (B), 4.10% and other chemical nutrientswere, Total N 0.06%, P_2O_5 0.01%, K_2O 0.07%, CaO 0.01%, S 0.04%, Fe 0.90%, Mn 0.80%, Zn 0.70% and water 99.48%.

The liquid organic boron fertilizer was applied weekly starting from week 2 after planting until six weeks after planting. The volume plant^{-1} was 200 mL for the basal treatment and 30-100 mL plant^{-1} for the foliar treatment.

Experimental design

This research experiment used a split-plot design. The main plot was the application method of liquid organic boron fertilizer applied to the base application. The subplot was the concentration of liquid organic boron fertilizer. Rates were 0, 25, 50, 75, 100, 125 and 150 mL L^{-1}.

Fertilization

Irrigation and fertilization were applied four times day^{-1}, at 6:00 and 10:00 am, 2:00 and 4:00 pm The duration of irrigation and fertilization was 10 min. The composition of AB mixed-tomato fertilizer is shown in Table 1.

Observations

For this experiment, all lateral branches were removed. A single stem was maintained until last harvest. Plant height data were collected weekly from week 2 to 9 after planting. Chlorophyll content analyzed three times, at weeks 2, 3 and 4 after planting using Spectrophotometer UV-VIS and 80% acetone solvents at λ=664.5 nm and λ=647 nm (Azia and Stewart, 2001). The number of flowers and flower clusters were recorded weekly from weeks 6 to 9 after sowing time. Tomato fruit collected, one every three days from 9 weeks after planting (WAP) to 15-WAP, and summed to obtain the total fruit yield plant^{-1}.

Table 1. AB-mixed fertilization for 0 to 6 weeks after planting.

	g 1,000 L^{-1}	N	P	K	Ca	Mg	S	Fe	Mn	Cu	Zn	B	Mo	Na
A														
$Ca(NO_3)_2.4H_2O$	1250.0[a]	148[a]			212[a]									
	1125.0[b]	133[b]			191[b]									
	1000.0[c]	119[c]			170[c]									
KH_2PO_4	205.0		46.6	59										
KNO_3	550.0[a]	76[a]		213[a]										
	650.0[b]	90[b]		251[b]										
	730.0[c]	101[c]		282[c]										
B														
$CuSO_4$	0.2						0.03			0.06				
$ZnSO_4.7H_2O$	1.5						0.17				0.34			
$MgSO_4.7H_2O$	655.0[a]					65[a]	85.24[a]							
	600.0[b]					59[b]	78.09[b]							
	655.0[c]					65[c]	85.24[c]							
Fe-EDTA	12.5							2.03						
H_3BO_3	1.6											0.28		
$MNSO_4$	1.5						0.32		0.55					
$Na_2MoO_4.H_2O$	0.1												0.04	0.02
Concentration		224[a]	47	272[a]	212[a]	65[a]	86[a]	2.03	0.55	0.06	0.34	0.28	0.04	0.02
		223[b]		310[b]	191[b]	59[b]	79[b]							
		220[c]		341[c]	170[c]	65[c]	86[c]							

[a]Plant age 0 to 6 weeks after planting.
[b]Plant age 6 to 12 weeks after planting.
[c]Plant age more than 12 weeks after planting.

RESULTS AND DISCUSSION

There was no interaction between the application method and the concentration of liquid organic boron fertilizer. The average of tomato height was 11.9, 17.2, 24.8, 40.6, 59.6, 99.8, 121.7 and 139.1 cm at 2, 3, 4, 5, 6, 7, 8 and 9 weeks after planting. Application method did result in a significant effect on plant height, but the concentration did. The lowest plant height was the control treatment (0 mL L^{-1}) of liquid organic boron. Meanwhile, at week 8 and 9 after planting, the treatment rate of 25 mL L^{-1} liquid organic fertilizer produced the tallest plants compared to other treatments. Overall, the application of liquid organic fertilizer increased plant height from 2 to 23% across the treatments and observation times (Table 2).

Table 2. The effect of liquid organic boron fertilizer on tomato plant height.

Treatments	Plant height (cm)							
	2 WAP	3 WAP	4 WAP	5 WAP	6 WAP	7 WAP	8 WAP	9 WAP
Methods								
Basal spray	11.9ns	17.2ns	24.9ns	40.3ns	58.6ns	98.7ns	123.3ns	136.7ns
Foliar spray	11.9	17.1	24.7	41.5	60.6	99.7	122.8	138.7
LOBF								
0 mL L^{-1}	11.2ns	15.0b	22.7ns	39.1ns	54.8b	99.2ns	114.4ns	132.2ns
25 mL L^{-1}	11.6	17.3ab	25.2	40.8	61.0a	101.0	149.9	140.0
50 mL L^{1}	11.8	17.3ab	25.0	40.8	59.0ab	96.9	117.4	137.7
75 mL L^{-1}	12.0	17.9a	25.9	41.4	62.2a	102.0	121.3	138.7
100 mL L^{-1}	12.3	17.6a	25.1	41.3	60.1ab	100.2	116.4	135.6
125 mLL^{-1}	12.1	17.5ab	24.3	41.3	59.5ab	98.9	118.7	138.5
150 mL L^{-1}	12.2	17.6ab	25.3	41.4	60.8ab	100.2	123.7	140.9
CV (%)	8.4	9.94	9.70	4.00	5.98	8.45	15.47	5.13

ns = no significance at 5%, WAP = weeks after planting.

The method of application and the dose of liquid organic fertilizer did not significantly affect the chlorophyll content of the plants (Table 3). The average of chlorophyll content was 31.9, 31.8 and 38.3 SPAD at 2, 3 and 4 weeks after planting. The lowest value obtained was for the control treatment, 31.0, 31.8 and 36.3 SPAD at 2, 3 and 4 weeks after planting. The highest value recorded was for the rate of 150 mL L^{-1} at 2-WAP and 75 mL L^{-1} at 3-WAP and 4-WAP. Overall, the liquid organic boron fertilizer increased the chlorophyll content from 5 to 8% across all treatments and observation times.

Table 3. The effect of liquid organic boron fertilizer on chlorophyll content and nutrient uptake.

Treatments	Chlorophyll content (SPAD)			Leaf uptake (%)			Fruit uptake (%)		
	2 WAP	3 WAP	4 WAP	N	P	K	N	P	K
Methods									
Basal spray	31.7ns	32.5ns	38.1ns	3.56ns	0.52ns	4.02ns	2.57ns	0.53ns	3.93ns
Foliar spray	31.9	33.1	38.4	3.72	0.56	4.14	2.43	0.54	3.72
LOBF									
0 mL L^{-1}	31.0ns	31.8ns	36.3ns	3.61ns	0.58ns	3.96ns	2.40ns	0.51ns	3.72ns
25 mL L^{-1}	31.4	33.1	38.5	3.41	0.52	4.11	2.73	0.55	3.91
50 mL L^{1}	32.2	32.2	38.1	3.59	0.51	3.98	2.45	0.51	3.72
75 mL L^{-1}	31.8	33.4	39.2	3.75	0.61	4.14	2.40	0.57	3.81
100 mL L^{-1}	32.7	33.0	38.6	3.82	0.55	4.28	2.64	0.56	4.01
125 mLL^{-1}	31.7	32.7	38.2	3.73	0.51	4.10	2.34	0.48	3.73
150 mL L^{-1}	32.7	33.2	38.7	3.68	0.53	4.10	2.53	0.57	3.86
CV (%)	6.03	3.64	4.94	9.56	28.76	12.20	13.76	13.79	13.88

ns = no significance at 5%, WAP = weeks after planting.

Meanwhile, there was no significant difference in nutrient uptake across all the treatments at 8 weeks after planting (Table 3). The leaf nitrogen uptake was 3.7%, and the fruit uptake was 2.5%. The leaf potassium uptake was 4.1% and the fruit potassium uptake was 3.8%. Furthermore, the average phosphorus uptake was similar, 0.5% for leaf and the fruit. The average concentration of phosphorus and potassium in this experiment was less than what has been reported before in other studies. (Davis et al., 2003), reported that the concentration of phosphorus in the shoot without foliar boron fertilizer was 0.70% and the concentration of phosphorus in the shoot with foliar boron fertilization was 0.67%.

In addition, the concentration of potassium was 4.74 and 5.30% with and without boron fertilization, respectively. Furthermore, the application of boron improved the boron concentration in the shoot and root sections.

The application of liquid organic fertilizer increased the number of flower clusters at 6-9 weeks after planting (Table 4). The control treatment had the lowest number of flower clusters. 0.7, 3.4, 4.5 and 5.2 clusters plant^{-1} at 6, 7, 8 and 9 weeks after planting. The application of 25 mL L^{-1} of liquid organic fertilizer resulted in the most significant improvement in flower clusters. It increased the number of flower clusters from 13 to 81% compared to the control treatment.

Table 4. The effect of liquid organic boron fertilizer on the number of cluster and flower plant^{-1}.

Treatments	Number of cluster plant^{-1}				Number of flower plant^{-1}			
	6 WAP	7 WAP	8 WAP	9 WAP	6 WAP	7 WAP	8 WAP	9 WAP
Methods								
Basal Spray	1.1ns	3.8b	5.0ns	5.5ns	4.4ns	12.0ns	22.4ns	12.4ns
Foliar Spray	1.0	4.1a	4.9	5.7	4.5	12.6	21.2	12.6
LOBF								
0 mL L^{-1}	0.7b	3.4b	4.5b	5.0b	2.9b	12.6ns	19.8ns	11.7ns
25 mL L^{-1}	1.3a	4.3	5.1a	6.1a	5.3a	12.9	24.9	13.3
50 mL L^{1}	1.1a	3.7	4.8ab	5.7ab	4.2ab	12.0	20.6	12.4
75 mL L^{-1}	1.1a	4.2	5.1a	5.9ab	5.2a	12.3	21.1	11.8
100 mL L^{-1}	1.1a	3.9	5.0a	5.2ab	4.4ab	11.4	19.6	11.1
125 mLL^{-1}	1.1a	4.0	5.0a	5.5ab	4.2ab	11.7	22.1	13.6
150 mL L^{-1}	1.1a	4.1	5.0a	5.8ab	4.7ab	13.0	24.2	13.5
CV (%)	22	12	13	10	27	17	19	23

ns = no significance at 5%, WAP = weeks after planting.

The greatest number of flowers plant^{-1} was obtained at eight weeks after planting (Table 4). After this age, the number of flowers reduced and fruit was produced. There was no significant difference between treatments on the number of flowers plant^{-1}. However, at eight weeks after planting, and a rate of 25 mL L^{-1} resulted in the highest number of flowers, 24.9 flowers plant^{-1} compared to control 19.8 flowers plant^{-1}.

The application of liquid organic fertilizer had a significant effect on fruit yield (Table 5). The greatest value was achieved by the application of 25 mL L^{-1} which increased the fruit yield by 18% from 16.31 to 19.19 kg plot^{-1}. In addition, it increased the number of fruit from 455.6 to 521.2 fruit plot^{-1}. Commonly, in tomatoes, increasing the number of fruit plant^{-1} would reduce the average fruit weight. However, in this experiment, increasing the number of fruit did not reduce the average fruit weight.

In many different crops, the application of boron and zinc, increased tomato yield and improved its protein content (Sarker et al., 2019). On the contrary, boron deficiency reduces the tomato fruit yield. For instance boron deficiency may cause disorders in the vascular bundle, xylem and phloem, and restrict root growth (Do Carmo Milagres et al., 2019), and reduces fruit yield.

In this experiment the application of organic foliar fertilizer significantly improved flower cluster number and increased fruit yield. The formatting of flower clusters was

influenced by the nutrient balance in the tomato plant. Foliar boron application altered the nutrient uptake in shoot and root which may affect the formation of flower clusters. Boron influences nitrogen and calcium concentration (Davis et al., 2003), thus, branching and formatting of flower clusters were significantly affected by nitrogen and calcium uptake (Ohta, 2017). Therefore, it was assumed that boron indirectly affects the tomato fruit yield by improving the nitrogen and calcium uptake.

Table 5. The effect of liquid organic boron fertilizer on fruit weight plant^{-1}, fruit weight fruit^{-1} and number of fruit plant^{-1}.

Treatments	Fruit yield (kg plant^{-1})	Fruit weight (g)	Number of fruits plant^{-1}
Methods			
Basal spray	3.67ns	37.91ns	98.14ns
Foliar spray	3.60	37.54	98.02
LOBF			
0 mL L^{-1}	3.26b	35.99ns	91.13b
25 mL L^{-1}	3.84a	36.70	104.2a
50 mL L^{1}	3.37b	35.79	94.3ab
75 mL L^{-1}	3.81ab	40.50	96.1ab
100 mL L^{-1}	3.66ab	40.08	93.5ab
125 mLL^{-1}	3.60ab	36.96	97.4ab
150 mL L^{-1}	3.69ab	38.06	102.2ab
CV (%)	13.19	14.58	11.88

ns = no significance at 5%.

However, the range between deficiency and toxicity levels of boron is small compared to other nutrients (Pandey and Archana, 2011). In this study, we have shown that the high concentration (150 mL L^{-1}) of liquid organic boron fertilizer produced lower fruit yield compared to the low concentration (25 mL L^{1}) (Table 5). The low concentration not only increased the yield but also had a greater efficienity compared to a high concentration. It can be concluded that the concentration of 25 mL L^{-1} was sufficient to support the growth and increase tomato yield. A further study is needed to investigate the effect of organic boron on nutrient uptake in tomato.

CONCLUSIONS

Application of 25 mL L^{-1} liquid organic fertilizer made from green manure increased the fruit yield by up to 18% and increased the number of fruits by 14% compared to the control treatment. The foliar sprays had greater efficiety compared to basal spray.

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Growth, yield, and postharvest quality of cabbage (*Brassica oleracea* L.) in response to different levels of nitrogen fertilizer

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Abstract

In the Philippines, vegetable farmers generally apply excessive amounts of fertilizer to maximize yield while not understanding the cost impacts and environmental effects. This study aimed to evaluate the effects of different rates of nitrogen fertilizer on the productivity of two cabbage cultivars. The experiment was conducted as a factorial using a randomized complete block design in two growing seasons. The two factors included level of nitrogen (21, 141, 191, 241, 291, 341 and 391 kg N ha^{-1}) and cultivar ('Resist Crown' and 'KEX-734') with each treatment combination replicated four times. The study showed that application of 291 kg N ha^{-1} resulted in the highest dry matter, largest heads (polar and equatorial) and highest average yield (35.49 t ha^{-1}) in the two growing seasons. At 391 kg N ha^{-1}, cabbage yielded the highest number of leaves and leaf count increased concomitantly with increasing N application rate. Results further showed that N application at the highest rate (391 kg N ha^{-1}) resulted in the highest postharvest weight loss (33.7%) and shrivelling of cabbage heads. The cultivar 'Resist Crown' produced a greater number of leaves, largest polar head size and consequently highest mean yield (25.7 t ha^{-1}) than cultivar 'KEX-734' in both cropping seasons. The results indicate that an application rate of about 291 kg N ha^{-1} is recommended for cabbage production since it increases yield through enhancing the size and weight of the cabbage head. 'Resist Crown' was a better cultivar than 'KEX734' having greater yield and postharvest quality.

Keywords: 'Resist Crown', nitrogen fertilizer, cabbage head size, dry matter, cultivar

INTRODUCTION

After water, nitrogen (N) is considered to be the most important limiting factor in crop production (FAO, 1985). In field-crop and forage cropping, N fertilization provides sufficient N to achieve maximum growth potential. However, in maximizing crop yield, potential N inputs are often higher than that required for maximum growth (Lemaire and Gastal, 1997) resulting in a surplus of N which increases the risk of environmental pollution (Dewi et al., 2010).

Genotype selection is also an important factor for maximizing crop yield and directly relates to crop nutrient requirement. Cultivars selected for high yield usually have a higher nutrient demand than traditional, local or wild cultivars (Huang, 2006). In cabbage, genotype and environment interactions can influence the marketable head and other key traits (Kleinhenz and Wszelaki, 2003). Research by Huang (2006) supports the latter findings that marketable head size depends on vegetative and reproductive characters. In another study, head size and weight per head increased with increasing N rate, reflecting a yield increase, with an associated reduction in the time to maturity (Hasan, 2010).

Cabbage production in Claveria, Northern Mindanao, Philippines is a significant vegetable crop with the most common variety being 'Resist Crown', a Capitata type. Farmers plant this cultivar as a preferred local choice without considering that there could be other

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better performing cultivars. Furthermore, despite the known benefits of fertilizer application, farmers often apply excessive amounts of nitrogen. Though these high rates can give high yields, it is not understood whether the same yields could be achieved with lower rates of N which would have reduced input and labor costs.

The present study was conducted to evaluate the effects of various rates of N fertilizer on the yield and postharvest quality of two cabbage cultivars.

MATERIALS AND METHODS

Cultural management practices

The study was conducted over two cropping seasons (1st cropping – May 3 to July 16, 2015 and 2nd cropping – August 1 to October 18, 2015) at the Agricultural Experimental Station of the University of Science and Technology of Southern Philippines Claveria (8°36'37.0"N; 124°52'59.9"E) at an elevation of 615 m a.s.l. Two cultivars, 'Resist Crown' and 'KEX-734', were evaluated. Cabbage seedlings were raised in an enclosed nursery to keep avoid insect infestations prior to transplanting. Seedling trays were filled with a sterilized soil mixture of garden soil, vermicast, lime and sand at a ratio of 4:5:1/2:1 and two seeds were planted in each seedling tray cell.

Prevailing agroclimatic data (rainfall and average daily temperature) were monitored over the duration of the studies using an automatic weather station (Figure 1). The monthly mean temperature and rainfall were 24.9°C and 18 mm (1st cropping) and 23.5°C and 13 mm (2nd cropping), respectively. Manual hand-watering was used to irrigate the experiment when needed.

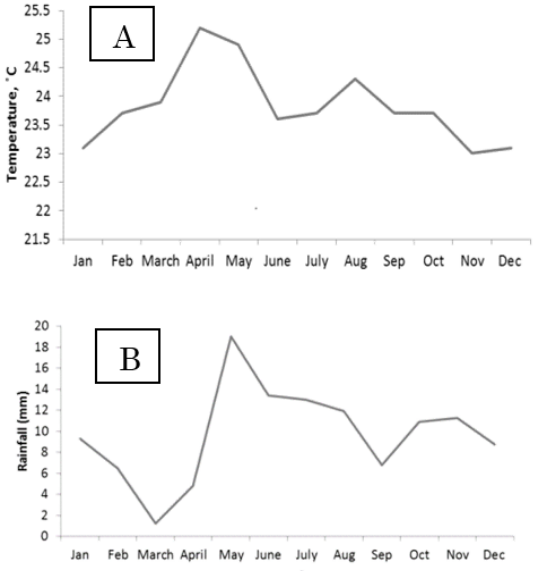

Figure 1. Average monthly temperature (a) and rainfall (b) at the USTP Claveria experimental area.

Preventive spraying with biological insecticide (Astron WDG) was done once every two weeks in rotation with (Lannate). Basal application of the commercial organic fertilizer WellGrow (2.1N:0.32P:1.0K with 5.78% Ca and trace elements of 0.87% Fe, 0.019% Cu, 8.312 ppm Mg, 927 ppm Mn, and 339 ppm Zn) and complete fertilizer (14-6-11) at a rate of 20 g and 10 g plant^{-1}, respectively, for those plots having 141-391 kg N ha^{-1}. On the 0 N treatment only WellGrow was applied. Urea (46-0-0), solophos (0-18-0) and muriate of potash (0-0-60) were fertilizers used as sources of nitrogen, phosphorus and potassium, respectively (Table 1). Application of fertilizer was divided into 3-split applications.

Table 1. Nitrogen treatments applied to two cabbage cultivars in experiments conducted at Claveria Northern Mindanao, Philippines.

Treatments	Fertilizer (g)		
	Urea	Solophos	Muriate of potash
21 kg N ha^{-1}	0	12.5	3.75
141 kg N ha^{-1}	2.5	12.5	3.75
191 kg N ha^{-1}	5.0	12.5	3.75
241 kg N ha^{-1}	7.5	12.5	3.75
291 kg N ha^{-1}	10.0	12.5	3.75
341 kg N ha^{-1}	12.5	12.5	3.75
391 kg N ha^{-1}	15.0	12.5	3.75

Cropping 1 was harvested 61 days after transplanting (DAT) when the head was full and compact. In the 2nd cropping, 'KEX-734' was harvested at 54 DAT and 'Resist Crown' at 61 DAT.

Data collection

The growth parameters measured included days to heading (from transplanting), plant stand (plant count relative to standard planting distance per unit area) and fresh and dry matter yield. The harvested vegetative parts were oven-dried at 70°C for 24 h or until constant weight. Data for yield and yield components were also collected, including number and weight of marketable and non-marketable heads per plot. Average head weight (g), head dimensions (polar and equatorial length), and yield (t ha^{-1}) were calculated. Postharvest quality was evaluated during the first cropping by taking initial head weight and the weight at each sampling period, which was then expressed as percentage of the initial weight. The fresh harvested samples (heads) were stored in an ambient room, away from adverse temperatures and protected from postharvest pests and diseases. The shriveling of heads over time was visually evaluated using a four point scale; 1) no shriveling, 2) slight shriveling 1-25% surface affected, 3) moderate shriveling 26-50% surface affected, and 4) severe shriveling greater than 50% surface affected.

Experimental and statistical design

The treatment plot had an area of 1×7 m. Seedlings were transplanted at about four to five weeks after sowing. Two rows were planted within each plot at a spacing of 0.5×0.4 m with a total of 35 plants per treatment replication.

The experiment was laid out in a randomized complete block design consisting of four replications with two factors including rates of nitrogen (21, 141, 191, 241, 291, 341 and 391 kg N ha^{-1}) and cultivars ('Resist Crown' and 'KEX-734'). Analysis of variance was used to test significance for differences across treatment means using ASSISTAT (version 7.0 beta). The Tukey's test was used to compare significant differences among means.

RESULTS AND DISCUSSION

Growth parameters

Time (days) to head formation was not affected by N application rate in either cropping season (Table 2). Plots with N applied at 141-341 kg N ha^{-1} recorded higher plant stands in the first cropping but did not affect stand density in the second cropping. In the first cropping this suggests that excessive application of N fertilizer contributed to nitrate build up in the soil and in the vegetables (Manchali et al., 2012), and that N was absorbed in the early growth stages and reutilized later (Fatimah et al., 2019). Application of 391 kg N ha^{-1} adversely affected plant stand (i.e., early plant growth and survival). Lim et al. (2007) claimed that salinity stress would likely decrease stomatal conductance limiting the growth in cabbage.

Table 2. Effects of nitrogen application rate (21-391 kg ha^{-1}) on time to heading and plant stand of two cabbage cultivars ('Resist Crown' and 'KEX-734') in two cropping seasons (wet and dry) in trials conducted at Claveria Northern Mindanao, Philippines.

Treatments	Time to heading (days)		Plant stand (%)	
	1st cropping	2nd cropping	1st cropping	2nd cropping
Rates of nitrogen (A)				
21 kg N ha^{-1}	53.9	54.9	94.5b	88.2
141 kg N ha^{-1}	54.2	55.8	100.0a	85.6
191 kg N ha^{-1}	54.4	55.0	100.0a	92.1
241 kg N ha^{-1}	54.9	55.2	100.0a	91.0
291 kg N ha^{-1}	55.8	56.2	100.0a	89.1
341 kg N ha^{-1}	55.5	55.8	99.0a	87.7
391 kg N ha^{-1}	56.0	55.5	96.0b	88.4
F-test	ns	ns	*	ns
Cultivars (B)				
Resist Crown	54.6	56.5a	98.7	91.9a
KEX-734	55.3	54.4b	98.3	85.9b
F-test	ns	**	ns	*
A×B				
F-test	ns	ns	ns	ns
CV (%)				
A	4.2	2.7	1.7	7.6
B	3.8	0.3	1.6	9.0

** significant at α=0.01, * significant at α=0.05, ns = not significant.

Head formation in 'KEX-734' occurred 2 days earlier in the second cropping while 'Resist Crown' had a 5.95% higher plant stand in that season (Table 2). Genotype and environment interactions have been shown previously to influence the head and core growth traits in cabbage (Kleinhenz and Wszelaki, 2003).

The highest marketable dry matter yield was recorded at a rate within the 291-341 kg N ha^{-1} range in the 1st cropping season but there was no consistent trend for the response to N rate in the 2nd cropping season (Table 3). 'Resist Crown' had the highest marketable dry matter yield in both seasons. In general, increasing N application affects leaf dry weight since N enhances plant photosynthesis and increases dry matter production (Novoa and Loomis, 1981).

Yield and yield components

There were no consistent significant differences in head size, in both the polar and equatorial dimensions, between rates of N in either cropping season (Table 4). An N rate of 391 kg N ha^{-1} produced the highest number of leaves in the first cropping season but leaf number was similar across the 241 to 391 kg N ha^{-1} range in the second season. Leaf number increased progressively with increasing N rate in the first season (Table 4). A quadratic response of yield to N rate was observed where the maximum yield was recorded at an application rate in the range of 241 to 291 kg N ha^{-1} (Table 5). Excessive N generally leads to an over emphasis on vegetative growth, often to the detriment of root and head development (Stefanelli et al., 2010). Leaf production in the 2nd cropping was lower than that in the 1st cropping due lower rainfall in the 2nd cropping (Table 4).

'Resist Crown' consistently produced heads with a greater number of leaves, had the largest head dimensions, and produced higher marketable yields in both cropping seasons (Tables 4 and 5).

Table 3. Effects of nitrogen application rate (21-391 kg ha^{-1}) on dry matter yield of two cabbage cultivars ('Resist Crown' and 'KEX-734') in two cropping seasons (wet and dry) in trials conducted at Claveria Northern Mindanao, Philippines.

Treatments	Dry matter yield (g plant^{-1})			
	1st cropping		2nd cropping	
	Marketable	Non-marketable	Marketable	Non-marketable
Rates of nitrogen (A)				
21 kg N ha^{-1}	224c	211a	189c	211a
141 kg N ha^{-1}	234c	183b	235a	151c
191 kg N ha^{-1}	226c	204a	187c	161c
241 kg N ha^{-1}	232c	205a	162d	172b
291 kg N ha^{-1}	265a	152d	207cb	175b
341 kg N ha^{-1}	267a	156d	219b	94e
391 kg N ha^{-1}	251b	165c	229ab	110d
F-test	**	**	**	**
Cultivars (B)				
Resist Crown	253a	192a	233a	119a
KEX-734	232b	172b	168b	180b
F-test	**	**	**	**
A×B				
F-test	**	**	**	**
CV (%)				
A	2.9	2.2	2.7	1.8
B	3.6	1.8	2.8	1.6

** significant at α=0.01.

Table 4. Effects of nitrogen application rate (21-391 kg ha^{-1}) on leaf count and head size of two cultivars ('Resist Crown' and 'KEX-734') of cabbage in trials conducted over two cropping seasons (wet and dry) at Claveria Northern Mindanao, Philippines.

Treatments	1st cropping			2nd cropping		
	No. of leaves	Head size (cm)		No. of leaves	Head size (cm)	
		Polar	Equatorial		Polar	Equatorial
Rates of nitrogen (A)						
21 kg N ha^{-1}	83.6e	8.0b	11.4 b	34.4b	9.4	12.1
141 kg N ha^{-1}	84.4e	9.2ab	12.0ab	34.4b	9.4	12.3
191 kg N ha^{-1}	86.0d	9.0ab	12.1ab	33.4b	10.0	12.9
241 kg N ha^{-1}	88.5c	9.3a	12.2ab	39.8a	9.7	12.9
291 kg N ha^{-1}	91.4b	9.8a	13.4a	38.9a	9.5	12.7
341 kg N ha^{-1}	92.2b	9.5a	13.1a	39.8a	9.7	12.9
391 kg N ha^{-1}	94.2a	9.3a	12.8ab	39.1a	10.1	13.0
F-test	**	**	**	**	ns	ns
Cultivars (B)						
Resist Crown	92.5a	9.6a	12.5	42.2a	9.8a	12.6
KEX-734	84.7b	8.7b	12.4	32.0b	9.6b	12.7
F-test	**	**	ns	**	*	ns
A×B						
F-test	ns	ns	*	*	ns	ns
CV (%)						
A	1.0	4.5	7.4	4.2	4.7	5.0
B	1.2	4.6	5.6	5.5	4.6	4.1

** significant at α=0.01, * significant at α=0.05, ns = not significant.

Table 5. Effects of nitrogen application rate (21-391 kg ha^{-1}) on marketable and non-marketable head weight (kg) and yield (t ha^{-1}) of two cultivars ('Resist Crown' and 'KEX-734') of cabbage in trials conducted over two cropping seasons (wet and dry) at Claveria Northern Mindanao, Philippines.

Treatments	1st cropping			2nd cropping		
	Head weight (kg)		Yield	Head weight (kg)		Yield
	Marketable	Non-marketable	(t ha^{-1})	Marketable	Non-marketable	(t ha^{-1})
Rates of nitrogen (A)						
21 kg N ha^{-1}	0.7d	0.6a	31.8d	0.4d	0.4a	19.6d
141 kg N ha^{-1}	0.7d	0.5b	33.1d	0.5c	0.3b	21.6c
191 kg N ha^{-1}	0.8c	0.5c	35.4cd	0.5c	0.2c	21.0c
241 kg N ha^{-1}	1.0a	0.4d	47.4a	0.6a	0.2d	23.3a
291 kg N ha^{-1}	1.0a	0.3e	45.6a	0.6a	0.2d	25.3a
341 kg N ha^{-1}	0.9b	0.5c	40.4b	0.5b	0.2d	23.6b
391 kg N ha^{-1}	0.8c	0.5c	37.4bc	0.5b	0.2c	23.6b
F-test	**	**	**	**	**	**
Cultivars (B)						
Resist Crown	0.9a	0.5a	38.9	0.6a	0.2b	25.7a
KEX-734	0.8b	0.4b	38.6	0.4b	0.3a	20.0b
F-test	**	**	ns	**	**	**
A×B						
F-test	**	**	**	**	**	**
CV (%)						
A	3.2	4.1	5.8	2.6	4.9	2.6
B	3.1	3.9	4.8	2.3	5.5	2.2

** significant at α=0.01, ns = not significant.

The highest head weight loss (33.8%) and shriveling rating (2.9) were recorded at 391 kg N ha^{-1} (Table 6). In general, the amount of weight loss progressively increased as N rate increased, indicating that higher N rates resulted in higher head weight loss and greater shriveling. Horticultural crops are inherently susceptible to rapid deterioration, particularly in the tropics such as Northern Mindanao, due to high moisture content. 'Resist Crown' tended to suffer greater weight loss and shriveling compared with 'KEX-734' (Table 6).

Table 6. Effects of nitrogen application rate (21-391 kg ha^{-1}) on postharvest quality of two cultivars ('Resist Crown' and 'KEX-734') of cabbage during the first cropping at Claveria Northern Mindanao, The Philippines.

Treatments	Weight loss (%)	Shriveling point
Rates of nitrogen (A)		
21 kg N ha^{-1}	24.5c	2.4b
141 kg N ha^{-1}	23.3c	2.4b
191 kg N ha^{-1}	24.5c	2.3b
241 kg N ha^{-1}	25.9c	2.4b
291 kg N ha^{-1}	25.0c	2.6ab
341 kg N ha^{-1}	29.7b	2.6ab
391 kg N ha^{-1}	33.8a	2.9a
F-test	**	**
Cultivars (B)		
Resist Crown	28.2a	2.7a
KEX-734	25.2b	2.4b
F-test	**	**
A×B		
F-test	**	ns
CV (%)		
A	6.0	9.5
B	5.9	9.0

** significant at α=0.01, ns = not significant.

CONCLUSIONS

The cultivar 'Resist Crown' had a higher plant stand, produced the greatest number of leaves, had a larger head (polar) and higher marketable yield than 'KEX-734'. Consequently, yield ha^{-1} for 'Resist Crown' was also greater. Application of 391 kg N ha^{-1} gave the highest number of leaves plant^{-1} and leaf count progressively increased with increasing N rate. Nitrogen rates in the range of 241 to 291 kg N ha^{-1} gave the highest marketable yield. At 391 kg N ha^{-1}, the highest weight loss and shriveling rating were recorded indicating that the high N application rates reduced the postharvest life of cabbage. 'KEX–734' recorded 3.68% earlier head formation, but only in the second cropping, and lower weight loss and shriveling compared with 'Resist Crown'. The study indicates that an N application rate of no higher than 291 kg N ha^{-1} can be recommended for cabbage production in Claveria Northern Mindanao as this rate produced the highest yield on both a size and weight basis. 'Resist Crown' was identified as the best variety because of its higher yield, especially under the drier conditions in the second cropping season.

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Comparative assessment of fertilization of marigold (*Tagetes erecta* L.) development, yield, quality and residual soil chemical properties

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Abstract

A field trial was conducted on the marigold cultivar 'Moon Sapphire' at the Naresuan University experimental farms in Phitsanulok, Thailand. The aim of this study is to test the hypothesis that mixed compound fertilizer can better improve marigold development, yield and induce an increase in total carotenoid content in the inflorescences. The experiment design is a randomized complete block design (RCBD) with four factors and four replications. The factors were: T1) control (no fertilization); T2) $N_{21}P_{21}K_{21}$+$N_8P_{24}K_{24}$ (1:1) = 625 kg ha^{-1}; T3) $N_{21}P_{21}K_{21}$+$N_8P_{24}K_{24}$ (1:1) + chemical and granular organic fertilizer with hormone mixed formula-HO (312.5 kg ha^{-1}) = 625 kg ha^{-1}; and T4) HO (625 kg ha^{-1}). Plant spacing was 0.5×0.5 m in a plot size of 4.0×2.5 m. The study demonstrated the significant effect fertilization can have on marigold yield, quality and residual soil chemical properties. The number of flowers ha^{-1} (2,254,666.67), flower shelf-life after 72 h (8.35 g plant^{-1}) and flower carotenoid (102.68 mg 100 g^{-1} FW) were greater for the HO treatment, but was not significantly different from the treatment $N_{21}P_{21}K_{21}$+$N_8P_{24}K_{24}$ (1:1) + HO. Upon completion of the study the soil chemical properties such as N, P, K, Fe, Cu, Zn, Mn, B, pH, CE and organic matter contents were improved by the HO and $N_{21}P_{21}K_{21}$+$N_8P_{24}K_{24}$ (1:1) + HO treatments. The results showed the HO fertilizer, and $N_{21}P_{21}K_{21}$+$N_8P_{24}K_{24}$ (1:1) + HO treatments produced the highest marigold yield and added significant value to the raw flower by increasing their total carotenoid content.

Keywords: carotenoid, fertilizers, marigold, yield, shelf-life, soil properties

INTRODUCTION

Fertilization is the most important and controllable factor affecting the productivity and biochemical value of marigold flowers. The type, value and rate of fertilizer application directly influence the color content level and size of flowers produced. Fertilization also indirectly influences plant physiology and the biosynthesis of metabolites and phytonutrients such as carotenoid in plants (Heaton, 2001). In South-East Asia, the ever-increasing demand for marigold flowers makes it imperative for science and technology to keep researching how to improve yield and product quality (Kumar et al., 2016). Globally, extracts form marigold play a major role in several cosmetic and pharmaceutical preparations. These include carotenoids, flavonoids, xanthophyll, sterols, triterpene saponins, essential oil, polyacetylenes, minerals, vitamin C and carbohydrates (Hussein et al., 2011). Amidst the current government restriction on the use of synthetic pigments in poultry diet, natural sources of xanthophylls, such as marigold is in high demand (Asif, 2008). For instance, the production of marigold seed and its consumption on an annual basis amounts to 12,000 million and 1,100 kg, respectively. The production area is about 250 ha. In addition, annual seed export to India, is about 1,200 kg and is forecasted to double in the coming years as demand increases from India and neighboring countries (Kumar et al., 2016).

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Despite marigolds enormous benefits and demand, the sustainability of the current production system is mainly based on synthetic fertilizer. This reliance on synthetic fertilizers is a concern for the future production of marigold flowers and seed (Keteku et al., 2019). Synthetic fertilizers are well known to have contributed immensely to soil pollution. This makes it crucial to incorporate renewable inputs which can maximize ecological benefits and reduce environmental hazards in a sustainable farming system (Kizilkaya, 2008). The development of organic and natural fertilizers or their integration with chemical fertilizer is on the rise (Gaskell and Smith, 2007). The major problem of chemical fertilization is the farmer's inability to balance the supply of nutrient elements, owing to rising costs and availability. Over the years the use of chemical fertilizers has resulted in a decrease in some important elements, leading to unproductive soils.

To help solve this issue, a new innovative fertilizer is making waves in Thailand. This fertilizer was developed at the Faculty of Agriculture, Naresuan University by integrating chemical fertilizer, compost powder, effective microorganism, soil amendments and plant growth hormones, and tested on soil properties (Intanon et al., 2017). Tests were conducted on maize and its potential yield (Keteku et al., 2019), and evaluated for use on many other crops. The purpose of our study is to explore the possibility of supplementing chemical fertilizer with this new product, that is ecofriendly and cost-effective. The test crop for this study is marigold, and effects on productivity and quality to minimize the usage of synthetic fertilizer and encourage the use of renewable materials.

MATERIAL AND METHODS

Research fertilizers

The chemical and granular organic fertilizer with hormone mixed formula (HO) was obtained from the Faculty of Agriculture, Natural Resources and Environment, Naresuan University, Thailand. Three samples of 50 g each were arranged for chemical analysis using the method developed by Lu (1999). Total nitrogen was determined by Kjeldahl analysis, and determination of other nutrient concentrations undertaken by the inductive coupled plasma emission spectrometry 4300 Optima DV (PerkinElmer Instruments, Norwalk, CT). The organic matter analysis was conducted using the potassium dichromate oxidation method, while pH was record at a 1:1 solution ratio with electrode (H19017 Microprocessor) pH meter. Electrical conductivity (EC) was measured with the EC meter. The presence of phytohormones (indole-3-acetic acid (IAA), gibberellic acid (GA_3), and cytokinins) were assessed by the high-performance liquid chromatograph (HPLC) system (Waters 2695 Separations Module, Waters, USA) equipped with a photodiode array detector (Waters 2996 Detector, Waters, USA) (Szkop and Bielawski, 2013). The compositional analysis of the HO fertilizer is shown in (Table 1). The NPK (21-21-21) and (8-24-24) were procured from the Soil and Land Development Department, Thailand.

Table 1. Compositional analysis of HO fertilizer.

N (%)	P (%)	K (%)	Fe (ppm)	Mn (ppm)	Zn (ppm)	Cu (ppm)	Cl (ppm)
10.64	10.71	9.83	2.51	222.00	181.00	27.24	3.14
OM (%)	**pH (1:1)**	**EC ($\mu S\ cm^{-1}$)**	**IAA ($mg\ kg^{-1}$)**	**GA_3 ($mg\ kg^{-1}$)**		**Cytokinins ($mg\ kg^{-1}$)**	
1.32	6.39	38.15	27.17	18.25		13.05	

Research site and plan

The experiment was conducted at the GPS coordinates of (16°55'0"N and longitude of 100°30'0"E), approximately 1,028 m a.s.l. in Phitsanulok, Thailand. The average annual rainfall is 1,339 mm, and approximately 85% of this rainfall occurs between June and October. The annual mean temperature was 27.8°C. The soil texture is a clayey loam. Approximately 50

g of the soil was collected from ten trial site locations for chemical analysis using the methods described above. The chemical composition of the soil before the study is indicated in Table 2.

Table 2. Compositional analysis of soil before study.

N (%)	P (%)	K (%)	Fe (ppm)	Cu (ppm)	Zn (ppm)	Mn (ppm)	B (ppm)	OM (%)	pH	EC (µS cm^{-1})
1.12	0.48	1.13	1011	1.09	37.64	30.64	0.21	0.89	5.8	69.84

The four fertilizer treatments and replications were arranged in a randomized complete block design (RCBD) using a 4×2.5 m plot size. The factors were: T1) control (no fertilization); T2) $N_{21}P_{21}K_{21}$+$N_8P_{24}K_{24}$ (1:1) = 625 kg ha^{-1}; T3) $N_{21}P_{21}K_{21}$+$N_8P_{24}K_{24}$ (1:1) + chemical and granular organic fertilizer with hormone mixed formula-HO (312.5 kg) = 625 kg ha^{-1}; and T4) HO (625 kg ha^{-1}) (Figure 1). Seeds of 'Moon Sapphire' marigold were raised in trays and the vigorous growing seedlings transplanted after 14 days at a row and inter row spacing of 0.5×0.5 m in each plot. Each plot had 40 seedlings. Before transplanting the seedlings, each ploy received approximately 4.7 kg (3,125 kg ha^{-1}) of cow dung as a basal treatment. Fertilizers were applied in four splits applications. Approximately 25% of each fertilizer applied on the 6th, 26th, 46th and 66th DAT (days after transplanting). The side placement method was used and the application rates were as per the experimental treatment design shown above.

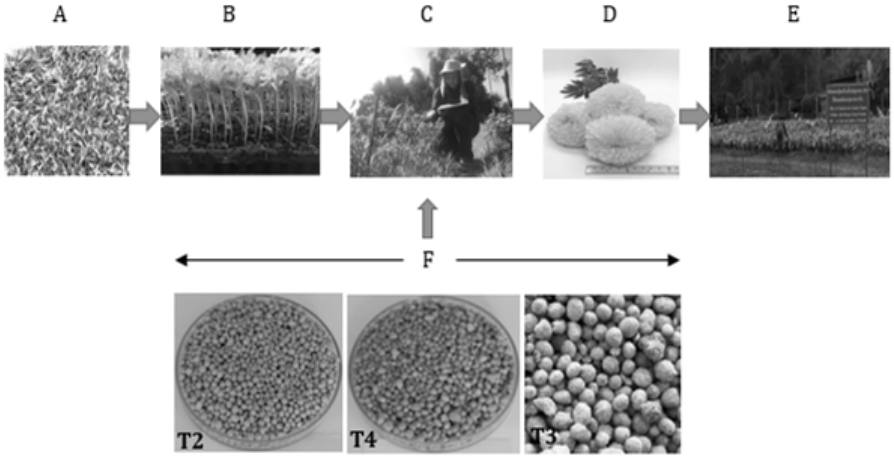

Figure 1. Marigold seeds (A), marigold seedlings (B), vegetative data collection (C), sampled flowers for carotenoid measurement (D), study site (E) and research fertilizers (F).

Yield and yield components

Data on day to first harvesting, flower height, flower size, flower number plant^{-1} and flower number plot^{-1} were recorded from the sample plants. All the matured flowers in each plot were collected at each data observation period and counted. The total flower number plot^{-1} was converted to hectare.

Quality assessment

Flower quality attributes such as, carotenoid content, hue angle, B value and flower shelf-life were measured using the flowers from the sample plants. Witham et al. (1971) method was adopted to determine carotenoid content and computed with the formulas in Equations 1-4. Flower color (Hue angle and B value) were measure with Chroma Meter CR-400.

$$\text{Chlorophyll a } (\mu g\ mL^{-1}) = (12.64 \times O.D.\ 664) - (2.99 \times O.D.\ 647) \quad (1)$$

$$\text{Chlorophyll b } (\mu g\ mL^{-1}) = (-5.6 \times \text{O.D. } 664) + (23.26 \times \text{O.D. } 647) \quad (2)$$

$$\text{Carotenoid } (\mu g\ mL^{-1}) = \frac{((1000\ \text{O.D.}470) - (3.27\ \text{x Chl a}) - (104\ \text{x Chl b}))}{229} \quad (3)$$

To convert to mg 100 g^{-1} FW:

$$\text{Carotenoid } (mg\ 100\ g^{-1}\ FW) = (\text{Carotenoid } (g\ mL^{-1}) \times 20.5 \times 100 \times 1 \quad (4)$$

where FW = fresh weight, Chl a = chlorophyll a, Chl b = chlorophyll b.

Plant tissue analysis

The shoots for tissue analysis were taken from the sample plants and ground with a Wiley mill (40 mesh) (Thomas Scientific, Swedesboro, NJ). Total N was determined by Kjeldahl analysis (Schuman et al., 1973). Concentrations of other nutrients were determined by inductively coupled plasma emission spectrometry 4300 Optima DV (PerkinElmer Instruments, Norwalk, CT). The phytohormones: indole-3-acetic acid (IAA), gibberellic acid (GA_3) and cytokinins analysis conducted using the High-Performance Liquid Chromatograph (HPLC) system.

Soil analysis after study

The experimental field site was rested for three months after the trial. The test the change in soil properties from the fertilizer applications soil samples were collected from each plot site for the analysis of nitrogen, phosphorus, potassium, iron, manganese, cupper, boron, zinc, organic matter, pH and electrical conductivity.

Statistical data analysis

Statistical analysis was carried out by analysis of variance (ANOVA) using SPSS 21.0 for Windows (SPSS Inc., Chicago, USA). The difference between treatments means were estimated by Duncan's multiple range test (DMRT) at a probability of 95% threshold.

RESULTS AND DISCUSSION

Yield and yield components

The results in Table 3 shows how the different fertilizers treatments influenced yield and its components. The shortest days to first flower harvesting, 71.25 and 71.27 days were recorded in T4 and T3, respectively. T2 and control (T1) treatment were slightly longer in days to reach first harvest. The days for T2 can be related to its higher nitrogen content (Agrios, 1997). Biesiada et al. (2006) documented that higher N can promote late emergence of flower buds in marigold and recommended 30-50 kg N ha^{-1} application. The tallest flower height and larget flower size were pronounced by T4 and T3, averaging 5.79, 5.19 and 6.17, 6.05 cm, respectively. T3 and T2 were also similar, while T1 produced the shortest flowers and smallest size. The percentage increase in flower size from T4, T3 and T2 were 25.1, 23.6 and 21.8% respectively, compared to the control (T1). Larger flower size attracts higher prices, this is an important economic attribute. If a fertilizer formula which can produce maximum flower size, this will be very useful to farmers. The number of flowers $plant^{-1}$, and $plot^{-1}$ ha^{-1} was not significantly ($p \leq 0.05$) different between the fertilizer treatments. However, an increasing trend in the number of flowers $plant^{-1}$ and flowers ha^{-1} were recorded for the treatments applied. Treatment T4 recorded 56.37, and 2,254,666.67, respectively. Treatment T4 was followed by T3 with 2,084,800 flowers ha^{-1}, and T2 at 2,040,800 flowers ha^{-1}.

These results may be due to amount t of available nutrient, efficient nutrient uptake and a greater translocation of assimilates from source to sink resulting greater flower yield. The nutrient elements, N, Fe, Cu, Zn, S and Mg are important in the synthesis of carbohydrate, and may be the reason behind the increase in flowers for T4 and T3. Generally, our study recorded a higher number of flowers $plant^{-1}$ than the 21.5 reported by Pacheco et al. (2013). In addition,

Król (2011) reported 60 and 45.6 flowers plant^{-1} for two successive years. Our study showed that high nitrogen levels for the T2 treatment did not yield the highest number of flowers. This is in line with Kumar et al. (2010), showing that plants under a 7% N + 60 kg P_2O_5 ha^{-1} + 60 kg K_2O ha^{-1} + Azotobacter 5 L ha^{-1} enriched banana pseudo stem sap 1% nourishment, produced the heaviest yield with enhanced attributes.

Table 3. Influence of fertilizers on marigold yield and yield components.

Treatments	Days to first harvesting	Flower height (cm)	Flower size (cm)	Flower plant^{-1}	Flower plot^{-1}	Flower ha^{-1}	Shelf-life after 72 h (g)
T1	72.91b	4.69c	4.62b	25.16b	1006.40b	1006400.00b	7.86
T2	73.16b	5.14b	5.91a	51.02a	2040.80a	2040800.00a	7.94
T3	71.27a	5.19ab	6.05a	52.12a	2084.80a	2084800.00a	8.24
T4	71.25a	5.79a	6.17a	56.37a	2254.67a	2254666.67a	8.35
CD at 5%	1.25	0.63	0.96	6.21	229.20	229204.82	ns

Mean values with different superscript letter within each column denotes significance difference at the p≤0.05 level.
ns = non significant, CD = critical difference.

This study found the storage of flowers for 72 h, T2 lost more weight flower^{-1}, compared to all other treatments. T4 and T3 maintained the heaviest flower weight of 8.32 and 8.24 g after 72 h. Furthermore, treatments differences were not significant (Table 3). This supports the report of Biesiada et al. (2006) that flower weight increases with N and P, but flower shelf-life reduces at a higher N rate.

Flower quality and tissue analysis

Table 3 illustrates the effect of the fertilizers on nutrient content, secondary metabolites and phytohormones present in the marigold after harvest. The type, quantity and fertilizer dose directly influenced the level of N, P and K available in the marigold shoots. Nitrogen was highest in T3 (0.15%), followed by T4 and T2. Furthermore, T4 recorded the highest phosphorus and potassium content, 0.36 and 2.26%, respectively. In addition, T3 and T2 showed comparable amounts to T4. Although lesser amounts of the three major nutrients were contained in T4 and T3, when compared to T2, their influence on plant nutrient uptake was greater than T2. This is due to the complementary interaction of major and minor elements (Heaton, 2001). Indirectly, high tissue nutrients affect the biosynthesis of secondary metabolites such as carotenoid which in turn enhances hue angle and B value. Furthermore, the amount of carotenoid in the flowers, due to the different fertilizers treatments T4, T3, T2 and control were 102.68, 96.04, 88.26, 85.15 mg 100 g^{-1} fresh weight (FW), respectively. Hue angle is an indication of color, which has a strong influence on the marketability of the flowers. It is expressed in degrees, with 0° corresponding to +A axis (red), and 90° for the +B axis (yellow). Consumers most often prefer yellow to gold flower color. Therefore a high hue angle and +B value are an indication of a bright yellow color. The hue angles recorded for T4, T3, T2 and control were 85.44, 85.28, 84.38 and 83.89°, respectively. The higher +B values for T4, T3, T2 and control were 105.36, 105.14, 103.13 and 100.41, respectively. Carotenoid content may have an influence on flower color. Hue angle and +B value were the highest in T4. Our study concurs with Moehs et al. (2001). In addition, our results showed an increase in amounts of carotenoid content, but no significant differences were found between the fertilizer treatments.

Nutrients and hormonal interaction can also enhance secondary metabolites production (Table 4). Auxins can enhance the activity of enzymes responsible for carotenoid biosynthesis. Our study found the auxin content was well expressed in the marigold shoots. However, GA_3 and cytokinin were in extremely low amounts. The difference between treatments was not significant. Nevertheless, the highest auxin content was 0.21 mg kg^{-1} in T4. Zaredost et al. (2014) also reported a high carotenoid of 3.903 mg g^{-1} dry weight in marigold under bio- and chemical fertilization.

Table 4. Compositional analysis of marigold.

Treatments	N (%)	P (%)	K (%)	Carotenoid (mg 100 g^{-1} FW)	Hue (°)	*B*	IAA (mg kg^{-1})	GA_3 (mg kg^{-1})	Cytokinin (mg kg^{-1})
T1	0.11b	0.21c	1.54c	85.15	83.89	100.41	0.08	0.0030	0.0003
T2	0.14ab	0.29b	1.73b	88.26	84.38	103.13	0.14	0.0050	0.0005
T3	0.15a	0.31ab	1.75b	96.04	85.28	105.14	0.19	0.0053	0.0006
T4	0.14ab	0.36a	2.26a	102.68	85.44	105.36	0.21	0.0057	0.0010
CD at 5%	0.03	0.06	0.17	ns	ns	ns	ns	ns	ns

Mean values with different superscript letter within each column denotes significance difference at the $p \leq 0.05$ level.
CD = critical difference.

Soil chemical properties after the study

Three months after the trial concluded, analysis of the soil revealed a significant ($p \leq 0.05$) improvement in the soil chemical properties (Table 5) for the treated plots. Soil N, P, K, were higher in the T4, T3 and T2 plots compared to the control treatment. The results also indicated that, Fe, Cu, Zn, B were also higher in T4 and T3 treatment plots. A decrease was observed in the micronutrient content of T2 plot compared to the initial values shown in Table 2. As a consequence, soil OM, pH and EC were also enhanced by T4 and T3 plot treatments. Generally, the most improved properties were found in T4 plot, followed closely by T3. These findings may be due to the EM compost included in the OM component of the HO fertilizer. EM compost is a promising soil supplement because it contains beneficial microbes which improves soil fertility through the slow release of organically bound nutrients (Dikr and Belete, 2017). According to Sharma et al. (2017), EC relates closely to other soil properties used to determine soil productivity. The EC of the soils were in the good range to promote good plant growth. For marigold, a pH between 5.0 and 6.5 is recommended (Robbins and Evans, 2010). However, a higher pH may promote micronutrients deficiency, especially Fe (Fisher et al., 2003). The results from this study showed the soil pH for all trial plots are within the recommended range.

Table 5. Influence of fertilizers on soil properties after the study.

Properties	T1	T2	T3	T4	CD at 5%
N (%)	1.03c	1.24b	1.58a	1.65a	0.13
P (%)	0.32c	0.50a	0.39b	0.52a	0.03
K (%)	1.14b	1.57a	1.49a	1.56a	0.21
Fe (ppm)	984.00b	997.33b	1175.33a	1206.33a	93.96
Cu (ppm)	0.95c	1.02c	1.97b	2.86a	0.10
Zn (ppm)	35.94d	37.49c	68.43b	77.77a	1.68
Mn (ppm)	30.75c	29.54c	74.62b	101.17a	2.57
B (ppm)	0.18c	0.24c	1.44b	2.23a	0.19
OM (%)	0.88b	0.94b	2.49a	2.64a	0.18
pH	5.77c	5.40d	6.10a	6.47a	0.17
EC (µs cm^{-1})	69.84b	60.72a	57.45a	55.49a	6.04

Mean values with different superscript letter within each row denotes significance difference at the $p<0.05$ level.
CD = critical difference.

CONCLUSIONS

Our study demonstrated the added benefits of combining chemical and organic fertilizers T4 and T3 over the sole chemical fertilization T2 treatment. Flower number $plant^{-1}$ and flower carotenoid contents were greater in the T4 and T3, compared to the T2 treatment. T4 produce 9.5% more flowers ha^{-1} and had 14% more carotenoid content, compared to that of T2. From our findings, the incorporation of micronutrients and PGR in the HO also proved very useful to enhance marigold yield, nutrient assimilation, and secondary metabolites production. After the investigation, the soil chemical properties were improved, especially in

T4 and T3. Therefore, we recommend the HO fertilizer (T4) for marigold production.

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Effect of nitrogen, potassium and calcium concentrations on growth, yield and nutritional quality of green oak lettuce

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Abstract

Plant production with adaptation of nutrient application rates may provide benefits to consumers, giving improved nutritional benefits and food functionality. This research studied the effect of nitrogen (N) potassium (K) and calcium (Ca) levels on yields and nutritional quality of green oak lettuce. Plants were grown in a NFT hydroponics system and supplied with nutrient solutions containing different levels of nitrogen (7.5, 15.0 and 22.5 mM L^{-1}), potassium (3.0, 6.0 and 9.0 mM L^{-1}) and calcium (2.5, 5.0 and 7.5 mM L^{-1}). At the harvest stage (four weeks after starting treatment), plant growth and yield for plant height, number of leaves $plant^{-1}$, total fresh weight, total dry weight and leaf area were recorded. The nutritional quality in terms of nutrient concentration (N, P and K), vitamin C, total carotenoids, total chlorophyll content, phenolic content, and total soluble solids were determined. The result showed that plant growth varied, due to the different concentrations of nitrogen, potassium, and calcium. For the nitrogen treatments, plants supplied with nitrogen at 15.0 mM L^{-1} had the highest number of leaves $plant^{-1}$. Plants supplied with nitrogen at 22.5 mM L^{-1} recorded the lowest of leaf area. For the potassium treatments, the total fresh weight and leaf area were highest when the plants were supplied with potassium at 6.0 mM L^{-1} compared to plants supplied with potassium at 3.0 and 9.0 mM L^{-1}. For the calcium treatments, the highest number of leaves $plant^{-1}$ and plant width was when plants were supplied with calcium at 5.0 mM L^{-1}. The results on nutritional quality i.e., vitamin C, total chlorophyll, plant mineral (NPK), total carotenoids, phenolic content and total soluble solids are discussed.

Keywords: nitrogen, potassium, calcium, nutritional quality, concentration, lettuce

INTRODUCTION

Mineral fertilization influences vegetable quality (Kaur and Kapoor, 2001). Understanding plant responses to different plant nutrients allows us to adjust fertilizer use to improve growth and their nutritional quality. Mozafar (1996) reported nitrate accumulation in spinach was reduced when vitamin C content was increased. This occurred when plants were transferred to a nitrogen-free solution for 2-3 days before harvest. In addition, Flores et al. (2004) studied the effect of different fertilization levels of Ca^{2+}, K^+ and NO_3^- on the bioactive nutrient content in red pepper (*Capsicum annuum* L.) fruit. The results showed lycopene and β-carotene content in pepper increased with increasing Ca^{2+} and NO_3^- concentrations in the nutrient solution, while vitamin C, sugar and total phenolic acid content were not affected by Ca^{2+} or NO_3^- treatment.

Nitrogen (N), potassium (K) and calcium (Ca) are important nutrient elements affecting growth, yield, and quality of plants. Soundy and Cantliffe (2001) reported that shoot growth in lettuce plantlets increased as N concentration increased from 0 to 60 mg L^{-1} in a floating irrigation system. For tomato plants, it was reported that the supply of nutrient solution with high proportion of potassium increased fruit dry matter, total soluble solids and the lycopene

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content in tomato fruit, whereas a high proportion of calcium improved tomato fruit yield (Fanasca et al., 2006). Moreover, lycopene concentration in tomato supplied with 8 mM L^{-1} of potassium concentration were 40% higher than tomato provided with 0 mM L^{-1} of potassium concentration (Trudel and Ozbun, 1971). Ruamrungsri and Inkham (2017) studied the effect of the addition of calcium nitrate on growth and bulb quality of *Hippeastrum.* They reported plants supplied with calcium nitrate at 150 and 200 mg L^{-1} had higher leaf number $plant^{-1}$ than plants supplied with calcium nitrate at 0 and 50 mg L^{-1}. In addition, Yuan et al. (2018) found increasing the total calcium content in lettuce cultivars from 60 to 180 mg L^{-1} was achievable by selecting and applying fertilizers with high calcium concentrations.

There is great interest in growing plants by adjusting nutrient concentrations to improve their growth and nutritional quality. The objective of this study is to determine the effects of nitrogen potassium and calcium concentrations on growth, yields and nutritional quality of green oak lettuce grown hydroponically.

MATERIALS AND METHODS

The experiments were conducted in a plastic greenhouse at Mae-Hia Agricultural Research, Demonstrative and Training Centre, Faculty of Agriculture, Chiang Mai University. Green oak lettuce (*Lactuca sativa* L.) seedlings were prepared by germinate seeds in sponge cubes. The seedlings were fertilized with Hoagland's standard nutrient solution for two weeks until the three true leaves stage. Seedlings were then randomly selected and transplanted into a nutrient film technique (NFT) hydroponic system. Seedlings were fertilized with different concentrations of nitrogen, potassium and calcium nutrient solutions. The experiment was divided into three sub-experiments: 1) N experiment; plants were fertilized with three levels of nitrogen (7.5, 15.0 and 22.5 mM L^{-1}) with potassium and calcium levels maintained at 6.0 and 5.0 mM L^{-1}, respectively, based on Hoagland's standard solution formula; 2) K experiment: plants were fertilized with three levels of potassium (3.0, 6.0 and 9.0 mM L^{-1}) with nitrogen and calcium levels maintained at 15.0 and 5.0 mM L^{-1}, respectively; 3) Ca experiment: plants were fertilized with three levels of calcium concentrations (2.5, 5.0 and 7.5 mM L^{-1}) with nitrogen and potassium levels maintained at 15.0 and 6.0 mM L^{-1}, respectively (Table 1). Treatments in each sub-experiment arranged in a complete randomized design (CRD) with three replicates and 30 plants per replication. The pH of all treatments was maintained at 6.0-6.5.

Table 1. Concentrations of the chemical compounds supplying the macronutrients (mM L^{-1}) and micronutrients (µM L^{-1}) in the nutrient solution of each treatments.

Nutrient sources	Treatments								
	Nitrogen (mM L^{-1})			Potassium (mM L^{-1})			Calcium (mM L^{-1})		
	7.5	15	22.5	3.0	6.0	9.0	2.5	5	7.5
Macronutrients (mM L^{-1})									
KNO_3	3.3	5.0	5.0	2.0	5.0	5.0	5.0	5.0	5.0
$Ca(NO_3)_2.4H_2O$	2.1	5.0	5.0	5.0	5.0	5.0	2.5	5.0	5.0
$MgSO_4.7H_2O$	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
NH_4NO_3	0.0	0.0	3.7	1.5	0.0	0.0	2.5	0.0	0.0
KCl	1.7	0.0	0.0	0.0	0.0	3.0	0.0	0.0	0.0
$CaCl_2.7H_2O$	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.2
$CaSO_4$	1.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.3
Micronutrients (µM L^{-1})									
H_3BO_3	46.3	46.3	46.3	46.3	46.3	46.3	46.3	46.3	46.3
$MnSO_4.4H_2O$	9.1	9.1	9.1	9.1	9.1	9.1	9.1	9.1	9.1
$ZnSO_4.7H_2O$	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
$CuSO_4.5H_2O$	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
FeEDTA	90.2	90.2	90.2	90.2	90.2	90.2	90.2	90.2	90.2

At harvest (four weeks after starting treatments), plant growth measurements were recorded, including plant height, number of leaves plant^{-1} and estimation of chlorophyll content (using a portable SPAD-502 Plus chlorophyll meter; Konica Minolta, Japan), total fresh weight, total dry weight, and leaf area (using LI-3100 leaf area meter; LI-COR, USA). The nutritional quality, concentration of nitrogen, potassium and phosphorus, total soluble solids, total carotenoids, total phenolic content, vitamin C and chlorophyll content determined.

Data analysis included analyses of variance (ANOVA) using generalized linear models by means of Statistic 8 analytical software package (SXW Tallahassee, FL). The significance of treatment effects presented as, not significant (ns), or significant at $p<0.05$. In the case of significant treatment effects, the comparison of means was performed by LSD at a significance level of 0.05.

RESULTS AND DISCUSSION

Growth and yields

At harvest, the nitrogen fertilizer concentration of 15 mM L^{-1} resulted in plants with significantly increased plant height, number of leaves plant^{-1}, total fresh weight and total dry weight compared to plants supplied with nitrogen concentrations of 7.5 and 22.5 mM L^{-1} (Table 2; Figure 1). This indicates a nitrogen concentration of 15 mM L^{-1} is the optimum level for growth and yield of green oak lettuces grown in hydroponic system. In addition, plants fertilized with a high concentration of nitrogen (22.5 mM L^{-1}) produced plants with the highest leaf color intensity but had the lowest growth and yield (Table 2; Figure 1).

Table 2. Plant growth and yields of Green Oak lettuce treated with different nitrogen, potassium and calcium concentrations at harvest stage (four weeks after starting treatments).

Treatments	Plant height (cm)	Plant width (cm)	No. of leaves plant^{-1}	Leaf color intensity (SPAD unit)	Leaf area (cm^2)	Fresh weight (g)	Dry weight (g)
Nitrogen (mM L^{-1})							
7.5	21.1b	26.5a	27.1b	16.3b	2,134.5a	190.3b	3.4b
15.0	25.3a	27.3a	31.0a	17.0b	2,395.1a	239.4a	5.2a
22.5	18.8c	23.1b	26.0b	25.0a	1,253.6b	104.0c	3.9b
$LSD_{0.05}$	*	*	*	*	*	*	*
Potassium (mM L^{-1})							
3.0	24.2a	26.8	24.3b	18.7	1,603.7c	148.8b	3.1b
6.0	25.3a	27.3	31.0a	17.0	2,786.1a	239.4a	5.2a
9.0	20.1b	26.6	25.4b	18.3	1,893.2b	177.3b	2.6b
$LSD_{0.05}$	*	ns	*	ns	*	*	*
Calcium (mM L^{-1})							
2.5	30.7a	27.0ab	24.4b	16.0b	1,808.3c	141.1	3.1
5.0	24.5b	27.2a	26.7a	20.3a	2,395.1b	139.0	2.9
7.5	26.7b	25.5b	22.5b	14.0c	2,842.1a	135.4	2.8
$LSD_{0.05}$	*	*	*	*	*	ns	ns

*significant different between means at 0.05 level of probability, according to analysis of variance. ns = not significant.
Means within the same column followed by different letters are significantly different.

Findings from this experiment and the high leaf color intensity found in plants due to fertilizing with a high nitrogen concentration supports the role nitrogen plays in increasing chlorophyll intensity. Hokmalipour and Darbandi (2011) also found increased nitrogen, significantly increased chlorophyll content. However, this experiment found that excessively high nitrogen concentrations lowered yields, possibly due to nutrient toxicity. Sheikh and Ishak (2016) reported that high concentration of nitrogen in the tissue of plants may cause

mineral toxicity and reduce physiological responses.

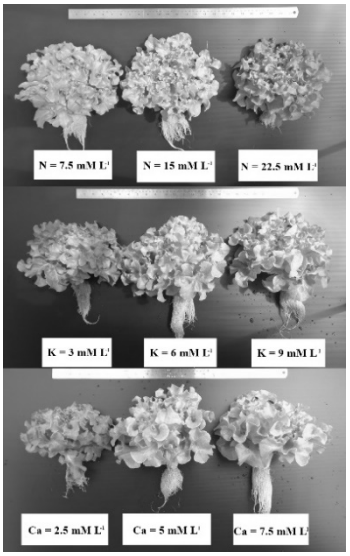

Figure 1. Growth of green oak lettuce treated with different nitrogen, potassium and calcium concentrations at harvest (four weeks after starting treatments).

Plants fertilized with potassium at 6.0 mM L^{-1} showed an increased leaf number $plant^{-1}$, leaf area and total fresh weight compared to those plants fertilized with 3.0 and 9.0 mM L^{-1} potassium concentrations (Table 2; Figure 1). These results indicate a potassium concentration at 6.0 mM L^{-1} may be an appropriate level to maximize growth and yield of green oak lettuces.

The different levels of calcium concentrations provided during this experiment had no effect on total plant fresh weight and total plant dry weight. However, this research found plants fertilized with a calcium concentration of 5 mM L^{-1} resulted in increased leaf number $plant^{-1}$, leaf color intensity and leaf area compared to plants fertilized at 2.5 mM L^{-1} (Table 2; Figure 1). Calcium is an essential element for physiological activity, particularly when cell division is occurring. Calcium is also required for cell wall structure and membrane permeability (Marschner, 1995). The supplement of calcium may have contributed to the significant increments in plant growth under high calcium concentration compared to the low concentration.

Nutritional quality

The nutritional quality of the green oak lettuces was analyzed for each treatment at harvest (four weeks after starting treatments). The results indicated the nitrogen application rates, even at the highest rate (22.5 mM L^{-1}) had no effect on phosphorus and potassium levels in green oak lettuce (Table 3). There was a decreasing trend for total soluble solids with increasing nitrogen concentrations from 7.5 to 15.0 mM L^{-1} (Table 3). Similar results were found by Xia and Cheng (2004) who reported sugar content decreased with increasing nitrogen levels. This is possibly due to the relationship between nitrogen and carbohydrate in plants. Evans (1983) described nitrogen as a direct factor which regulates carbon balance that is the basic element for sugar construction.

Plants fertilized with a high potassium concentration of (9.0 mM L^{-1}) showed the best results for potassium concentration in plant at harvest. The different application levels of potassium had no effect on nitrogen content in the plants of oak lettuce (Table 3). Plants fertilized with the highest calcium concentration (7.5 mM L^{-1}) showed significantly lower potassium content than plants fertilized with calcium concentrations of 5.0 mM L^{-1} (Table 3). These findings are possibly due to the antagonism between the nutrients, calcium, and potassium. Nguyen et al. (2017) reported that potassium, calcium, and magnesium are

strongly antagonistic to each other. In case of excess concentration of one element, the uptake of the other elements is inhibited.

Table 3. Nitrogen (N), phosphorus (P), potassium (K) and total soluble solids (TSS) contents in green oak lettuces treated with different nitrogen, potassium and calcium concentrations at harvest (four weeks after starting treatments).

Treatments	N (mg gDW^{-1})	P (mg gDW^{-1})	K (mg gDW^{-1})	TSS (°Brix)
Nitrogen (mM L^{-1})				
7.5	57.31b	30.50	75.32	1.85ab
15.0	59.87b	36.10	71.08	1.50b
22.5	75.07a	36.41	49.49	2.05a
$LSD_{0.05}$	*	ns	ns	*
Potassium (mM L^{-1})				
3.0	81.76	51.37a	30.16c	1.95a
6.0	75.07	36.10b	71.08b	1.50b
9.0	80.81	46.01a	0.87a	1.95a
$LSD_{0.05}$	ns	*	*	*
Calcium (mM L^{-1})				
2.5	60.62b	49.21a	58.38ab	2.20ab
5.0	81.67a	36.10c	71.08a	1.50a
7.5	31.96c	42.19b	39.33b	1.90b
$LSD_{0.05}$	*	*	*	*

*significant different between means at 0.05 level of probability, according to analysis of variance.
ns = not significant. Means within the same column followed by different letters are significantly different.

In addition, there was less nitrogen content in the lettuce fertilized with the high calcium rate of 7.5 mM L^{-1} than the low calcium rates of 2.5 and 5.0 mM L^{-1} (Table 3). This is possibly due to secondary effects of calcium on the senescence pattern in leaves. Pal and Laloraya (1973) reported that the soluble-nitrogen content is generally less in high calcium plants.

The total carotenoid and vitamin C content in the oak lettuce did not differ with increasing nitrogen applications. However, total chlorophyll content was higher in plants fertilized with a nitrogen rate of 22.5 mM L^{-1} compared to the lower nitrogen treatments rates of 7.5, and 15.0 mM L^{-1} (Table 4). This result is understandable, as nitrogen is a structural element of chlorophyll. In the experiment, there was a dramatic decrease of total phenolics when the nitrogen concentration increased from 15.0 to 22.5 mM L^{-1} (Table 4). This is a result of excess nitrogen uptake by the plants. Li et al. (2008) reported under high nitrogen levels, the synthesis of phenols, phenylalanine is preferentially applied into chain protein synthesis rather than for phenol compounds.

Plants fertilized with potassium at 6.0 mM L^{-1} showed the highest results in total phenolic at harvest, while plants fertilized with potassium at 9.0 mM L^{-1} had the highest chlorophyll content (Table 4).

The calcium fertilizer rate of 5.0 mM L^{-1} showed the highest total phenolic content compared to the calcium rate of 2.5 mM L^{-1}. Furthermore, plants fertilized with a calcium rate of 2.5 mM L^{-1} had the highest vitamin C content compared to plants fertilized with calcium rates of 5.0 and 7.5 mM L^{-1} (Table 4). Plants fertilized with low calcium rates, showed high vitamin C levels. This is possible due to the increase in ascorbic acid, which improves tolerance to nutrient deficiency stress. As an antioxidant, ascorbic acid may affect tolerance to environmental stress (Gallie, 2013). Furthermore, increasing ascorbic acid content provides greater tolerance to other environmental stresses. Wang et al. (2010), reported *Arabidopsis* with increased ascorbic acid content and redox state, was a result of increased dehydroascorbate reductase expression, which retained more ascorbic acid and chlorophyll with lower membrane damage following exposure to high light and temperature.

Table 4. Total carotenoid, total phenolic, vitamin C and total chlorophyll contents in green oak lettuces treated with different nitrogen, potassium, and calcium concentrations at harvest (four weeks after starting treatments).

Treatments	Total carotenoid (µg g FW^{-1})	Total phenolic (µg g FW^{-1})	Vitamin C (mg 100 g FW^{-1})	Total chlorophyll (mg 100 g FW^{-1})
Nitrogen (mM L^{-1})				
7.5	4.05	656.64ab	2.76	0.17b
15.0	3.23	723.61a	2.76	0.13c
22.5	4.08	556.94b	2.30	0.22a
$LSD_{0.05}$	ns	*	ns	*
Potassium (mM L^{-1})				
3.0	4.05a	520.16b	2.76	0.13b
6.0	3.23b	730.04a	2.76	0.14b
9.0	3.88ab	446.09c	2.76	0.23a
$LSD_{0.05}$	*	*	ns	*
Calcium (mM L^{-1})				
2.5	4.58	355.56b	3.37a	0.19b
5.0	4.38	534.57a	2.76ab	0.14c
7.5	4.53	479.01ab	2.33b	0.27a
$LSD_{0.05}$	ns	*	*	*

*significant different between means at 0.05 level of probability, according to analysis of variance. ns = not significant. Means within the same column followed by different letters are significantly different.

CONCLUSIONS

Nitrogen applied at 15 mM L^{-1} and/or potassium at 6 mM L^{-1} increased the fresh plant weight yield of green oak lettuce. The supply of different levels of nitrogen and potassium concentrations did not affect the vitamin C content. Whereas plants supplied with calcium at 2.5 mM L^{-1} had higher vitamin C levels then plants fertilized with calcium at 7.5 mM L^{-1}. This study revealed that nitrogen and/or potassium content of green oak lettuce plants increased with the application of high nitrogen and/or potassium applications.

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Comparison of two methods for the determination of nitrogen in leaf and yield in banana, jujube and rubber tree

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Abstract

Nitrogen is an essential element for plant growth and development. Generally, the standard technique of nitrogen analysis is done by the Kjeldahl method. However, this method is a long and time-consuming procedure. A faster, alternative technique for nitrogen analysis is colorimetry. The objective of this work was to evaluate the potential of colorimetry for nitrogen analysis. The investigation was conducted using three plants species, banana (*Musa* sp.), jujube (*Zizyphus mauritiana* Lam.) and rubber tree (*Hevea brasiliensis*). In each plant, the nitrogen contents in both leaf and yield were analyzed. The comparisons of two methods were analyzed using a t-test, analysis of variance and liner regression analysis. The results demonstrated a highly positive correlation between the Kjeldahl method and the colorimetry technique (r^2=0.88, $p<0.01$). This identified colorimetry as a prospective technique for the measurement of nitrogen analysis in plant tissue. Also, the nitrogen concentrations in the leaves of all plants were higher than those nitrogen concentrations in their respective products. The nitrogen concentrations in leaves analyzed by colorimetry were 1.47% for the banana, 2.30% for jujube and 2.01% for rubber tree plants while the nitrogen in yields was 0.35% for banana pulp, 1.07% for jujube pulp and 0.62% for dry latex.

Keywords: Kjeldahl method, colorimetry technique, *Musa* sp., *Zizyphus mauritiana*, *Hevea brasiliensis*

INTRODUCTION

Leaf analysis is a very useful tool for plant nutritional diagnosis and capable of providing data for analysis. The dynamic nature of leaf tissue composition, which is strongly influenced by leaf age, maturation stage, and the interactions involving nutrient absorption and translocation. The practice of tissue diagnosis and its utilization is sometimes difficult to gain a clear understanding. Methods for nutritional diagnosis using leaf tissue analysis, such as that proposed by Mourão Filho (2004), is growing in importance, and its interpretation is usually based on the total contents of nutrients in leaves (Putra et al., 2010).

Nitrogen (N) fertilization is a major agronomic practice that affects both the yield and quality of the crop. While crop N fertilizer requirements vary widely between and within fields, the amount of N applied is often the same (Morón et al., 2007). However, nutrition and fertilization practices are important factors in determining fruit yield and quality. Among the several methods of plant nutritional status diagnosis (Mourão Filho, 2004), the Kjeldahl (standard) technique is generally the most common. The particles are first digested, representing a rather time-consuming process, which requires several reagents and titration (Dalai et al., 1984; Attanandana et al., 1989; Bekers et al., 1996; Bilbao et al., 1999; Mills and Jones, 1996; Rossi et al., 2004).

An alternative technique for nitrogen analysis, colorimetry is a viable, less time-consuming technique (Baethgen and Alley, 1989; Bekers et al., 1996), whose potential derives from acid-wet digestion, which causes organic nitrogen and nitrate-nitrogen compounds to transform into ammonium as a free cation (Suwanwong, 2004). The reagent solutions in this

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method can produce the color of ammonium cation for measurement by spectrophotometer.

The objective of this work was to evaluate the potential of the colorimetry technique for nitrogen analysis through comparison with the Kjeldahl method. To obtain samples of different N contents in plant tissues, three plant species were selected; banana (*Musa* sp.), jujube (*Zizyphus mauritiana* Lam.) and rubber tree (*Hevea brasiliensis*). We also examined the difference between leaves and products for more distributed N concentrations in each sample.

MATERIALS AND METHODS

Plant materials

Ten samples of leaves and products (pulp and dry latex) of banana, jujube and rubber tree were collected, washed by distilled water, dried at 65°C for 72-96 h, and then mashed to a powder for N analysis (total 60 samples).

Digestion process

The plant tissue samples were digested by the wet acid method (Attanandana et al., 1989; Mills and Jones, 1996), using sulfuric acid with sodium sulfate and selenium in a digestion block at 380-400°C. The clear color of the digested solutions was adjusted to 50-mL volumes via deionized water. Comparisons of the colorimetry technique and Kjeldahl method were conducted from a single solution bottled from each digestion.

Nitrogen analysis

The first method employed, the standard Kjeldahl method (Horwitz, 1980; Attanandana et al., 1989) and each of the 60 digested solutions analyzed. The digested plant samples were also analyzed by the colorimetry technique (Baethgen and Alley, 1989; Bilbao et al., 1999; Suwanwong, 2004) as detailed below:

1. Preparation of the two reagents.

- Reagent A: dissolve 20 g phenol and 0.1 g sodium nitroprusside in deionized water, and adjust the final volume to 2,000 mL by adding deionized water;
- Reagent B: dissolve 10 g sodium hydroxide and 16.8 mL sodium hypochlorite in deionized water and adjust the final volume to 2,000 mL by adding deionized water. Protect the Reagent B solution from light;
- The N standard solution: prepare N concentrations at 0, 50, 100, 150 and 200 mg L^{-1} from 1,000 mg L^{-1} ammonium chloride.

2. Analysis procedure.

- Sampled solution: pipet 20 µL of the sampled solution into 15 mL test tubes; then, add Reagent A (2.5 mL), Reagent B (2.5 mL) and mix the solution. Wait 20 min for a complete reaction of the mixed solution and then measure the development of color via spectrophotometer at a wavelength of 625 nm;
- Standard solution: prepared in the same manner as the sample solution and the standard curve was calculated as a linear regression.

Data analysis

Data obtained were analyzed using a t-test, analysis of variance and liner regression analysis (SPSS 11.5, SPSS Inc., New York, USA).

RESULTS AND DISCUSSION

Nitrogen in leaves and products

The nitrogen in the banana leaves ranged from 0.67 to 1.62% (Kjeldahl method) and 0.89-1.86% (colorimetry technique). Similar readings were obtained from the other leaf samples (Table 1). The average N concentrations in the banana, jujube, and rubber tree leaves were 1.28, 2.02 and 2.15%, respectively (Kjeldahl method) and 1.47, 2.30 and 2.01,

respectively (colorimetry technique).

Table 1. Comparison of leaf nitrogen concentrations (%) of banana, jujube and rubber tree determined by the Kjeldahl method and colorimetry technique.

Sample	Banana		Jujube		Rubber tree	
no.	Kjeldahl	Colorimetry	Kjeldahl	Colorimetry	Kjeldahl	Colorimetry
1	1.528	1.496	2.326	2.020	2.205	2.305
2	1.575	1.849	2.291	1.754	2.013	2.190
3	0.700	0.998	2.147	2.578	2.555	2.816
4	1.482	1.794	1.598	1.980	2.594	2.421
5	1.517	1.734	2.473	2.412	2.123	2.506
6	1.027	1.582	2.112	1.781	1.614	1.843
7	1.108	0.967	2.018	2.027	1.925	2.196
8	1.587	1.551	2.053	1.973	1.937	2.500
9	1.622	1.855	2.380	2.007	1.995	2.177
10	0.665	0.894	2.100	1.582	1.272	2.050
Range	0.67-1.62	0.89-1.86	1.60-2.47	1.58-2.58	1.27-2.59	1.84-2.82
Mean	1.28±0.12	1.47±0.12	2.02±0.08	2.30±0.09	2.15±0.12	2.01±0.09
T-test	ns		ns		ns	
SD	0.37	0.38	0.25	0.30	0.39	0.27

ns = no significant difference in a couple N analyses.

The N in the product samples showed lower concentration values than those of the leaves, in both techniques. They exhibited the same pattern as in the leaf analysis. The average N concentrations in banana, jujube and rubber tree products were 0.33, 0.96 and 0.47%, respectively (Kjeldahl method). Results from the colorimetry technique were 0.35% N for banana pulp, 1.07% N for jujube pulp and 0.62% N for dry latex (Table 2).

Table 2. Comparison of nitrogen concentrations (%) of the banana and jujube pulp and the dry latex of the rubber tree determined by the Kjeldahl method and colorimetry technique.

Sample	Banana		Jujube		Rubber tree	
no.	Kjeldahl	Colorimetry	Kjeldahl	Colorimetry	Kjeldahl	Colorimetry
1	0.082	0.220	1.085	0.871	0.525	0.721
2	0.350	0.341	1.089	0.951	0.688	0.423
3	0.373	0.384	0.922	1.084	0.35	0.522
4	0.385	0.408	0.949	1.416	0.362	0.741
5	0.362	0.286	0.817	0.732	0.362	0.443
6	0.152	0.165	0.957	0.977	0.373	0.522
7	0.268	0.438	1.015	1.383	0.525	0.901
8	0.303	0.396	0.922	1.031	0.49	0.702
9	0.373	0.347	0.863	1.177	0.49	0.682
10	0.665	0.554	0.933	1.124	0.56	0.582
Range	0.08-0.67	0.17-0.44	0.82-1.09	0.73-1.42	0.35-0.69	0.42-0.90
Mean	0.33±0.05	0.35±0.04	0.96±0.03	1.07±0.07	0.47±0.03	0.62±0.05
T-test	ns		ns		ns	
SD.	0.16	0.11	0.09	0.21	0.11	0.15

ns = no significant difference in a couple N analyses

The statistical comparisons in each sampled set (six sets) was not significantly different in all couple N analyses (Tables 1 and 2).

Relationship between the two nitrogen analysis methods

The relationship between N concentrations analyzed for the Kjeldahl method and the colorimetry technique showed a linear correlation (Figure 1) expressed by the equation; y = 0.941×-0.0268 (r^2=0.88, p<0.01). This evidence confirms the ability of the colorimetry technique for N analysis in plant tissues as an alternative to the Kjeldahl method. This evidence supports corresponding work of Bilbao et al. (1999) which determined the effectiveness of colorimetric analysis of *Passalum fasciculatum* and Standard Reference Material (SRM 1547 peach leaves). In addition, the alternative technique also reduced the time required to analyze a large number of samples, as well as reducing the costs for the use of specialized equipment and reagents. However, the colorimetric method produced slightly higher values in the upper N concentrations as reported by Baethgen and Alley (1989). The variation in N values was possibly caused by: 1) the hand-titration technique within the Kjeldahl method, and 2) the standard N preparation for the colorimetry technique in higher concentrations (>100 mg L^{-1}). This may have reduced the responses of the absorbance value of the spectrophotometer. Therefore, further N analysis is needed to refine these procedures.

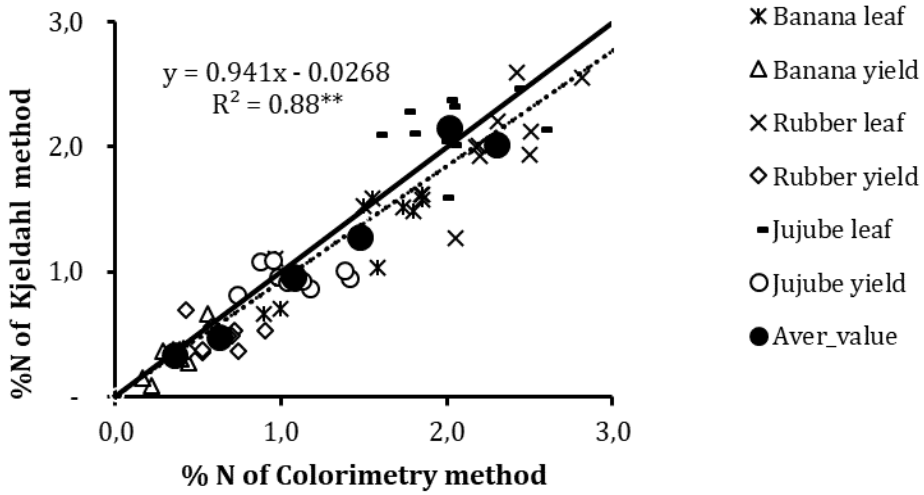

Figure 1. Correlation of nitrogen concentrations analyzed by the Kjeldahl method and colorimetry technique of leaves and products of banana, jujube and rubber tree plants (*n*=60). Continuous and dotted lines indicate the 1:1 reference and trend line, respectively.

CONCLUSIONS

The comparison of nitrogen (N) analysis techniques between the Kjeldahl and colorimetry methods showed similar N concentration values in leaves and products of banana, jujube, and rubber tree plants with a strong positive correlation (r^2=0.88, p<0.01). The results demonstrated the viability and timeliness of the colorimetric method for N analysis in the plant tissues.

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Antifungal effect of zinc oxide nanoparticles against disease in durian caused by *Phytophthora palmivora*

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Abstract

Durian (*Durio zibethinus*) is one of the most important economic fruit crops in Thailand with high international demand. However, this commercially important crop is affected by the fungus, *Phytophthora palmivora* which causes fruit, stem and root rot disease and significant economic losses. Zinc oxide nanoparticles (ZnO-NPs) have recently shown capability as an antimicrobial agent being biocompatible, economic, and having a low toxicity. Their potent ability can stimulate the production of excess reactive oxygen species, the release of zinc ions, and the induction of cell apoptosis. The objective of this study was to investigate the antifungal effect of ZnO-NPs, with 25-50 nm size range, at concentrations of 0, 500, 1000 and 2000 µg mL^{-1} on growth inhibition of *P. palmivora* denominated as Phy001 and Phy002. A poisoned food technique with potato dextrose agar (PDA) was used in the study together with an investigation of the inhibition of infection on durian leaves. The results showed that ZnO-NPs at a concentration of 2,000 µg mL^{-1} could significantly inhibit mycelial growth of Phy001 and Phy002 by 56.6 and 53.6%, respectively. Moreover, the nanoparticles could reduce the disease severity on durian leaves in both isolates by 50.5 and 43.7%, respectively. These results suggest that the use of ZnO-NPs could be a satisfactory and environmentally safe alternative to the current fungicides that are used to control durian disease caused by *P. palmivora*.

Keywords: zinc oxide nanoparticles, antifungal agent, *Phytophthora palmivora*, durian

INTRODUCTION

The international durian market is dominated by Thailand, which accounts for 90% of global share through its exports (Bais, 2016), which largely go to China, Hong Kong, Malaysia, Taiwan, and the USA (Parichatnon et al., 2017). In 2018, the total area under cultivation was 108,060 ha, which generated 752,760 t of durian fruit. The total export value was US$ 1,168.65 million, which was earned from 516,008 t of durian products (Office of Agricultural Economics, 2019). The biggest challenge to the production of durian is *Phytophthora* disease, which is a fungus that attacks all parts of the durian tree in all of its different growth phases including after harvest. Typical symptoms are stem rot, root rot and fruit rot, thus restricting yield (Drenth and Guest, 2004; Suksiri et al., 2018). While fungicides do exist, which have the capacity to control this disease, there are practical difficulties associated with their application because the pathogen involved tends to develop resistance. Furthermore, there are also adverse side effects which make such fungicides a danger to both people and animals, as well as the wider environment. Accordingly, interest is growing in various metal oxide nanoparticles which have the potential to control such plant diseases due to particular properties, which include a large specific surface area and the ability to block the activity of a wide range of microorganism types (Sabir et al., 2014; Jiang et al., 2018).

One potential alternative is the use of zinc oxide nanoparticles (ZnO-NPs), since these offer a means of preventing plant disease that is less invasive in the environment (Navale et al., 2015; Wagner et al., 2016; Al-Dhabi and Valan Arasu, 2018). Zinc oxide is an inorganic compound which typically takes the form of an insoluble white powder (Sabir et al., 2014). There are a number of mechanisms by which ZnO-NPs are able to perform their antimicrobial

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activity. For example, they release Zn^{2+} ions which have antimicrobial properties. They also interact with light to form reactive oxygen species (ROS) which include hydrogen peroxide (H_2O_2), hydroxyl radicals (^{-}OH) and superoxides (O_2^{-}). Antimicrobial outcomes can also result from electrostatic interactions. ZnO-NPs are capable of entering microbial cell walls via carrier proteins or through ion channels, whereupon they are able to form bonds with various organelles, thereby allowing them to damage the cells (Espitia et al., 2012; Hou et al., 2018; Jiang et al., 2018; Martínez-Carmona et al., 2018). However, the full specifics of the ways in which ZnO-NPs carry out their antifungal activity are not yet completely understood. Recently, a number of researchers have reported that ZnO NPs can inhibit the growth of fungi by interfering with cell function causing deformation in the fungal hyphae. It was observed that the intensity of nucleic acids and carbohydrate bands increased significantly in fungal hyphae treated with ZnO NPs (Al-Dhabi and Valan Arasu, 2018).

The antifungal activity of ZnO-NPs has been studied on different plant fungal pathogens, such as *Botritis cinerea* and *Penicillium expansum* causing postharvest fruit diseases (He et al., 2011); *Peronospora tabacina* causing tobacco disease (Wagner et al., 2016); *Aspergillus flavus* and *A. fumigatus* (Navale et al., 2015); *A. niger* and *A. flavus* (Al-Dhabi and Valan Arasu, 2018). However, the effects of ZnO-NPs on *P. palmivora*, which causes durian diseases, does not appear to have been studied previously.

The objectives of this study were: 1) to isolate *P. palmivora* which naturally infects durian crops and to conduct pathogenicity tests; 2) assess the antifungal properties of ZnO-NPs sized 25-50 nm at concentrations of 500, 1,000 and 2,000 μg mL^{-1} on mycelial growth of *P. palmivora* and its infection on durian leaves. The findings can be expected to assist in determining strategies for the future management of diseases affecting durian.

MATERIALS AND METHODS

The isolation of *P. palmivora* and pathogenicity testing

1. Isolation of *P. palmivora.*

Samples of naturally infected durian showing indications of fruit rot were collected from plantations and brought to the Plant Disease Clinic of the Department of Agricultural Technology, King Mongkut Institute of Ladkrabang, Prince of Chumphon Campus, Chumphon, Thailand. A PAR(PH)-V8 selective medium was prepared using fungicides and antibiotics, along with cooled clarified V8 juice, in accordance with the protocol of Jeffer (2006). The infected parts of the durian plant were sliced into small pieces and then surface sterilized with 10% Clorox® for 2-3 min (sodium hypochlorite, a.i. 6.0%) before washing in sterilized distilled water and dried with sterilized blotting paper. Once dry, the plant tissue was placed on the prepared PAR(PH)-V8 selective medium for incubation at room temperature. After three days, the hyphal tips of the culture were removed and placed upon clarified V8 juice agar. The identification of *P. palmivora* followed the process of Gallegly and Hong (2008).

2. Pathogenicity testing.

Pathogenicity of the *P. palmivora* isolates were tested on the leaves of 'Monthong' durian. Firstly, healthy durian leaves were collected and rinsed carefully under running tap water prior to sterilization using 10% Clorox®. Blotting paper was then used to remove any surface moisture, and the leaf bases were wrapped with damp cotton wool to ensure that the leaves would not dry out. The leaves were then put in a plastic container. Six wounds were made on each leaf using a sterile needle. For each of the isolates, a mycelium disc of 5 mm diameter was cut from the colony margin of the isolates which was then cultured on PDA for three days. The discs were then inverted and placed on a wound site. Damp paper towels were then put in the plastic container, along with the inoculated leaves, prior to incubation for four days at room temperature, whereupon lesion diameters were measured.

The influence of ZnO-NPs on *P. palmivora* mycelial growth

The ZnO-NPs of size 25-50 nm required for the experiments were obtained from the

College of Nanotechnology at King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. A poisoned food method was used to assess the effects of these nanoparticles on the Phy001 and Phy002 *P. palmivora* isolates. The two *P. palmivora* isolates were cultured individually on PDA plates for four days at room temperature. Mycelial discs of 5 mm diameter were then cut from the margins of colony. These discs were placed on PDA as a control or on PDA amended with ZnO-NPs at concentrations of 500, 1,000, and 2,000 µg mL^{-1} using five replications. The colony diameters were measured after four days of incubation at room temperature, in order to calculate the percentage of mycelial growth inhibition accomplished by the ZnO-NPs treatment, using the following formula: [(mean of colony diameter on the control medium – mean of colony diameter on the medium with ZnO-NPs)/(mean of colony diameter on the control medium)]×100.

The influence of ZnO-NPs on *P. palmivora* infection of durian leaves

In order to examine the influence of ZnO-NPs on *P. palmivora* infections, a number of leaves were detached from 'Monthong' durian. A total of 12 leaves were rinsed in sterilized water and surface sterilized with 10% Clorox® before air drying. Three pairs of wounds were made for inoculation using a sterile needle. These leaves were then soaked for 10 min, either in sterilized water as the control group, or in ZnO-NPs at concentrations of 500, 1,000 and 2,000 µg mL^{-1}. Mycelial discs, with a diameter of 5 mm, were cut from the margin of a 3-day-old colony of *P. palmivora* and were then placed on the wounds. These inoculated durian leaves were incubated four days at room temperature in a plastic container under moist conditions after which the lesions were measured. The percentage of reduction in disease severity was calculated using the following formula: [(mean lesion diameter on water-treated durian leaves – mean lesion diameter on ZnO-NPs-treated durian leaves)/mean lesion diameter on water-treated durian leaves]×100.

Data analysis

For statistical analysis, a one-way ANOVA was performed followed by a least significant difference test (LSD). Probability values $p \leq 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Pathogens and pathogenicity testing

1. Pathogens.

Naturally infected durian exhibiting signs of fruit rot provided two isolates which were identified as Phy001 and Phy002. The morphological characteristics were examined following seven days of growth on clarified V8 agar. The colony morphology of both isolates had a stellate pattern. The isolates produced sporangia which were ovoid to limoniform in shape with papilla. Identification as *P. palmivora* was confirmed. Thailand has been shown to have similar growing conditions to other major durian producers including Indonesia, Malaysia, and Vietnam, which also suffer from losses due to *P. palmivora* infections in durian crops (Drenth and Guest, 2004; Abad and Cruz, 2013).

2. Pathogenicity testing.

Pathogenicity testing was able to confirm that the Phy001 and Phy002 *P. palmivora* isolates acted as pathogens when introduced to durian leaf wounds. Initially, the visible signs included a dark brown necrotic appearance after two days. Four days after inoculation, these dark brown necrotic lesions on the leaves had mean diameters of 12.96 and 12.34 mm for the Phy001 and Phy002 isolates, respectively (Figure 1).

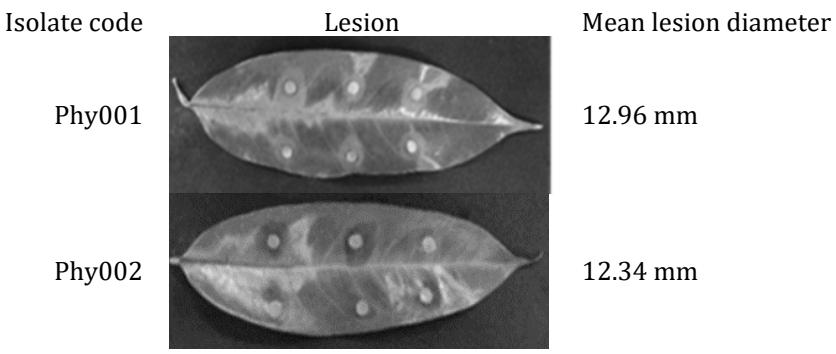

Figure 1. Lesions on detached leaves of durian cv. 'Monthong' and mean of lesion diameter after inoculation with Phy001 or Phy002 of *Phytophthora palmivora* isolates for four days.

Antifungal influence of ZnO-NPs on *P. palmivora* mycelial growth inhibition

The antifungal effects of ZnO-NPs on mycelial growth of *P. palmivora* on PDA was assessed. Various different concentrations of ZnO-NPs were tested using the Phy001 and Phy002 isolates. Results showed that ZnO-NPs were able to restrict mycelial growth in both isolates and the effectiveness increased with an increase in concentration. The best results of the Phy001 and Phy002 isolates were found at 2000 μg mL^{-1}, according to Table 1 and Figure 2. Inhibition achieved by the ZnO-NPs at concentrations of 500 and 1000 μg mL^{-1} was less than that at 2000 μg mL^{-1}. These findings indicate that ZnO-NPs might be effective in restricting the growth of *P. palmivora* and that the overall effectiveness of ZnO-NPs in terms of their antifungal properties is depended on the concentration used. Earlier studies have shown that ZnO-NPs can readily bind to the surface of eukaryotic cells through pinocytosis allowing the cells to absorb the ZnO-NPs (Espitia et al., 2012; Hou et al., 2018; Jiang et al., 2018; Martínez-Carmona et al., 2018). The accumulation of ZnO-NPs can take place either in the outer membrane or in the cytoplasm of a fungal cell, which will then result in the release of Zn^{2+} which, in turn, destroy the fungal cell membrane, damage the membrane proteins, and causing genomic instability, all of which ultimately inhibit fungal growth (Rajiv et al., 2013; Navale et al., 2015; Jamdagni et al., 2018; Siddiqui et al., 2018; El-Waseif, 2019). This process overall tends to be more effective at higher concentrations because there are more zinc particles to exert their influence. Furthermore, a clear zone appeared around the circumference of the colony in each of the treatments, with the exception of the control. This may be the result of the fungi absorbing the ZnO-NPs. This pattern was also observed in the case of *Phytophthora* sp. which causes leaf fall disease in rubber trees (Laohasakul et al., 2016), and a number of other fungal pathogens in plants, including *Fusarium* sp. (Sharma et al., 2010), *Botrytis cinerea* and *Penicillum expansum* (He et al., 2011), *Aspergillus niger* and *Botrytis cinerea* (Erazo et al., 2019), and *Rhizopus stolonifera* (Nafady et al., 2019).

Table 1. Inhibitory effects of of ZnO-NPs at 500, 1,000 and 2,000 μg mL^{-1} on mycelial growth of *Phytophthora palmivora* Phy001 and Phy002 after incubation for four days on PDA.

Concentration ZnO-NPs (μg mL^{-1})	Inhibition of mycelial growth (%)	
	Phy001	Phy002
Control	0d	0d
500	44.92c	42.30c
1,000	49.12b	49.19b
2,000	56.64a	53.58a

Means within the same column followed by the different letters are significantly different at $p<0.05$ by LSD.

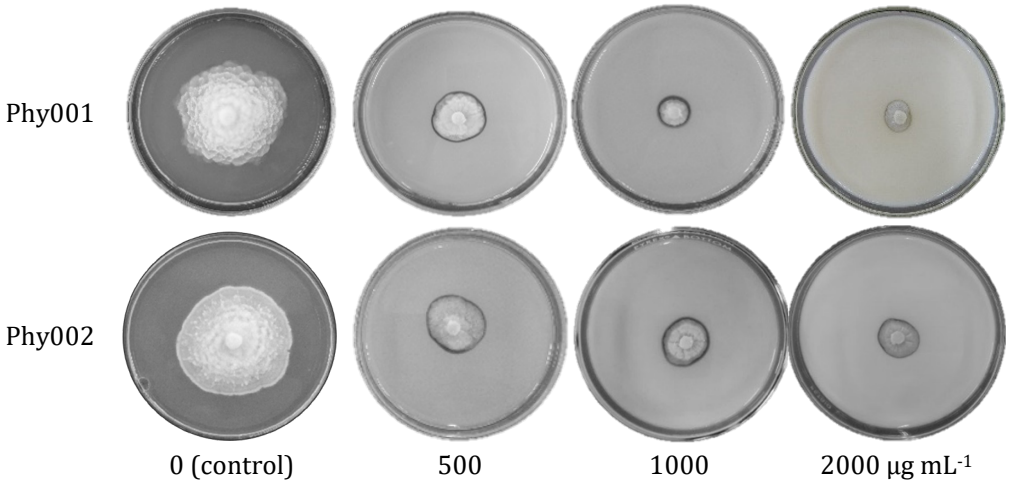

Figure 2. Inhibitory effects of of ZnO-NPs at 500, 1,000 and 2,000 µg mL^{-1} on mycelial growth of *Phytophthora palmivora* Phy001 and Phy002 after incubation for four days on PDA.

The antifungal influence of ZnO-NPs on the inhibition of *P. palmivora* infection in durian leaves

Assessments were made of the antifungal influence of ZnO-NPs on *P. palmivora* in infected durian leaves. Significant differences in the lesion diameters between the various ZnO-NPs treatments occurred at the different concentrations. As the concentration increased, the lesion diameter was reduced in both isolates. Therefore, the greatest lesion diameter reduction in both the Phy001 and Phy002 isolates were achieved at 2000 µg mL^{-1}, according to Table 2 and Figure 3. In comparison to the effects involving a reduction in mycelial growth, increased ZnO-NPs concentrations were necessary to accomplish similar reductions in leaf infection. These findings are similar to those of other researchers who showed that ZnO-NPs were able to reduce the infection from *P. tabacica* in tobacco leaves. In addition, Nafady et al. (2019) examined the effects of ZnO-NPs against *Rhizopus* soft rot on sweet potato tubers, finding that the shelf-life of the produce could be extended after harvest. It is thus clearly apparent that ZnO-NPs are able to serve as an effective antifungal agent which can protect crops from *P. palmivora* infections.

Table 2. Inhibitory effects of of ZnO-NPs at 500, 1,000, and 2,000 µg mL^{-1} on *Phytophthora* sp. infection of durian leaves after three days of inoculation.

Concentration ZnO-NPs (µg mL^{-1})	Inhibition of disease severity (%)	
	Phy001	Phy002
Control	0c	0c
500	40.87b	32.17b
1,000	45.39b	34.71b
2,000	50.52a	43.70a

Means within the same column followed by the different letters are significantly different at $p<0.05$ by LSD.

CONCLUSIONS

In this study, an evaluation was made of the antifungal capabilities of ZnO-NPs, of size 25-50 nm, for their ability to counteract the oomycete fungal pathogen *P. palmivora,* which is known to cause diseases in durian. It can be concluded that ZnO-NPs are able to significantly reduce mycelial growth and thus restrict *P. palmivora* infection under laboratory conditions. These findings indicate, therefore, that ZnO-NPs may have considerable potential for

development as a fungicidal agent which may find applications as a replacement for synthetic fungicides which have been previously used in the management of disease in durian crops in the field. Such current fungicides have some unwanted side effects which would warrant their replacement. Future research will initially be directed toward an examination of the efficacy of ZnO-NPs in commercial durian plantations.

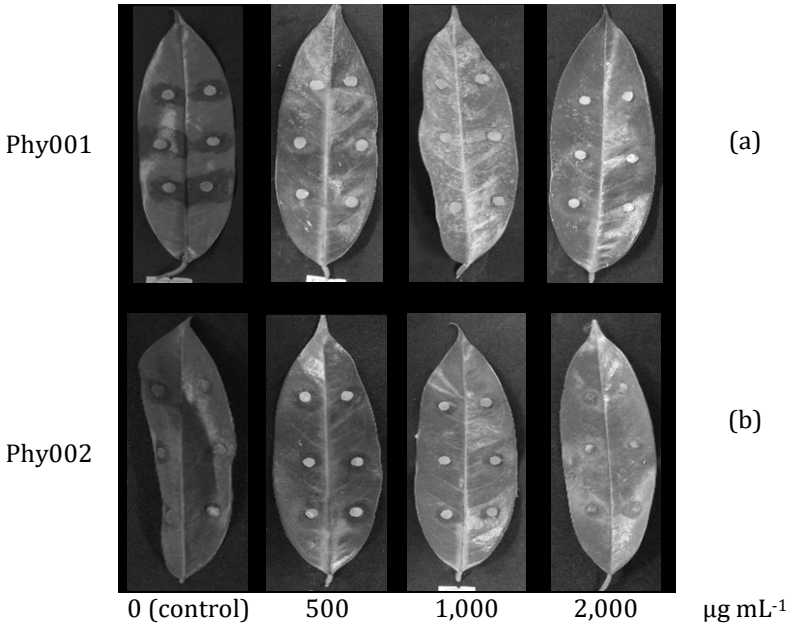

Figure 3. Comparative development of lesions on detached leaves of 'Monthong' durian pretreated with 500, 1,000 and 2,000 μg mL^{-1} ZnO-NPs or untreated three days after inoculation with Phy001 and Phy002 of *Phytophthora palmivora* isolates.

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Factors associated with occurrence of target leaf spot of cucumber

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Abstract

Target leaf spot, caused by *Corynespora cassiicola*, is a severe disease affecting cucumber cultivation. Most Japanese cultivars are susceptible to this disease. Additionally, the pathogen often has fungicide resistance making it difficult to control. We investigated factors associated with the occurrence of cucumber target leaf spot in order to predict and prevent the disease. The pathogen can grow at 10-35°C on potato dextrose agar medium. The optimum temperature for mycelial growth was approximately 30°C. Conidia of the pathogen germinated well at 25-35°C. Optimum temperatures for pathogen infection on cucumber leaves were approximately 25-30°C. Slight infection was observed at 10 and 35°C. More than 18 h of leaf-wetness duration at 20-30°C was necessary for obvious infection of the pathogen. Disease symptoms were enhanced by extension of the wetness duration. Symptoms especially became severe after more than 30 h of wetness. The results of inoculation tests showed that the number and/or area of lesions varied among cucumber cultivars. Some cultivars showed reduced symptoms in both the number and area of lesions compared with a susceptible control cultivar. These cultivars appeared to be resistant on pathogen infection and development. The findings we obtained from this study are useful for prediction and control of cucumber target leaf spot.

Keywords: *Corynespora cassiicola*, *Cucumis sativus*, cultivar, environmental factor, plant disease

INTRODUCTION

Corynespora cassiicola causes severe leaf spot disease on many different horticultural crops. Target leaf spot, caused by *C. cassiicola*, is a severe disease affecting cucumber. In Japan, recent popular cultivars of cucumber are generally susceptible to this disease (Hasama, 1993). Furthermore, employing bloomless rootstock reportedly enhances disease occurrence (Hasama et al., 1993). Fungicide use is, therefore, extremely important for the control of this disease. However, the occurrence of resistance to fungicides has made target leaf spot management difficult (Miyamoto et al., 2009, 2010).

Infection, expansion, and sporulation of a fungal plant pathogen are usually affected by environmental factors such as temperature, wetness, and humidity (Rowlandson et al., 2015; Tantau and Lange, 2003). Identifying the environmental conditions that are suitable for the establishment and growth of pathogens is critically important in predicting and preventing disease occurrence. Previously, Hasama (1993) investigated the conditions that were suitable for the occurrence of cucumber target leaf spot. However, gene mutation frequently occurs in the pathogen population (Miyamoto et al., 2009, 2010). It is important, therefore, that the conditions that are suitable for the target leaf spot pathogen that currently occurs in Japan, are further investigated. Additionally, the resistance of a cucumber cultivar is an important biological factor affecting target leaf spot occurrence. The use of resistant cultivars provides an effective means for controlling cucumber target leaf spot, especially where there is fungicide resistant *C. cassiicola*. Information about resistance against target leaf spot among Japanese cucumber cultivars is lacking, although resistance traits of cultivars have been reported elsewhere (Abul-Hayja et al., 1978; Liu et al., 2017; Strandberg, 1971; Wang et al.,

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2010; Wen et al., 2015). This study investigated the effects of environment factors and cultivars on the incidence and development of cucumber target leaf spot caused by *C. cassiicola*.

MATERIALS AND METHODS

Assays of mycelial growth

Isolates of cucumber target leaf spot pathogen (*C. cassiicola*) were maintained on potato dextrose agar (PDA) slants at room temperature. Three isolates of *C. cassiicola* (MAFF_242434 from Kochi Pref., FCCC1716 from Fukuoka Pref., and Ibrk1 from Ibaraki Pref.) were cultured initially on PDA plates at 25°C in the dark for a week. A mycelial disk (5 mm diameter) obtained from the cultured plate of each fungal isolate was then placed at the center of another PDA plate (90 mm diameter). Plates were incubated in the dark at several temperatures; 5, 10, 15, 20, 25, 30 and 35°C. A week later, the colony diameter was measured. Three PDA plates were used for each isolate. The average and standard error were calculated.

Assays of conidial germination

Two isolates (FCCC1716 and Ibrk1) of *C. cassiicola* were cultured on PDA plates at 25°C in the dark for a week. Sterile distilled water was poured onto the cultured plate to collect fungal conidia. A conidial suspension was dropped onto a water agar plate and was incubated at each temperature (5, 10, 15, 20, 25, 30 and 35°C) in the dark. After 2, 4, 6, 8, 10, 12 and 24 h, conidia on the water agar plate were stained with lactophenol cotton blue. A sample of 100 conidia was observed using a compound microscope and conidia with a germ tube that was clearly visible were counted.

Temperature effects on pathogen infection

Seeds of cucumber ('Tsuyamidori') were sown in plastic pots filled with commercial potting mix and were cultivated in a controlled environment chamber (25°C, 12 L/12 D). Conidia of *C. cassiicola* (isolate FCCC1716) were collected using the method described in the section above. Subsequently, a conidial suspension (103 conidia mL^{-1}) was prepared using distilled water. Leaves, except for the second true leaf, of a 12-leaf cucumber plant were removed. A conidial suspension of *C. cassiicola* was sprayed (15 µL cm^{-2}) onto the adaxial surface of the second true leaf. Inoculated cucumber plants were put into a sealed clear plastic container and were incubated for 48 h at each temperature (10, 15, 20, 25, 30 and 35°C). After the leaf surface was dry, plants were maintained in the controlled environment chamber (25°C, RH 50% and 12 L/12 D). Lesions on the inoculated leaf were counted seven days after inoculation. Additionally, digital photographs of inoculated leaves were recorded and the leaf areas and lesion areas were measured using specific software (freeware ImageJ; Schneider et al., 2012). For each temperature, three or four plants were used.

Temperature and wetness duration effects on pathogen infection

Cultivation of cucumber seedlings and preparations of conidial suspensions of *C. cassiicola* were prepared as in the section above. However, five-leaf cucumber plants were used for the assay. The conidial suspension of *C. cassiicola* was sprayed (15 µL cm^{-2}) onto the adaxial surface of the third true leaf (in experiment 1), or the second and third true leaves (in experiments 2 and 3). Inoculated cucumber plants were put into a sealed clear plastic container and were incubated at each temperature (10, 15, 20, 25, 30 and 35°C) for various times (6, 12, 18, 24, 30, 36, 42 and 48 h). After the leaf surface was dry, plants were maintained in the controlled environment chamber (25°C, RH 50% and12 L/12 D). Lesions on the inoculated leaf were counted five days after inoculation. For each experimental treatment, 3-5 plants were used.

Inoculation test of cucumber cultivars

Cucumber cultivars that were used in the tests were: 'Sagami-hanjiro Fushinari (#1)', 'SR22 (#2)', 'Fresco 100 (#3)', 'Chinatsu (#4)', 'Excellent Fushinari 353 (#5)', 'Courage (#6)',

'Dessor (#7)', 'Enka (#8)', 'Select I (#9)', 'ZQ-7 (#10)', 'Status Summer III (#11)', 'PS-2 (#12)', 'ViewStar (#13)', 'Senka (#14)', and 'Tsuyamidori (#15)'. Seed supply companies of these cultivars were Takii & Co., Ltd. (#1), Saitama Gensyu Ikuseikai Co., Ltd. (#2, 4, 5 and 10), Kurume Vegetable Breeding Co. (#3, 9, 11, 12 and 13), Tokiwa Co., Ltd. (#6, 7 and 8), Nakahara Seed Co. Ltd. (#14), and Tokita Seed Co., Ltd (#15). 'Sagami-Hanjiro Fushinari (#1)', a Japanese traditional resistant cultivar, was used as a resistant control. 'Tsuyamidori (#15)' was used as a susceptible control. All of the other cultivars are current commercially used cultivars in Japan. Cultivation of cucumber seedlings, preparation of conidial suspension of *C. cassiicola*, and inoculation were performed similarly to the description in the section "Temperature effects on pathogen infection". However, the second and fourth true leaves of five-leaf cucumber plants were inoculated with the pathogen and three plants of each cultivars were used for the tests. Inoculated cucumber plants were put into a sealed clear plastic container and were incubated at 25°C for 48 h. After removal from the containers, the cucumber plants were cultivated in the controlled environment chamber (25°C and 12 L/12 D). At 14 days after inoculation, the numbers and areas of lesions on the inoculated leaves were evaluated. For experiment 1, the leaf area and lesion area were measured using ImageJ (Schneider et al., 2012). For experiment 2, the lesion area was evaluated using the following index: 0 = no symptoms, 1 = lesion area was slight, 2 = lesion area was less than one-eighth of the total leaf area, 3 = lesion area was less than one-fourth of the leaf area, 4 = lesion area was less than half of the leaf area, and 5 = lesion area was more than half of the leaf area. Numbers and areas of lesions were compared using Mann-Whitney U-tests. Statistical analyses were performed using EZR v.1.32 (Kanda, 2013; http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files /statmedEN.html).

RESULTS AND DISCUSSION

All three Japanese isolates of *C. cassiicola* were able to grow at 10-35°C on potato dextrose agar medium (Figure 1). No growth was observed at 5 or 40°C. Optimum temperatures for mycelial growth were approximately 30°C. Differences among isolates were very small.

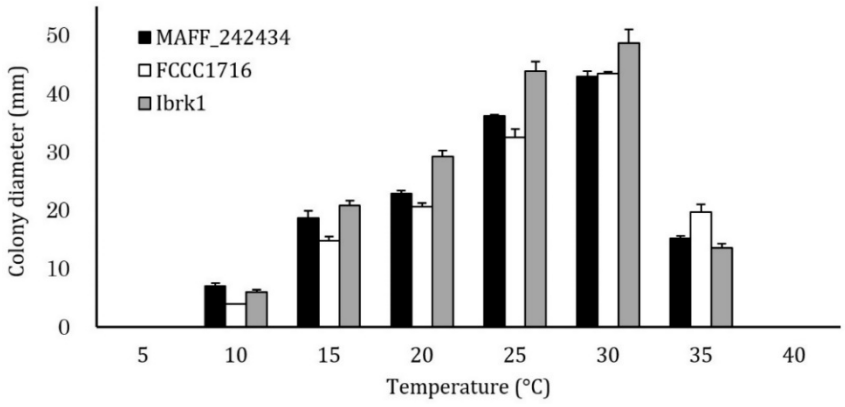

Figure 1. Effects of temperature on mycelial growth of three isolates (MAFF_242434, FCCC1716 and Ibrk1) of cucumber target leaf spot pathogen, *C. cassiicola*. The fungus was cultured on potato dextrose agar for a week in the dark. The average of three plates was calculated for each isolate. Bars show the standard error.

Conidial germination rates of *C. cassiicola* FCCC1716 and Ibrk1 at each temperature were similar. The results obtained for isolate FCCC1716 are presented in Figure 2. The optimum temperatures for conidial germination was 25-35°C. At this temperature range, most of the conidia germinated within four hours. Germination was delayed at other temperatures and was almost totally suppressed at 5°C. These results for mycelial growth and conidial germination are similar to those reported by Hasama (1993). However, approx. 40%

of conidia germinated after 24 h of incubation at 40°C in our study, whereas Hasama (1993) observed no germination at 39°C. In addition, the germination rate after 4 h of incubation at 28°C reported by Hasama (1993) was no greater than 35% which was about half of the rate determined in our study. It is apparent that the isolates in our study could germinate more quickly and could tolerate hotter conditions.

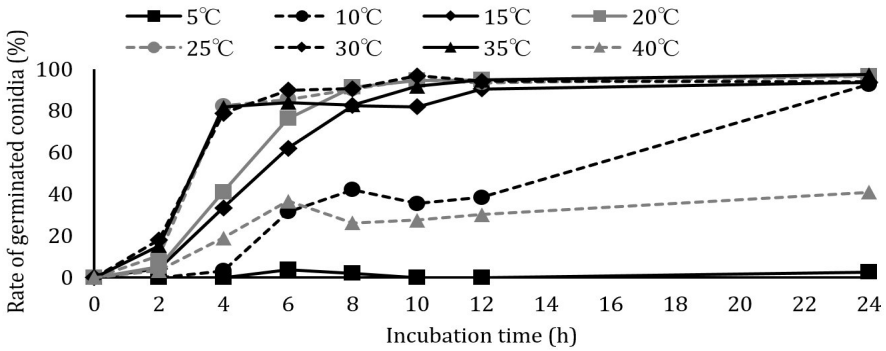

Figure 2. Temperature effects on conidial germination of cucumber target leaf spot pathogen, *C. cassiicola* (isolate FCCC1716). Fungal conidia on water agar plates were incubated for 2-24 h in the dark. One hundred conidia were observed for estimation of the germination rate.

When cucumber plants inoculated with *C. cassiicola* were kept wet for 48 h at 20-30°C, 20-30% of the leaf area was infected (Figure 3). In contrast, the lesion area and number were both small when inoculated plants were kept wet at 10 and 35°C. Development of infection of *C. cassiicola* was apparently suppressed at these temperatures. In contrast, the pathogen germinated and grew well at 35°C (Figures 1 and 2). However, Hasama (1993) reported suppressed formation of an appressorium-like structure at 35°C and this suppression may have inhibited infection.

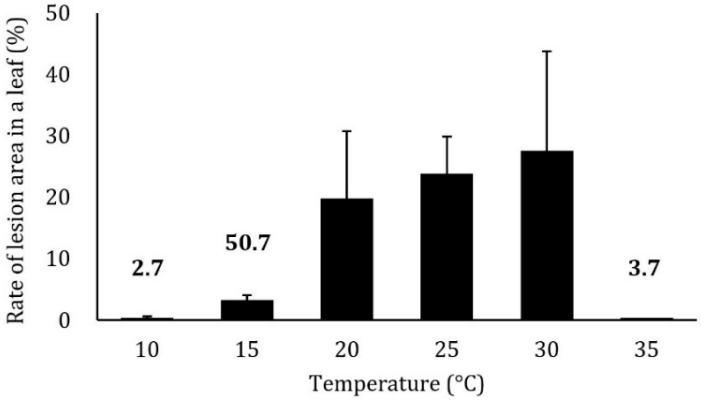

Figure 3. Temperature effects on infection of *C. cassiicola*. Inoculated cucumber plants (*Cucumis sativus*) were kept in a wet condition for 48 h at each temperature. After drying, plants were maintained at 25°C. Lesion areas and numbers were evaluated at seven days after inoculation. The average number of lesions per leaf is shown in each column at 10, 15 and 35°C. Lesions in 20-30°C were mutually fused and uncountable. The error bar shows the standard error.

Effects of leaf-wetness duration on infection of *C. cassiicola* were investigated. Inoculated cucumber plants were kept wet for 6-48 h at 15-30°C, the temperature range where there is a risk of infection. Among the assays conducted, the shortest wetness duration

for lesion formation and the greatest number of lesions were observed in the 25-30°C (Figure 4). This temperature appeared to be optimum for infection of *C. cassiicola* on the cucumber leaves. At this temperature, pathogen infection was observed slightly at 12 h of wetness duration. However, more than 18 h of wetness duration was necessary for infection to be obvious. Extending the period of leaf wetness increased lesion number (Figure 4). Where there were more than 30 h of wetness, the disease symptoms were particularly severe. Hasama (1993) reported that there was slight and obvious infection of *C. cassiicola* on cucumber leaves that were induced by 12 and 24 h of wetness duration, respectively which is consistent with our results. Jones and Jones (1984) reported similar results for target leaf spot of tomato caused by *C. cassiicola*. However, no infection of *C. cassiicola* was observed with a wetness duration of less than 18 h on sweet pepper leaves (Shimomoto, 2010) where 24-48 h of wetness were necessary for obvious symptom development. Cucumber and tomato appear, therefore, to be infected more readily than sweet pepper.

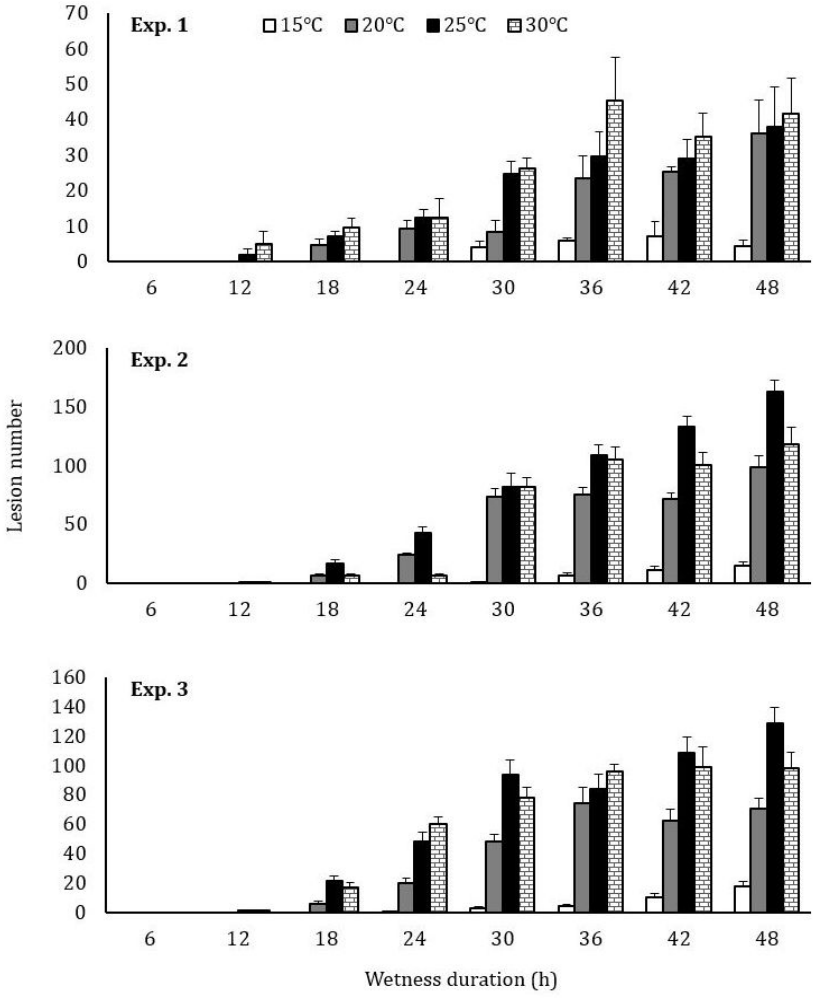

Figure 4. Temperature and wetness duration effects on the infection of cucumber target leaf spot pathogen, *C. cassiicola*. Inoculated cucumber plants (*Cucumis sativus*) were kept wet for 6-48 h at each temperature. After drying, plants were maintained at 25°C. The lesion number was evaluated five days after inoculation. The average (per leaf) was calculated. The bars shows the standard errors.

Resistance of cucumber cultivars against *C. cassiicola* was assessed by evaluating the number and area of lesions on inoculated leaves (Figure 5). 'Sagami-Hanjiro Fushinari (#1)' is a Japanese traditional resistant cultivar whereas the others are recent Japanese commercial

cultivars. In our experiments, the lesion number varied among cultivars. For example, cultivars 'Sagami-Hanjiro Fushinari (#1)' and 'Chinatsu (#4)' showed low lesion numbers in both experiments 1 and 2. By contrast, cultivars 'ViewStar (#13)', 'Senka (#14)' and 'Tsuyamidori (#15)' showed numerous lesions in both experiments 1 and 2. Assessment of the lesion area was not always consistent with lesion number in the respective cultivars. For example, the degree of lesion area on 'SR22 (#2)' was higher than that of lesion number in both experiment 1 and 2, which indicates that cucumber cultivars have resistance of two distinct types: against infection and against the expansive spread in the leaf tissue. The disease resistance of cultivars should, therefore, be evaluated both by the number and by the areal size of the lesions. In our experiments, some cultivars such as 'Sagami-Hanjiro Fushinari (#1)' and 'Chinatsu (#4)' appeared to have resistance of both types against *C. cassiicola*. Three loci controlling highly effective resistance against target leaf spot of cucumber have been reported worldwide (Abul-Hayja et al., 1978; Wang et al., 2010; Wen et al., 2015). However, the resistance of Japanese cultivars used for this study was not absolute, but rather close to tolerance. Additional investigations must be conducted to elucidate the characteristics of the target leaf spot resistance of these Japanese cultivars.

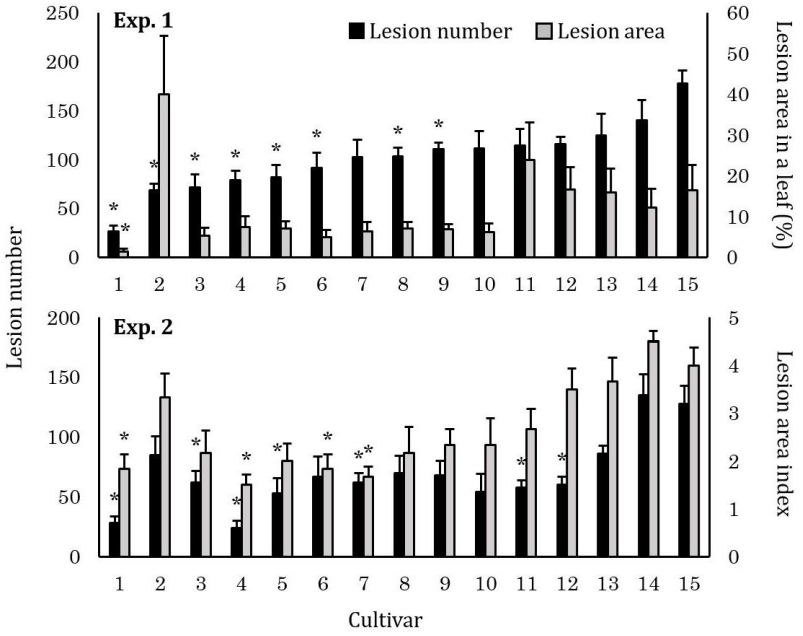

Figure 5. Results of inoculation tests using various cucumber (*Cucumis sativus*) cultivars. Numbers and areas of lesions on leaves were evaluated 14 days after inoculation with *C. cassiicola*. The number (#) of each cultivar corresponds to the number presented in the Materials and Methods section. Black bars represent the average of lesion number per leaf. Gray bars represent average lesion area expressed as a percentage of leaf area (experiment 1) or the average lesion area index (experiment 2). Bars shows standard errors. An asterisk denotes significant difference by Mann-Whitney U test ($p<0.01$) compared with a susceptible control cultivar, 'Tsuyamidori (#15)'.

The findings obtained from this study are expected to be useful as basic information for predicting the occurrence of target leaf spot on cucumber. Disease occurrence prediction permits efficient disease management using minimum amounts of fungicides at optimal timing. Reduction of fungicide usage is expected to reduce labor, costs, and the risk of development of fungicide-resistant pathogens. Additionally, control of environmental and biological factors based on disease occurrence prediction is expected to reduce the disease efficiently. Information about cultivar resistance is useful for controlling *C. cassiicola*,

including avoiding the development of fungicide resistant populations.

CONCLUSIONS

- *C. cassiicola* grew at 10-35°C. The optimum temperature for mycelial growth was 30°C;
- Optimum temperature for conidial germination of *C. cassiicola* was 25-35°C;
- *C. cassiicola* highly infected cucumber leaves at 20-30°C whereas infection at 10 and 35°C was slight;
- Optimum temperatures for infection of *C. cassiicola* on cucumber leaves were in the 25-30°C range. A leaf wetness duration of more than 18 h resulted in obvious infection at these temperatures and wetness of more than 30 h caused severe symptom development;
- Some cucumber cultivars resisted both infection and expansion of *C. cassiicola*.

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Changes in abscisic acid, antioxidant concentration and the activities of antioxidant enzymes in Japanese apricot (*Prunus mume*) under short-term anoxic storage conditions

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Abstract

Application of an anoxic treatment is used as a chemical free and environmentally friendly technique to delay ripening, eliminate physiological disorders, promote antioxidant capacity and extent storage shelf-life of some fruits and vegetables. The effects of an anoxic treatment on lipid peroxidation, abscisic acid (ABA) concentration, antioxidant activity, and the activities of selected antioxidant enzymes in Japanese apricot (*Prunus mume*) were evaluated. In the anoxic treatment used in this study, mature green fruit were exposed to N_2 with a flow rate 200-250 mL min^{-1} for 6 h at 20°C. Non-treated fruit were used as a control treatment. Fruit were kept in corrugated boxes and subsequently stored at 20°C and 90-95% RH. They were randomly collected every two days for analysis. The anoxic treatment delayed changes in fruit color, induced 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, and ferric reducing antioxidant power (FRAP), and increased total phenolic concentrations (TPC). The anoxic treatment moderated the integrity of fruit membranes by delaying malondialdehyde (MDA) accumulation, increasing ABA concentrations, delaying the decline of superoxide dismutase (SOD) and catalase (CAT) activity, and promoting peroxidase (POD) activity. The results suggest that an anoxic treatment may delay the ripening processes in Japanese apricot by promoting the activities of antioxidants and the levels of antioxidant enzymes.

Keywords: abscisic acid, anoxic treatment, ripening, SOD, CAT

INTRODUCTION

Apricot (*Prunus mume*) is classified as a climacteric fruit and its shelf-life is generally limited. Ripening of the fruit can be delayed with low temperatures, controlled atmospheres or chemical treatments (Fagundes et al., 2015; Park et al., 2018; Wang et al., 2018). An anoxic treatment is an alternative technology that can be used for regulating the ripening process (Pesis, 2005), reducing decay development in tomatoes (*Lycopersicon esculentum*) (Fallik et al., 2003) and maintaining postharvest quality in peaches (*Prunus persica*) (Lara et al., 2011). Furthermore, Li et al. (2013) reported that application of a low oxygen treatment promoted antioxidant enzyme activities by reducing reactive oxygen species (ROS) levels and lipid peroxidation in mushroom (*Pleurotus eryngii*). It is widely considered that anoxic treatment is attractive because of its effectiveness and the relatively low cost of implementation.

ROS such as hydrogen peroxide (H_2O_2), the hydroxyl radical ($^{\cdot}OH$) and the superoxide radical (O_2^{-}) generally originate in the mitochondria, chloroplast and apoplast during respiration and photosynthesis (Tovar-Méndez et al., 2011). The formation of ROS causes cell damage by interfering with the activity of proteins, nucleic acids, and the lipid membrane (Choudhary et al., 2012). An excess of ROS also influences the concentrations of some plant hormones such as abscisic acid (ABA), which respond to various environmental stresses (Hu

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et al., 2005). Recently, Kowitcharoen et al. (2015) showed that endogenous ABA concentrations and antioxidant activities increased significantly under abiotic stress in sugar apple (*Annona squamosa*). Moreover, the application of ABA induced an increase in the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR) (Choudhary et al., 2012).

An anoxic treatment was shown to significantly delay fruit softening in parallel with suppression of ethylene production in Japanese apricot (Phonyiam et al., 2016b). However, there are no reports on the role of an anoxic treatment on the ripening processes and antioxidant systems in Japanese apricots. The study, therefore, aimed to investigate the effect of anoxic conditions on the changes in ABA concentrations and antioxidant systems during ripening in 'Inazumi' Japanese apricot.

MATERIALS AND METHODS

Fruit materials and treatment

Fruit of Japanese apricot 'Inazumi' at 105 days after full bloom (DAFB) (before the ripening stage), were obtained from an experimental field located at 35°N; 140°E at Chiba University, Japan.

The fruit were randomly assigned to two groups of 150 fruits each. The apricots were placed into plastic chambers and then exposed to pure nitrogen. The oxygen concentrations in the chambers was measured in the headspace with a gas analyzer to be 0.05% (v/v). The treatments were left for six hours with humidified N_2 flow (200-250 mL min^{-1}). Untreated fruit were used as a control. Fruit were kept in corrugated boxes and then subsequently stored at 20°C and 90-95% RH. Samples were randomly collected every two days. Fresh samples were freeze-dried and stored at -30°C until analysis. Sample weight was expressed on a dry weight basis.

Changes in skin color were measured with a Chroma meter (Mini Scan EZ 45/0 Hunter Lab). Following Ziosi et al. (2008), a and b values were calculated and expressed as hue angle ($h° = tan^{-1}$ b/a). In order to interpret the color, red was determined as an angle of 0°, yellow as 90°, and green as 180°.

Measurement of lipid peroxidation

Lipid peroxidation was determined by the production of malondialdehyde (MDA) following Song et al. (2009) with some modifications. A freeze-dried sample (0.4 g) was mixed with 3 mL of 10% trichloroacetic acid (TCA). The sample was incubated overnight at 4°C. The homogenate was centrifuged at 6,600 *g* for 10 min at 4°C. A supernatant of 0.4 mL was mixed with 1.6 mL of 0.5% (w/v) thiobarbituric acid (TBA) in 10% (w/v) TCA, incubated for 30 min in boiling water and then cooled quickly. Absorbance was measured at 532 nm by spectrophotometer (GENESYS 10S, USA). The non-specific turbidity was corrected by deducting the absorbance at 600 nm. An extinction coefficient of 155 mM cm^{-1} was used for determining the MDA concentrations.

Measurement of antioxidant activity and total phenolic concentrations

Freeze-dried samples (500 mg) were extracted in 10 mL of 80% methanol and then shaken overnight at room temperature. The mixtures were filtered using Whatman filter paper No.1, and the methanolic extract was stored at -30°C until analysis.

Free radical scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) was assayed following Phonyiam et al. (2016a) with modifications. A sample of the extract (10 µL) was mixed with 3 mL 0.1 mM DPPH-methanolic solution and was then incubated in the dark for 30 min at room temperature. The decrease of absorbance was assayed at 517 nm by spectrophotometer (GENESYS 10S, USA) and scavenging of DPPH radical activity was expressed as mmol of trolox kg^{-1}.

Ferric reducing antioxidant power (FRAP) activity was investigated according to Phonyiam et al. (2016a). A sample of the extract (400 µL) was added to 2.6 mL of FRAP reagent and the mixture was incubated for 30 min at 37°C. After incubation, the mixture was

monitored at 595 nm and concentrations of FRAP were expressed as mmol of Fe^{2+} kg^{-1}.

Total phenolic concentrations were determined following Salem et al. (2013) with slight modifications. A methanolic extract of 250 µL and 1.25 mL of the Folin-Ciocalteu's phenol reagent were mixed. One mL aliquot of 7.5% (w/v) sodium carbonate (Na_2CO_3) was added to the mixture after incubation for 3 min. The mixture was then incubated for 1 h at room temperature. The phenolic concentrations were determined at 765 nm and results were expressed as g of gallic acid kg^{-1}.

Extraction and determination of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) activities

A 500-mg sample of the freeze-dried sample was dissolved in 5 mL of extraction buffer containing 1% (w/v) polyvinyl pyrrolidone (PVP) and 1 mM ethylenediaminetetraacetic acid (EDTA). The mixture was incubated for 3 h at 4°C, and then centrifuged at 15,000 *g* for 10 min at 4°C. The supernatant was used for determining the SOD, CAT and POD activities by spectrophotometer (GENESYS 10S, USA) assay.

The SOD activity was evaluated by inhibiting the nitrobluetetrazolium (NBT) reduction, which was monitored at 560 nm according to the recent method of Wu et al. (2014) with slight modifications. Sodium phosphate buffer of 0.1 M (pH 7.8) was used for extraction. The reaction mixture (3 mL) contained 200 µL of crude extract, 1.6 mL of 50 mM phosphate buffer (pH 7.8) and 300 µL for each of 100 mM EDTA, 130 mM *L*-methionine, 750 µM NBT and 20 µM riboflavin. The mixed solutions were illuminated with luminescent lamps for 10 min and monitored at 560 nm. The reaction mixture was placed in the dark, and it served as a blank. The SOD activities were expressed as enzyme units kg^{-1} protein.

CAT activity was evaluated by measuring the decomposition of H_2O_2 according to the assay developed in Wu et al. (2014) with slight modifications. A 0.1 M sodium phosphate buffer (pH 7.0) was selected for extraction. The assay mixture (2 mL) comprised 1.5 mL of 5 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0) and 0.5 mL of crude extract. The spectrophotometric measurement was monitored at 240 nm after incubation for 10 min. The specific activities of CAT were expressed as enzyme units kg^{-1} protein by using an extinction coefficient of 0.0436 mM^{-1} cm^{-1}.

The POD activity was assayed according to the method of Wu et al. (2014), using guaiacol as the substrate. The reaction mixture consisted of 0.5 mL of crude enzyme, 1 mL of 100 mM Na-phosphate buffer (pH7.0) and 0.5 mL of 8 mM guaiacol. The mixture was incubated for three minutes at room temperature. One mL aliquot of 24 mM H_2O_2 was then added to the mixture. The increase in absorbance at 470 nm was recorded every 30 s for 3 min using a spectrophotometer. The POD activity was expressed as enzyme units kg^{-1} protein.

Protein content was evaluated following the method of Bradford (1976) using bovine serum albumin (BSA) that served as the standard.

Extraction and determination of abscisic acid (ABA)

ABA concentration was analyzed according to the method of Kowitcharoen et al. (2015). Freeze-dried sample (1 g) was homogenized in 20 mL of 80% (v/v) methanol with 0.1 g butylatedhydroxy toluene (BHT) antioxidant, 0.1 g ascorbic acid, and 0.5 g polyvinylpolypyrrolidone (PVPP). A 200 µL of ABA-d_6 (an internal standard) was then added. The homogenate was centrifuged at 15,000 *g* for 15 min at 4°C. The solution was filtered to obtain an aqueous solution. The extracted sample was adjusted to a pH of 2.5 by 0.1 M hydrogen chloride (HCl) and partitioned three times with 100% (v/v) ethyl acetate. The ethyl acetate was removed through evaporation. The dried sample was re-dissolved using 1.5 mL of 100% ethyl acetate. The samples were purified by high performance liquid chromatography (HPLC; Spectroscopic, Tokyo, Japan) and identified using gas chromatography mass spectrometry (GC-MS-SIM; model QP5000; Shimadzu, Kyoto, Japan).

Statistical analysis

The statistical analyses were carried out using SPSS version 16.00 software (IBM Institute, NC). The analysis of data was expressed as mean ± standard deviation (SD). The

differences were compared with T-tests at $p<0.05$.

RESULTS

Coloration

The anoxic treatment was able maintain a light green peel color for up to six days of storage (DAS). The hue value was measured at 107.7 (Figure 1) whereas the untreated fruit which turned yellow, had a hue value of 89.4.

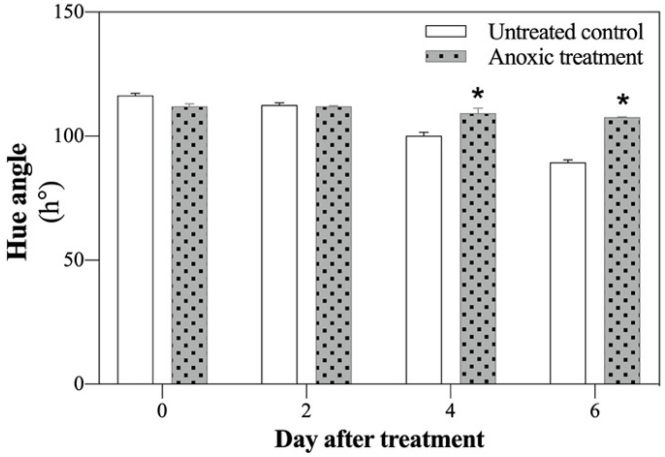

Figure 1. Changes of hue angle in 'Inazumi' Japanese apricot during storage at 20°C. Values are the means ± SD of three replications, * significant at $p<0.05$.

MDA concentration, DPPH and FRAP activities, and TPC concentration

The concentration of MDA in the untreated fruit gradually increased throughout the storage period (Figure 2A). In contrast, the MDA levels in the anoxic-treated fruit significantly declined to four DAS and then increased but remained below the untreated value.

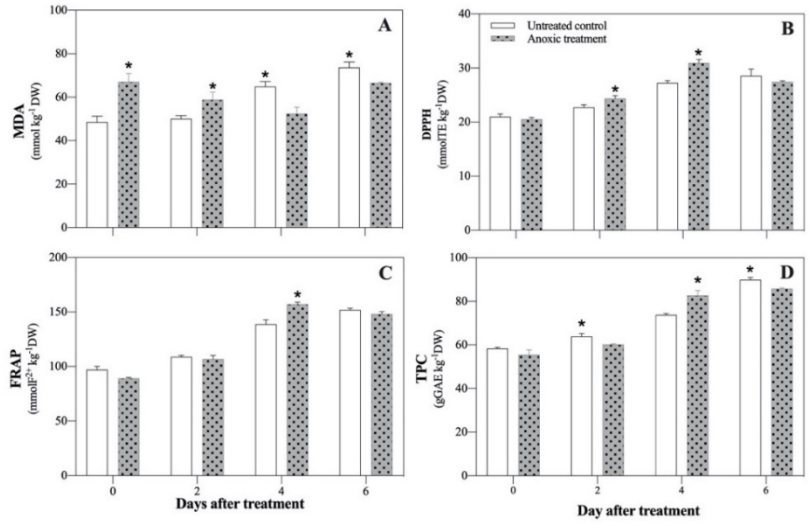

Figure 2. Changes of MDA (A), DPPH (B), FRAP (C) and TPC (D) in 'Inazumi' Japanese apricot during storage at 20°C. Values are the means ± SD of three replications, * significant at $p<0.05$.

The DPPH radical scavenging activities in the anoxic treated fruit were higher than those in the untreated fruit (Figure 2B). FRAP did not show significant differences except for four DAS (Figure 2C) when the FRAP level in the anoxic-treated fruit was higher than that in the untreated fruit. The total phenolic concentration in both treatments gradually increased during storage (Figure 2D). However, total phenolic concentration did not show any clear or consistent differences between anoxic-treated fruit and the untreated fruit.

SOD, CAT, and POD activities and ABA concentration

SOD activities in both treated and untreated fruit gradually decreased during storage (Figure 3A). However, SOD activities in the anoxic-treated fruit were higher than those in the untreated fruit throughout. CAT activities in anoxic-treated fruit were also higher than those in the untreated control (Figure 3B) except at six DAS. POD activities continuously increased throughout the storage period (Figure 3C) and were higher in the anoxic-treated fruit than in the untreated control at four and six DAS.

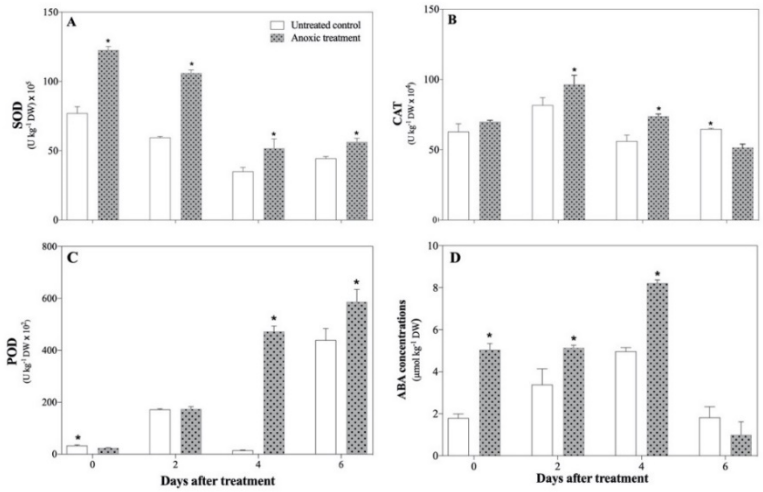

Figure 3. Changes of SOD (A), CAT (B), POD (C) and ABA (D) in 'Inazumi' Japanese apricot during storage at 20°C. Values are the means ± SD of three replications, * significant at $p<0.05$.

The anoxic treatment significantly increased ABA concentration (Figure 3D) except at six DAS where values were low. The highest concentrations of ABA in both the anoxic-treated and untreated fruit were observed at four DAS.

DISCUSSION

In general, color development, due to chlorophyll degradation, is the primary physiological change that occurs during ripening of various fruits and vegetables (Kasampalis et al., 2020). Recently, it has been shown that anoxic conditions could effectively delay color development in tomatoes and pineapples (*Ananas comosus*) when compared with untreated fruit stored at room temperature (Techavuthiporn et al., 2017). The results in this study showed that anoxic treatment greatly delayed fruit color development. This finding may relate to the enhanced concentrations of antioxidants and the activities of enzymatic antioxidants, resulting in a delay in the ripening of Japanese apricots during storage at 20°C. These results correlate with previous research which showed that anoxic application for six hours delayed ripening of Japanese apricot via inhibition of ethylene and 1-aminocyclopropane-1-carboxylic acid (ACC) production (Phonyiam et al., 2016b).

MDA is a secondary by-product of lipid peroxidation caused by abiotic stress, which is used as an indicator of cell oxidative damage (Xu and Liu, 2017). A previous study of jujube fruit (*Ziziphus mauritiana* Lamk.) indicated that MDA concentration increased at the ripening

stage with a concomitant increase of oxidative stress (Kumar, 2008). The results presented here show that, although suppressed by the anoxic treatment, an increase in MDA was found not only in anoxic-treated fruit, but also in the untreated fruit at the ripening stage. These results suggest that the increase in MDA concentration was induced by both anoxic stress and senescence.

It has been shown previously that anoxic application enhances the increase in antioxidant activity, as determined by measures such as ascorbic acid, glutathione, DPPH and FRAP values, in pineapple fruit during storage at room temperature (Phonyiam et al., 2016a; Techavuthiporn et al., 2017). The concentrations of total phenolic compounds are enhanced in plants as a resistance mechanism against many stress conditions (Setha, 2012). In this study, the increase in antioxidant activities and phenolic concentrations were higher at 4 DAS in the anoxic treated fruit. These results are consistent with those of You et al. (2012) who reported higher levels of antioxidant activity and phenolic concentrations in anoxic-treated Chinese water chestnuts (*Eleocharis tuberosa*) slices. In addition, the high antioxidant activities and phenolic concentrations may be associated with resistance to lipid peroxidation under short anoxic conditions.

The activities of both enzymatic and non-enzymatic antioxidants are affected by the disruption of ROS activities. This study showed that SOD and CAT activities in the fruit decreased in the later stages of ripening during storage. Previous studies have also shown that SOD and CAT activities decrease during ripening (Kumar, 2008; Murshed et al., 2013). Furthermore, Resende et al. (2012) showed that the decrease of CAT activity coincided with an increase in MDA concentrations during ripening in papaya (*Carica papaya*). The result presented here show that POD activity in anoxic treated fruit was generally higher than that in untreated fruit. Song et al. (2009) similarly showed that anoxic stress stimulated an increase in POD activity in kiwifruit (*Actinidia deliciosa*) during low temperature storage. Lotfi et al. (2015) reported that an increase of POD activity under anoxic conditions may be related to the protection of cell walls against lignification and crosslinking damage. These results in this study, overall, indicate that several enzymatic antioxidants, such as SOD, CAT and POD, may have a role in regulating ROS levels in Japanese apricot under short anoxic conditions.

ABA is correlated to the ripening process in both climacteric and non-climacteric fruit (Saito et al., 2018) and Bulgakov et al. (2019) reported that ABA plays a role in the regulation of plant stress defenses. In this study, the ABA concentrations in Japanese apricot were enhanced, together with induction of SOD, CAT and POD activities, under anoxic conditions. It is possible, therefore, that the increase in ABA concentration was correlated with enzymatic antioxidant activities which may be essential for activating plant defenses against stress.

CONCLUSIONS

The anoxic treatment increased ABA concentrations, enzymatic antioxidants (SOD, CAT and POD), antioxidant activities and total phenolic concentrations, while decreasing MDA production and delaying the changes of fruit color during storage.

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Effect of storage temperature and light irradiation on respiration rate, red color development, and gene expression involved in carotenoid synthesis in green bell pepper (*Capsicum annuum* L.)

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Abstract

Bell pepper is a popular vegetable and a good source of beneficial elements for the human diet, such as vitamins, minerals, and carotenoids. The environmental condition surrounding the bell pepper is an important factor affecting its external and internal quality. During the external color change phase, the chlorophyll degradation and carotenoid synthesis is an important consumer attribute of green bell pepper after harvest. The objective of this study was to investigate the effect of storage temperatures (10, 20 and 30°C) and light irradiation on the quality, respiration rate and gene expression of fresh bell pepper. Measurements were conducted every five days throughout the storage period (25 days). The result showed that low storage temperature reduced the respiration rate and delayed the color development in green bell pepper. Light irradiation, stimulated the color change from green to red, when compared to the dark condition. Higher storage temperature and light irradiation showed high expression of the genes encoding phytoene synthase (*CaPsy*), ζ-Carotene desaturase (*CaZds*), β-carotene hydroxylase (*CaCrtZ-2*), and capsanthin/capsorubin synthase (*CaCcs*), involved in carotenoid synthesis. These findings indicate that high storage temperature combined with light irradiation stimulated carotenoid synthesis in harvested green bell peppers.

Keywords: green bell pepper, respiration rate, storage temperature, light irradiation, carotenoid synthesis, gene expression

INTRODUCTION

Bell pepper (*Capsicum annuum* L.) is an important vegetable crop that is cultivated in warm climates. It can be consumed at many color stages, green, red, yellow, or orange (Baenas et al., 2019; Ilić et al., 2017). Bell peppers are rich sources of antioxidants and bioactive compounds such as vitamin A, C, phenolic compounds, carotenoids, and flavonoids that are necessary in the human diet. Bell peppers are a perishable vegetable and require proper management to maintain their quality and shelf-life. Respiratory activity and shelf life depend on surrounding environmental factors influenced by the stage of maturity, storage temperature, storage condition, gas concentrations, and storage time. All affect the physical decay and rapid senescence, water loss, and its susceptibility to chilling injury (Baenas et al., 2019; Cisternas-Jamet et al., 2020; Keshri et al., 2019).

The external color is the first attribute that determines the commercial quality and consumer acceptance of bell pepper. Temperature is the key environmental factor affecting the quality in the postharvest process. Bell peppers are commonly sold from temperature-controlled shelves in supermarkets or at ambient temperature in the local wet markets. The difference in temperature affects the color change in green bell pepper. The lighting in the supermarkets may also affect the color change of the bell pepper. Not only is color affected,

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but the temperature and light irradiation can also affect the physiological response by regulating cell function and metabolism. In plants, light does not only cause stress in plant tissues, but also influences the postharvest behavior and gene expression. Especially when fruits and vegetables are irradiated during storage. This stimulates biosynthesis in fruit and vegetables such as carotenoid synthesis (Denoya et al., 2020; Loi et al., 2019; Yuan et al., 2017).

Carotenoids are visual signals of bell pepper maturation. The color of the pepper may change from green to yellow, orange, or red, depending on the type of carotenoids synthesized and accumulated (Martínez-López et al., 2014). Carotenoid genetic differences and degree of maturation is influenced by environmental factors.

Figure 1 shows the carotenoid biosynthetic pathway in plants. The first step of carotenoid biosynthesis is the formation of phytoene from geranylgeranyl pyrophosphate catalyzed by phytoene synthase (Psy). Phytoene desaturase (Pds) carries out a two-step desaturation reaction of phytoene, which is subsequently converted into phytofluene and ζ-carotene, the first visible carotenoid (Berry et al., 2019; Gómez-García and Ochoa-Alejo, 2013; Liu et al., 2020). The two-step desaturation reaction catalyzed by Zds conversion of ζ-carotene into lycopene through neurosporene. Lycopene is the precursor of cyclic carotenoids such as β-carotene, an important component of the reaction centers and antenna for the photosynthetic apparatus. Lycopene is also a substrate for the biosynthesis of various other carotenoids (Gómez-García and Ochoa-Alejo, 2013). Capsanthin/capsorubin synthase (Ccs) is the enzyme catalyst that transforms the 5,6-epoxycarotenoids antheraxanthin and violaxanthin into capsanthin and capsorubin, respectively (Baenas et al., 2019; Gómez-García and Ochoa-Alejo, 2013).

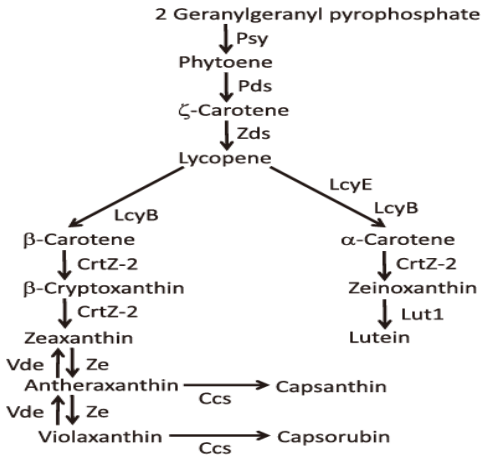

Figure 1. Carotenoid biosynthetic pathway of sweet pepper (*Capsicum annuum* L.) (Nagata et al., 2015).

The aim of this study is to investigate the change in physical properties and expression of genes involved in the carotenoid biosynthesis of green bell pepper during storage at different temperatures with or without continuous light irradiation.

MATERIALS AND METHODS

Plant materials

Pepper fruits (*Capsicum annuum* L.) were obtained from Ibaraki Prefecture, Japan. Peppers were transported to the laboratory at Matsudo campus, Chiba University, Japan. Pepper fruit with two ripening stages: fully ripe (red pepper) and commercial maturity stages (green pepper) were selected based on uniformity of size without damage, decay, and defects. 170 fruits were used in this experiment. Each pepper fruit was randomly packed in polypropylene (PP) bag (20 μm in thickness) with five fruits per bag. Then stored at three

different temperatures (10, 20 and 30°C) in dark condition as well as a light irradiation condition at 30°C. In light condition, green bell pepper was exposed to continuous light irradiation throughout the storage period. The five pepper fruits were removed from the bag every five days to check mass, respiration rate, color and gene expression throughout the storage (25 days). Samples for the continuous non-destructive measurements were repacked and stored after each measurement.

Respiration rate measurement

The mass value of the pepper fruit was taken at the beginning of each experimental sample day. After the mass value measurements, fruits were put in a 1-L acrylic jar set in the respective temperature chamber. One mL of the headspace gas was withdrawn using the gastight glass syringe. CO_2, O_2 and N_2 were analyzed using the gas chromatograph equipped with TCD detector (GC-8APT, Shimadzu, Japan). Gas analyses were done 1, 2 and 3 h after closing the jar. The respiration rate was calculated using the following equation:

$$\mathrm{Qc} = \frac{\Delta Cc \times (V_0 - V) \times Pc \times T_0}{10^2 \times T \times M \times t} \times 1000$$

where, Qc: gas production rate (mg kg^{-1} h^{-1}), ΔCc: gas concentration difference (%), V_0: volume of the jar (L), V: sample volume (L), Pc: gas density, T_0: 273.15 (K), T: measurement temperature (K), M: sample mass (kg), and t: measurement time (h).

Color measurement

The skin color of the bell pepper fruit measured using a colorimeter (CR-300, Minolta, Japan). Measurements were conducted at three points vertically (top, middle, and bottom) and three points on the circumference. The L*, a*, and b* values were recorded for each measurement.

Processing of reverse grayscale image (showing red color "CIE a*" by black color)

Plates of the color image from green bell pepper were imported to the image analysis software (Adobe, Photoshop CC 2019). In this software, L*, a*, b* color mode was selected. The red color (a*) information was extracted from the image and invert. Therefore, the red color information can be seen by black color in the processed image.

Gene expression analysis

Gene expression analysis was carried out by the method described in Thammawong et al. (2014). Primers for four target genes and a house keeping gene (*β-Tubulin*) used as a normalizer are shown in Table 1.

Table 1. Primers used for the quantitative RT-PCR.

Gene name	Accession no.	Forward primer	Reverse primer
CaPsy	X68017	5' GCAGGTCTATCCGACGAAGA 3'	5' GAAGATTCTCCATTTATCGGTCA 3'
CaZds	X89897	5' AGATGGGTCCGCTGGATT 3'	5' GTCACTAACCGGTTCGACAAA 3'
CaCrtZ-2	Y09225	5' CGAGCTGAACGATATTTTTGC 3'	5' ATGGTTGAAACCGAATGAAAA 3'
CaCcs	X77289	5' AGTGCACTGTCCCCTTGGT 3'	5' TAATTCAAAGGCTCTCTATTGCTAGAT 3'
CaβTub[a]	EF495259	5' GGAGATGTTCAGGAGGGTGA 3'	5' AAGAAAGCCTTGCGCCTAA 3'

[a]*β-Tubulin* was used as normalizer.

Statistical analysis

All data were collected, and analyzed for the mean and ± standard deviation of three replicates except for the respiration measurement. Results were compared by two-way analysis of variance (ANOVA) and Tukey test using the software SPSS 20 (SPSS Inc., USA). Differences at $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

Respiration rate

Temperature is an important factor that affects the respiration rate of bell pepper. Figure 2 shows the effect of storage temperatures on the respiration rate of bell pepper stored at 10, 20 and 30°C. Green bell pepper stored at 30°C had the highest respiration rate compared with the other storage temperatures. Hameed et al. (2015) reported that under low temperatures, slow respiration rates and every 10°C increase, the respiration rate doubled. Singh et al. (2014) also observed reduced respiration rate in green mature capsicum, tomato and guava by decreasing storage temperature.

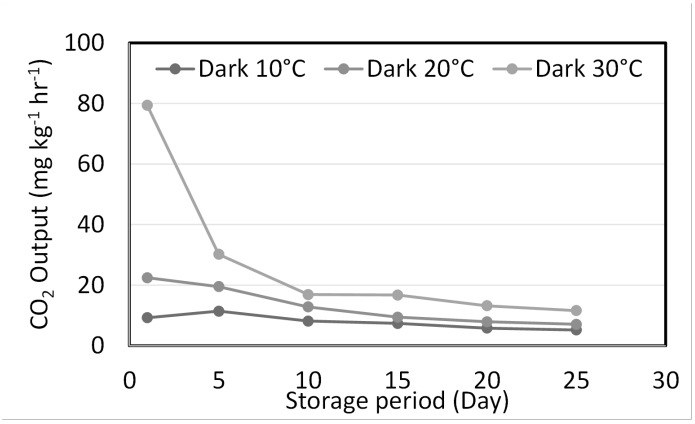

Figure 2. Respiration rate of green bell pepper at 10, 20 and 30°C during storage.

The storage period also affected the respiration rates of green peppers. Under 30°C, the highest respiration rate of 79.40 (CO_2 mg kg^{-1} h^{-1}) was observed after one day from harvesting and it decreased sharply. Except for the earlier stage of storage at 30°C, the respiration rates gradually declined until the end of storage period for all temperatures. At the end of the storage, respiration rates of bell pepper were 5.2, 7.1 and 11.6 CO_2 mg kg^{-1} h^{-1}, respectively. These results were similar to that reported by Singh et al. (2014). Respiration rate is an indicator of the overall physiological activity. Thus, the storage of green bell pepper at 10°C will be beneficial for quality maintenance compared to other storage temperatures.

Color

Carotenoids are visual markers of bell pepper maturation. Figure 3 shows the color change for fruit stored at 10°C (dark), 20°C (dark) and 30°C (light/dark). It was obvious that the red color developed in the fruits stored at 30°C (Figure 3C, D) was enhanced by light irradiation (Figure 3D). There was no visible color change of the fruit stored at 10 and 20°C during 25 days of storage.

Figure 4 shows the change in CIE a* value of green bell pepper during storage at 10, 20 and 30°C. The fruit stored at 30°C with light irradiation showed a sharp increase in CIE a* value and a significant difference after 20 days and onwards. The fruit stored at 30°C without light irradiation showed a slight increase in CIE a* value with a significant difference after 25 days of storage. Statistical analysis between with light and without light showed a significant difference after storage for 20 and 25 days. This proved that light irradiation promoted red color development of bell pepper at a storage temperature of 30°C. There was no statistical difference in color values among the fruit stored at 10 and 20°C during 25 days of storage.

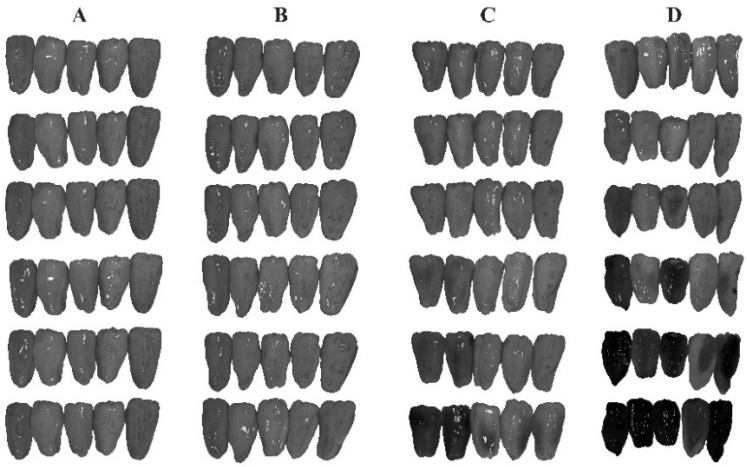

Figure 3. Reverse grayscale image (showing the red color (a*) intensity by black color) of the green bell pepper before and after storage at 10°C (A), 20°C (B) and 30°C (C) under dark and 30°C light (D)conditions stored for 0, 5, 10, 15, 20 and 25 days from top to bottom.

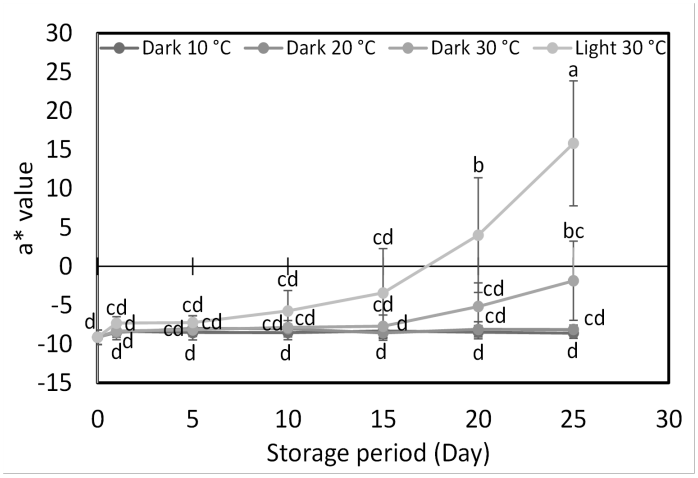

Figure 4. Color (a* value) for green bell pepper storage at 10, 20 and 30°C in dark and light conditions during storage. Means denoted by different letters indicate significant differences between treatments ($p<0.05$).

The color change in green pepper involves chlorophyll degradation and red color development. Hameed et al. (2015) reported that the retention of chlorophyll was higher at lower temperatures. It was found that irradiation with light plays a role in stimulating the development of red color in green peppers during storage. Under lighting and a storage temperature of 30°C, a slight color change can be seen after 10 days (Figures 3 and 4), whereas no obvious changes were observed under the dark conditions. Therefore, this confirmed that light irradiation can promote the color change of green bell pepper. Takahashi et al. (2018) investigated the use of light irradiation for promoting color development in sweet pepper and reported an increase in the carotenoid content by lighting. Light irradiation is also reported to have an effect on de-greening the peel of citrus fruit (Yuan et al., 2017). Green peppers contain yellow carotenoid pigments but are usually masked by chlorophyll. The degradation of chlorophyll may play role in color change in green bell pepper. In tomato fruit, a decrease in chlorophyll and biosynthesis of lycopene are promoted by ethylene. However, for sweet pepper, research is unable to confirm that ethylene promotes coloring (Hornero-Méndez et al.,

2000; Takahashi et al., 2018). The mechanism of color synthesis in green bell pepper needs to be determined.

Gene expression

To identify the effects of temperature and light irradiation on the expression of genes involving carotenoid biosynthesis in green bell pepper during storage, four target gene were selected for the quantitative RT-PCR. Table 1 shows the primers used for qRT-PCR (Nagata et al., 2015).

Figure 5 shows the results of qRT-PCR of the four genes from two maturity stages (mature green and red ripe) of non-stored green bell pepper and that for stored mature green bell pepper. There are big differences between mature green and red ripe. Red ripe bell pepper showed significantly higher levels in the expression of all four genes.

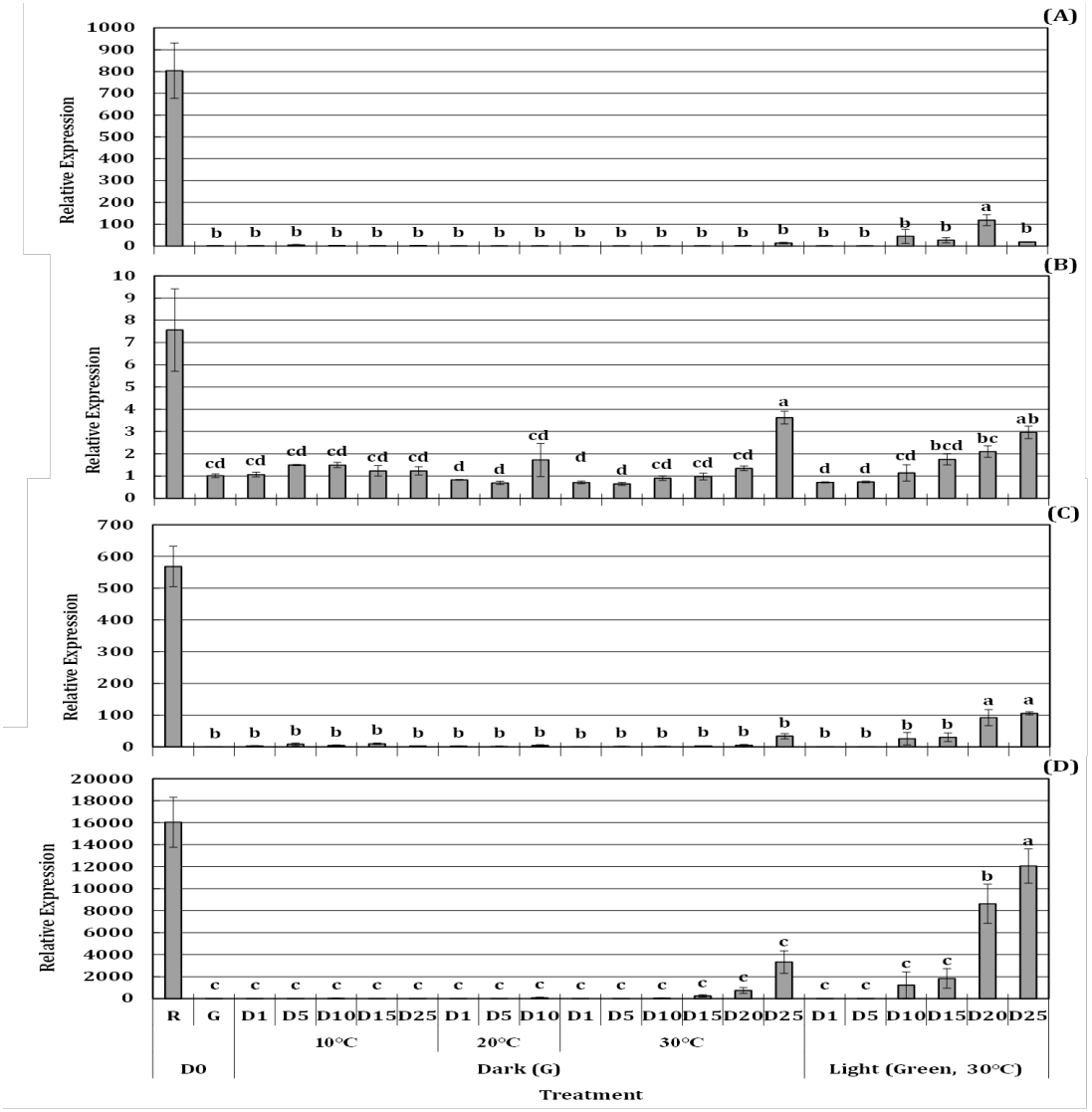

Figure 5. Expression levels of *CaPsy* (A), *CaZds* (B), *CaCrtZ-2* (C), and *CaCcs* (D) normalized against β-Tubulin in bell pepper based on quantitative real-time PCR analysis. Means denoted by different letters indicate significant differences between green bell pepper (excluding full ripe (red) bell pepper) ($p<0.05$). (Dx means days of storage. R and G in D0 are red bell pepper and green bell pepper without storage).

Phytoene synthase encoding gene (*CaPsy*) is known to contribute to carotenoid biosynthesis during fruit ripening (Takahashi et al., 2018). In Figure 5A, light irradiated green bell pepper showed a transient increase of *CaPsy* expression. No significant increase was observed from fruit stored under dark conditions at 10, 20 and 30°C.

The increasing trend, during storage was observed for *CaZds* in the fruit stored at 30°C (Figure 5B). Gómez-García and Ochoa-Alejo (2013) reported that the expression of *Zds* was low in green fruits and a slight increase before the detection of carotenoid synthesis was observed, and levels then remained constant throughout ripening.

In Figure 5C, the increase in *CaCrtZ-2* expression during storage was found in bell pepper stored at 30°C in light conditions. Under dark conditions (30°C), slight increase (no significant difference) was observed at the end of the storage period.

Capsanthin or capsorubin synthase (Ccs) is the key enzyme producing the capsanthin. This is the major pigment in red bell pepper. At a storage temperature of 30°C, Ccs encoding gene (*CaCcs*) significantly increased, especially under light conditions (Figure 5D). These observations are consistent with Gómez-García and Ochoa-Alejo (2013) who reported that *CaCcs* expression was observed in mature chili peppers during ripening.

The relationship between the content of carotenoids including capsanthin and the gene expression level should be investigated further to fully understand the mechanism of carotenoid metabolism in green bell pepper. The chlorophyll degradation and its transcript control should also be studied to understand the color change in green bell pepper after harvest.

CONCLUSIONS

The respiration rate of bell pepper fruits was affected by storage temperature and storage period. The color change in bell pepper was enhanced by high storage temperature and light irradiation. In terms of the gene expression levels, involving the carotenoid pathway, results indicated that bell peppers exposed to light condition at 30°C had significantly high level of expression. This suggests higher capsanthin (the major carotenoid in the bell pepper) content and red color development.

ACKNOWLEDGEMENTS

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Developing the typical ripening pattern curve model for tomato harvested at mature green stage based on the pericarp color using the CIE a* value

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Abstract

This study aims to develop a model predicting the red color development for tomatoes that were harvested at the mature green stage of development as a function of the storage period. The model predicting change in CIE a* value based on the storage period was developed by modifying the original sigmoid-type function model with cumulative ethylene production as a free variable based on a previous study. The modification was conducted by changing the free variable from cumulative ethylene production to storage period. However, the modified sigmoid-type function model was only able to describe the change in red color development in the range where CIE a* value is greater than zero. Therefore, data were divided into two parts based on the CIE a* value, below zero (P1) and greater than zero (P2). For P1, it is possible to know the time lag. This is the time taken for mature green tomatoes to reach the onset of the red color development. For P2, it is possible to apply the modified sigmoid-type function model for the change in CIE a* value in time. The typical ripening pattern curve of tomato was obtained by combining all the parameters for P1 and P2. Findings showed that this model can predict the ripening process of tomato clearly compared to our previous model. The previous model developed was based on cumulative ethylene production. Therefore, this typical ripening pattern curve will enable us to predict the ripening process of tomato harvested at the mature green stage based on the storage period.

Keywords: mature green tomato, modeling, ripening, ripening pattern, sigmoid, tomato

INTRODUCTION

Tomato is one of the world's most popular, economically significant, and widely irrigated vegetable crops that contain important specific nutritive (Gallardo et al., 2006). It has been broadly reported that the ripening process in tomato is associated with the development of red color and the onset in ethylene production. Compared to the onset of ethylene production, red color development is a visible parameter that can be evaluated without complicated methods or equipment. Furthermore, it was reported by Kim et al. (2015) that red color development is the most critical external characteristic for visual assessment of ripeness in tomato fruit.

Nowadays, the non-destructive evaluation method has been a prominent research study. The determination of fruit maturity and postharvest qualities based on non-destructive methods has been widely reported (Gorbe and Calatayud, 2012; Kasampalis et al., 2020; Lien et al., 2009; Liñero et al., 2017; Wanitchang et al., 2011). Evaluation of the maturity stage of tomato by using color image analysis is also well known (Choi et al., 1995; Liñero et al., 2017).

Furthermore, prediction approaches for change in color of tomato at different conditions to estimate the maturity stage have been reported for over a decade (Schouten et al., 2007; Thai et al., 1990; Tijskens and Evelo, 1994). Those ripening prediction models were based on the color parameters and only Ciptaningtyas et al. (2020) and Nakamura et al. (2010)

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developed the color prediction method based on the cumulative ethylene production. Nakamura et al. (2010) modified the sigmoid function to describe the change in CIE a* value as a function of cumulative ethylene production for mature green tomato stored at 25°C. Ciptaningtyas et al. (2020) demonstrated that the modified sigmoid type-function model was applicable for storage temperature range 10-35°C. However, the determination of cumulative ethylene production is not simple. It requires a complex technique to obtain data with high validity and will be expensive to implement commercially (Kader, 2002).

In the previous study (Ciptaningtyas et al., 2020), the relationship between the CIE a* value and storage period (day) of 'Momotaro York' tomato harvested at the mature green stage of development was found to follow a sigmoid type-function. Therefore, it is possible to develop a ripening prediction model based on red color development CIE a* value as a function of the storage period by modifying the sigmoid-type function model. Hence, this study aims to develop a typical ripening pattern curve for 'Momotaro York' tomato harvested at the mature green stage of development and stored at 25°C. By using the proposed typical ripening pattern curve, it is possible to predict the ripening stages of tomato based on the storage period. The findings of this study will support a predictive non-destructive ripening process model for tomato. This model will support best practice in the tomato industry and the ripening stages of tomatoes in the supply chain can be precisely predicted based on their storage period.

MATERIALS AND METHODS

Data acquisition

In this study, five tomato fruits (*Solanum lycopersicum* L. 'Momotaro York') were used as the samples. The tomatoes were grown hydroponically in a greenhouse in Matsudo campus, Matsudo City, Chiba Prefecture, Japan. The fruits were hand-picked on January 16, 2018 at the uniform maturity stage (mature green stage of development). Fruits that were free from external defects and almost uniform in size (157.80±16.72 g) were selected as the samples. Without washing or treatment with any fungicide, each fruit was numbered. To prevent water loss from the fruit, unsealed plastic pouch (20 μm anti-fogging-oriented polypropylene) was used to pack each fruit individually and stored under dark conditions at 25°C until the fully ripe stage of development.

A reflectance spectrophotometer (CM-600d, Konica Minolta, Tokyo, Japan) was used for determining the pericarp color of the tomato using the CIE L*, a* and b*. The spectrophotometer was calibrated against a white reference plate. The same three parts on the fruit equator and one part on fruit blossom end were measured for determining the pericarp color. The CIE a* value, which denotes colors ranging from green (negative CIE a* value) to red (positive CIE a* value), was used to determine the red color development during storage. The average CIE a* value at the four locations on each fruit was calculated. The CIE a* value of the tomatoes was recorded every 24 h since it was harvested until it reaches the fully ripe stage of development.

Sigmoid-type function

In this study, the modified sigmoid-type function model was used to predict the CIE a* value of tomato, as shown in Equation 1. Originally, the free variable x denotes cumulative ethylene production (Nakamura et al., 2011). However, in this study, the free variable x is expressing the storage period (days). While α, β, and γ are the optimized parameters obtained from a nonlinear least square method using solver function in Microsoft Excel (Microsoft Corporation, Redmond, USA).

$$\text{Estimated CIE a}^{*}\text{value} = \frac{\alpha}{(1+e^{\beta x})} + \gamma \quad (1)$$

Time lag

The time lag is described as the time required by the tomato to begin the development

of red color since it was harvested at the mature green stage of development. According to six ripening stages established by USDA (United States Department of Agriculture), tomato will start the development of red color at the breaker stage of development. Hypothetically, the CIE a* value at the breaker stage of development will equal zero. However, since red color was already developed at the breaker stage of development, the CIE a* values at that maturity stage is always higher than zero. Therefore, to optimally determine the time lag when the CIE a* value is equal to zero, the time lag was estimated using the transformation of Equation 1. The time lag was determined by calculating the day of storage when CIE a* value is equal to zero, shown by Equation 2.

$$x = \frac{\ln\left[\frac{\alpha+\gamma}{-\gamma}\right]}{\beta} \quad (2)$$

Fitting approach and goodness of fit

A nonlinear least square method using solver function in Microsoft Excel was used to obtain the parameters α, β, and γ. The goodness of the proposed mathematical models was evaluated by the coefficient of determination (R^2) and root means square error (RMSE) between the predicted and experimental data.

RESULTS AND DISCUSSION

The relationship between CIE a* value and storage period of an individual tomato sample (25°C-Y1) is shown in Figure 1. At the beginning of the ripening process, CIE a* values of the tomato were below zero (P1). Negative CIE a* sample values, the green indicator appeared before the red color developed. After eight days of storage, CIE a* sample values turned positive, indicating the red color development. Experimentally, eight days is the time lag of the tomato sample (25°C-Y1). According to Tijskens and Evelo (1994), storing tomatoes at 25°C allows tomato fruit to ripen normally. One of the indicators of the normal ripening process in tomato, other than the onset of the rise in ethylene production, is red color development. Red color development in tomato was shown by the increase in CIE a* value in P2. During the ripening process of the sample, the CIE a* value increased until it reached the equilibrium value (CIE a* value ≈26). The fully ripe stage of development occurred after 11 days of storage.

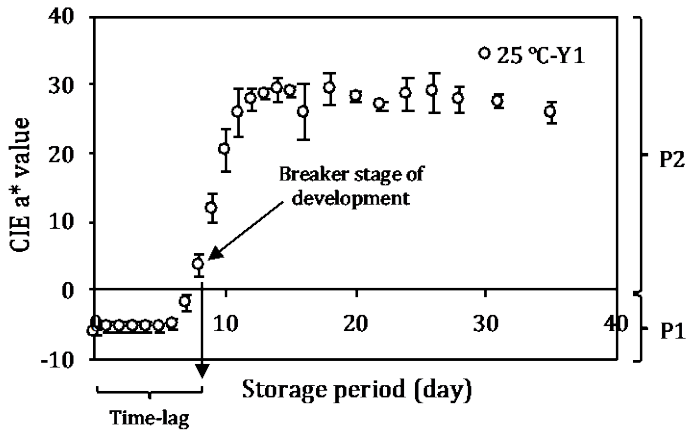

Figure 1. Change in color (CIE a* value) of one tomato sample during the ripening phase, showing the breaker stage of development, time lag, and the area of P1 and P2 for the 'Momotaro York' tomato harvested at the mature green stage and stored at 25°C.

Individual variation in horticultural product does exist (Hertog et al., 2007), therefore there will be a difference in the characteristic of individual tomato fruit during red color development. The proposed ripening pattern curve needs to be able to accommodate those

differences in this characteristic. According to Ciptaningtyas et al. (2020) and supported by the findings of Biswas et al. (2012), the onset of red color development among individual tomatoes were different even though the tomatoes were harvested at the same time, at the same maturity stage, and stored at the same storage temperature condition. To be able to account for those differences, at least three biological replications were required to developed a typical ripening pattern curve. Figure 2 shows the comparison of the experimental and predicted CIE a* value of five tomato samples in P2. The predicted CIE a* value as a function of storage period was calculated based on Equation 1 for each tomato sample. By inputting the day of storage and optimizing the parameters α, β, and γ using a nonlinear least square method, the CIE a* values were predicted. Each prediction function was accompanied by parameters α, β, and γ, shows in Table 1. The value of R^2 and RSME showed in Table 1 also attested that the proposed model has an acceptable goodness of fit. Furthermore, the value of RSME certifies that the deviation between the experimental data and predicted data has a narrow range, which means it is near the expected value. This result confirms that the red color development in 'Momotaro York' tomato harvested at the mature green stage of development was able to be predicted by using the proposed modified sigmoid type function model based on the storage period at 25°C.

Table 1. Values of the parameters α, β, and γ, the coefficient of determination (R^2), and the root mean square error (RMSE) between the experimental and predicted data for the relationship of CIE a* value and storage period.

Samples	α	β	γ	R^2	RMSE
25°C - Y-1	3743.27	-0.62	-3714.95	0.958	1.383
25°C - Y-2	1300.92	-0.54	-1271.91	0.963	1.467
25°C - Y-3	3606.05	-0.64	-3579.47	0.954	1.208
25°C - Y-4	194.67	-0.44	-167.95	0.948	1.699
25°C - Y-5	2718.93	-0.69	-2691.38	0.962	1.095
Average	2312.77	-0.59	-2285.13		

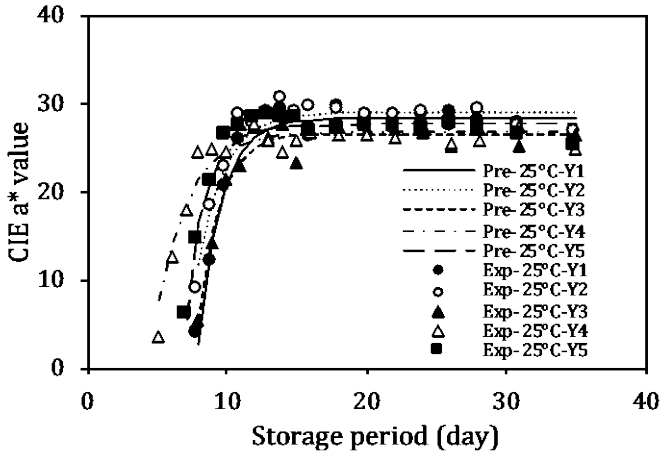

Figure 2. Comparison between predicted (Pre) and experimental (Exp) data for five individual 'Momotaro York' tomatos stored at 25°C, based on the proposed sigmoid-type function model.

To obtain the typical ripening pattern curve that can accommodate different characteristics of individual tomato, a universal sigmoid-type function model that can predict the red color development based on the storage period was established. By computing the average value of parameters α, β and γ from five tomato samples (Table 1) and storage period,

into Equation 1, the universal typical ripening pattern curve was developed as shown in Figure 3. This curve is capable of representing the red color development in 'Momotaro York' tomato stored at 25°C.

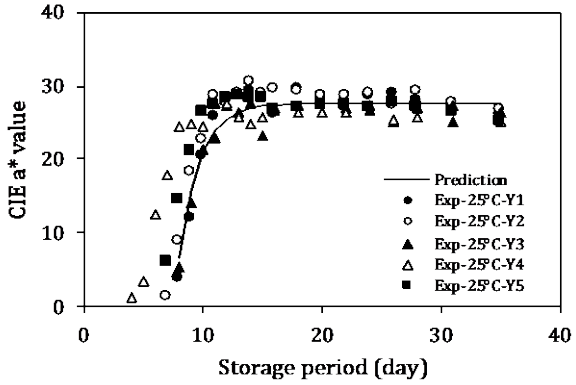

Figure 3. Comparison between the universal typical ripening pattern curve (Prediction) and experimental data (Exp.) from five individual 'Momotaro York' tomatoes harvested at the mature green stage of development and stored at 25°C.

The proposed sigmoid-type function model has a limitation. The predicted CIE a* value starts above zero, which affects the proposed sigmoid-type function model to adequately predict a typical ripening pattern curve. This condition is due to the determination of the starting point for the estimation method being based on the experimental time lag data for breaker stage of development. Whereas the breaker stage of development (red color development) has already occurred. Therefore, finding the time lag when CIE a* value is equal to zero is crucial. Hence, the time lag was calculated by using Equation 2.

Equation 2 shows the requirement of parameters α, β, and γ to find the estimated time lag. Since the universal typical ripening pattern curve was established based on the average value of parameters α, β, and γ from five tomato samples, the same average value of parameters α, β, and γ were also used to estimate the time lag when CIE a* value was equal to zero. Based on the calculation, it was able to be determined. The onset of the red color development for 'Momotaro York' tomato stored at 25°C occurs at seven days and 12.72 h from harvest at the mature green stage of development. By combining the proposed sigmoid-type function model, and the estimated time lag, a typical ripening pattern curve that predicts the red color development (whole range of P2) of 'Momotaro York' tomato harvested at the mature green stage of development and stored at 25°C, was established (Figure 4).

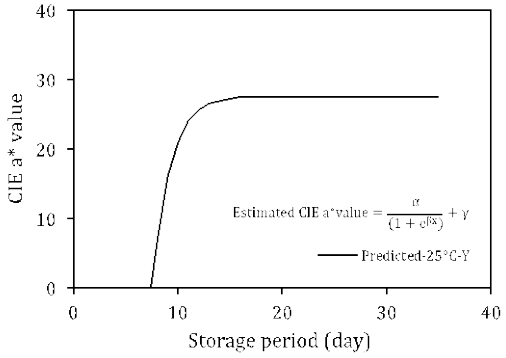

Figure 4. Typical ripening pattern curve of 'Momotaro York' tomato harvested at the mature green stage of development and stored at 25°C.

CONCLUSIONS

The findings in this study are the first to describe the relationship between CIE a* value and storage period. It follows a sigmoid-type function for ripening of tomato at 25°C across the whole color range during development (from breaker stage to full ripe stage). The typical ripening pattern curve that was successfully developed in this study will enable us to predict the ripening process of tomato harvested at the mature green stage during storage at 25°C based on the storage period.

ACKNOWLEDGEMENTS

This research was supported by grants from the Project of the NARO Bio-oriented Technology Research Advancement Institution (the special scheme project on vitalizing management entities of agriculture, forestry and fisheries) and by JSPS KAKENHI Grant Number JP17H01499.

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Effect of propionic acid on fruit decay and postharvest quality of Vietnamese purple passion fruit during low temperature storage

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Abstract

The impact of propionic acid on fruit decay and postharvest quality of Vietnamese purple passion fruit during low temperature storage was studied by soaking fruits in 0.25, 0.35 and 0.45% propionic acid for 3 min at room temperature, and then storing them at 5±1°C for 42 days. Untreated fruits were used as control. The total microorganisms, percentage of fruit decay and weight loss; total soluble solids (TSS) content; total titratable acidity; vitamin C content were monitored during the storage time. The results showed that the 3-min soaking treatment in 0.35% propionic acid solution had a reduced percentage of fruit decay for 42 days in storage. Moreover, this treatment maintained low total microorganism levels, low weight loss, and the TSS content, total titratable acidity and vitamin C content of the passion fruit remained unchanged when compare to control fruits.

Keywords: total microorganism, TSS content, vitamin C content, total titratable acidity

INTRODUCTION

Purple passion fruit (*Passiflora edulis* Sims) is a tropical climacteric fruit (Biale, 1975; Wills et al., 1982). The fruit has the unique flavor thanks to its juicy pulp, and has great commercial potential due to demand for both fresh fruit and processed juice is on the rise. However, one of the challenges in the passion fruit value chain is the short shelf life of the fruit, which leads to postharvest decaying, shriveling, and wilting, and contributes to postharvest losses. Postharvest deterioration is mainly caused by the loss of moisture content, peel color darkening, microbial contamination, and nutritional loss. These factors contribute to the unacceptable appearance of fresh produce that includes symptoms such as wrinkles, non-preferable color, postharvest decay and lack of nutritional content (Pruthi 1963; Arjona and Matta, 1991; Bora and Narain, 1997). Moisture condenses on the fruit surface under consistently high RH, creating conditions favorable for pathogen growth (Zagory and Kader, 1988). The common fungal attack is by *Alternaria passiflorae* with circular, sunken, and brown spots on the fruit surface, and septoria blotch caused by *Septoria passiflorae* (Rodriguez-Amaya, 2003). Pruthi (1963) noted that during long-term storage at 6.5°C, passion fruit was attacked by white (*Fusarium oxysporum*), blue (*Penicillium expansum*) and black (*Aspergillus niger* and *Rhyzopus nigricans*) fungi.

Propionic acid (PA) is a naturally occurring carboxylic acid with chemical formula $CH_3CH_2CO_2H$. As a food additive, it is approved for use in the EU (UK Food Standards Agency), USA (US Food and Drug Administration) and Australia and New Zealand (Australia New Zealand Food Standards Code). Propionic acid can inhibit the growth of fungi, yeast (Haque et al., 2009; Selwet, 2009), and bacteria (Wang et al., 2009). The antifungal activity of propionic acid against *Candida albicans, Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Streptococcus* spp., *Candida* spp., *Sporothrix* spp., *Fusarium* spp., *Penicillium* spp., *Paecilomyces* spp. and *Aspergillus* spp. has been reported by Müller and Thaler (1981), Razavi-Rohani and Griffiths (1999), Larous et al. (2007) and Huang et al. (2011). Higgins and Brinkhaus (1999) found that PA at the concentration of 0.20% or less was able to reduce the growth rate of *Aspergillus* spp., *Geotrichum* spp., *Mucor* spp.,

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Fusarium spp., *Penicillium* spp., and *Scopulariopsis* spp. to 50%. PA at the concentration of 0.15% reduced the growth of the *Penecillium expansum* by more than 70% (Larous et al., 2007). Recommended storage temperatures for passion fruit are 3-5°C (Wills et al., 1982), 7-10°C (McGregor, 1987), 5-7°C (Pruthi, 1963), and 5±1°C at a relative humidity of 75-80% (Van and Hue, 2017).

The main purpose of this study was to investigate the effects of propionic acid on fruit decay and postharvest quality of Vietnamese purple passion fruit during low temperature storage.

MATERIALS AND METHODS

Plant materials

In 2019, mature purple passion fruits (85-90 days after fruit set) harvested from a commercial orchard in the Son La province, Vietnam were used for this research. The purple passion fruits were harvested in the morning and packaged in 20-kg plastic baskets, lined with paper and transported to the laboratory within three hours. Fruits were then selected for uniformity of shape, size, color, with nodefects prior to use in this experiment.

Studying methods

The optimal and feasible concentrations of PA (0.25, 0.35 and 0.45%) were selected after preliminary tests. The fruits were first soaked in 0.25, 0.35 and 0.45% PA solutions for 3 min and dried for 6 h at room temperature while the control fruits were not soaked. Then ten soaked and control fruits were packed in polypropylene bags of 305×457 mm in size, and 0.035 mm thick with four holes of 0.8 cm^2 bag^{-1}. The fruits were then stored at 5±1°C in a cold room, sampled and analyzed at 7-day intervals. Each treatment had three replications. A completely randomized design was used for the experiment. T0 was the control; T1, T2 and T3 fruits were soaked in 0.25, 0.35 and 0.45% PA solutions, respectively.

The total soluble solids content was determined using a digital refractometer (PAL-1, Atago, Japan). The titratable acidity (TA) was determined as citric acid by titrating against 0.1 NaOH (AOAC, 2000). Vitamin C content was determined by using the detective dye 2.6 dichlorophenol-indophenol by standardizing 0.1% standard 2.6 dichlorophenol-indophenol dye solution against 0.1% ascorbic acid solution (AOAC, 2000).

Percentage of weight loss was calculated by weighing the whole fruits packed in PP bags before and after storage (taken as 100%). Fruit decay was assessed as the percentage of fruit showing decay as follows:

$$\text{Percentage of fruit decay} = \frac{\text{Number of fruit decay}}{\text{Total fruit}} \times 100$$

The total microorganism was determined according to the method of Whangchai et al. (2006). The sampled fruits were immersed in sterile distilled water and shaken at 180 rpm for 30 min at room temperature. For each treatment, a sample (1 mL) of the suspension was spread over a PDA medium. The PDA plates were incubated at 25°C for 72 h and the survival of microorganisms expressed as the mean number of colony forming units (CFU mL^{-1}).

Statistical analysis was carried out using the SPSS software (version 20.0) and Duncan's multiple range test ($p \leq 0.05$) used to determine the significant difference of means between the treatments and control.

RESULTS AND DISCUSSION

Change in total microorganism populations on fruit surface, percentage of fruit decay and weight loss

Total microorganism populations on the passion fruit surface including fungi, yeasts, and bacteria of treated and control fruits increased with time spent in storage (control fruits increased from 2.3 to 15.3×10^6 CFU mL^{-1} and treated fruits increased from 2.1×10^3 to

13.2±0.3×10^4 CFU mL^{-1} after 42 days in storage) (Table 1). There was a marked difference in the total microorganism between control and treated fruits. The control fruits had much higher total microorganism counts than treated fruits during the storage period. This result demonstrates that PA significantly reduced total microorganism population on the surface of passion fruit. The mechanism of inhibition of growth of fungi by organic acids is generally not considered a pH phenomenon. However, it is well known that growth and morphology of fungi is influenced by the pH of the media (Sacks et al., 1986). Propionic acid was effective in inhibiting fungal growth in a laboratory assay. It is superior to several other organic acids tested (Higgins and Brinkhaus, 1999). Table 1 shows that T3 treatment maintained the lowest total microorganism populations on fruit surface of passion fruit during storage. This result justified the concentration of 0.45% PA, which markedly prevented the development of total microorganism on the passion fruit surface.

Table 1. Change in total microorganism populations on fruit surface during storage period (CFU mL^{-1}).

Treatments	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
T0	2.3±0.6×10^6	2.6±0.9×10^6	4.9±0.6×10^6	7.4±0.5×10^6	10.3±0.8×10^6	15.3±0.6×10^6
T1	1.1±0.5×10^4	1.4±0.4×10^4	2.9±0.5×10^4	9.8±0.7×10^4	10.8±0.5×10^4	13.2±0.3×10^4
T2	1.4±0.7×10^4	1.9±0.5×10^4	2.2±0.3×10^4	3.0±0.4×10^4	4.7±0.5×10^4	6.0±0.4×10^4
T3	2.1±0.4×10^3	2.9±0.2×10^3	1.6±0.4×10^4	2.4±0.3×10^4	3.6±0.6×10^4	4.7±0.5×10^4

For the control treatment 3.5% of fruit started to decay after 7 days in storage, and thereafter the decay amount accelerated with increased storage time (after 35 and 42 days it was 61.4 and 79.4%, respectively) (Figure 1). As shown in Figure 1, 1.5% of fruits in T2 decayed after 28 days and 1.6% of fruit in T3 started to decay after 30 days in storage. In contrast, 1.3% of fruit in T1 started to decay after 14 days and reached 24.4% after 42 days in storage. This study indicates that low fruit decay correlated with low total microorganism populations (Table 1; Figure 1). Treatments T2 and T3 maintained low fruit decay percentages, and there was no significant difference between these treatments (p≤0.05) during storage. Results for T2 and T3 showed that the dosage of PA was effective to control fruit decay (9.5-9.7%) in passion fruit for 42 days. Higgins and Brinkhaus (1999) research confirmed the efficacy of propionic acid to inhibit the growth of various fungi commonly found in feed ingredients. This acid inhibited fungal growth effectively at the concentration range of 0.1-0.2%, which agrees well with commonly used application levels.

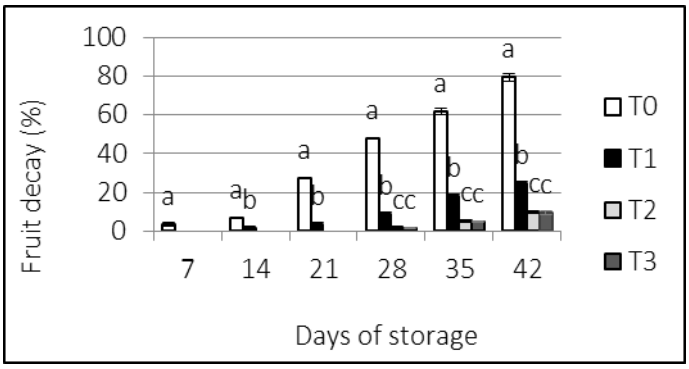

Figure 1. Changes in percentage of fruit decay of passion fruit either treated or untreated, during storage at 5°C. Columns with different letters indicate significant differences by Duncan's multiple range test (p≤0.05).

After harvest, purple passion fruit lose weight rapidly, which triggers shriveling under conventional packaging conditions. Higher moisture loss usually occurs in the fruit peel than from the pulp. Thus, fruit peels appear wrinkled and becomes visually unacceptable, even if

sensory evaluation of the pulp inside the fruit is acceptable to consumers (Pruthi, 1963; Shiomi et al., 1996a, b). Kader et al. (1989) suggested that higher moisture loss in horticultural commodities corresponding to higher respiration and ethylene response. To avoid peel desiccation and excessive weight loss, the application of special plastic films could help to minimize fruit shriveling (Kader, 1986; Kader et al., 1989). After 21 days in storage at different cold temperatures, weight loss of treated passion fruits ranged from 10.1 to 24.02% (Van and Hue, 2017).

The weight loss of stored passion fruit is shown in Figure 2. During the first 7 days in storage, the percentage of weight loss in the control fruit was 0.7%, and it reached 3.2% by day 28. The weight loss of treated fruits ranged from 0.3 to 0.6% by day 7, and from 2.9 to 3.5% by day 42 in storage. As seen in Figure 2, the weight loss of treated and control fruits tended to rise with increasing storage time, and the control fruits had higher weight loss than treated fruits during storage. Results from this study show that high weight loss correlated with high fruit decay and high total microorganism populations (Table 1; Figures 1 and 2). These results show the PA rates used in this study was effective in reducing the weight loss in passion fruit during storage.

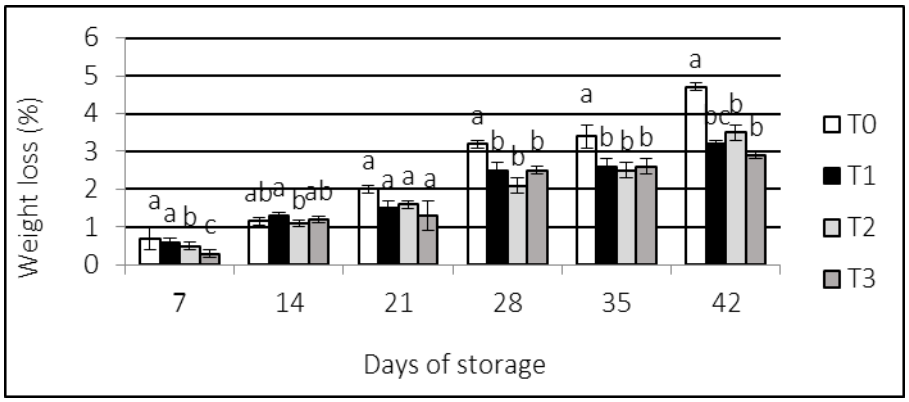

Figure 2. Changes in percentage weight loss of passion fruit either treated or untreated, during storage at 5°C. Columns with different letters indicate significant differences by Duncan's multiple range test ($p \leq 0.05$).

Change in total soluble solids content (TSS), titratable acidity and vitamin C content

There was no significant difference in TSS content between treated and control fruit during the storage period, except by day 35 ($p \leq 0.05$). Overall, the TSS content of treated and control fruits tended to decrease with increasing storage time. After 42 days in storage the TSS content of control fruits and treated samples ranged from 12.7 to 13.5% (Figure 3). Our results were consistent with the findings of Maniwara et al. (2015) who concluded that passion fruit packaged under all conditions showed a decrease of total soluble solids over storage time. The passion fruit lost about 10% of its initial TSS. Typically, fully ripe purple passion fruit accumulated soluble solids in the pulp, mostly organic acids, and sucrose molecules. However, once picked, passion fruit could consume and convert certain compounds by aerobic respiration or anaerobic processes (Arjona and Matta, 1991; Shiomi et al., 1996a, b). The TSS in passion fruit ranged from 10.8 to 12.5% and did not differ significantly among treatments as reported by Arjona and Matta (1991). TSS increases gradually in all passion fruit as ripening progresses. Yumbya et al. (2014) reported that packaging significantly ($p=0.05$) reduces the rate of TSS increase for fruits harvested at both stage 2 and 3. For the unpackaged fruits (stage 2), TSS rose rapidly from an initial 12.4 °Brix peaking at 13.9 °Brix on day 6 of storage and declined gradually until the end of storage (day 9). Furthermore, MAP packaged fruits maintained significantly low TSS levels until the end of storage (Yumbya et al., 2014).

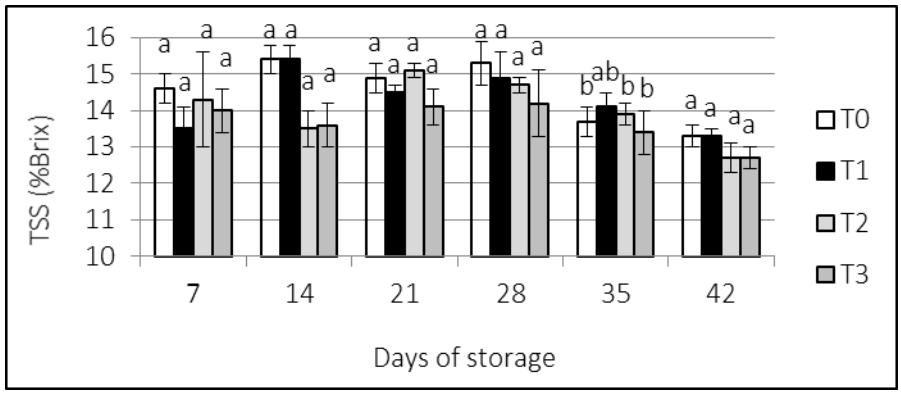

Figure 3. Changes in TSS content of passion fruit either treated or untreated, during storage at 5°C. Columns with different letters indicate significant differences by Duncan's multiple range test ($p \leq 0.05$).

The total titratable acidity (TTA) of treated and control fruits tended to decrease with the increase in storage time (from 4.3 to 5.1% by day 7 and declined from 3.1 to 3.4% by day 42 in storage). There was no difference in TTA between treated and control fruits after 21 to 42 days in storage ($p \leq 0.05$) (Figure 4). Maniwara et al. (2015) reported, like TSS, TTA from passion fruit juice notably decreased over time under all packaging conditions and passion fruit juice lost about 40% of organic acids during storage from an initial value of 4.0% and ending at 2.5%. The diminishing of organic acids such as ascorbic and citric acids in purple passion fruit usually occurs because of acid metabolism and degradation (Arjona and Matta, 1991). After harvest, purple passion fruit lose acids and moisture rapidly in ambient temperatures and atmosphere due to the high level of respiration and the increase of related enzymatic acidic degradation (Shiomi et al., 1996a, b). TA of passion fruit reduced during storage period and TTA of control fruit dropped strongly (from 6.85% to below 3%) after 14 days in storage, reported by Van and Hue (2017) and confirmed in this study. Yumbya et al. (2014) reported levels of TTA decreased gradually with storage time in fruits harvested at both stages of maturity. Fruits harvested on stage 3 represented significantly low initial levels of TTA (0.43% citric acid) compared to that of stage 2 fruits (0.55% citric acid). Packaging significantly slowed down the decrease in TTA levels throughout storage (Yumbya et al., 2014).

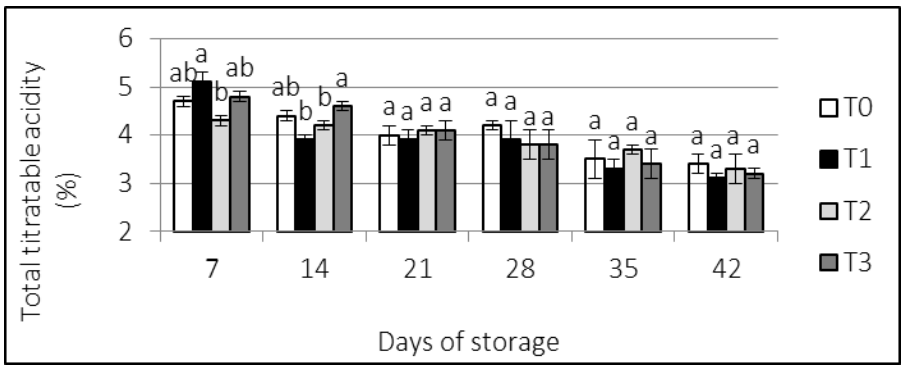

Figure 4. Changes in total titratable acidity of passion fruit either treated or untreated, during storage at 5°C. Columns with different letters indicate significant differences by Duncan's multiple range test ($p \leq 0.05$).

Figure 5 indicated the changes in vitamin C content of treated and control fruits during the storage. Overall, vitamin C content of treated samples and control fruits gradually decrease with increasing storage time. There was difference in vitamin C content between treated and control fruits by day 7 and 35 in storage ($p \leq 0.05$) (Figure 5). After 42 days in storage, there

was no difference in vitamin C content of treated and control fruits ($p \leq 0.05$), and it ranged from 33.1 to 36.1 mg 100 g^{-1}. This result demonstrated that the dose of PA used in this study did not influence the vitamin C content in passion fruit during the storage. Maniwara et al. (2015) concluded that the passion fruit juice lost vitamin C (one of the major organic acids enriching the juice) during storage. Vitamin C degradation was mainly caused by the exposure to O_2, light and high temperatures (Maniwara et al., 2015). Vinci et al. (1995) reported that passion fruit stored at 4°C lost 40% of its ascorbic acid after only one week of storage in a conventional cool room. Vitamin C content of passion fruit significantly decreased during storage period and vitamin C content of control fruit declined more than 50% (Van and Hue, 2017). Levels of ascorbic acid dropped gradually with ripening in both the packaged and unpackaged fruits from an initial of 42.9 to 29.0 mg 100 mL^{-1} at the end of storage (day 9) (Yumbya et al., 2014). The decrease in the vitamin during ripening is partly due to degradation of ascorbic acid through oxidation (Appiah et al., 2011). In addition, Vitamin C being a water-soluble vitamin, correlated positively with that of water loss through transpiration as reported by Valero and Serrano (2010) and Siddiqui et al. (2011).

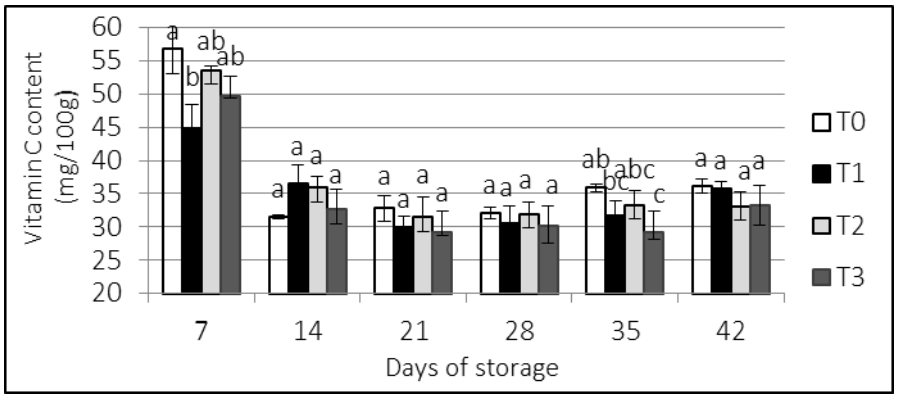

Figure 5. Changes in vitamin C content of passion fruit either treated or untreated, during storage at 5°C. Columns with different letters indicate significant differences by Duncan's multiple range test ($p \leq 0.05$).

CONCLUSIONS

The application of 0.45% PA by dipping, effectively prevented the development of microorganism on the fruit surface. For in purple passion fruit it reduced the percentage of fruit decay and weight loss compared to the control fruit and fruit in the other treatments. Moreover, fruit maintained nutrient ingredients expressed as total titratable acidity, TSS content and vitamin C content for 42 day at 5°C. This is a feasable treatment for storage of passion fruit on a commercial scale.

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Non-destructive detection of granulation in stored 'Magallanes' pummelo [*Citrus maxima* (Burm. Ex Rumph.) Merr.] fruit

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Abstract

Granulation is a common physiological disorder found in citrus fruit. It is characterized by discolored, dry and hardened juice vesicles. It lowers the overall quality of the fruit. This study assessed two non-destructive methods, flotation and a capacitance-based technique, for determining granulation. This research included the degree of granulation, along with some physico-chemical characteristics of small, medium, and large 'Magallanes' pummelo fruit. The two non-destructive methods tested did not correctly determine granulation in 'Magallanes' pummelo and therefore cannot be used to detect granulation. The entire pummelo fruit did not float, nor sink in water or in 10% salt solution. The distance between conducting plates in the capacitance measurement and the thick pummelo rind could have prevented this technique in differentiating the granulated from the non-granulated fruit. Granulation was evident in a few fruit even at harvest, as well as during storage with large fruit presenting greater granulation than small fruit. Though degree of granulation did not differ, the percentage of granulation was greater in large fruit (91.48%) at nine weeks of storage in ambient conditions. Granulation developed further with storage. Granulation was slight at three weeks of storage. At six weeks of storage, medium and large fruit had lower hue and TSS. Large fruit had lower TA while chroma, visual quality and pH did not vary among the three fruit sizes. Fruit size may be used as a factor in determining granulation in ambient-stored 'Magallanes' fruit, wherein large fruit were more granulated.

Keywords: internal dryness, non-destructive method, vesicle drying, visual quality

INTRODUCTION

The pummelo [*Citrus maxima* (Burm. Ex. Rumph) Merr.] is the largest of all citrus species. In the Philippines, 'Magallanes' is the most common cultivar grown in the Davao region. However, pummelo quality is often reduced because of granulation, a physiological disorder caused by gel formation within the juice vesicle (Ritenour et al., 2004). Granulated vesicles have elevated respiration, increased juice pH, and less soluble sugars and acids (Bartholomew et al., 1941). Several preharvest factors affecting granulation in citrus have been reported such as time of harvest, fruit size, rootstock and tree age, season of harvest, irrigation, fertilizers, and prevailing temperatures during production (Burns and Albrigo, 1998; Bartholomew et al., 1941). Granulation in citrus has been observed in large fruit with inferior physicochemical characteristics, such as lower in juice content, acidity, and ascorbic acid (Sharma et al., 2006). Evaluation of these characteristics in different fruit size, would give some insights on the granulation of pummelo and whether the fruit size could be a determining factor for granulation.

Granulation in pummelo is detected with less certainty unless fruit is opened. A non-destructive method of detecting granulation is preferred over a destructive one in assessing fruit internal quality. Internal citrus fruit quality can be detected nondestructively using X-ray and nuclear magnetic resonance imaging (Ladaniya, 2008). However, these methods make use of expensive equipment which are not readily available. For this study, two cheaper alternative

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methods such as flotation and capacitance-based techniques were evaluated to non-destructively assess pummelo granulation. The flotation method using 1 and 10% salt solution has been used to determine mango harvest maturity, based on specific gravity of 'Carabao' mango (Lizada, 1991). Salt is added to control the specific gravity of the solution. Teerachaichayut et al. (2012) reported that the capacitance-based technique was able to discriminate normal tangerine fruit from the granulated fruit. Capacitance measures the ability of fruit to store electric charge (Burubai, 2014).

This research aimed to assess whether granulation can be determined by non-destructive methods such as the flotation and capacitance-based techniques, and to evaluate whether the fruit size could be a determining factor for granulation through assessing the different physico-chemical characteristics in relation to degree and percentage of granulation in small, medium and large 'Magallanes' pummelo fruit.

MATERIALS AND METHODS

Commercially mature (190 days from flower opening) 'Magallanes' pummelo fruit were harvested during the wet season in Samal in Davao del Norte, Southern Philippines. Newly-harvested pummelo fruit were first visually assessed and then classified into small (544.58±73.34 g), medium (768.34±64.62 g) and large (1107.23±158.68 g) fruit. Fruit were stored in ambient conditions (27.36±0.46°C, 88.23±8.01% RH) for three months and sampled every three weeks. Fruit (25 pieces) from each size were procured and treated with 500 mg L^{-1} thiabendazole for 3 min to minimize decay. Five fruit samples from each size were observed for physical, chemical, physiological and granulation qualities for each sample time.

The non-destructive methods used for determining granulation were the flotation and capacitance-based techniques. In the flotation method, fruit were placed first in water then next in 10% salt solution. A modified capacitance-based device modeled after Teerachaichayut et al. (2012) method was used to determine the capacitance of the pummelo fruit. The values obtained in the two methods were compared with the actual percentage and degree of granulation.

The physical data gathered included color using a chromameter (CR400 Minolta, Japan) which measured the a*, b* coordinates to determine chroma and hue. Visual quality was scored from 8 to 9=excellent to 1=poor; degree of granulation from a scale (1 = no granulation, 2 = 1-10%, 3 = 11-25%, 4 = 26-50%, 5 = 51-75% and 6 = 76-100%) was used on each fruit segment. Percentage of granulation was obtained by counting the number of segments with granulation in each fruit over the total number of segments per fruit. In addition, titratable acidity (TA), pH (Hanna combo pH and EC meter) and total soluble solids (Atago digital refractometer) were measured. This study was set up on a completely randomized design. We analyzed the data using analysis of variance (ANOVA) while the least significant difference (LSD) at 5% was used for the comparison of treatment means.

RESULTS AND DISCUSSION

Non-destructive methods used to assess granulation

1. Flotation method and capacitance-based technique.

The entire pummelo fruit did not float nor sink in water or in salt solution. Therefore, the percentage of the fruit's surface that was not under water or salt solution was estimated instead. No difference in the percentage of fruit surface that was above the water or solution was found (Table 1). However, the percentage of granulation varied at 9 and 12 weeks of storage and degree of granulation varied at 6 and 12 weeks of storage (Figure 1). This indicates that the flotation method failed to discriminate between granulated and non-granulated fruit. The density of a 10% salt solution may not be enough to discriminate granulated fruit. Increasing the amount of salt in the solution can increase the density of water (Meylor and Finn, 1994). This may allow discrimination of granulated fruit. In 'Navel' orange, less dense fruit had moderately high granulation, however, denser fruit still developed granulation but only to a slight degree (Ritenour et al., 2004).

Table 1. Percentage surface area of fruit that was above the water or 10% salt solution and capacitance readings (nF) of various 'Magallanes' pummelo fruit sizes held in ambient storage conditions (27.36±0.46°C and 88.23±8.01% RH) for 12 weeks.

Size	Time (weeks)							
	3	6	9	12	3	6	9	12
	Percentage surface area exposed above the water[a]				Percentage surface area exposed above salt solution[a]			
Small	49.50a	58.75 a	52.50a	53.75a	47.50b	58.75a	48.75a	51.25a
Medium	47.50a	56.25 a	50.00a	57.50a	46.25b	52.50a	48.33a	52.50a
Large	53.75a	50.00 a	52.50a	50.00a	53.75a	47.50a	47.50a	46.25a
	Vertical capacitance[a]				Horizontal capacitance[a]			
Small	0.41a	0.34a	0.39a	0.42a	0.46a	0.58a	0.51 a	0.54a
Medium	0.40a	0.39a	0.42a	0.56a	0.51a	0.56a	0.50 a	0.51a
Large	0.39a	0.44a	0.37a	0.40a	0.49a	0.59a	0.43 a	0.56a

[a]Per parameter, means in a column with common letters are not significantly different using LSD at 5% level of significance.

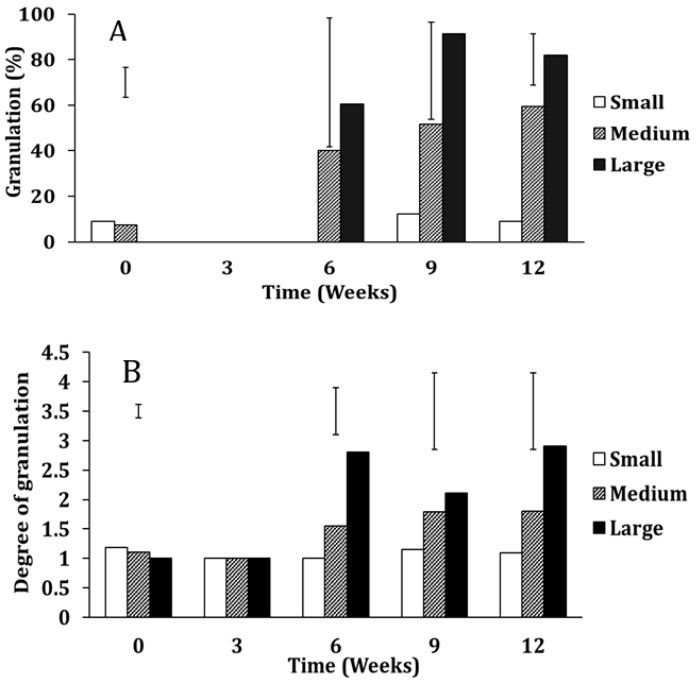

Figure 1. Percentage of segments per fruit exhibiting granulation (A) and degree of granulation (B) in three sizes of 'Magallanes' pummelo fruit stored under ambient conditions. LSD bars indicate significance at $p<0.05$.

Also, there was no difference between vertical or horizontal capacitance readings when compared with percentage and degree of granulation of the fruit (Table 1; Figure 1). This non-destructive method cannot also be used to determine granulation in 'Magallanes' pummelo. Accuracy of capacitance readings is affected by the gap distance between the conducting plates (Terzic et al., 2011). Compared to the smaller-sized tangerines, the bigger-sized pummelo fruit has shown a greater distance between conducting plates in capacitance measurement. In addition, the thicker pummelo rind compared to the thinner rind of tangerines could have prevented this technique in differentiating the granulated from the non-granulated pummelo fruit.

2. Granulation.

Large pummelo fruit were double the weight of the small fruit. A very small amount of granulation was evident, even at harvest in small and medium fruit (Figure 1). Granulation had already developed in medium and large pummelo fruit by six weeks. It took another three weeks before small fruit exhibited granulation. The percentage of granulation for medium and large fruit continued to progress until the end of the storage period. Regardless of fruit size, storage duration affects the development of granulation in 'Magallanes' pummelo. The trend observed in the percentage of granulation of fruit can be due to the unique samples used for each storage period. Large fruit exhibited a higher percentage of granulation but only differed from small fruit at 9 and 12 weeks (Figure 1A). The percentage of granulation is not directly proportional but complementary (Figure 1B) to the degree of granulation. For instance, 100% granulation meant all segments in a fruit exhibited granulation, regardless of the degree of granulation, whether rated slight, moderate, or excessive. Large fruit was more granulated at 6 weeks compared to medium fruit. The degree of granulation did not vary at 9 weeks of storage. The percentage of granulation in large fruit was higher in week 9 but the degree of granulation was lower compared to 6 and 12 weeks. This indicates that there were more fruit segments showing granulation in large fruit at 9 weeks however each segment only showed slight degree of granulation (i.e., affected segments showed 1 to 10% dryness). On the other hand, at 6 and 12 weeks of storage, fruit segments of large fruit showed 11 to 25% dryness of juice vesicles.

The reports on Valencia oranges (Bartholomew et al., 1941), Satsuma mandarin (Wen et al., 2013), grapefruit (Burns and Albrigo, 1998) and other citrus fruit (Sharma et al., 2006) confirm that granulation is most prevalent in large fruit. Faster senescence or advanced maturity in large fruit compared to small fruit of the same bloom or batch of harvested fruit (Burns and Albrigo, 1998) may contribute to higher incidence of granulation. This could imply the need not to delay pummelo harvesting. Moreover, granulation is affected by a complex interaction of different factors such as storage, fruit size and maturation (Burns and Albrigo, 1998) as well as other preharvest factors. Furthermore, Ritenour et al. (2004) reported a short bloom period and severe high temperature likely stressed trees of 'Navel' orange, resulting in very low fruit set and subsequent development of large fruit which is associated with granulation.

Physico-chemical evaluation

1. Color and visual quality.

Chroma showed a slight increase for all medium and large fruit as the storage period progressed, thus, indicating an increase in saturation from greenish yellow to yellow (Table 2). At three weeks, large fruit were already yellow. This is reflected by hue angles that are closer to 90, while small and medium fruit became more yellow later (Table 2). In Valencia orange, off-color fruit from a regular bloom were found to be more granulated (Bartholomew et al., 1941). We found there was a greater decline in the visual quality of the large fruit as storage progressed from 3 to 6 weeks of storage (Table 2). This was due to the more yellow rind color increasing in intensity.

2. Total soluble solids (TSS).

TSS of medium and large fruit was lower than the TSS of small fruit (Table 2). The higher granulation in large fruit may have consequently reduced the TSS. The reduction of TSS in large, granulated fruit could be due to the utilization of sugars as substrates in the process of cell wall thickening during granulation (Hofman, 2011). Likewise, the TSS of some granulated citrus fruits such as oranges, lemons, grapefruit, and lime were observed to be lower compared to non-granulated fruit (Sharma et al., 2006).

3. Titratable acidity (TA) and pH.

Large fruit had lower TA compared with medium and small fruit at 6 weeks (Table 2). There was lower incidence of granulation in small fruit resulting in higher TA. The utilization

of acid as substrate during granulation could affect the decreasing TA in large fruit (Hofman, 2011). The reduction in TA during granulation can lead to an increase in pH which was observed in the later period (data not shown).

Table 2. Color, visual quality, and quality characteristics of 'Magallanes' pummelo fruit size held in ambient storage conditions (27.36±0.46°C and 88.23±8.01% RH).

Size	Time (weeks)					
	3	6	3	6	3	6
	Chroma[a]		Hue		Visual quality	
Small	29.50a	29.47a	102.83a	99.74a	6.25a	6.00a
Medium	27.38b	31.10a	106.37a	88.08b	6.50a	6.25a
Large	30.24a	31.00a	95.41a	89.75b	7.25a	6.00a
	TSS[a] (°Brix)		TA[a] (%)		pH[a]	
Small	12.40a	13.73a	0.70a	0.78a	5.12a	4.88a
Medium	11.80a	11.30b	0.72a	0.64ab	5.25a	4.95a
Large	11.03b	11.63b	0.65a	0.56b	4.93a	4.96a

[a]Per parameter, means in a column with common letters are not significantly different using LSD at 5% level of significance.

The pH of fruit at 3 and 6 weeks did not vary with fruit size (Table 1). The acidity was lower in non-granulated pummelo fruit, as observed in small 'Sai Nam Phueng' tangerines (Boonyakiat et al., 2012; Sharma et al., 2006).

CONCLUSIONS

The two non-destructive methods tested, namely the flotation and capacitance-based techniques, were unable to correctly determine the occurrence of granulation in pummelo fruit. Thus, the two methods cannot be used in detecting granulation at harvest or during storage. In the flotation method, the entire pummelo fruit did not float nor sink in water or in 10% salt solution. Therefore, the percentages of the fruit surface that was not under water or for the salt solution were estimated instead. The 10% salt solution is not adequate for discriminating granulated fruit. For the capacitance method, fruit shape, and the greater distance between the conducting plates in capacitance measurement and the thicker pummelo rind could have prevented this technique from differentiating the granulated from the non-granulated fruit. The fruit least affected by granulation were the small fruit, whereas medium and large fruit were more granulated, which increased during storage. At 6 weeks of storage, granulation in large fruit was characterized by lower hue, lower total soluble solids and lower TA relative to the small fruit. On the other hand, chroma, visual quality and pH did not vary. Thus, the size of pummelo fruit may be an indicator in determining granulation particularly during ambient storage wherein large fruit were more granulated for the cultivar 'Magallanes' in the Philippines.

ACKNOWLEDGMENTS

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Quality of 'Lakatan' banana (*Musa acuminata*) as influenced by 1-methylcyclopropene and maturity stage

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Abstract

Banana, a climacteric fruit, produces ethylene gas which promotes ripening. Fruit ripening can be controlled by ethylene antagonists such as 1-methylcyclopropene (1-MCP) to extend shelf life. We determined the effect of various 1-MCP concentrations (0, 100 or 1,000 nL L^{-1}) on 'Lakatan' banana at the mature green and light green stages. Fruit were sanitized with 200 µL L^{-1} NaOCl, air-dried, dipped in 200 µL L^{-1} ethephon for 5 min, and fumigated with 1-MCP (immediately for mature green fruit once dried, and two days later for light green banana) inside an air-tight chamber for 24 h. All fruit were stored in 20.7±0.3°C, 85.3±6.4% RH after 1-MCP treatment until end of shelf life. Fruit weight loss, firmness, total soluble solids (TSS), peel color, and visual quality were examined at four days interval. Treating mature green or light green fruit with 100 or 1,000 nL L^{-1} 1-MCP resulted in lower weight loss, slower peel color change, retention of firmness, and lower TSS compared to the control. Further, fruit ripening was better delayed in those treated with 1,000 nL L^{-1} 1-MCP as it slowed change to full yellow stage albeit less vibrant by 2.3 days than those treated with 100 nL L^{-1} 1-MCP, and 19 days longer than the untreated fruit. On day 25, 1,000 nL L^{-1} 1-MCP treated fruit attained the almost full yellow stage with a 9.75 °Brix TSS whereas the 100 nL L^{-1} 1-MCP treatment appeared to show the preferred fruit quality that is less firm and sweeter (15 °Brix). Thus, fumigation of 100 nL L^{-1} 1-MCP for 24 h can potentially extend shelf life of 'Lakatan' banana.

Keywords: 1-methylcyclopropene, maturity, ripening, shelf life

INTRODUCTION

Banana (*Musa* spp.) is an important fruit crop. Worldwide, it is one of the most widely cultivated and consumed fruit. The Philippines is an important banana producer (Department of Agriculture, 2018). In 2018, the overall production of banana in the country reached 9.4 million metric tons with an estimated annual growth rate of 2% (Philippine Statistics Authority, 2018). 'Lakatan' banana (*Musa acuminata*) is a distinct banana fruit widely grown in the Philippines comprising 10% of the total banana production. In recent years, 'Lakatan' has been exported to Japan, China, other East Asian and Middle Eastern countries (Herradura et al., 2013). 'Lakatan' banana is a diploid cultivar (AA Group) and one of the most popular dessert bananas in the Philippines. It has higher β-carotene content and in the domestic market it is more expensive than the other local banana cultivars.

Banana is typically a climacteric fruit that shows an increase in ethylene production and respiration rate during ripening stage. Ripening enhances color uniformity and palatability of the fruit; however, around 3-30% of the fruit is estimated to be wasted due to premature ripening, weight loss, mechanical damage, disease, and/or rotting (Nuevo and Apaga, 2010). Previous studies have shown potential benefits of ethylene inhibitors in controlling ripening. The synthetic gaseous compound, 1-methylcyclopropene (1-MCP) is said to be an effective ethylene antagonist compound which can extend the shelf life of fresh produce by controlling ripening-related changes such as drastic changes in color, texture, flavor, and aroma of the

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flesh (Blankenship, 2001). It is an ethylene blocker that prevents ethylene-dependent processes such as ripening and senescence in many horticultural crops (Blankenship and Dole, 2003). By competing with the hormone ethylene for the same sites, 1-MCP blocks the ripening of some climacteric fruits. In 'Cavendish' banana, the combined 300 nmol mol^{-1} 1-MCP + 1,200 or 2,400 nmol mol^{-1} ethylene treatment appeared to be a promising treatment to extend storage, following overseas shipping (Botondi et al., 2014). In another study, the shelf life of 'Cavendish' banana stored at 20°C was also extended when treated with 15 µL L^{-1} 1-MCP and subsequently exposed to ethylene (Macnish et al., 1997). Jiang et al. (1999) demonstrated that 1 µL L^{-1} 1-MCP for 1 h at 20°C delayed the ripening effects induced by 100 µL L^{-1} ethylene.

As ripening proceeds quickly in banana, the shelf life and quality of the fruit also deteriorates. Therefore, regulating the ripening of fruit to slow down its deterioration rate may be beneficial. This will be useful for bananas bound to far destinations by applying treatments that counteract the effect of ethylene, such as 1-MCP. This study evaluated the effect of different concentrations of 1-MCP on the ripening and quality of 'Lakatan' banana at two maturity stages that were dipped in ethephon prior to treatment.

MATERIALS AND METHODS

Plant material preparation

Freshly-harvested 'Lakatan' banana fruit were obtained from Libertad, North Cotabato, Philippines (8°31'57.8"N; 124°21'29.9"E) and transported in cushioned trays to the Postharvest Biology Laboratory in the University of the Philippines Mindanao, Davao City (7°05'03.4"N; 125°28'36.5"E). A portion of mature green fruit were treated immediately at the day of harvest while the rest of the fruit were stored in 20°C for two days until it reached light green stage. Per bunch of banana fruit, only the first three hands were utilized for the experiment. Each hand of banana was further divided into three clusters with 3-4 fingers per cluster while discarding fingers at the opposite ends of the hand as well as damaged fruit. Each cluster was washed with tap water to remove dirt and debris, then sanitized with 200 µL L^{-1} NaOCl for three minutes and air-dried. Next, fruit were dipped in 200 µL L^{-1} ethephon for 5 min prior to treatment with 1-MCP.

1-Methylcyclopropene treatment

The 1-MCP powder needed for the treatment of 100 and 1,000 nL L^{-1} were calculated based on the Ideal Gas Law (Semat and Katz, 1958). Distilled water (5 mL) was injected in the chamber through a tube to release the 1-MCP into its gaseous form. Fruit were treated in an air-tight chamber (95.57 L volume) devised with a fan to facilitate circulation of gases inside it. Fruit inside a static chamber without 1-MCP treatment served as control. The fruit were treated for 24 h under ambient room conditions (26.5±0.7°C, 80.1±4.3% RH) and stored in 20.7±0.34°C, 85.3±6.4% RH.

Quality evaluation

Quality of 'Lakatan' banana was evaluated every four days for up to 29 days. Weight loss (%) was determined by calculating the proportion of weight lost from the initial. Peel and pulp firmness (kgf) were measured at the middle portion of the fruit using a penetrometer (Fruit Tester FT 327 Pressure Tester, Wagner Instruments, USA). Total soluble solids (TSS) was measured by chopping banana fruit flesh and blending ~15 g of it with 45 mL distilled water using an Osterizer blender. The supernatant was filtered using a fine mesh then a drop of the liquid was placed onto the refractometer prism (HI 96801, Hanna Instruments, Romania) to measure the TSS (°Brix). Necessary temperature corrections were applied.

The peel color of the fruit was determined through an index developed by Kader (1996). Using a seven-point scale; the peel color was evaluated as; 1 = mature green, 2 = light green, 3 = more green than yellow, 4 = more yellow than green, 5 = yellow with green tips, 6 = full yellow, 7 = yellow with brown freckles. The visual quality was evaluated using the scale; 1 = excellent (field fresh, no defects), 2 = very good (slight defects), 3 = good (defects progressing, limit of saleability), 4 = fair (onset of decay, limit of edibility), 5 = poor (severely deteriorated)

(Ekman et al., 2019). Shelf life is expressed as the number of days until the fruit is deemed saleable (visual quality of 3).

Experimental and statistical analysis

Six replicate-clusters from different banana hands (1-3) were distributed among treatment lots using a completely randomized design (CRD). Data were analyzed using factorial (two-way) analysis of variance (ANOVA) and interactions with 1-MCP concentration and maturity stage as factors. Difference in means was detected using Fisher's LSD at $p \leq 0.05$.

RESULTS AND DISCUSSION

Weight loss

The treatment of 1-methylcyclopropene (1-MCP) resulted in a significantly lower weight loss in 'Lakatan' banana relative to the control fruit (Figure 1A). In terms of maturity stage, a lower weight loss was incurred when fruit were treated at a light green stage compared to those treated earlier at the mature green stage (Figure 1B). This could be due to a more suitable maturity stage of fruit at 1-MCP treatment which is light green in comparison with mature green. Fruit weight loss can be attributed to water loss, transpiration, respiration, and storage conditions such as temperature and relative humidity (Lownds et al., 1993). The high energy required to perform metabolic processes also adds up to the fruit's weight loss (Krishnakumar and Thirupathi, 2014). In this study, the fruit that were stored at low temperature and high RH (20.7±0.3°C and 85.3±6.4% RH) retained more moisture, lower respiratory activity, and delayed senescence thereby maintaining the quality of the fruit (Blankenship and Herdeman, 1995). 1-MCP might have also played a significant role in delaying the ripening in 'Lakatan' banana fruit as it has similarly reduced fruit weight loss in 'Rasthali' banana (Krishnakumar and Thirupathi, 2014). No interaction effect between 1-MCP concentration and maturity stage on the fruit weight loss was noted (Table 1).

Table 1. Interaction effect of 1-methylcyclopropene treatment and maturity stage on the quality of 'Lakatan' banana treated for 24 h and stored at 20.7±0.3°C, 85.3±6.4% RH.

Parameter	Storage period (days)						
	1	5	9	13	17	21	25
Weight loss	ns	ns	ns	ns	ns	ns	ns
Firmness	ns	ns	ns	*	*	*	ns
Total soluble solids	ns	*	*	*	*	ns	ns
Peel color index	*	*	ns	ns	*	*	ns
Visual quality	ns	ns	ns	*	ns	ns	ns
Days to full yellow stage				*			
Shelf life				ns			

* significantly different at $p \leq 0.05$; ns = not significant.

Firmness

The treatment of 1-MCP, especially at 1,000 nL L^{-1} followed by 100 nL L^{-1}, was able to delay the softening of 'Lakatan' banana compared to the untreated fruit (Figure 2A). As early as 24 h after 1-MCP treatment, a big difference between the firmness of treated fruit was already observed compared to the control. Further reduction in the rate of softening was observed when fruit were treated at mature green stage (Figure 2B).

Thus, softening of the fruit was repressed by 1-MCP treatment. Similar results were observed in the study of Pelayo et al. (2003) where 1,000 nL L^{-1} 1-MCP significantly delayed softening of stage 3 (more green than yellow) and stage 4 (more yellow than green) bananas. The interaction between 1-MCP concentration and maturity stage was observed between 13 and 21 days after treatment with retention of firmness in favor of the higher 1-MCP concentration (1,000 nL L^{-1}) treatment of the mature green stage (Table 1). Banana fruit

softens as it ripens, especially during the climacteric to post climacteric phases. When ripening proceeds, pectin substances located in the cell wall and middle lamella of the cell solubilize thus the fruit reduces its firmness (Trivedi, 2012). On softening, Feng et al. (2000) showed that polygalacturonase and cellulase activities were lowered by 1-MCP due to its antagonistic effect on ethylene-induced ripening response.

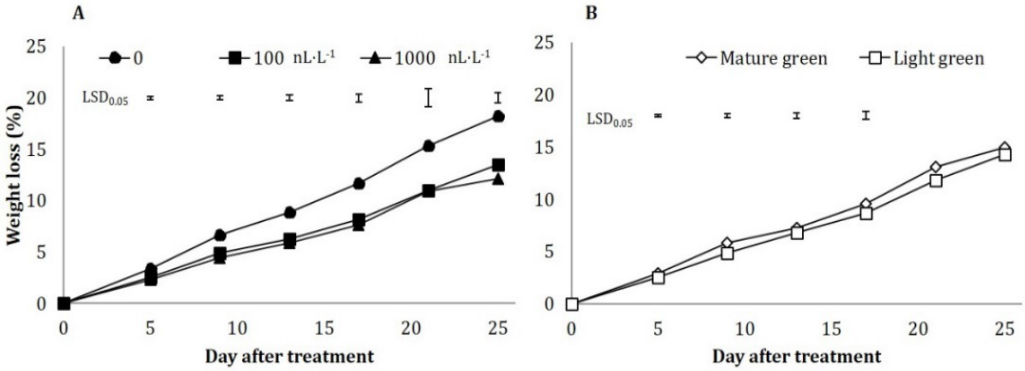

Figure 1. Weight loss of 'Lakatan' banana as influenced by 1-methylcyclopropene concentration (A) and maturity stage (B) during storage at 20.7±0.3°C, 85.3±6.4% RH. Bar represents difference in means using Fisher's LSD at p≤0.05.

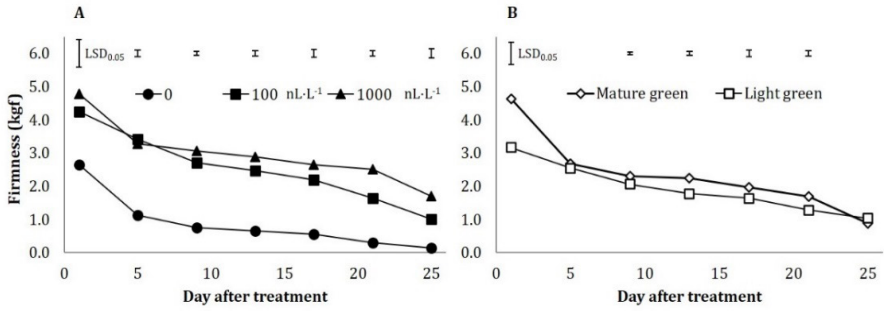

Figure 2. Firmness of 'Lakatan' banana as influenced by 1-methylcyclopropene concentration (A) and maturity stage (B) during storage at 20.7±0.3°C, 85.3±6.4% RH. Bar represents difference in means using Fisher's LSD at p≤0.05.

Total soluble solids

The accumulation of total soluble solids (TSS) in the fruit was significantly delayed by the treatment of 1-MCP especially at a concentration of 1,000 nL L^{-1} (Figure 3A). After 24 h of 1-MCP treatment, the difference between the treated and untreated fruit was observed with TSS content lower in 1-MCP treated fruit. There was a gradual increase in TSS for treated bananas compared to the untreated control, and a substantial upsurge in TSS after five days for the control fruit and 21 days for those treated with 100 or 1,000 nL L^{-1} 1-MCP. A slight delay in the accumulation of TSS was recorded when the fruit was treated at the mature green stage (Figure 3B). On the 25th day, 1,000 nL L^{-1} 1-MCP treated fruit attained the almost full yellow stage with a low TSS of 9.75 °Brix whereas the 100 nL L^{-1} 1-MCP treatment appeared to show the preferred fruit quality that is less firm and sweeter (15 °Brix). There was an interaction effect of the factors on the TSS content of the fruit at 5 to 17 days after treatment (Table 1).

In a study by Rahman et al. (2013), 1-MCP effectively delayed the increase of TSS on 'BARI Kola-1' bananas. The TSS content of banana increases as the fruit ripens due to the breakdown of starch into sugar (Trivedi, 2012). As the banana fruit ripens, the starch hydrolyzes into sugar which gives a sweet flavor and enhances the edibility of the fruit. The

hydrolysis of starch is mainly catalyzed by the activity of α-amylase and β-amylase as a part of the ripening process (Cordenunsi and Lajolo, 1995).

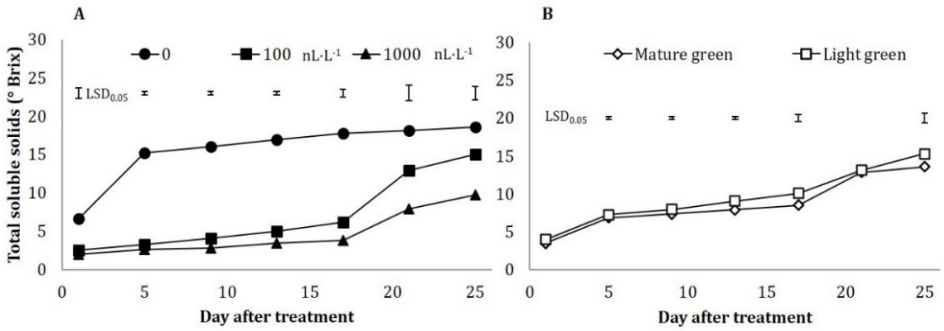

Figure 3. Total soluble solids of 'Lakatan' banana as influenced by 1-methylcyclopropene concentration (A) and maturity stage (B) during storage at 20.7±0.3°C, 85.3±6.4% RH. Bar represents difference in means using Fisher's LSD at p≤0.05.

Peel color

Color development is one of the manifestations of the ripening process initiated by ethylene in fruits. Peel color change in 'Lakatan' banana was delayed by the 1-MCP exposure compared to the untreated fruit (Figure 4). The delaying effect of 1,000 nL L^{-1} 1-MCP was more apparent than 100 nL L^{-1} concentration at 17 to 25 days after treatment. The control fruit already reached the full yellow stage (stage 6) after nine days while 1-MCP treated fruit were still at stages 5 (yellow with green tips) to 6 after 25 days.

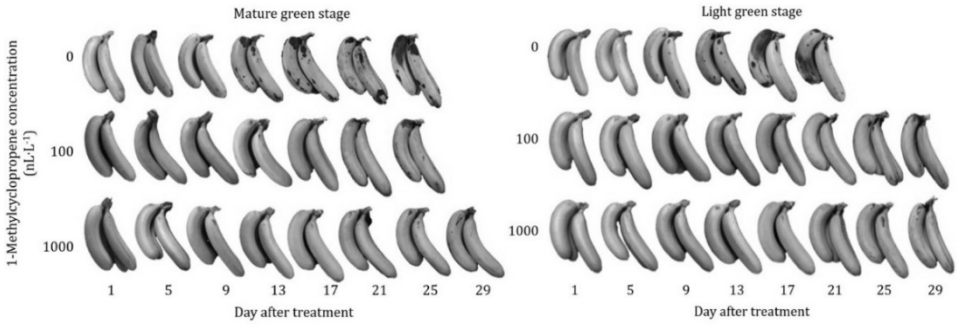

Figure 4. Visual appearance of 'Lakatan' banana treated with 1-methylcyclopropene at mature green or light green stage, then stored at 20.7±0.3°C, 85.3±6.4% RH for 29 days.

1-MCP blocks the ethylene receptors thereby preventing ethylene-dependent processes to occur including color changes. 1-MCP can prevent the activation of enzymes related to the degradation of the chlorophyll hence delaying the loss of green color (de Martino et al., 2007). The present findings concur with the previous results when banana at ripening stage 3 (more green than yellow) and 4 (more yellow than green) treated with 300 or 1,000 nL L^{-1} 1-MCP had slower peel color change compared to untreated banana (Pelayo et al., 2003). However, as the banana fruit ripened in this study, some fruit exhibited uneven color development. This could be due to the inconsistent ethylene biosynthesis present in the peel (de Martino et al., 2007).

In terms of maturity stage, light green banana fruit maintained its green color slightly longer than those treated at mature green stage. According to Lurie (2007), the length of inhibition of color change could be determined by the stage when the banana fruit was treated. It can then be inferred that the response of the fruit to the 1-MCP was dependent on

concentration and time (Jiang et al., 1999). In this study, the interaction of 1-MCP concentration and maturity stage influenced the peel color development from 24 h after the treatment until 21 days of storage except at day 9 and day 13 (Table 1). Untreated fruit was first to reach the full yellow stage (8.7 days, data not shown) followed by those treated with 100 nL L^{-1} 1-MCP (26 days), and by those treated with 1,000 nL L^{-1} 1-MCP (28.3 days).

However, fruit treated with 1-MCP did not produce a bright yellow color upon reaching the full yellow stage in contrast to the control fruit. A vibrant yellow color was attained by the control fruit after nine days compared to the 1-MCP treated fruit at 25-29 days which was described as pale and dull yellow. 1-MCP may have slowed down the peel color change, but it did not maintain the color intensity. This phenomenon could also be attributed to the storage conditions of this study (i.e., 20.7±0.3°C and 85.3±6.4% RH) which was different from the recommended temperature for natural and uniform ripening of banana fruit which is at 25°C (Hu et al., 2014). Furthermore, Fu et al. (2019) reported that carotenoid content which is responsible for the yellow peel color is affected by temperature.

Visual quality and shelf life

The treatment of 1-MCP (100 or 1,000 nL L^{-1}) at mature green stage maintained 'Lakatan' banana fruit's visual quality up to a saleable stage until 21 days of storage (Figure 5). The deterioration of visual quality was delayed by 12 days in 1-MCP-treated fruit compared to the control. Defects started to progress (i.e., having visual quality rating of 3) after 17 days of storage for the control fruit whereas it was after 21 days for the 1-MCP treated fruit. This result accords with the study of Trivedi (2012) in which 1-MCP-treated 'Cavendish' banana fruit developed less brown spotting and yellowing.

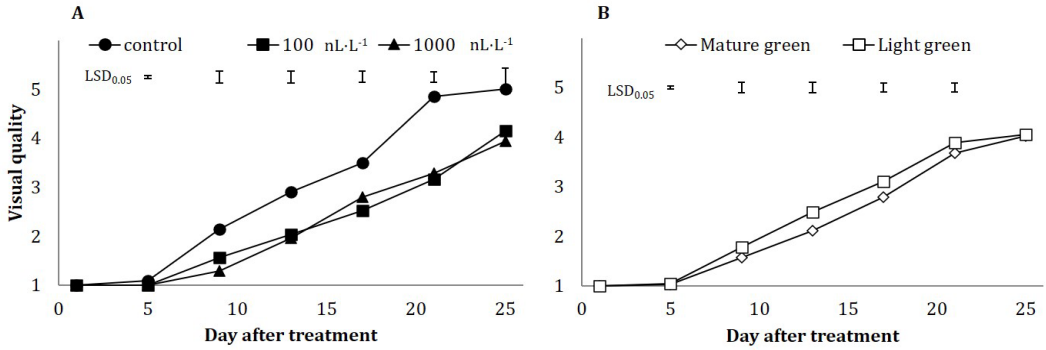

Figure 5. Total soluble solids of 'Lakatan' banana as influenced by 1-methylcyclopropene concentration (A) and maturity stage (B) during storage at 20.7±0.3°C, 85.3±6.4% RH. Bar represents difference in means using Fisher's LSD at p≤0.05.

Brown spots became evident on the peel as fruit attained a visual quality rating of 4 (i.e., onset of decay). The 'Lakatan' banana fruit developed browning, rot, and presence of crown rot disease toward the end of storage. The development of disease could be attributed to the growth of fungi which has been stimulated by the high relative humidity in the storage room (i.e., 85.3±6.4% RH). Generally, if the RH reaches over 86%, it may lead to conidia of most fungi to germinate (Badger, 1965). Crown rot disease is caused by fungal pathogens in the genera *Colletotrichum*, *Fusarium*, *Acremonium*, *Verticillium*, and *Curvularia* (Nelson, 2008).

'Lakatan' banana fruit treated with 1-MCP, regardless of its concentration, had longer shelf life (20 days, data not shown) compared to the untreated fruit which only lasted for 15 days. Further, fruit treated at mature green stage had longer shelf life (19.4 days) compared to those treated at light green stage (17.4 days). However, there was no interaction effect between 1-MCP concentration and maturity stage on the shelf life of the fruit (Table 1).

CONCLUSIONS

The treatment of 100 or 1,000 nL L^{-1} 1-MCP and subsequent storage at 20.7±0.3°C,

85.3±6.4% RH maintained the shelf life of 'Lakatan' banana for up to 20 days while the untreated fruit had a shelf life for 15 days. Treating the fruit with 100 or 1,000 nL L^{-1} 1-MCP resulted in lower weight loss, slower peel color change, retention of firmness, and lower TSS when it was treated at either mature or light green stages. Further, fruit ripening was best delayed in those treated with 1,000 nL L^{-1} 1-MCP as it slowed down the transition to full yellow stage by 2.3 days than those treated with 100 nL L^{-1} 1-MCP, and 19.6 days longer than the untreated fruit. The lower 1-MCP treatment resulted in sweeter fruit after 25 days. Fumigation of 100 nL L^{-1} 1-MCP for 24 h can potentially extend shelf life of 'Lakatan' banana.

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α-L-arabinofuranosidase activity and gene expression in two tomato cultivars showing different flesh textures

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Abstract

In ripe fruit, depolymerization/solubilization of hemicellulose and pectin is controlled by a set of cell wall-modification enzymes. A decrease of mechanical strength and the determination of flesh texture in the fruit are caused by metabolic changes associated with cell wall polysaccharide. In tomato, it has been shown that pectate lyase has a central role in fruit softening. However, in spite of flesh texture being an important factor in determining fruit quality, little is known about the enzyme(s) relating to the control of flesh texture. Changes in the strength of cell-to-cell adhesion in fruit are considered to be one of the factors involved in changes in fruit texture. Several reports have been shown that arabinosyl-containing polysaccharides play important roles in cellular attachment or cell wall flexibility. α-L-arabinofuranosidase is an enzyme which is capable of releasing arabinofuranosyl residues from polysaccharides. To examine the contribution of α-L-arabinofuranosidase to fruit texture formation, we measured α-L-arabinofuranosidase activity and transcription levels of α-L-arabinofuranosidase-encoding genes in two tomato cultivars: 'Shonan Pomoron Red' and 'Reiyo'. Compared to fruit showing normal texture, a higher activity of α-L-arabinofuranosidase was observed in fruit showing abnormal texture. However, no significant difference in fruit firmness was found between the fruit of the two cultivars. These results indicate that α-L-arabinofuranosidase potentially caused the abnormal flesh texture in tomato fruit. Among six α-L-arabinofuranosidase-encoding genes, the transcripts of two genes were not detected in the both cultivars. Other genes, which showed differential expression patterns, would be responsible for the formation of abnormal flesh texture in tomato fruit.

Keywords: arabinose, cell adhesion, firmness, juiciness, mealiness, softening

INTRODUCTION

Tomato (*Solanum lycopersicum*) is one of the most highly produced vegetable crops worldwide. The production value of tomato is the highest of all vegetable crops in Japan. Although most tomato fruit grown in Japan are the fresh-market type, consumption of the processing type of fruit has also recently increased. Generally, the fruit of the processing type are characterized as being firmer with a low-juiciness flesh texture while fresh-market cultivars are softer and have higher juiciness. 'Shonan Pomoron Red' ('SPR'), which has been released from Kanagawa Agriculture Research Center, is an F_1 hybrid from a cross between a processing type and a fresh-market type. It was developed in response to recent demand for freshly-harvested processing fruit instead of canned fruit (Kita, 2004; Hoya et al., 2013). The fruit flesh of 'SPR' is relatively non-juicy compared to the normal fresh-market type but is not as firm as the processing types. It is a long ellipse type (Kita, 2004; Hoya et al., 2013).

As fruit ripening proceeds, flesh firmness decreases and a unique texture is formed because of the modification of the architecture of the cell wall polysaccharides. The modifications of the polysaccharides are caused by a number of enzymes found in the apoplast.

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Studies of fruit softening enzyme(s) have been done for a long time. Recently it has been revealed that pectate lyase plays a central role in fruit softening during tomato ripening (Uluisik et al., 2016; Yang et al., 2017). However, there is little information about the enzyme(s) that are related to the determination of flesh texture. A lack of juiciness, that is often termed mealiness or woolliness, is one of the undesirable fruit traits and can occur as the formation of abnormal texture during ripening and after low-temperature storage. In the case of tomato fruit, chilling injury symptoms, such as an alteration of the ripening phenomena, pitting and decay, are generally observed when fruit have been exposed to temperatures below 12.5°C (Hobson, 1987; Cheng and Shewfelt, 1988). Altered cell wall metabolism under low temperatures can lead to mealy texture in tomato fruit (Rugkong et al., 2010).

Pectin, a major component of the middle lamella that mainly consists of homogalacturonan and rhamnogalacturonan, contains arabinosyl-polysaccharides such as arabinogalctan and arabinan. Modification of arabinan side-chains has been found in mealy fruits of tomato (Orfila et al., 2001, 2002), apple (Peña and Carpita, 2004; Nara et al., 2001; Nobile et al., 2011) and peach (Yoshioka et al., 2011). Arabinose-containing polysaccharides in the cell wall play an important role in cellular attachment (Iwai et al., 2001; Orfila et al., 2002) and cell wall flexibility (Jones et al., 2003; Moore et al., 2008). Thus, the action of α-L-arabinofuranosidase, which hydrolyzes arabinofuranosyl residues from various polysaccharides, is likely to cause development of the mealy texture.

In this study, we measured fruit firmness and juiciness in two tomato cultivars: 'Shonan Pomoron Red' which has an abnormal texture and 'Reiyo' which has a normal texture. Further, to address the influence of low temperature on fruit texture, fruit were exposed at 10°C to develop chilling injury. A potential role of α-L-arabinofuranosidase in the development of abnormal texture under low temperature is discussed.

MATERIALS AND METHODS

Plant materials

Tomato (*Solanum lycopersicum*) cultivars 'Reiyo' (Sakata Seed Corp., Kanagawa, Japan) and 'Shonan Pomoron Red' (Kanagawa Agricultural Technology Center, Kanagawa, Japan) were used in this study. The plants were grown in a greenhouse at Kanagawa Agricultural Technology Center under conventional conditions. Fruit were harvested at the pink stage and ripened to red ripe or over ripe stage at either 10 or 20°C. After measurement of fruit firmness and flesh properties, the pericarp was frozen using liquid nitrogen and stored at -80°C until use.

Measurement of fruit firmness and juiciness

Fruit firmness was measured using a handy hardness meter (Fujiwara Scientific Co., Ltd., Tokyo, Japan). Equatorial positions were examined at least three times per fruit. For measurement of juiciness, six discs (10 mm diameter) were prepared from pericarp tissue of one fruit using a cork borer and both exocarp and endocarp were removed with a scalpel. The disk was placed on three-layered filter paper and crushed with a plunger (40 mm diameter) equipped with a creep meter (RHEONER II, Yamaden Co., Ltd., Tokyo, Japan) to 90% deformation. Released juice from the disc was absorbed on the filter papers and the weight was measured after removal of the residues from the paper. The ratio of excluded juice amount per entire amount was determined.

Enzyme extraction and assay of α-L-arabinofuranosidase activity

Extraction of crude enzyme from tomato fruit was carried out according to the method described by Sozzi et al. (2002). 4-Nitrophenyl α-L-arabinofuranoside or 4-nitrophenyl β-D-xylopyranoside was used as a substrate.

RNA extraction and quantitative RT-PCR

Fruit pericarp was ground using a mortar and pestle in the presence of liquid nitrogen. Total RNA from the powdered sample was isolated with a Plant RNA Isolation Reagent (Life

Technologies Japan Ltd., Tokyo, Japan) according to the manufacturers' instructions. Contaminating genomic DNA in total RNA was removed by treatment with DNase I (Takara Bio Inc., Shiga, Japan). cDNA was synthesized using PrimeScript reverse transcriptase (Takara Bio Inc.) with an oligo d(T) primer (5'-ACCTGGAAGAATTCGCGGCCGCAGGAA(T)18-3').

Quantitative RT-PCR

The reactions were performed in a final volume of 20 µL consisting of 1 µL of diluted cDNA, 0.4 µM of specific primers and 10 µL of SYBR Premix Ex *Taq*II (Takara Bio Inc.). PCR conditions were 95°C for 30 s, and 40 cycles of 95°C for 5 s and 60°C for 30 s. The expression levels were normalized using the clathrin adaptor complexes medium subunit/endocytic pathway (CAC) (Expósito-Rodríguez et al., 2008). Gene-specific primers for *SlArf/Xyl1*, *SlArf/Xyl2*, *SlArf/Xyl3* and *SlArf/Xyl4* were used as described in Tateishi et al. (2014). The primers for *LeXYL1* were 5'-CTTCACCACCCTCAGTCCAT-3' and 5'-GGTCCTAATGCAACTTTC CTG-3', and for *LeXYL2* were 5'-CCCTTGAGGAAGGACATGCT-3' and 5'-TGTGAACTGAGGGTGGT GAA-3'.

RESULTS AND DISCUSSION

Changes in fruit firmness and flesh juiciness were measured in two tomato cultivars, 'Shonan Pomoron Red' ('SPR') and 'Reiyo', which showed different flesh textures, during ripening. 'Reiyo' is a typical fresh table tomato while 'SPR' is an intermediate type resulting from a cross between a fresh type and a processing one. Fruit firmness decreased in both cultivars as fruit ripened at 20°C (Figure 1A). Juiciness of fruit flesh was assessed by a measurement of the rate of released juice from a pericarp disk when collapsed with a plunger. No significant difference in released juice was observed between cultivars at the pink stage. However, the released juice was more abundant in 'Reiyo' than 'SPR' at the red ripe and over ripe stages (Figure 1B). This difference reflects a distinct difference in flesh texture between the two cultivars; 'Reiyo' is a juicier cultivar than 'SPR'.

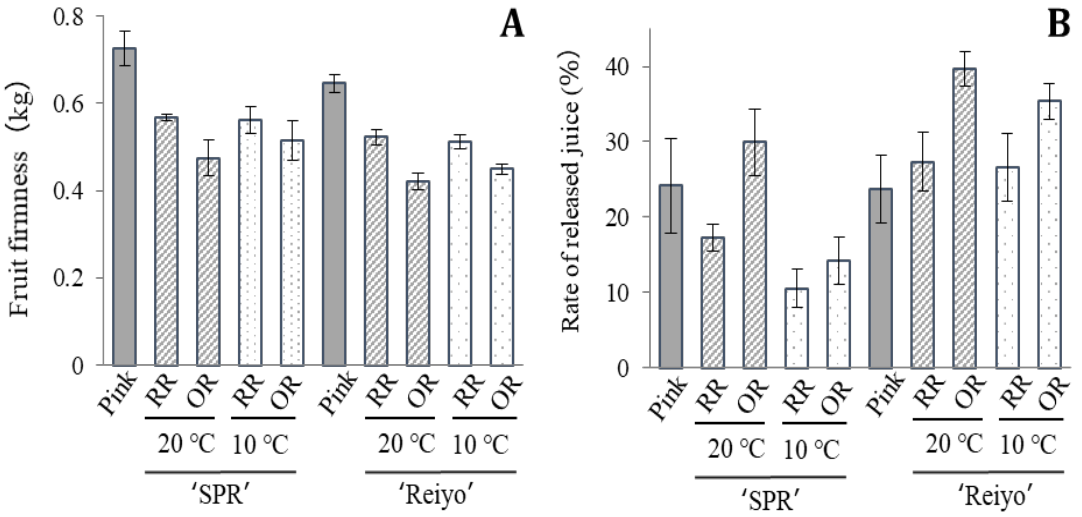

Figure 1. Changes in fruit firmness (A) and rate of released juice (B) during fruit ripening at 10 or 20°C. Pink, RR (red ripe) and OR (over ripe) indicate ripening stage. The values are the means of five fruits. Vertical bars indicated the standard error (*n*=5).

Ripening progress was suppressed when the fruit were ripened at 10°C. However, there were no significant differences in fruit firmness at the same stage of the progression in ripening between the fruit ripened at 10 and 20°C (Figure 1A). In the case of tomato fruit, chilling injury symptoms, such as disruption of the ripening phenomena, pitting and decay,

are generally observed when the fruit has been exposed below 12.5°C (Hobson, 1987; Cheng and Shewfelt, 1988). The symptoms and the extent of chilling injury seem to vary depending on exposure time to chilling temperature, ripening stage, and situations before and after exposure to low temperature. In this study, when the flesh texture of fruit ripened at 10°C was checked it had developed an abnormal texture, determined as a dry-feeling in the mouth (data not shown). In order to assess juiciness, the rate of release of juice from the collapsed pericarp disc was measured. In 'SPR', the rate of released juice was markedly reduced when the fruit was ripened at 10°C at both the red ripe and over ripe stages (Figure 1B). In contrast, in 'Reiyo', the decrease was observed only at the over ripe stage. The lower juiciness implies that there had been a separation between each cell due to weakened cell-to-cell adhesion rather than to cell rupturing; the resultant characteristic is recognized as mealy fruit tissue (Arefi et al., 2016). These results indicate that development of the mealy texture was caused by ripening at 10°C while fruit firmness was not influenced.

Arabinose-containing polysaccharides in the cell wall play an important role in cellular attachment (Iwai et al., 2001; Orfila et al., 2002) and in wall flexibility (Jones et al., 2003; Moore et al., 2008). A modification of arabinosyl side chains has been observed in tomato fruit showing a mealy texture (Orfila et al., 2001, 2002). Thus, we measured α-L-arabinofuranosidase activity. The activity slightly decreased during ripening at 20°C in 'SPR' fruit while the activity declined markedly at the over ripening stage in 'Reiyo' fruit at this temperature (Figure 2). In contrast, the activity increased when ripened at 10°C in both cultivars compared to that at 20°C (Figure 2). It has been reported previously that several α-L-arabinofuranosidase simultaneously possesses β-xylosidase activity (Tateishi, 2008). In this study, changes in β-xylosidase activity were also measured and they showed almost the same pattern as occurred with the changes in α-L-arabinofuranosidase activity (data not shown).

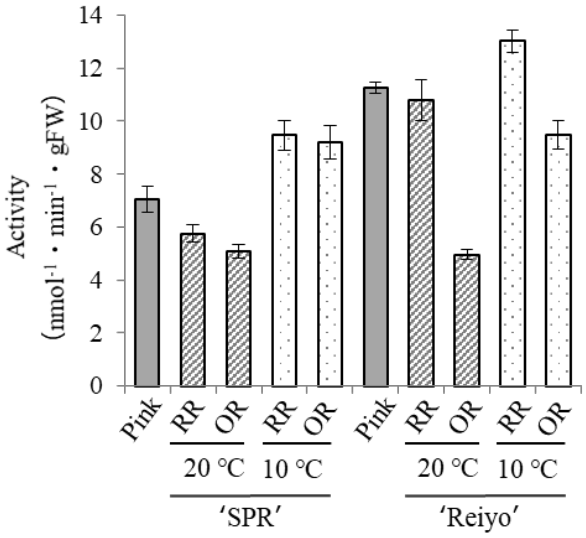

Figure 2. Changes in α-L-arabinofuranosidase activity during fruit ripening at 10 or 20°C. Pink, RR (red ripe) and OR (over ripe) indicate ripening stage. The values are the means of five fruits. Vertical bars indicated the standard error (*n*=5).

The expression levels of six α-L-arabinofuranosidase-related genes that are classified as being in the glycoside hydrolase family 3 were monitored: *SlArf/Xyl1-4* and *LeXYL1-2*. *SlArf/Xyl1* expression level was nearly constant in 'SPR' and decreased in 'Reiyo' during ripening at 20°C. However, the expression increased in the fruit ripened at 10°C in both cultivars (Figure 3A). The different accumulation pattern of *SlArf/Xyl1* between 'SPR' and 'Reiyo' fruit ripened at 20°C may influence the difference in juiciness found between the two cultivars. Since the expression was affected by temperature, SlArf/Xyl1 would also also be responsible for the juiciness that was lost under the cool temperature. In addition to

SlArf/Xyl1, *SlArf/Xyl3* expression also increased in the fruit ripened at 10°C in both cultivars (Figure 3B). The temperature-dependent increase was also found in *SlArf/Xyl3* expression. However, this gene has reported to be predominantly expressed in floral organs (Tateishi et al., 2014). The absolute expression level of *SlArf/Xyl3* is predicted to be low in fruit tissue, meaning that there would be less contribution to the increased α-L-arabinofuranosidase activity. For *LeXYL1*, the highest expression was observed in red ripe fruit in both cultivars (Figure 3C). The result is in agreement with the results described by Itai et al. (2003). The expression pattern was not affected by ripening temperature (Figure 3C). From the results it would appear that *LeXYL1* was not responsible for the increase α-L-arabinofuranosidase activity and the development of abnormal texture at 10°C. Expression of *LeXYL2* was very low in red ripe and over ripe fruit in both cultivars ripened at 20°C; the transcript was hardly detectable especially in over ripe fruit (Figure 3D). However, when the fruits were ripened at 10°C, accumulation of the transcript was observed in both red ripe and over ripe fruit in both cultivars (Figure 3D). This accumulation would have been due to de novo expression of *LeXYL2* induced by the cool temperature. *LeXYL2* would, therefore, be responsible for the increase in α-L-arabinofuranosidase activity under 10°C conditions and would lead to the development of mealy texture. Among the six α-L-arabinofuranosidase-related genes, which are classified in the glycoside hydrolase family 3, SlArf/Xyl1 and SlArf/Xyl2 have been shown to have both α-L-arabinofuranosidase and β-xylosidase activities. SlArf/Xyl4 has β-xylosidase activity alone (Tateishi et al., 2014). Expression of either *SlArf/Xyl2* or *SlArf/Xyl4* could not be detected in ripening tomato fruit even though the fruit ripened at 10°C. Although it is not elucidated whether LeXYL2 possess both the activities or not, increases of both α-L-arabinofuranosidase and β-xylosidase activities in the fruit ripened at 10°C would be attributed to the expression of *SlArf/Xyl1* and *LeXYL2*.

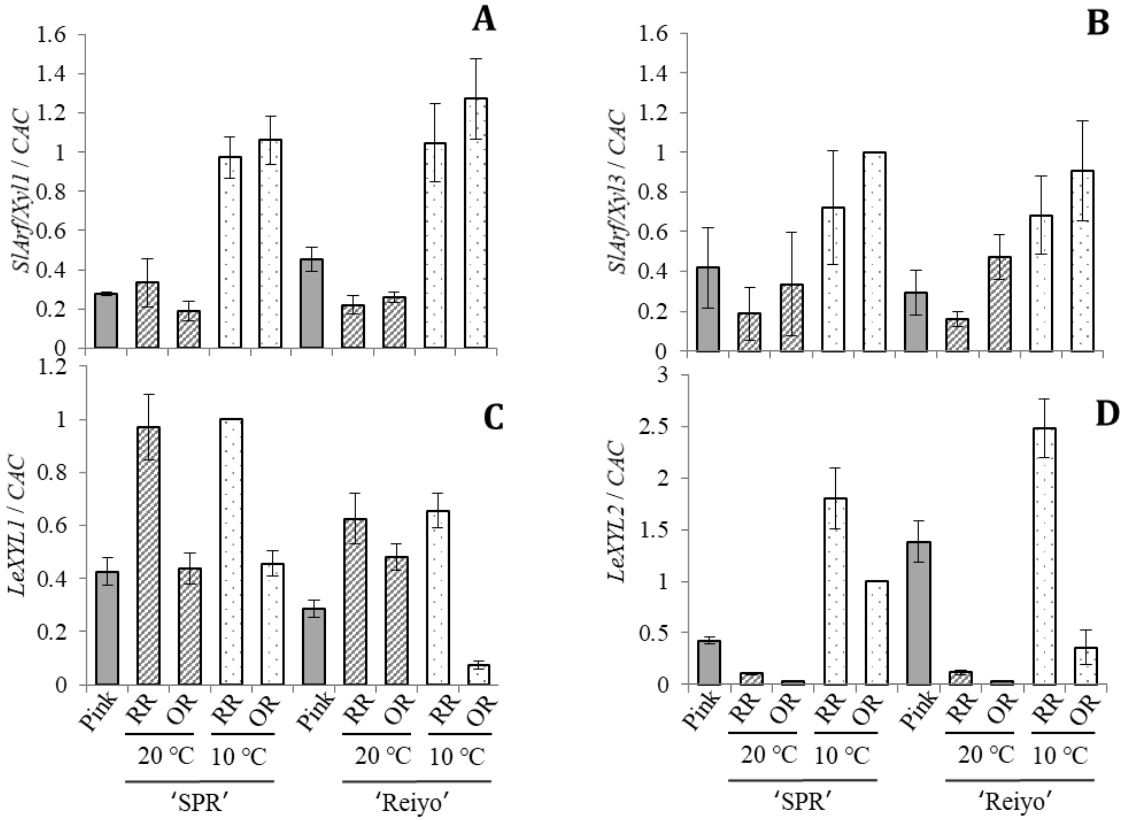

Figure 3. Relative expression of α-L-arabinofuranosidase-related genes during fruit ripening at 10 or 20°C. Pink, RR (red ripe) and OR (over ripe) indicate ripening stage. The values are the means of five fruits. Vertical bars indicated the standard error (*n*=5).

Modifications of arabinose-containing polysaccharides are found in fruits with a mealy texture. The action of α-L-arabinofuranosidase is potentially responsible for the development of the abnormal texture via cell wall modification. Although, in this study, we did not confirm the changes in arabinose-containing polysaccharides in the mealy fruit, the juicy-less texture found in 'SPR' may be the result of a higher expression level of *SlArf/Xyl1.* The development of mealiness texture when the fruit was ripened at 10°C might have been caused by both an increase of *SlArf/Xyl1* expression and de novo expression of *LeXYL2*.

CONCLUSIONS

The following conclusions can be drawn from this study:

1. A lower rate of release of juice from the pericarp disks of 'SPR' than from 'Reiyo' indicates a textual difference between cultivars that has a genetic basis;
2. Juiciness is lost when fruit are ripened at 10°C, even though the fruit softness are at the same level;
3. α-L-arabinofuranosidase seems to be responsible for the juicy-less property and mealiness, rather than fruit firmness;
4. Among the α-L-arabinofuranosidase-related genes, *SlArf/Xyl1* and *LeXYL2* appear to be involved in the development of the non-juiciness and abnormal texture that is induced by cool temperature.

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The impact of harvest time on the quality of Indian jujube 'Nomsod' produced in northeastern Thailand

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Abstract

Indian jujube (*Zizyphus mauritiana* Lamk.) is an economic fruit crop and 'Nomsod' is a promising new jujube cultivar for production in northeastern Thailand. The fruit is high in vitamin C content and has a sweet yet slightly acidic taste. However, there is no scientific information in relation to its quality, especially regarding harvest maturity under production conditions in Thailand. This study investigated the effect of harvest time on the quality of Indian jujube 'Nomsod'; specifically, at 90, 105, 120, and 135 days after full bloom (DAFB). Fruit growth at 120 DAFB had the highest values for fruit weight and diameter, fruit width, fruit length, and pulp thickness. L* and b* values increased during development. The total soluble solids (TSS) concentration increased to a maximum of 10.40 °Brix at 120 DAFB. Titratable acidity content (TA) and firmness both increased gradually to 0.26% and 62.65 N, respectively, also at 120 DAFB. The ratio of TSS/TA at this stage was 40.60. In contrast, the amount of ascorbic acid gradually decreased until 120 DAFB. It was concluded, therefore, that to achieve a high TSS it is best to harvest 'Nomsod' Indian jujube at 120 DAFB.

Keywords: jujube, *Ziziphus mauritiana* Lamk., harvest maturity, quality

INTRODUCTION

'Nomsod' jujube (*Ziziphus mauritiana* Lamk.), a member of the *Rhamnaceae* family, was developed from a hybrid between the cultivar 'Jujube Honey' from Taiwan and a special varietal species from India. Established by cleft grafting on a wild type rootstock, the resultant plant is resistant to diseases and insects, and very suitable for cultivation in Thailand's northeast region. 'Nomsod' jujube has been produced in Kalasin Province, Thailand. Dalal et al. (2019) and Li et al. (2007) described the jujube fruit's rich flavor and high nutritional value as being an important source of energy, protein, and essential minerals. The nutritional value is equivalent to aonla and guava, and slightly better than citrus fruit and apple (Bal and Uppal, 1992).

The 'Nomsod' jujube cultivar is found locally at Baan Pon, Tambon Pon, Amphur Kham Muang, in Kalasin Province in Thailand's northeast region. The fruit is oval in length and round in circumference, and the skin is smooth, glossy, and thin. When ripe, the flesh is white, crisp, and sweet (Pareek et al., 2009). In its first phase of development, the fruit skin is green and gradually turns yellow. When ripe, it turns burnt-orange, red-brown, or red. If overripe, the fruit emits a sweet and fragrant flavor (Hocking, 1993). The jujube's harvesting period is an important factor in determining maturity, as a harvest that is too early or too late may reduce the fruit's quality and durability (Junior et al., 2010). Changes that affect the quality of the product, both external and internal, may arise due to improper timing of the harvest, which may also have a significant impact on yield.

This study was conducted to determine the most suitable harvest time for 'Nomsod' jujube. The research determined the effects of harvest time on jujube quality, including the changes that occur in total soluble solids concentration and total titratable acidity during fruit growth, and the timing to produce the best harvest maturity.

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MATERIALS AND METHODS

Sample preparation

Samples of 'Nomsod' jujube fruit were harvested from an orchard at Baan Pon, Tambon Pon, Amphoae Kham Muang, Kalasin Province in the northeast of Thailand. The fruits were collected at 90, 105, 120 and 135 days after full bloom (DAFB).

Determination of physical characterization

Physical characteristics measured included weight (g), and width, length, fruit pulp thickness, and polar diameter (mm) using a vernier caliper. Fruit peel color, determined as L*, a* and b* values, was measured using a color meter (HunterLab MiniScan EZ 1043).

Determination of chemical characteristics

Analysis of the ascorbic acid content followed a modified version of Wira and Srivikhanyothin (2003). Total soluble solids (TSS) concentration was determined using a digital hand-held refractometer (PAL-1). Titratable acidity (TA) was measured on a 5 mL sample of juice that was titrated with 0.1 N NaOH, using 1% phenolphthalein as an indicator. Percentage titratable acidity was calculated as being equivalent to the concentration of citric acid.

RESULTS AND DISCUSSION

Fruit weight and polar diameter

Fruit weights and diameters of 'Nomsod' jujube between 90 and 135 DAFB were significantly different. Fruit weight generally increased concomitantly with an increase in fruit age, producing the highest results at 120 DAFB, followed by a harvest period of 135, 105, and 90 DAFB. The fruit diameter was statistically greatest at 135 DAFB (Table 1). These results confirm those of Choi et al. (2011) who showed that fruit weight and polar diameter in 'Mechu' jujube increased with increasing fruit age but subsequently decreased when the fruit was fully ripe. While polar diameters gradually increased, weight loss occurs at the later harvest periods due to dehydration, which results in the loss of turgor in tissues and is associated with increased ethylene production (Fadda and Mulas, 2010). Argenta (2006) reported that weight loss may be due to respiration, as well as overall fruit degeneration.

Table 1. Changes of physical properties of Indian jujube (*Z. mauritiana* Lamk.) 'Nomsod' fruit.

Days after full bloom	Fruit weight (g)	Polar diameter (mm)	Width (mm)	Length (mm)	Pulp thickness (mm)	Peel color		
						L*	a*	b*
90	12.73c	25.80b	26.95b	29.87c	7.93b	58.42b	-9.57	28.64b
105	22.03b	31.79a	32.37a	35.32b	11.27a	62.46a	-7.24	29.94a
120	27.40a	32.25a	34.55a	40.47a	11.56a	63.13a	-4.87	30.4a
135	23.13ab	32.44a	33.20a	35.98b	11.64a	63.47a	-8.6	30.29a
F-test	**	**	**	**	**	*	ns	**
CV (%)	11.93	4.13	4.26	4.38	5.39	3.36	38.53	2.09

Means followed by the same letter within each column were non-significant (ns), significantly different at p=0.05 (*) or 0.01 (**).

Fruit width, length and pulp thickness

The changes in fruit width, length, and overall growth of the jujube fruit followed a double sigmoid pattern. The study of Lu et al. (2012) found that jujube fruit 'Changhong' growth curve displayed a double sigmoid with a very short lag phase, will be found during at the last three ripening stages at 72, 80 and 88 days after petal fall. Which is similar to that reported for other jujube cultivars (Bal and Singh, 1978a, b; Abbas et al., 1994; Abbas and Fandi, 2002). Fruit length and fruit width were greatest at 120 DAFB (Table 1; Figure 1). Pulp

thickness was statistically the same from 105 to 135 DAFB although it did progressively increase over this time period. 'Changhong' jujube fruit requires approximately 80 DAFB to reach maturity, whereas 'Zaytoni' jujube fruit takes as long as 124 DAFB (Abbas and Fandi, 2002). The rapid ripening of 'Changhong' fruit is due to its rapid growth in the first phase of development, perhaps due to rapid cell elongation and division (Bollard, 1970), and maybe associated with high levels of auxins, gibberellins and cytokinins (Pareek, 2013). After an initial period of rapid growth, the fruits of 'Çhanghong' entered a delayed period of growth, similar to that of 'Zaytoni' (Abbas and Fandi, 2002). In this study, 'Nomsod' jujube fruit appeared to develop similarly to 'Zaytoni' jujube.

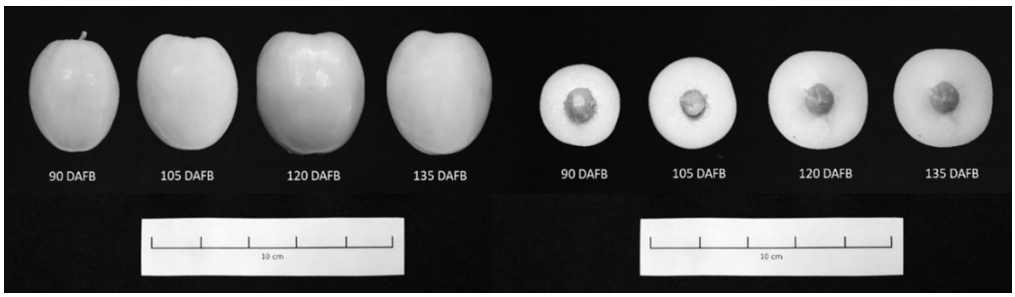

Figure 1. Indian jujube (*Z. mauritiana* Lamk.) 'Nomsod' fruit growth from 90 to 135 days after full bloom (DAFB).

L*, a* and b* values

Changes in fruit peel color in 'Nomsod' from 90 to 135 DAFB were determined as changes in luminosity or lightness (L*), green-red contrast (a*), and blue-yellow contrast (b*). Both the L* and b* values did not change significantly from 105 to 135 DAFB although there was a trend for both to be highest at 135 DAFB (Table 1). The increases in L* and b* values during fruit development may be due to reduced synthesis of chlorophyll and carotenoids (Jat et al., 2012). The value of the green-red contrast (a*) showed no statistical difference over the period of fruit development. Kaewjulapat (2008) reported that jujube peel color generally changes from green to yellow as the fruit develops. The initial green color of the immature jujube fruit is caused by the amount of chlorophyll present. However, as the fruit ripens, it creates a yellow pigment through the increase of β-carotenes, and a respective decrease in chlorophyll concentration.

Firmness, total soluble solids (TSS) and titratable acidity (TA)

The firmness of the jujube fruit was highest at 120 DAFB and then declined markedly (Table 2). Kaewjulapat (2008) also determined that fruit firmness decreased with increasing fruit age late in development. However, changes in actual firmness may also depend on species. The total soluble solids concentration and titratable acidity were also highest at 120 DAFB and both TSS and TA then significantly declined at 135 DAFB. (Table 2). Lu et al. (2012) found that the TSS contents of fruit were generally low at the early stages of growth, increasing throughout the growing period until reaching a maximum value when the fruit ripens physiologically. Coincidentally, the titratable acidity decreased (Bal and Singh, 1978a, b) (Table 2). Kaewjulapat (2008) showed that the jujube species *Z. spina-christi* had low acid content in the initial stages of growth, but increased when the fruit was fully ripened. In contrast, several cultivars of the species *Z. mauritiana* had high acid content in the early stages of fruit growth, but these high levels gradually decreased with maturity (Bal and Singh, 1978a, b). It may be concluded, therefore, that changes in acid contents at various growth and maturity stages vary according to the cultivar and species of jujube. The highest TSS/TA ratio was found at 120 DAFB (Table 2).

Table 2. Harvest maturity and postharvest quality of Indian jujube (*Z. mauritiana* Lamk.) 'Nomsod' fruit.

Days after full bloom	Firmness (N)	TSS (°Brix)	TA (%)	TSS/TA	Ascorbic acid (mg 100 g^{-1} FW)
90	43.93ab	3.10c	0.16b	19.93c	2.00b
105	57.77ab	9.90a	0.26a	38.65ab	1.74b
120	62.65ab	10.40a	0.26a	40.60ab	0.80c
135	32.72b	4.13b	0.14c	31.07b	3.09a
F-test	*	**	**	**	**
CV (%)	23.47	4.67	5.66	14.20	18.00

Means followed by the same letter within each column were non-significant (ns), significantly different at p=0.05 (*) or 0.01 (**).

Ascorbic acid

The ascorbic acid concentration in 'Nomsod' fruit was decreased at 90-120 DAFB, but became highest at 135 DAFB (Table 2). Ascorbic acid has previously been shown to increase until the fruit ripens (Abbas, 1997). Islam et al. (2015) and Pareek (2013) determined that Chinese jujube had the highest vitamin C concentration early in the ripening stage, which then decreased to its lowest value at 90 DAFB. Lu et al. (2012) also noted that ascorbic acid in 'Changhong' jujube increased until 56 DAFB and then subsequently decreased. In another study, increases of ascorbic acid in jujube fruit varied and were dependent on the cultivar (Wu et al., 2010).

CONCLUSIONS

The results determined from this study, considering both the physical and chemical characteristics of 'Nomsod' jujube fruit, showed that the optimum age for harvest was between 120 and 135 days after full bloom. An increase in harvest age affected several physiological characteristics in the fruit, including weight, chemical quality, and an increase in the soluble solids concentration.

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Utilization of dragon fruit (*Hylocereus polyrhizus*) peel and passion fruit (*Passiflora edulis*) juice for fruit jam development

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Abstract

This study was conducted to utilize dragon fruit (DF) peel (100% blended, 50:50 blended and strips, 50:50 blended with fruit bits) and passion fruit (PF) juice (10, 15, 20% w/w) into dragon-passion jam. Sensory qualities (color, aroma, taste, mouthfeel and general acceptability) were evaluated using a 9-point hedonic preference score. A consumer acceptability test was done to validate the acceptance of the optimum formulation. The optimum formulation (50:50 DF blended peel and fruit bits, 15% PF juice) was bounded by an aroma and taste acceptability score of ≥7 by sensory panelist. Parameter estimates from response surface regression (RSREG) analysis indicated that the utilization of DF peel into dragon-passion jam provided a positive response to color acceptability. It was found that 20.22% PF juice was the maximum level required to attain a predicted color acceptability score of 7.12 (moderately liked) and when exceeding 20.22% the predicted color acceptability of the product decreased. Furthermore, the optimum formulation had a potential to compete in the marketplace with a consumer general acceptance score of 8.35 (liked very much) which was higher than the predicted acceptability score.

Keywords: jam, peel, passion fruit, dragon fruit, sensory evaluation

INTRODUCTION

Dragon fruit (*Hylocereus polyrhizus*) has a pleasant taste and exotic appearance, as well as nutritional and functional properties, which makes it attractive for cultivation. Among the known dragon fruit species, the red-fleshed cultivar (*Hylocereus polyrhizus*) is prominent due to its functional properties, attracting the interest of consumers and producers (Magalhães et al., 2019). It has a bright red peel with overlapping green fins or bracts that cover the fruit, a fact that has gained popularity in a number of countries (Jaafar et al., 2009).

Dragon fruit has been commercially grown in Claveria, Misamis Oriental for at least five years. Recently, its cultivation is expanding, as recorded in the local agroecosystem, both in Claveria and in Northern Mindanao, in general. As a result, the volume of fruit production has also increased resulting to the need to produce value-added products considering that dragon fruit is highly perishable, with a shelf life up to only 10 days (Hoa et al., 2006). If the harvest is delayed by 2-3 days, the quality loss in the fruit is accelerated and they easily deteriorate (Kishore, 2016).

Dragon fruit are rich in naturally-occurring flavonoids, which are primarily found in dragon fruit peel. Flavonoids have a wide range of biological activities, such as the inhibition of cell proliferation, the induction of apoptosis, the inhibition of specific enzymes, antibacterial action, and antioxidant effects. Dragon fruit peel tastes like spinach having a slimy and soggy texture. Currently, dragon fruit peel is considered as a food waste in most areas but in the mid-west of Philippines it is used with vegetables. Pharmacology and cosmetology research on the peel has been conducted but only a few studies have been carried out on food processing options. Hence, it would be advantageous if dragon fruit peel could be utilized in a value-added product like jam. However, its acceptability in such a product remains

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to be determined.

Jam is usually made from pulp and juice of one fruit (whole fruit). It can be defined as a cooked and gelled fruit preserve that is able to be stored long-term. It is normally used as a spread, filling or food jelly. The preparation of fruit jam traditionally involves the use of pectin as a gelling agent, although sugar or honey and citric acid may be added as well (Patten, 2004).

The measurement of hedonic responses is fundamental to understanding the relationship of chemical properties to food preference and selection. For more than half a century, the traditional 9-point hedonic scale (Peryam, 1952; Peryam and Pilgrim, 1957), in its various formats (e.g., labels only, labels with numbers), has been widely used to assess the average degree of liking or disliking of foods or consumer products across a large number of subjects. In recognition of the positive aspects of this scale (Lawless and Heymann, 1998), it has been adopted by many researchers and has become the dominant tool for measuring hedonic perception.

The study aimed to utilize dragon fruit peel in the production of jam, and to evaluate the products sensory acceptability and consumer acceptance.

MATERIALS AND METHODS

The peel of red-fleshed dragon fruit (*Hylocereus polyrhizus* Britton and Rose) and intact passion fruit (*Passiflora edulis*) were procured from the Research, Development and Extension Office, USTP-Claveria, Philippines. Sugar, gulaman (Philippine agar extracted from seaweed) and citric acid were procured at Monster Kitchen, Cagayan de Oro City, Philippines.

The dragon fruit peel was collected in a clean container. The peel and passion fruit were dipped immediately in sanitizing solution (20 ppm sodium hypochlorite). The peel was then boiled until it became tender, and then divided into two parts. One part was sliced into strips (approximately 4.0×0.5 cm), and the other part was blended using a food blender (NUTRiWORKS model JZ999, China). The blended peel was mixed with the peel strips or with fruit bits (1.5 cm cubes of dragon fruit pulp) for the treatments shown in Table 1. Passion fruit juice was manually extracted through a strainer with muslin cloth.

Table 1. Treatments applied in producing dragon-passion fruit jam.

Treatment	Passion fruit juice (%)	Dragon fruit peel
T1	10	100% blended
T2	15	100% blended
T3	20	100% blended
T4	10	50:50 strips & blended
T5	15	50:50 strips & blended
T6	20	50:50 strips & blended
T7	10	50:50 fruit bits & blended
T8	15	50:50 fruit bits & blended
T9	20	50:50 fruit bits & blended

A 3×3 factorial design was used with the treatments shown in Table 1 together with the standard jam formulation of 60% white sugar (w/w), 3% gulaman (w/w) and 0.5% citric acid (w/w). The mixture of sugar and dragon fruit was boiled for 3 min and then passion fruit juice, gulaman and citric acid were added with continuous stirring. A cold plate method was used to test the jam endpoint. The jam was hot-filled into jars which were then tightly closed.

The dragon-passion jam was evaluated for its color, texture, sweetness, flavor and general acceptability. An incomplete block design (IBD), following Cochran and Cox (1957), was used with the set plan of t=9, k=6, r=8, b=12 and E=0.94. A Type II design was used where t was the number of treatments, k the number of samples that were given to a panelist, r the number of replications based on IBD, b the number of blocks, and E the efficiency factors. The set plan was replicated four times to make a total of 48 panelists, each evaluating only 6 samples. The panelists were a combination of 10 professionals (aged 31-50 year-old), 10 young professionals (aged 21-30 year-old) and 28 food processing university students (aged

15-20 year-old). A 9-point hedonic preference scale was used in evaluating the sensory acceptability of the samples for parametric data analysis. At the same time, the panelists were asked to evaluate descriptive quality attributes of the samples by assigning a designating nominal value (Table 2). Descriptive data on sensory attributes were cross tabulated to get the frequency of the perceived color, aroma, taste and mouthfeel.

Table 2. Descriptive quality attributes with designated nominal value for dragon-passion fruit jam.

Color	Aroma	Taste	Mouthfeel
1 – red violet	1 – only perceptible as dragon fruit	1 – bland	1 – watery
2 – violet	2 – more perceptible as dragon fruit than as passion fruit	2 – sweet	2 – jelly
3 – dull red	3 – well-blended as passion and dragon fruit	3 – good-blend of sweet and sour	3 – viscous
4 – reddish brown	4 – more perceptible as passion fruit	4 – just sour	4 – very sticky
5 – brown	5 – only perceptible as passion fruit		

Consumer tests were carried out to determine the acceptance and preference toward the optimum formulation. The test employed 100 general consumers from the university. For the consumer acceptance test, consumers were presented with the optimum formulation and were asked to rate the product based on their own judgement. For this test, the optimum formulated product was presented together with a commercial strawberry jam product under blind testing conditions. Consumers were asked to rate the samples for color, aroma, taste, mouthfeel and general acceptability.

Statistical analysis was carried out using STATISTICA 8.0 software. The data obtained from the 9-point hedonic rating in the sensory evaluations were subjected to analysis of variance (ANOVA) and response surface regression analysis (RSREG) to determine the effects of independent variables on the sensory qualities of the dragon-passion jam formulations. A superimposed surface plot derived from RSREG on each of the sensory parameters was developed for determining the optimum acceptability formulation with each of the sensory attributes that had an evaluation score greater than seven. Consumer acceptance and preference test results were subjected to a paired t-test and a chi-squared test, respectively.

RESULTS AND DISCUSSION

The mean acceptability scores and cross tabulation for the quality descriptions of the sensory attributes of dragon-passion jam with varying combinations of dragon fruit peel and passion fruit juice are presented in Tables 3 and 4, respectively. The parameter estimates for the acceptability of each sensory attribute of dragon-passion jam are shown in Table 5.

Dragon-passion fruit jam was perceived as being dull red regardless of the levels of passion fruit (PF) juice and whether the dragon fruit (DF) was 100% blended or in the form of strips (Table 4). However, it was perceived as being red violet when blended peel was added with dragon fruit bits (pulp). The red color is derived from the natural pigments in DF and jam produced from DF peel with no DF pulp was perceived as being dull red. This relates to the findings of Chia and Chong (2015) who reported that drum-dried DF peel powder is a dull red color.

There were no consistent differences in color, aroma and mouthfeel acceptability among any of the treatments (Table 3). However, T7 (10% PF, 50:50 blended peel and fruit bits) had the highest color acceptability of 7.44 which means “like moderately” in the 9-point hedonic scale. Treatment 3 (20% PF and 100% blended DF) had the highest aroma acceptability of 7.37 (like moderately) which, considering the peel has no distinct aroma (Magalhães et al., 2019), the 20% PF content is likely to have enhanced the aroma acceptability (Table 4). Treatment 3 was perceived as being a well-blended PF and DF mix with a viscous mouthfeel (Table 4).

Table 3. Summary of mean acceptability scores for each sensory attribute of different blends of dragon-passion jam.

Treatments	%PF	DF	Sensory attributes				
			Color	Aroma*	Taste*	Mouthfeel	Gen. acc.*
1	10	100% blended	5.84b	6.50b	5.97c	5.81b	5.97c
2	15	100% blended	6.78a	6.81ab	6.47bc	6.62a	6.66b
3	20	100% blended	7.06a	7.37a	7.44a	7.19a	7.19ab
4	10	50:50 strips & blended	6.75a	6.97ab	6.91ab	6.56a	6.72b
5	15	50:50 strips & blended	6.75a	6.97ab	7.09ab	7.00a	7.06ab
6	20	50:50 strips & blended	6.75a	7.09ab	7.22ab	7.16a	6.84ab
7	10	50:50 fruit bits & blended	7.44a	6.91ab	7.09ab	6.94a	7.16ab
8	15	50:50 fruit bits & blended	7.28a	7.09ab	7.22ab	7.16a	7.50a
9	20	50:50 fruit bits & blended	6.91a	7.00ab	7.34a	7.25a	7.41ab
Overall response mean			6.84	6.97	6.92	6.80	6.94

n=64. Values with the same letter are not significantly different, * significant sensory attribute.
9-point hedonic scale: 9 = like extremely, 8 = like very much, 7 = like moderately, 6 = like slightly, 5 = neither like nor dislike, 4 = dislike slightly, 3 = dislike moderately, 2 = dislike very much, 1 = dislike extremely.

Table 4. Summary of cross tabulation for quality description on the sensory attributes of dragon-passion jam.

Treatments	Sensory attributes			
	Color	Aroma	Taste	Mouthfeel
1	Brown	Well-blended passion and dragon fruit	Well-blend sweet and sour	Jelly
2	Dull red	More perceptible dragon fruit than passion fruit	Well-blend sweet and sour	Jelly
3	Dull red	Well-blended passion and dragon fruit	Well-blend sweet and sour	Viscous
4	Dull red	Well-blended passion and dragon fruit	Sweet	Very sticky
5	Dull red	Well-blended passion and dragon fruit	Well-blend sweet and sour	Very sticky
6	Dull red	Well-blended passion and dragon fruit	Well-blend sweet and sour	Very sticky
7	Red violet	Well-blended passion and dragon fruit	Sweet	Viscous
8	Red violet	Well-blended passion and dragon fruit	Well-blend sweet and sour	Viscous
9	Reddish brown	Well-blend passion and dragon fruit	Well-blend sweet and sour	Viscous

Table 5. Summary of parametric estimates for the acceptability of each sensory attribute of dragon-passion jam.

Parameter	Color	Aroma	Taste	Mouthfeel	Gen. acc.
Mean/Interc.	4.189**	6.306**	6.684**	6.009**	6.132**
(1) %PF (L)	-0.839*	-0.297	0.126	-0.621	-0.457
%PF(Q)	-0.279	-0.055	0.079	0.533	0.365
(2) DF (L)	-6.306**	-1.625	-0.103	-1.536	-1.453
DF (Q)	2.341**	0.667*	0.333	0.706	0.763*
1L by 2L	-2.058**	-1.172**	-1.343*	-1.996**	-1.675**
1Q by 2L	-0.607	0.070	0.313	0.408	0.287
R^2	0.144	0.057	0.104	0.089	0.093

ns = not significant, ** significant at p<0.01, * significant at p<0.05.
L = linear model; Q = quadratic model

There was a consistent trend for the taste acceptability of the jam being attributed to the increasing level of PF juice in each mixture. For example, with 20% PF in the jam of the T3 mix, the taste acceptability was rated as 7.44. This result is likely to be because PF has a very acidic pulp, a highly intense aroma and a strong flavor with its juice having good acceptability

in different markets (Righetto et al., 1999).

Response surface regression models of the different sensory attributes which produced an acceptability rating of ≥7 was superimposed on a contour plot (Figure 1). This optimum formulation (shaded zone) was bounded mainly by aroma and taste acceptability, which were the main reasons for the use of the different DF peel and PF juice combinations in the preparation of the dragon-passion jam. The mouthfeel and color ratings established limits for acceptability such that, when exceeding 18.4% PF, the acceptance of the product in terms of mouthfeel would reduce and, similarly, when the mix had less than 11% PF the color acceptability of the product would be reduced.

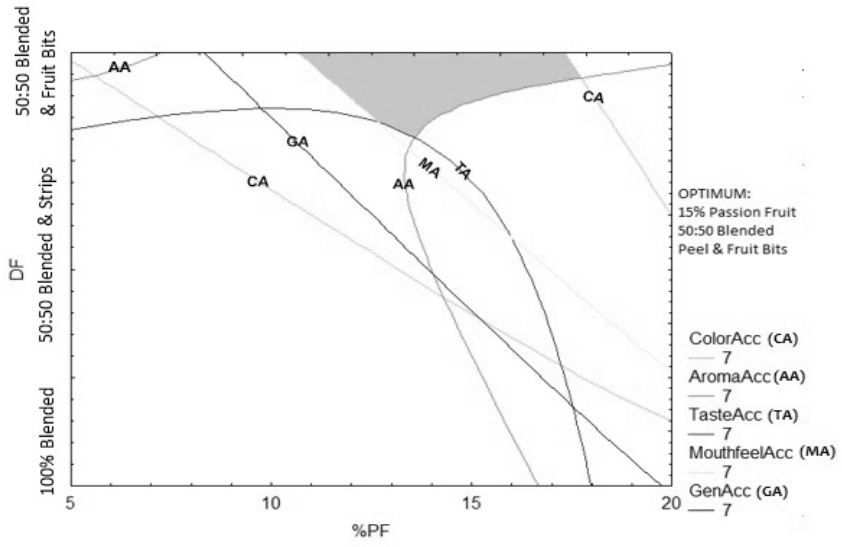

Figure 1. Superimposed acceptability plot of color, aroma, taste, mouthfeel and general acceptability with an acceptability value of ≥7.

Response surface regressions showed negative linear, quadratic and interaction effects between color acceptability and the levels of PF juice and DF peel in the jam mixes (Table 5). Parameter estimates indicated that the use of DF peel in the dragon-passion jam provided a positive quadratic response to color acceptability.

A content of 20.22% PF juice was found to be the maximum required to attain a predicted color acceptability rating of 7.12 (like moderately) and that exceeding this content would decrease the color acceptability of the product (Table 6). At a content of 8.91% PF juice, the dragon-passion jam received a predicted aroma acceptability of 6.8 (like moderately).

Table 6. Critical point of acceptability on sensory qualities of dragon-passion jam analyzed by response surface methodology (STATISTICA 8.0).

Response	Critical value		Predicted acceptability	Type
	% w/w Passion fruit juice	Dragon fruit peel^{ns}		
Color acceptability	20.225**	1.642	7.129	Maximum
Aroma acceptability	8.913**	1.850	6.808	Saddle
Taste acceptability	5.039**	21.311	9.115	Saddle
Mouthfeel acceptability	18.390	2.355	7.043	Maximum
General acceptability	19.721	2.295	7.169	Maximum

ns = not significant, ** significant at p<0.01, * significant at p<0.05.

Consumer acceptability testing of the product gave a mean general acceptance score of 8.35 (like very much) which was higher than the predicted general acceptability value of 7.17 derived from the response surface regression. It was anticipated that the predicted sensory

attributes' acceptability and the acceptability determined by the actual consumer test would produce similar results. Instead, the result of the paired t-test concluded that the actual consumer acceptance of dragon-passion jam was significantly higher than the anticipated predicted value (Table 7). A possible reason for this outcome was that the coefficient of prediction (R^2 Table 5) generated from response surface models of the different sensory attributes was very low.

Table 7. Paired t-test of the surface response predicted acceptability and the actual consumer acceptability.

Pair; predicted vs. actual	Mean	t	df	Sig. (2-tailed)
Color	1.39	-20.190	99	0.000**
Aroma	1.33	-13.388	99	0.001**
Taste	0.91	8.340	99	0.000**
Mouthfeel	1.11	-11.199	99	0.000**
General acceptance	1.15	-11.953	99	0.000**

n=100; ** highly significant.

When compared with the commercial strawberry jam product, the consumer preference test resulted in 68% liking the optimum formula product and 90% liking the existing product; although, 54% preferred the optimum formula product more than the existing product. The Chi-square test concluded that there was no significant difference between the optimum formulation and the existing commercial product (Table 8).

Table 8. Chi-square test on consumer preference test between optimum formula product versus existing product.

Product	Like	Dislike	Preferred	Chi-value	X^2 at 0.01
Existing	90	10	46	0.640	0.423711^{ns}
Optimum	68	32	54		

n=100; ** highly significant.

CONCLUSIONS

Dragon fruit peel and passion fruit juice can be utilized together in the formulation of a dragon-passion jam. Passion fruit juice improved the taste acceptability of the product. The optimum formulation, which was identified through a superimposed contour plot, was 15% PF juice in combination with 50:50 blended peel and fruit bits. A consumer general acceptance rating of the optimum formulation was 8.35 (like very much) which was higher than the predicted general acceptability (7.17, like moderately).

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Production and free radical scavenging activity of guyabano (*Annona muricata* L.) peel tea with varying levels of honey and calamansi (*Citrus fortunella microcarpa* B.) juice

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Abstract

The production of tea drinks typically uses tea leaves but now various sources, like the bark and peel of certain plants, are being used. Guyabano (*Annona muricata* L.) peel has a higher free radical scavenging activity (FRSA) than its pulp. This enables the use of guyabano peel as a medium for tea production. This study was conducted to formulate guyabano peel tea with varying levels (0, 5 and 10% v/v) of honey and (0, 2.5 and 5% v/v) of calamansi (*Citrus fortunella microcarpa* B.) juice. Physico-chemical properties of the product, such as total soluble solids concentration (TSS), pH and titratable acid (TA) were determined and sensory qualities (color, aroma, taste, flavor, general acceptability) were evaluated using a 9-point hedonic scale. Consumer preference testing and free radical scavenging activity were determined on the optimum formulation and on an existing product. The optimum formulation has the potential to compete with existing green tea products in the market. The addition of honey and calamansi juice increased the overall acceptability score to be≥6.8 (like slightly to like moderately) of guyabano peel tea. The TSS and pH of guyabano peel tea decreased at a high concentration of calamansi juice, while TSS and pH increased with added honey. The FRSA values of the control (0% honey and 0% calamansi juice), the highly acceptable product (10% honey and 5% calamansi juice) and the existing green tea product were 246.84, 342.91 and 333.96 µmol Trolox Equivalent 100 mL^{-1}, respectively. The optimum formulation of guyabano peel tea, with 8.4% honey and 3.0% calamansi juice, had an FRSA of 324.64 µmol Trolox Equivalent 100 mL^{-1}.

Keywords: *Annona muricata* L., calamansi juice, free radical scavenging activity, honey, sensory evaluation, tea

INTRODUCTION

Modern consumers no longer consider beverages as thirst quenchers but rather as health products that contain specific ingredients which form part of their lifestyle (Santiago, 2010). The high rising health consciousness of consumers has led to the emergence of various functions food products. One such product is tea which has been released into markets with several compositions and types. Beverages such as herbal infusions and various teas constitute an important source of antioxidants (Warren, 1999) and could be taken as a good complement for antioxidant intake in the human diet.

One of the promising peels that can be used for tea production is from guyabano. Guyabano (*Annona muricata* L.) is a watery fruit consisting of a white edible pulp that is high in nutritive value. This includes vitamins, potassium and dietary fiber (Asian Journal, 2013). Kangleon et al. (2011) observed high free radical scavenging activity (FRSA) in guyabano peel which can be correlated to high antioxidant content. This opens the possibility that guyabano peel could be used as a medium for tea production.

The bitter taste of tea made from guyabano peel has a negative perception by consumers.

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The addition of another dominant taste that could suppress the bitterness would be a good augmentation to such a product. The sour taste of calamansi (*Citrus fortunella microcarpa* B.) is a potential flavoring for such a tea. Calamansi is composed of 170 phytochemicals, that served as cancer preventing compounds (Wang et al., 2007), making it attractive for use as such an ingredient. Further, combining honey into tea and calamansi could promote favorable tea consumption. Honey is naturally high in dextrose and levulose sugars, with small amounts of at least 22 other complex sugars. Research has also indicated that honey's unique composition makes it useful as an antimicrobial agent and as an antioxidant (White and Doner, 1980).

Several studies have been conducted on the medicinal and nutritional values of the fruits used in this study but none have been conducted on the exploration and utilization of guyabano peel as a tea with honey and calamansi juice used as tea blends. Hence, this study was conducted to evaluate such combinations.

MATERIALS AND METHODS

Collection of raw materials and tea preparation

Ripe guyabano fruit and calamansi were purchased from Baybay Public Market, Baybay City, Philippines, and the honey with a proper label was purchased in a Department Store at Ormoc City, Philippines. The ripe guyabano was thoroughly inspected, washed, sanitized with 10-15 ppm sodium hypochlorite, and then peeled. The peel was water blanched (90-100°C) for 1 min then filtered using cheese cloth to remove the peel and its refined powder (dust-like texture). To make the guyabano peel tea, the peel was dried in a convective dryer at 60°C for 4-6 h until brittle. The dried product was ground and stored in an airtight container and/or polyethylene bag, and then kept in a cool, dry, dark place. The prepared calamansi juice was stored in a refrigerator (4°C) until needed but for not more than a week.

A 3-g sample of dried guyabano peel was infused in 200 mL (equivalent to 1 tea cup) boiled water (approximately 100°C) for 10-15 min. For the tea blends, 0, 5 and 10% (v/v) honey and 0, 2.5 and 5% (v/v) calamansi juice were added to the guyabano peel tea.

Sensory quality evaluation

Sensory evaluation was carried out to determine the effects of the levels of calamansi juice and honey on the different sensory qualities of the combined product. Evaluation was done employing 48 food technology students of Department of Food Science and Technology (DFST), VSU, Baybay City, Philippines as panelists. An incomplete block (IBD) experimental design by Cochran and Cox (1957) was used for the sensory evaluation since there were nine treatments to be tested; the set plan was t=9, k=6, r=8, b=12, E=0.94 and type II. Each panelist was presented with six samples coded with randomized 3-digit numbers and were asked to rate the product, based on their own judgment, using an appropriate score sheet. The taste, flavor, color, and aroma were evaluated, using a combination of quality descriptors, assigning a nominal value as perceived by the panelists. Simultaneously, the panelists were asked to assign a sensory acceptance score using a 9-point hedonic preference scale. The sensory scores were subjected to statistical analysis using STATISTICA 8.0 software (Stat Soft, Inc.).

Physico-chemical analysis

A 15-mL aliquot of each sample was placed in a small cup. The pH was measured using a digital pH meter (Milwaukee, SM 102). The total soluble solids concentration (TSS) was determined using a calibrated hand refractometer (Reichart Analytical Instrument, °Brix 65Hp) and titratable acidity (TA) was determined by titration using standard 0.1 N NaOH solution.

Selecting the optimum formulation and consumer tests

The results from the 9-point hedonic scores were analyzed in the Response Surface Regression (RSREG) module using STATISTICA software (Stat Soft, Inc.) to determine the effects of the main variables on the sensory qualities of the product. Contour plots were made

for the optimum acceptability of each of the sensory attributes evaluated. The region having an acceptable combination of calamansi and honey was selected through super-imposing the different contour plots, resulting in the region showing optimum formulation. A new sample was then made from the combination that fell within this optimum formulation region (being 8.4% honey + 3% calamansi juice + guyabano peel tea). This optimum formulation was subjected to a comparison acceptance test (t-test) with a treatment outside of the optimum region (selected as 5% honey + 2.5% calamansi juice + guyabano peel tea). This was to verify that the selected optimum formulation was best perceived compared with treatments beyond the optimum region.

Consumer testing was carried out to determine the consumer's acceptance and preference toward the product. The optimum formulation (8.4% honey + 3% calamansi juice + guyabano peel tea) was subjected to a consumer acceptability test using a 9-point hedonic scale. This consumer acceptance testing employed 100 general consumers from students and staff of the DFST, VSU. Consumers were presented with the optimum formulation sample and were asked to rate the product based on their own judgement. Consumer's preference was further assessed by comparing the sensory score of the optimum formula sample with a commercial iced tea product. Consumer preference data for the two treatments were analyzed using a Chi-square test.

Determination of the free radical scavenging activity (FRSA) using the DPPH method

The antioxidant activity of the teas was determined using DPPH as the source of free radicals. A stock solution of DPPH (60 µM) was prepared using 95% ethanol and distilled water. The initial absorbance was measured using a UV-Vis Spectrophotometer (Shimadzu model). The assays were performed using the modified procedure of Prakash (2007) as cited by Salas et al. (2013). A 0.1-mL aliquot of the test samples was pipetted into a test tube containing 3.9 mL DPPH solution to initiate the reaction; the mixture was shaken, the test tube wrapped with carbon paper and then left to stand at ambient temperature in a dark room for 1 h. The free radical scavenging assay was done by reading absorbance at the established λmax. A 95% ethanol solution served as a blank and Trolox was the reference standard. Scavenging of the DPPH free radical was measured using a standard calibration curve.

Experimental design and statistical analysis

The experiment was a 3×3 factorial design. Statistical analysis was done using STATISTICA 8.0 software (StatSoft, Inc.). Results for the different sensory attributes and the physico-chemical activity were analyzed using an analysis of variance. Differences between treatments were determined using Duncan multiple range test (DMRT). The descriptive quality score was analyzed in terms of frequency and cross tabulation with the hedonic acceptability scores to describe the product's quality attributes.

The response surface regression analysis (RSREG) module in STATISTICA 8.0 (StatSoft, Inc.) was used to determine the effects of the independent variables on the defined parameters. Analysis of regression coefficients and parameter estimates were calculated to describe the regression models for each attribute.

Consumer verification acceptability test and preference test results were subjected to a paired t-test and a chi-squared test, respectively.

RESULTS AND DISCUSSION

Sensory evaluation

The guyabano peel tea was perceived as being clear before the addition of honey and calamansi juice. When honey and calamansi juice were incorporated, they effectively blended or even masked the aroma and flavor of guyabano, which was considered desirable. The honey that was used in the mixtures had an amber color and the calamansi juice had yellow color. When honey was added at 5%, the color of the tea became light yellow and when the amount was doubled to 10%, its color became yellow to dull yellow. The yellow color of the tea was perceived in all treatments with calamansi juice and the color intensity was strengthened by

the addition of honey. Hence, at the low amount of honey (5%), with any levels of calamansi juice, the color of the tea was generally perceived as light yellow. Conversely, with honey at the 10% level, the color of the tea was perceived as yellow at all levels of calamansi juice. At 5% honey with no calamansi juice the flavor of guyabano still dominated. At 10%, it provided a pronounced honey flavor. Whenever the amount of honey was at 10%, the honey flavor was perceived as dominant while calamansi and guyabano flavor was considered to be well-blended (Table 1).

Table 1. Summary of the sensory attributes mean scores of guyabano peel tea with different levels of honey and calamansi juice (CJ).

Treatments	Variable		Sensory attributes*				
	Honey (%v/v)	CJ (%v/v)	Color	Aroma	Taste	Flavor	General acceptability
1	0	0	5.84b	5.95d	4.69c	5.36d	5.28f
2	0	2.5	5.97b	6.31bcd	5.05c	5.41d	5.50ef
3	0	5	6.06b	6.50abcd	5.06c	5.56cd	5.75def
4	5	0	6.17b	6.02cd	5.69b	5.80cd	5.94cde
5	5	2.5	6.36b	6.50abcd	6.05b	6.23b	6.19cd
6	5	5	6.44b	6.69ab	5.88b	6.03bc	6.47bc
7	10	0	7.02a	6.58abc	6.70a	6.81a	6.88b
8	10	2.5	7.08a	6.97a	7.17a	7.30a	7.53a
9	10	5	7.22a	6.92a	7.13a	7.34a	7.56a
Over-all response mean			6.46	6.49	5.94	6.20	6.34

n=64. Values with the same letters are not significantly different; * significant sensory attribute.
9-point hedonic scale: 9 = like extremely, 8 = like very much, 7 = like moderately, 6 = like slightly, 5 = neither like nor dislike, 4 = dislike slightly, 3 = dislike moderately, 2 = dislike very much, 1 = dislike extremely.

The calamansi had a significant linear effect in the aroma acceptability of the formulation. Calamansi was twice as responsible for the aroma acceptability than honey. However, the interaction of the two variables had no significant effect on aroma acceptability (Table 2). Honey had a significant linear effect on the taste acceptability of the formulation. Increasing the amount of honey provided a significant increase in the taste acceptability of guyabano peel tea (Table 2). Consequently, honey became the dominant factor of taste acceptability of the tea formulation and not the calamansi juice. Calamansi juice should be limited only to an amount of 2.76% to achieve a high taste acceptability score (Table 2). Moreover, RSREG analysis revealed that calamansi had linear and quadratic effects but there was no linear and/or quadratic effect of honey on the general acceptability response. This implies that calamansi made a greater impact then honey on the acceptability of the formulation but there was no significant interaction between the two variables.

Table 2. Critical point of acceptability on sensory qualities of guyabano peel tea with different levels of honey and calamansi juice.

Response	Critical value		Acceptability
	% Calamansi juice	% Honey	
Color acceptability	16.87	-1.61	6.28
Aroma acceptability	5.01	0.78	6.51
Taste acceptability	3.05	-21.89	3.26
Flavor acceptability	2.76	-2.81	5.47
General acceptability	2.33	-5.27	5.50

Flavor acceptability increased to a small extent with increasing amounts of calamansi but increased significantly with increasing honey concentration (Table 2). However, the predicted computed flavor acceptability of 5.47 falls as "neither like nor dislike" in the 9-point hedonic scale. The predicted response value for calamansi juice was 2.76% at any level of honey. The general acceptability of the formulation at ≥7.5 was affected by both process variables (Table 2), and the predicted general acceptability of 5.50, which is "neither like to like slightly" in the 9-point hedonic scale, had a predicted response value of 2.33% for calamansi juice at any level of honey.

Physico-chemical properties

The relationship between the levels of honey and TSS values followed the basic mass effect law – as the level of honey increased, the TSS value also increased. The TSS value was based mainly on the amount of honey used in the formulation since honey contains a high soluble solids concentration of 95-99% (White and Doner, 1980). For every 1% increase of honey, there was a corresponding 0.2 °Brix increase in TSS (Table 3).

Table 3. Summary of parametric estimates of physico-chemical evaluation of guyabano peel tea with different levels of honey and calamansi juice.

Parameter	Parametric estimates		
	TSS	pH	TA
Intercept	1.857**	4.304**	0.021**
% Calamansi (A)	-0.842**	-0.569**	0.076**
A*A	0.190**	0.055**	-0.002**
% Honey (B)	0.199**	-0.071**	0.003^{ns}
B*B	0.116**	0.000^{ns}	0.000^{ns}
A*B	-0.001^{ns}	0.013**	-0.000^{ns}
R^2	0.9935	0.979	0.996

ns = not significant, ** significant at p<0.01.

Calamansi juice is naturally acidic (3.5≤pH≤4.0). Guyabano peel teas with 5% calamansi had a pH<3. The decrease in the pH of the tea was, therefore, primarily due to the addition of the calamansi juice. For every 1% change in the amount of calamansi, the pH was expected to drop by 0.6. In contrast, a small decrease (0.07) in pH was expected in every 1% change in honey content (Table 3). These combined effects of honey and calamansi juice on the pH value of the formulation meant that, as the level of calamansi juice increased, the pH of the formulations decreased, thus making the product more acidic. The predicted value of pH acceptability at the critical point of the response surface was pH 2.84 which occurred at a critical value of 5.54% for calamansi and at any value for honey (Table 4).

Table 4. Critical point of acceptability on sensory qualities of guyabano peel tea with different levels of honey and calamansi juice.

Response	Critical value		Acceptability at critical point
	% Calamansi juice	% Honey	
TSS	2.21	-0.85	0.84 min
pH	5.54	-3.17	2.84s
TA	20.53	13.96	8.26s

min = minimum, s = saddle.

Higher titratable acidity was found in those teas with a high content of calamansi. Results of the RSM regression indicated that for every 1% increase in calamansi the TA values of the tea increased by 0.076% (Table 3). This direct relationship between TA and the level of calamansi was expected because calamansi primarily contributed to the acid content of the tea. A contoured surface plot of the TA, at various levels of calamansi (0-5%) and honey (0-

10%), showed that there was a constant increase in TA with increasing amounts of honey and calamansi. Hence, calamansi was the major contributor to the TA of the tea.

Selecting the optimum formulation and consumer acceptability test

Models of different sensory attributes that produced an acceptability rating of ≥6.8 were superimposed on the data from the production cost model (data not presented) to determine the least-cost combination and the optimum formulation of guyabano peel tea supplemented with honey and calamansi. A predicted acceptability of 6.8 was found to intersect at 3.0% calamansi juice and 8.4% honey with an expected break-even price of Php 5.16 (Philippines peso) per 300 mL, excluding packaging costs (Figure 1).

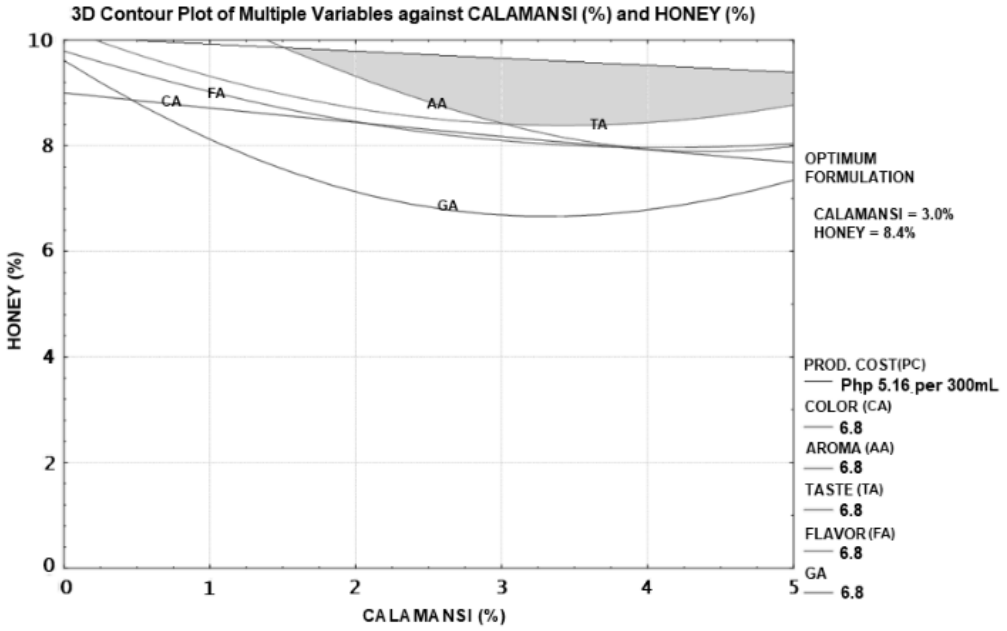

Figure 1. Superimposed acceptability plot of color, aroma, taste, flavor and general acceptability with an acceptability value of ≥6.8 and at Php 5.16 (Philippines peso) product cost.

This optimum formulation was bounded by aroma and taste acceptability which was the main reason for the incorporation of both the calamansi juice and the honey. The selected optimum formulation above (8.4% honey + 3% calamansi juice + guyabano peel tea) was made into a new product and the physico-chemical tests gave results of pH 3.4, TSS 9.8 °Brix, and TA 0.036.

The difference between the acceptability test of the actual optimum formulation (8.4% honey + 3% calamansi juice + guyabano peel tea) versus T5 (5% honey and 2.5% calamansi juice which was nearest to the optimum) was subjected to a paired t-test. It indicated that the sensory acceptability of the optimum formulation was significantly different from T5 which was beyond the optimum region predicted by the RSREG analysis.

In the consumer tests of the optimum formulation, 83% liked the product and 79% liked the existing commercial iced tea product, which provided a good indicator of the potential marketability of the new guyabano peel tea product. The result of the Chi-square test on the preference of the two products, showed that there was an equal chance for either produce to be selected when displayed in the market (Table 5).

Table 5. Chi test on consumer preference between the best formulated guyabano peel tea (supplemented with honey and calamansi) and the existing iced tea product.

Treatment	Like	Dislike	Preferred	χ value	X at 0.01	X at 0.05
Optimum	83	17	48	0.160	3.841^{ns}	6.635^{ns}
Existing	79	21	52			

ns = not significant.

Free radical scavenging activity of the optimum formulation

The optimum formulation, together with the acceptable formulations (T_1 and T_9) of the guyabano peel-honey-calamansi tea and the existing iced tea product were subjected to a DPPH assay (Table 6). The free radical scavenging activity of the optimum formulation was similar to the existing product implying that the optimum formulation had a similar antioxidant activity to the commercial iced tea product. Hence, the guyabano peel tea was not only similar in terms of sensory attributes but it could also compete with the existing product in terms of antioxidant benefits. Nonetheless, some previous research has indicated that a higher FRSA was not necessarily indicative of a higher antioxidant value (Wang, 2011).

Table 6. Free radical scavenging activity of selected guyabano peel tea honey-calamansi formulations and the existing product.

Treatment	Calamansi juice (%)	Honey (%)	FRSA (µmol TE 100 mL^{-1})[a]
T1	0	0	246.84b[b]
T9	5	10	342.91a
Optimum	3	8.4	324.64a
Existing	-	-	333.96a

[a]TE = Trolox equivalent.

[b]Values with the same letter are not significantly different.

CONCLUSIONS

It was possible to formulate a guyabano peel tea, with added honey and calamansi juice, that had a high sensory acceptance. Increasing the amounts of honey and calamansi juice improved the quality of guyabano peel tea by increasing its sensory acceptability.

Physico-chemical properties of the teas were affected by the incorporation of both honey and calamansi juice. TSS and pH decreased with increasing calamansi juice and increased with increasing honey, while TA increased with increasing calamansi juice and decreased with increasing honey content. Guyabano peel tea could be best produced at optimum levels of honey and calamansi juice of 8.4 and 3% v/v, respectively. This formulation had an FRSA of 324.64 µmol TE 100 mL^{-1} and a production cost of Php 5.16 300 mL^{-1}, excluding packaging costs.

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Dynamics of East Asian flower economies: China, Japan and Korea

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Abstract

Since 2000, flower production and trade in East Asia have developed dynamically. China has greatly increased flower production, exports and imports, whereas Japan has decreased its production. South Korea has now become an importer. We investigate in detail the development of the flower industry in each nation, as well as its production, consumption, and trade. In China we identify the importance of pot plants and nursery stock in production, compared with Japan. Increases in imports of cut flowers and flower bulbs are apparent. Since 2000 the Japanese flower market has stagnated. Japan is still a large flower importer. We identified a low level of flower consumption by individual consumers, small-scale production by a large number of producers, and a large number of small-scale wholesale markets. Since 2010 the South Korea flower market has declined and exports have decreased. The "Act on the Development of the Flower Industry and Flower Culture" was enacted on August 20, 2019. We investigate the establishment process of the law and discuss some problems of the Korean flower industry.

Keywords: flower industry, flower export, flower trade, flower production, flower consumption

INTRODUCTION

Since 2000, flower production and trade in East Asia have developed dynamically. China has greatly increased flower production, exports and imports, whereas, Japan has decreased its production. Thailand, a traditional flower exporter, has been eclipsed by China and Malaysia, whereas South Korea has now become an importer. This paper investigates the dynamics and the contours of the flower industry in the important flower-consuming nations, namely, China, Japan, and South Korea, which are big flower consumers.

Since 2010, China has ranked #1 in the export of flowers and plants in East and South-East Asia and has greatly increased its volume (Table 1). China's exports in 2017 are 11 times of those in 2000, and more than double compared with those of Malaysia in 2017. Thailand declined from #1 in 2000 to #4 in 2017, and Malaysia increased in ranking from #5 in 2000 to #2 in 2010. Vietnam was #5 in 2015. South Korea increased from 2000 to 2005, decreasing in 2010, before disappearing from the table in 2015. Japan increased from #5 in 2010 to #3 in 2017.

In relation to imports, China increased from #5 in 2000 to #2 in 2017, an increase in 1400%. Korea increased from #4 in 2000 to #3 in 2015. Korea turned from a net exporter to importer. Japan is a net importer, ranked #1, although its import volume has decreased from 2010 to 2017. Vietnam increased its imports in 2015 and 2017.

CHINA

China's total production of flowers and plants increased from 334,000 ha in 2002 to 1,392,000 ha in 2017, with an average annual growth rate of 9.97%. Cut flowers (roses, lilies, chrysanthemums, gerbera and gladiolus) increased from 18,800 ha in 2002 to 69,800 ha in 2017 at an annual growth rate of 9.12%. Pot plants increased from 39,100 to 118,600 ha at 7.69%. Nursery stock increased from 163,800 ha to 800,600 ha at an annual rate of 11.16%

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and flowers (used in medicine and food) from 28,500 to 270,500 ha at an annual rate of 16.20%, whereas production of seedlings and flower seeds decreased. Bulbs increased from 2,700 ha in 2002 to 6,100 ha in 2015 but declined to 2,600 ha in 2016 (International Association of Horticultural Producers, 2012, 2018).

Table 1. Ranking of exports and imports in East and South-East Asia for flowers and plants; HS code 0600 (unit: million dollars). Source: United Nations Comtrade Database (2019).

	Exports				
	2000	**2005**	**2010**	**2015**	**2017**
1	Thailand 42.2	Thailand 88.3	China 205.6	China 299.7	China 338.5
2	China 31.8	China 77.1	Malaysia 130.4	Malaysia 126.8	Malaysia 139.8
3	Singapore 31.4	Korea 55.2	Thailand 111.1	Thailand 110.9	Japan 123.8
4	Korea 30.6	Malaysia 52.7	Korea 105.1	Japan 70.4	Thailand 123.4
5	Malaysia 22.5	Singapore 34.2	Japan 77.4	Vietnam 48.8	Vietnam 65.6
	Imports				
	2000	**2005**	**2010**	**2015**	**2017**
1	Japan 390.3	Japan 463.8	Japan 630.5	Japan 553.3	Japan 585,7
2	Hong Kong 52.7	China 68.7	China 104.0	China 217.3	China 280,9
3	Singapore 44.9	Hong Kong 53.1	Singapore 77.0	Korea 97.4	Korea 107,8
4	Korea 28.8	Korea 50.7	Korea 61.6	Singapore 88.4	Vietnam 103.2
5	China 20.7	Singapore 45.0	Hong Kong 46.4	Vietnam 77.0	Singapore 87.4

The production of flowers and plants in 2017 in China and Japan are shown in Table 2. As indicated by the data, China's total area is 50 times that of Japan. China's nursery stock and pot plants are more important than Japan in both scale and proportion. In Japan cut flowers are important in scale and proportion whereas in China, flowers are used in medicine, food, and industry and other usages are important. It is understood that Chinese people decorate their rooms primarily with pot plants, whereas Japanese display cut flowers.

China is a net exporter, increasing both in exports and imports (Table 1). Flower bulbs are increasingly imported, mainly from the Netherlands, whereas live plants (pot plants, nursery stock) are increasingly exported to the Netherlands, South Korea, Myanmar, and Japan (Figure 1). Cut flowers (carnations, roses and lilies) are exported to Japan, Thailand, Singapore, and Malaysia (Cheng et al., 2012). Imports of cut flowers have greatly increased, and the net exports have decreased since 2012. This is an effect of westernization of flower consumption in China. Foliage and branches are mostly exported to Japan.

Table 2. Production of flowers and plants in China and Japan. Source: International Association of Horticultural Producers (2018) and Ministry of Agriculture, Forestry and Fisheries (2020a).

2017	China		Japan[a]	
	1,000 ha	%	1,000 ha	%
Total area	1392	100.0	26.8	100.0
Cut flowers, foliage and branches	70	5.0	14.5	53.9
Pot plants	119	8.5	1.6	6.1
Nursery stock	801	57.5	5.0	18.7
Flowers (medicine, food)	271	19.4		
Flowers (industry, other usage)	65	4.7		
Flower seeds	6	0.4		
Turf	50	3.6	5.3	19.8
Seedlings	9	0.6		
Bulbs	3	0.2	0.3	1.1
Dry flowers	1	0.0		

[a]In Japan nursery stock includes flowering trees (3,624 ha).

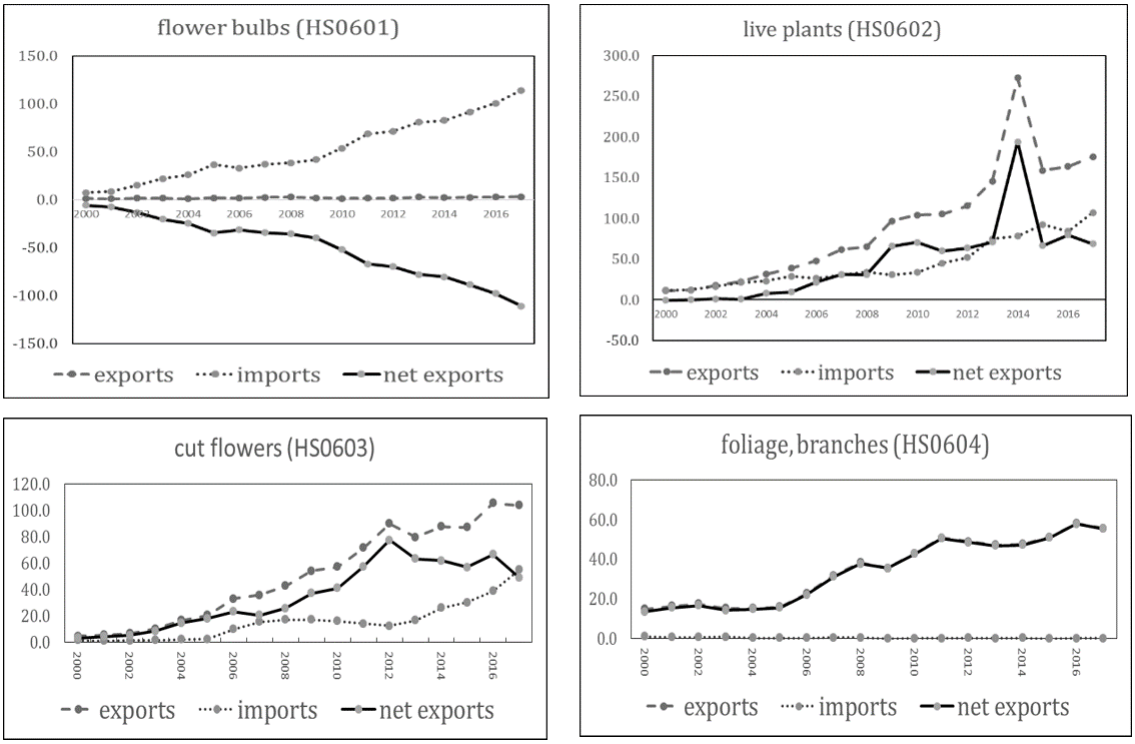

Figure 1. China's exports and imports of flowers and plants (unit: million dollars). Source: United Nations Comtrade Database (2019).

The following problems with the cut flower and plant industry in China have been noticed in previous publications (Cheng et al., 2012; Monnikhof, 2015; China Flower Association, 2017): lack of uniformity of flower quality; poor logistics, including distribution methods such as insufficient cooling systems; week competitiveness in export markets; shortage of new cultivars; insufficient market research; and insufficient networking among consumers, retailers, and wholesalers. Niisato (2018) identified the same problems in the Chinese flower bulb sector. In brief, the Chinese flower industry is young and developing.

JAPAN

In Japan, the flower and plant industry has stagnated.

Consumption

The share of business demand, such as ceremonial funeral and wedding occasions, in the domestic consumption of flowers and plants is relatively high. However, consumer demand, as represented by individual households and personal gifts, is currently low and not showing any increases in consumption. The annual purchase of cut flowers per household decreased from 11,551 yen in 2000 to 8,255 yen in 2018, according to the Ministry of Internal Affairs and Communications (2020). By age, the amount purchased by those 60 years or older exceeded 11,000 yen annum^{-1}, whereas that in the age group of 20-30 years was less than 2,500 yen. In addition, the purchase amounts differed by region.

Distribution

In the retailing stage, retail stores specializing in flowering plants account for approximately 70% in terms of both the number of stores and the sales amount (Ministry of Economy, Trade and Industry, 2020). Many of the specialty stores are small scale and there is an absence of large-scale retailers with multiple stores and franchise management. Wholesale markets decreased from 251 in 1990 to 109 in 2018. Almost all wholesale markets have been wholesale merchants, conventional privately operated markets, with the trading volume of each wholesale market being generally small.

The trading volume of wholesale markets decreased from 558 billion yen in 1997 to 362 billion yen in 2018, and the number of trading flowers also decreased. These trends are strongly observed in small-scale wholesale markets located in the countryside. However, some wholesale markets have maintained a consistent trading volume and several top-selling wholesale agents are expanding their market share.

Production structure

The number of commercial farmers producing flowering plants decreased by 34% from 88,000 in 2000 to 58,000 in 2015. Farm managers over 60 years of age accounted for 63% of the total and this progression has accelerated (Ministry of Agriculture, Forestry and Fisheries, 2020b). The output of flowers and plants decreased from 630 billion yen in 2000 to 370 billion yen in 2017 because of the reduced capacity of producers.

In relation to the output of cut flowers, there are differences in production volume by plant variety. Chrysanthemums, roses, and carnations dominate production. However, showy prairie gentians and lilies have been promoted recently as new plant variety developments, along with a diversity of other different types.

Imports and exports

Cut flowers account for the majority of the total volume for flowering plant imports. Along with the decline in domestic production, the ratio of imports has continuously increased from 12.9% in 2000 to 26.6% in 2017 (Table 3), especially for carnations (increasing from 34% in 2007 to 60% in 2017) and chrysanthemums, because of quality improvements such as long duration transport and storage.

Table 3. Data on cut flowers (unit: billion pieces). Source: Ministry of Agriculture, Forestry and Fisheries (2020c).

Data	2000	2005	2010	2015	2016	2017
Production	55.9	50.2	43.5	38.7	37.8	37.0
Import	8.3	10.4	13.2	12.7	13.1	13.4
Total	64.2	60.6	56.7	51.4	50.9	50.4
Import ratio (%)	12.9	17.2	23.3	24.7	25.7	26.6

However, national strategies for export expansion have been implemented resulting in an increase from 6.3 billion in 2010 to 13.5 billion yen in 2017 with 90% of the export value being in garden plants, bonsai or dwarfed potted plants, and other potted plants (Table 4). China accounts for 60% in the total exports from Japan.

Table 4. Data on cut flowers, pot plants, and bulbs; import value (unit: billion yens). Source: Ministry of Finance (2020).

Data	2000	2005	2010	2015	2016	2017
Production	5,867	4,997	3,816	3,798	3,788	3,687
Import	421	512	552	669	634	657
Total	6,288	5,509	4,368	4,467	4,422	4,344
Export	1	2	6	8	9	14
Import ratio (%)	6.7	9.3	12.6	15.0	14.3	15.1

SOUTH KOREA

Production and consumption

The South Korean flower industry grew steadily during the period of 1975-2005 and has since declined. Production in 2018 has fallen to about half that of 2005. By category, cut flowers and potted flowers (called bonsai in Korea) account for 33.2 and 36.6% of production, respectively. All types differ in the timing of their peak production, but all have been trending downward (Table 5).

Table 5. Production value of flowers and consumption value per capita (unit: million won). Source: Ministry of Agriculture, Food and Rural Affairs (2020).

Year	Total	Cut flowers	Pot plants	Ornamental trees	Consumption value/capita (won)
1990	239,348	59,224	99,516	55,779	5,646
1995	508,970	225,757	189,046	67,317	11,472
2000	664,997	301,245	268,499	58,526	13,859
2005	1,010,532	451,661	435,532	60,874	20,870
2010	850,995	297,561	431,755	42,515	16,098
2015	633,207	217,409	221,491	28,316	13,310
2016	560,248	177,350	194,678	24,309	11,722
2017	565,788	183,264	192,796	25,824	11,906
2018	538,543	178,647	196,909	20,858	11,888

In 2018, of cut flowers, roses accounted for 29.4%, chrysanthemums 25.5% and lilies 6.7%. Consumption per capita also peaked in 2005 and then has declined significantly, similar to production. This large decrease in per capita spending is thought to be a major contributor to the significant decrease in production. It should be noted, however, that in 2018 there was hardly any difference in GDP per capita between South Korea and Japan (approximately $ 44,000 and 43,000, respectively,). The Japanese people spent $ 100 person^{-1} year^{-1} on flowers, whereas Korean people spend only $ 12.

Trade

Exports can be divided into establishment (the late 1990s): stagnation (early 2000s) boom (late 2000s) with the peak in 2010. The embedded cycle has delayed production value by 5 years. Imports have risen sharply since 2010 and have continued to grow year-on-year. Trade has been in the deficit since 2015, with the deficit increasing annually.

The law on the development of the flower industry in South Korea

On August 20, 2019, South Korea enacted a Law No.16473 (Ministry of Government Legislation, 2019). According to Chapter 1, "establishes the necessary items for fostering and supporting the flower industry, lays the foundation for the development of the flower industry, and promotes the culture of flowers. The purpose is to contribute to the development of the national economy and the improvement of the quality of life of the people." The law has six chapters and 21 articles. Important chapters include the Comprehensive Plan for Flower Industry Development (Chapter 2), the cultivation of flower industry (Chapter 3), and the promotion of flower culture (Chapter 4). This law, when it comes into force in August in 2020, plans to revitalize the domestic flower consumption market (Table 6).

Table 6. Trade of cut flowers and flower buds; HS code 0603 (unit: $ 1,000). Source: Korea International Trade Association (2020).

Year	Exports	Imports	Balance
1990	161	351	▲ 190
1995	2,685	2,246	438
2000	21,594	1,614	19,980
2005	32,717	1,757	30,959
2010	79,961	3,631	76,330
2015	17,348	21,402	▲ 4,054
2016	16,826	24,740	▲ 7,914
2017	14,623	30,097	▲ 15,475
2018	11,405	34,772	▲ 23,367
2019	10,078	36,906	▲ 26,828

CONCLUSIONS

The developments of production, consumption, and trade of the flower industries in China, Japan, and South Korea are compared in this paper and the problems that the industry in each country faces are presented. Collaboration and joint research between these three countries will be necessary to solve all of the problems that each of them faces.

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Farmers' perception on fig (*Ficus carica*) cultivation in southwest Bangladesh

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Abstract

Exotic fig cultivars have shown the potential to replace the wilds that grow spontaneously in Bangladesh. These exotic fig cultivars bear fruit all year round. Exotic figs would be a new fruit crop in Bangladesh, having the capability of meeting some of the nutritional requirements of Bangladesh citizens. However, assessing the farmers' perception about fig (*Ficus carica* L.) cultivation is necessary, prior to introducing these exotic figs as a new fruit crop. A survey was conducted using a pretested interview questionnaire from 300 randomly selected respondents from six unions of Khulna, Jashore and Satkhira district in Bangladesh. This study found the majority of respondents are willing to grow figs commercially (73%). About 57% could identify production problems. This signifies that a majority of respondents (57.3%) bears a higher level of perception in identification of the problems associated with fig cultivation. A good portion of the respondents showed from moderate (39%) to high (61%) level of perception, having the capabilities of selecting possible solutions to problems identified. Lack of quality seedlings was identified as major problem, scoring 739 out of 900 total possible score. Approximately 57% of respondents considered the problems identified as moderate severe. About 54.3% of respondents possess an overall clear perception in problem identification and possible solutions regarding fig cultivation. Among selected characteristics, age, extension media contact, educational qualifications, agricultural and fruit farming experience and cosmopolitanism, there was a significant correlation with the perception of the farmers.

Keywords: fig, perception, problems, solutions, minor fruit

INTRODUCTION

Among 800 species of fig, *Ficus carica* L. is the most cultivated species. It is a syconium (numerous flowers embedded in a fleshy receptacle) and belongs to the family *Moraceae*. The history of fig dates back over 2,000 years. The religious importance of fig is also significant. It is mentioned in The Holy Quraan, Hadith and The Bible (Washburn and Brennand, 2010). Figs are very rich in mineral content and possess traditional therapeutic benefits (Soni et al., 2014). The people of Bangladesh suffer from malnutrition (Uddin et al., 2016), on average each person consumes only 44.7 g (HIES, 2010) of fruit everyday instead of 100 g as recommended by FAO/WHO (2003).

About 54% of fruits in Bangladesh is produced during the four months of the summer (April to July), with 46% produced during the remaining eight months of the year (Banglapedia, 2015; Pasha and Uddin, 2019). The introduction of new fruit crops such as fig for Bangladesh would increase the availability of fruit for consumption there (Mehraj et al., 2013). However, no attempt for managed cultivation of fig in Bangladesh has been undertaken. This may be due to, unavailability of suitable cultivars, a lack of knowledge of production methods, or a lack of knowledge about its nutritional value. This study was designed to collect information on the farmers' perception about fig cultivation, and its acceptability as a fruit crop in Bangladesh. In addition, data were collected on farmers' perceptions of possible problems related to cultivation and possible solutions.

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MATERIALS AND METHODS

Locale and time of the study

A survey was conducted using pretested interview questionnaire from 300 randomly selected respondents at 24 villages, from 12 unions and six upazilas of Khulna, Jashore and Satkhira districts, in Bangladesh. Face-to-face interviews were conducted during September 2 to December 30, 2018. The sample selection method was purposive random sampling (50 farmers from each of the upazilla), a technique established by Tongco (2007).

Measurement of variables

Personal and socio-economic characteristics – age, education, family size, experience in agriculture and fruit cultivation, farm size, annual income, extension media contact, organizational participation and cosmopolitanism – were considered as independent variables and the dependent variable was perception (in respect to problem, solution and overall which denotes the average perception considering perception in identifying the problem and solution).

Data calculation

1. Problem severity according to the respondents.

Respondents were asked to rate each of the six possible problems mentioned in the questionnaire and the score of the respondents calculated using the following formula: PS = $(N_1 \times 0) + (N_2 \times 1) + (N_3 \times 2) + (N_4 \times 3)$; where PS = problem severity, N_1 = no problem, N_2 = less severe problem, N_3 = moderately severe problem, and N_4 = highly severe problem.

Thus, the problem severity score of a respondent may vary from 0 to 18. From the obtained score, farmers were categorized into three groups, respondents who mentioned the problems as, less severe (score 0-6), moderately severe (score 7-12) and highly severe (score >12).

2. Calculating the extent of problem severity and solution suitability.

According to the respondents' rating, the problem severity and solution suitability scores were calculated as follows: PS or SS = $(N_4 \times n) + (N_3 \times n) + (N_2 \times n) + (N_1 \times n)$ (Bashar, 2006); where PS = problem core, SS = solution core, n = number of respondents, N_1 = no problem (score 0) or not a suitable solution at all (score 1), N_2 = less severe problem (score 1) or less suitable solution (score 2), N_3 = moderately severe problem (score 2) or moderately suitable solution (score 3), and N_4 = highly sever problem (score 3) or highly suitable solution (score 4).

Every respondent (total 300) may assign a minimum 0 and maximum 3 for a specific problem and for a specific solution from 1 to 4. Therefore, the problem total score may vary from 0 to 900 and for probable solutions the total score may range from 1 to 1,200.

The extent of possible problem severity (%) and probable solution suitability % related to fig cultivation in southwest Bangladesh was measured by using the following formula:

$$\text{\% Problem severity or \% Solution suitability} = \frac{\text{Observed problem severity or the solution suitability score} \times 100}{\text{Maximum possible problem severity (900) or the solution suitability score (1,200)}}$$

3. Calculation of farmers' perception.

Four-point Likert type rating scale was assigned as 4, 3, 2 and 1 against both problem and solution related perceptions (Likert, 1932; Pervin et al., 2018). The scores were assigned, based on the response of the respondents (on the possible problem and solution). The minimum and maximum score for the problem and solution (four possible solutions for each of the six problems) identified by respondents were assigned as 1 to 4. This progressively gave a minimum score of 6 and maximum score of 24. This score was again categorized in three categories as, a less clear perception (score 6-12), moderately clear perception (score 13 to 18) and a clear perception (score >18). Overall perception was calculated by calculating the

mean of perception in problem perspective and perception in solution perspective.

Data analysis

Data analyzed using Microsoft Excel and Statistical Package for Social Science (SPSS). The analysis that explored the relationship between dependent and independent variables was carried out using Pearson's product moment correlation coefficient 'r'.

RESULTS AND DISCUSSION

Socio-economic status of the respondents

The socioeconomic status of the respondents (data not included) signifies that, majority of them are young to middle aged having at least secondary level of education. However, they prefer government or non-government jobs or run their own business rather than practicing in agriculture. They think that agriculture will degrade their social status. Moreover, they are interested in adopting modern technologies through extension media or internet, but still unwilling to form an organization or hold any position in organization, which confirms Huque et al. (1996) research findings. This study revealed that people, who are more cosmopolite, are not solely dependent on agriculture. People prefer an easy but luxurious life, which is not possible from agriculture as a profession in Bangladesh.

Present status and prospects of fig cultivation in southwest Bangladesh

From Table 1, findings showed that all fig plants in each locality (*F. racemosa* or *F. hispida*) are natural grown tree types. Contrary to this, *F. carica* is shrub type, which is also easy to grow, even in the homestead of the farmers (Brien, 2002). No respondents cultivated or eat locally grown tree type fig as fruit. More than half of the respondents (67%) already know about *F. carica* as an edible fruit, as it is mentioned in The Holy Quraan, Hadith (as 'Teen') and also in Bible (Borhany, 2005). About 92% of respondents are willing to eat Teen as fruit, if grown in their locality. All respondents are willing to cultivate teen as fruit, if appropriate production technology is developed and good cultivars are available. Approximately 75% of the farmers were willing to cultivate it commercially, because it is a very high value fruit and praised for its eating quality all over the world (Reddy et al., 2008).

Categorization of the respondents based on obtained problem score

Six possible problems related to fig cultivation were placed before the respondents to measure their severity, as described in methodology section. The problem severity score ranged from 4 to 18, in a scale of 0 to 18. Most of the respondents (57%), considered the problems as moderately severe, followed by acutely severe (39.3%) and of low severity (3.7%) (Table 2).

Possible problems and their rank order in respect of fig cultivation

Lack of quality seedlings was identified as the most severe problem, scoring 739 out of a possible 900 (Table 3). This was followed by the high price of seedlings (score 707), lack of knowledge about production technology (score 498) and nutritional value of fig (score 386) and no idea about edible fig (score 361). They considered uncertainty about marketing as the least severe problem regarding fig cultivation (score 272 out of a possible 900).

Rank order of the possible solutions related to *F. carica* cultivation

Mass circulation by extension workers was the most preferred solution among the 15 suggested solutions, scoring 1049 (87.42%) out of a possible 1200. Other possible solutions identified by the respondents ranked in order were, producing and distributing seedlings at reasonable price, followed by mass circulation of cultivation practices through the media, establishing demonstration plots, developing production technology and providing training to the farmers, etc. (Table 4).

Table 1. Present status and prospects of fig cultivation in southwestern Bangladesh.

Sl. no.	Questions	Answer			
1	What type of fig plant grown in your locality?	Tree		Shrub	
		Number	%	Number	%
		300	100	0	0
2	How does fig grown in your locality?	Naturally		Cultivated	
		Number	%	Number	%
		300	100	0	0
		Yes		No	
		Number	%	Number	%
3	Do you eat fig as fruit grown in your locality?	0	0	300	100
4	Do you cultivate fig in your orchard?	0	0	300	100
5	Do you know about edible fig (Teen)?	201	67	99	33
6	Are you willing to eat fig as fruit?	276	92	24	8
7	Would you cultivate edible fig if it is possible in your locality?	300	100	0	0
8	Would you cultivate edible fig if you get seedlings of good variety?	300	100	0	0
9	Would you cultivate fig commercially if it is possible in your locality?	219	73	81	27

Table 2. Categorization of the respondents based on the obtained problem score.

Problem category	Score	Number of respondents	Percentage (%)	Mean	SD.	Range of score
Less severe problem	≤6	11	3.7	12.34	2.93	4-18
Moderate severe problem	7-12	171	57			
Severe problem	>12	118	39.3			

Table 3. Possible problems and their ranking in relation to *F. carica* cultivation.

Possible problems	Obtained score (out of 900)	% Problem severity	Rank order
Lack of quality seedlings	739	82.11	1st
High price of seedlings	707	78.56	2nd
Lack of knowledge about production technology of fig	498	55.33	3rd
No knowledge about nutritional value of fig	386	42.89	4th
Have no idea about edible fig	361	40.11	5th
Uncertainty about marketing of fig	272	30.22	6th

Possible problems and solutions in teen (*F. carica*) cultivation as perceived by the respondents

More than half (57.3%) of the selected farmers perceived the problems clearly, while 41.7% perceived the problems as moderate and only 1% with less clarity. Perception score in problem perspective ranged from 10 to 23, with a mean of 18.81 and a standard deviation of 2.11 (Table 5). For the solution perspective, perception varied from 4 to 24 with a mean and standard deviation of 17.83 and 2.50, respectively. Majority of the respondents (58.7%) indicated a moderate clear perception about the probable solutions followed by clear (39%) with only 2.3% of respondents having a less clear perception. About 54% of the farmers possessed an overall clear perception i.e., the perception of both the problem and solution perspective.

Table 4. Rank order of the solutions related to teen (*F. carica*) cultivation.

Suggested solution	Score (out of 1200)	Solution preference (%)	Ranking
Mass circulation by extension workers	1049	87.42	1st
Seedling production and distribution at reasonable price	1027	85.58	2nd
Mass circulation by electronic and print media	1005	83.75	3rd
Training on production technology of fig	958	79.83	4th
Establishing demonstration plots	944	78.67	5th
Training for seedling production at farmers level	940	78.33	6th
Developing suitable production technology by research for Bangladesh	899	74.92	7th
Inclusion of fig in the fruit list and extension of its production technology	726	60.5	8th
Cultivation by farmers' organizations and selling though collection points	700	58.33	9th
Creation of markets for fig	634	52.83	10th
Fixing reasonable price of seedlings by the government	605	50.42	11th
Harmonizing supply chain between the super shops and producers	588	49	12th
Involving different NGOs for making fig popular as a fruit	545	45.42	13th
Training up the nursery owners for seedling production	514	42.83	14th
Arranging seminar/symposium/conference/ workshops/training	468	39	15th

Table 5. Perception of the respondents regarding problems, solutions and overall.

Category		Score	Number	Percentage (%)	Mean	SD	Range
Perception (problem perspective)	Less clear	6-12	3	1	18.81	2.11	10-23
	Moderately clear	13-18	125	41.7			
	Clear	>18	172	57.3			
Perception (solution perspective)	Less clear	6-12	7	2.3	17.83	2.50	4-24
	Moderately clear	13-18	176	58.7			
	Clear	>18	117	39			
Overall perception	Less clear	6-12	6	2.0	18.33	1.93	10-23
	Moderately clear	13-18	132	44.0			
	Clear	>18	162	54			

Correlation between selected characteristics of the respondents and their perception

Among the ten selected socioeconomic characters of the respondents, age, extension media contact, educational qualification, agricultural and fruit farming experiences, and cosmopolitanism, showed a significant correlation with the perception of the farmers (Table 6). However, the cosmopolitanism showed a negative correlation. That means, the more cosmopolite a person is, the more likely they are to be involved in professions other than farming. Also, they possess low perceptions about problems related to farming and their solutions. However, the other parameters showed a non-significant correlation with perception, in terms of identification of problems, solutions, and overall perception.

Table 6. Pearson's product moment correlation coefficient (r) between the selected characteristics of the respondents and their problem confrontation and perception.

Sl. no.	Characteristics (independent variable)	Pearson's product moment correlation coefficient (r)			
		Problem confrontation in fig cultivation	Perception (problem perspective)	Perception (solution perspective)	Perception (overall)
1	Age	0.049 ns	0.308**	0.158 **	0.286**
2	Educational qualification	0.34 ns	0.045 ns	0.172**	0.141*
3	Family size	0.073 ns	0.051 ns	-0.027 ns	0.18 ns
4	Farming experience	0.063 **	0.057 **	0.012*	0.025**
5	Experience in fruit farming	0.358 **	0.088 ns	0.107 ns	0.123**
6	Income	0.251 ns	0.128 ns	0.059 NS	-0.078 ns
7	Farm size	0.160 ns	-0.126 ns	0.114 ns	-0.044 ns
8	Extension media contact	0.254 **	0.167**	0.33**	0.140**
9	Organizational participation	-0.063 ns	0.189 ns	0.128 ns	-0.048 ns
10	Cosmopolitanism	- 0.142*	0.095 ns	0.133 ns	- 0.130*

ns = non-significant, ** Significant at the 0.01 level (2-tailed),* Significant at the 0.05 level (2-tailed).

CONCLUSIONS

The majority of the respondents (67%) have knowledge about edible fig (Teen) and all respondents are willing to eat and cultivate this fruit. However, a lack of quality seedlings was ranked as acute problem. Commercial fig cultivation would be possible in southwest Bangladesh if the availability of quality seedlings is ensured.

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Roles of site facilitators in improving farm income by vegetable growing in South Cotabato and Maguindanao, Philippines using the Livelihood Improvement through Facilitated Extension (LIFE) model

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Abstract

Mindanao is considered as the "food basket" of the Philippines. However, it is home to the poorest provinces in the country. In 2018, the University of the Philippines Mindanao scaled out the "Livelihood Improvement through Facilitated Extension" (LIFE) Model. This is a agricultural entrepreneurship extension strategy program, undertaken in two conflict-vulnerable areas, namely Lambukon, Canahay, Surallah in South Cotabato and Bisang, Talisawa, Datu Abdullah Sangki in Maguindanao. Each site is assigned two local facilitators as extension agents. Monthly reports, responses to a questionnaire, and a focus group discussion were assessed. This is to relate facilitator roles and tasks to the improvement of two farmer groups livelihoods. A farmer group at each site, identified vegetable growing as their priority livelihood. Group members attended Farmer Field Schools, worked in their communal demonstration area and on individual farms. They also visited markets and successful farms to learn farming technologies. The program and partner agencies provided some of the farm inputs. Facilitators visited farmers often, teaching farming technologies. Approximately 45% of the facilitators time was interacting with the farmers to develop social capital. Linkages with at least six local partners at each site strengthened institutional support for the farmer groups. Farmers earned additional income, approximately 44 to 64% from the sale of vegetables. The two groups were registered with the Department of Labour and Employment. This allowed them to access government services. Household food security was addressed with vegetables harvested from their farms. Drought and typhoons disrupted their vegetable growing but the farmers replanted. Likewise, the program re-established connections with the new ministers of ministries under the Bangsamoro Autonomous region in Muslim Mindanao, and was proactive in procurement matters.

Keywords: Livelihood Improvement through Facilitated Extension, Mindanao, agricultural extension, vegetables

INTRODUCTION

The Philippines is an agricultural country with low adoption of technologies; planting of traditional crops; weak research, development, extension and delivery systems by local government agencies; and unclear policies (FAO, 2012). Mindanao supplies 40% of the country's food requirements. However, its provinces are among the poorest in the nation (NEDA, 2012). It suffers from conflicts in vulnerable areas and has the highest poverty levels (Adriano and Parks, 2013). However, government services in Mindanao are perceived to be low compared to Luzon and Visayas (Concepcion et al., 2003).

Established in 1995 through the Republic Act 7889, the University of the Philippines Mindanao (UP Mindanao) aims to provide responsive and relevant extension services to

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marginalized sectors in Mindanao (University of the Philippines Mindanao, 2019). Agricultural productivity in Mindanao needs to be improved (NEDA, 2012) to help alleviate poverty (Coxhead and Warr, 1995). UP Mindanao, with funding from the Department of Science and Technology-Philippine Council for Agriculture, Aquatic and Natural Resources Research and Development and in partnership with the Landcare Foundation Philippines, Inc. (LFPI), scaled out the Livelihood Improvement through Facilitated Extension (LIFE) model developed by ACIAR Mindanao Agricultural Extension Program (AMAEP). The model aims to: 1) improve access to technical innovation, 2) build community social capital, and 3) collaborate closely with local institutional partners (Vock et al., 2018). It has been tried in communities with socio-cultural or political conflict. Lambukon, Canahay, Surallah, South Cotabato and Bisang, Talisawa, Datu Abdullah Sangki, Maguindanao were chosen after consultation and validation with the local government units (LGUs). These communities are in dire need of agricultural assistance. Two local facilitators for each site serve as extension agents and are guided by UP Min Project Leaders. Shah et al. (2013) defines the role of extension agents as knowledge workers who can offer advisory and consultancy services to farmers. This paper focuses on the roles of the facilitators in enhancing farmers' livelihood through vegetable growing using the LIFE Model. As extension agents, defining and identifying their roles in the LIFE Model can aid future implementers in preparing support and training for farmers.

MATERIALS AND METHODS

Two facilitators who were residents of South Cotabato and Maguindanao were chosen per site, and trained prior the start of the program. Progress at each site was monitored through teleconferences, face to face meetings, and field visits. Two farmer groups were organized: the Lambukon Amligan T'boli Integrated Farmers Association (LATIFA) in South Cotabato and the Teduray Farmers and Producers Association (TFPA) in Maguindanao. These groups were registered with the Department of Labour and Employment (DOLE).

Facilitators' tasks as reflected in their contracts were reviewed via their monthly accomplishment reports that indicated the number of hours' undertaking extension tasks. The facilitators' journal entries were used to determine and tally the duration and frequency of visits to the sites. Site facilitators also answered a questionnaire, assessing their competencies upon entry into the program, the usefulness of training provided, and additional training needs. Focus group discussion sessions were undertaken to validate their responses.

The baseline income of farmers was established, and their income monitored until year 2. Separate incomes were estimated for corn, their traditional crop, and for different vegetable types. The farmers trained in how to record their sales and the produce used at home or given away. We listed the various partnerships established, technologies disseminated, and crops grown.

RESULTS AND DISCUSSION

Site facilitators as agricultural extension agents

Site facilitators help farmers build confidence in research and fact-based decisions. Facilitators connect them to private and government institutions to improve their livelihoods. Qualified and competent agents are key to the effectiveness of an extension service as they bring new information to the farmers (Shah et al., 2013). They must also understand agriculture to communicate new technologies to farmers effectively (Terblanché, 2008). Two facilitators are Agricultural Technology graduates, one has earned graduate units in Masters in Agricultural Science Technology and the fourth has a degree in Agricultural Education, majoring in extension. For this project they are expected to facilitate: 1) implementing the LIFE Program, 2) planning community workshops, 3) forming and strengthening community groups, 4) training farmer-facilitators, and 5) developing social capital through workshops, farmer cross-visits, and knowledge sharing. They also have to: 6) initiate activities, 7) engage with community stakeholders and help them form partnerships, 8) gather baseline information on existing agricultural livelihood activities, social capital, and extension

practices, and document observed changes, 9) identify gaps or opportunities in existing agricultural livelihood activities, 10) work with partner agencies to review progress and improve outcomes, 11) report progress and implementation issues, 12) attend seminars/trainings/meetings and present results, and 13) review research findings. The facilitators rated "always" and "often" on most of the functions they performed, except for "sometimes" on review of research findings. This is because the research papers are still being prepared. Mwangi (1998) highlighted the seven principles to the delivery of new agricultural technology is through extension: 1) consultation, 2) building mutual trust, 3) establishing rapport with stakeholders, 4) being sensitive to farmers' needs, constraints, and opportunities, 5) using appropriate terminology to teach farmers, 6) having good technical preparation and self-confidence, and 7) being a good listener.

Roles of site facilitators

For eight months in Year 1 and 2, the time in hours for each declared task was collated from the facilitators' reports and journals. However, for Year 2, the data were collated from three site facilitators as the team's composition had changed. Table 1 shows the time spent on each task for Year 1 and 2. Interaction and activities involving farmers was approximately 45% of the facilitators' time (48.28% for South Cotabato and 41.28% for Maguindanao).

Table 1. Time (%) facilitators were involved in various tasks for 8 months of interaction with farmers in 2018 and 2019.

Tasks	South Cotabato		Ave.	Maguindanao		Ave.
	Y1	Y2		Y1	Y2	
Interaction with community partners	48.95	47.61	48.28	36.18	46.38	41.28
Journal writing and preparation of accomplishment report	11.94	4.90	8.42	21.16	3.97	12.57
Planning/meeting with UP Mindanao team and co-site facilitators	18.08	9.18	13.63	19.79	7.21	13.50
Engagements with institutional partners	14.07	9.05	11.56	10.10	13.02	11.56
Administrative matters	2.37	12.04	7.20	5.73	14.86	10.30
Updating/planning/consultation with LFPI[a]	4.39	9.18	6.78	3.34	5.50	4.42
Community literacy program	-	3.64	1.82	-	6.79	3.40
Teleconference with farmers/partner agencies/UP Mindanao team	0.21	4.41	2.31	3.69	2.27	2.98

[a]LFPI – Landcare Foundation of the Philippines, Inc.

1. Interaction with community partners.

Interaction with community partners usually involves tasks such as; 1) field monitoring, 2) preparation of activities and farm work, 3) Farmer Field School training, 4) planning for upcoming activities, and 5) consultation with farmers. The two farmer community groups, LATIFA and TFPA have 27 and 34, registered members, respectively. Extension agents encounter problems in propagating new technologies. This is due to lack of resources and costly inputs (Farooq et al., 2010), which can cause failure during the implementation phase. The present program provides some farm inputs and taps into local partners who assist in providing other needs for the farmers. Facilitators also assist farmers in accessing government aid.

Due to the facilitators' constant presence, they reported improved rapport with the farmers and now receive invitations to community events. Facilitators spent almost half of their working period interacting with farmers for the past two years. They conducted individual interviews, which helped them get to know each member. They learned the farmers' background and family composition which led to knowing their needs and priorities. Individual farm monitoring also enabled one on one conversations with the farmers mostly delving into their farm activities and technologies. Farmers developed trust toward the facilitators and in turn, the program. The farmers have gained confidence in sharing their concerns, which helped identify additional support needed. Cultivating strong relationship,

mutual trust, and strong communication between the farmers and the extension agents is essential, to ensure the success of the extension model (Mwangi, 1998; Farooq et al., 2010).

Both sites reported improved participation and ownership on the part of the farmers. Farmers were initially shy with only a few participating in discussions. Farmers have become more participative in trainings and meetings. Farmers reported improved relationship with neighbors, as more time is spent together. Project extension agents tailored their facilitation style toward the community's culture, like learning and using local language. Facilitators reported receiving inquiries from farmers through text messages and calls. With aid from institutional partners, the facilitators conducted the various trainings on climate change and basic knowledge on vegetable gardening; soil and water conservation; farm planning; field layout; bed preparation; soil nutrient and moisture management; transplanting, vermicomposting, trellising and other horticultural practices; plant propagation; pest and disease management; record keeping; harvesting, postharvest handling, marketing; and market exposure and enterprise development, including price monitoring.

Figure 1 shows the number of visits by site facilitators in Year 1 and 2 for eight months, respectively. The facilitators spent more time (18 times per month) onsite in Year 2, This was due to an increase in training and data gathering compared to Year 1 (14 times per month). South Cotabato facilitators conducted on average 14.25 and 18.25 visits per month in Year 1 and 2, respectively. Maguindanao facilitators logged an average of 13.62 visits per month for Year 1, and 17.25 visits per month for Year 2.

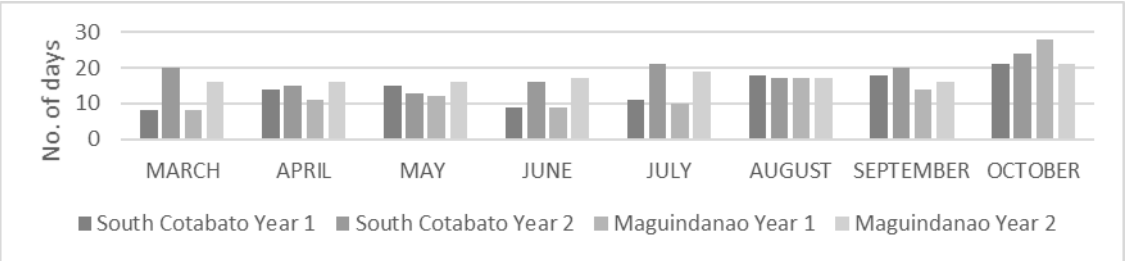

Figure 1. Number of days per month of facilitators' visits to the sites for eight months each in 2018 and 2019.

The demonstration farms for South Cotabato and Maguindanao were damaged due to a typhoon and drought, respectively. This led to more site visits to re-establish the farmers' communal farms. The facilitators also partnered with agencies to conduct training and distribution of farm inputs. Due to the distance from one individual farm to the next, facilitators can only visit two to three farms a day for monitoring. Facilitators' time spent onsite prior and during May, 2019 elections and during Ramadan was considerably less (April to May).

2. Journal writing and preparation of accomplishment report.

Site facilitators submitted their journals and accomplishment reports. These were used to track their interactions with farmers and institutional partners as well as challenges encountered.

3. Planning/meeting with UP Mindanao staff and co-site facilitators.

The facilitators are in constant communication with the UP Mindanao staff. In particular, to arrange stakeholders' forums, meetings with partners; to draft trainings; and to discuss procurement, reports and problems encountered in the field. They also held meetings between themselves to discuss activities, challenges and ways forward.

4. Engagements with institutional partners.

Engagements with institutional partners involved courtesy calls/meetings, attendance at trainings and activities hosted by partner agencies, and project updating. The LIFE model

emphasizes fostering good relations with partners through continuously updating them of the concerns and success stories of the farmers. This enabled institutional partners to tailor their response to meet the needs of the community. Active institutional partners (six in South Cotabato and seven in Maguindanao; Table 2) provided personnel and farm supplies.

Table 2. Institutional partners of the program at each site.

Lambukon, Canahay, Surallah in South Cotabato	Bisang, Talisawa, Datu Abdullah Sangki in Maguindanao
Department of Agriculture XII	Ministry of Agriculture Fisheries and Agrarian Reform
Philippine Coconut Authority	Philippine Coconut Authority
Bureau of Fisheries and Aquatic Resources	Ministry of Indigenous Peoples Affair
Department of Social Welfare and Development	Ministry of Social Services and Development
	Ministry of Basic, Higher and Technical Education
Municipal Local Government Unit of Surallah	Municipal Local Government Unit of Datu Abdullah Sangki
Barangay Local Government Unit of Canahay	Barangay Local Government Unit of Talisawa

Vock (2018) highlights by organizing and registering with DOLE, farmers develop linkages with institutions resulting in better access to government programs. They also receive financial aid and ensure the local political commitment to the sustainability of their farmer group programs. Courtesy calls increased the sites' visibility and enabled more assistance toward the community as well as introduced the Model to agencies that may be capable of adopting it. The Bureau of Fisheries and Aquatic Resources established a tilapia demonstration farm in Canahay, Surallah and the Department of Agriculture XII provided poultry (chicks). Agricultural technicians from the Municipal Agriculture Office of Surallah and Datu Abdullah Sangki join the facilitators in conducting training. Farmers were able to converse with experts while enabling partners to identify possible assistance they can provide. Their constant presence increased farmers' trust toward their institutions.

5. Administrative matters.

Preparation of purchase orders for farm supplies, venues and van rentals were undertaken. In addition, transactions with suppliers, and other administrative matters were also conducted.

6. Engaging with Landcare Foundation of the Philippines, Inc. (LFPI).

LFPI facilitators who used the LIFE Model before mentored the site facilitators for this study. Facilitators discuss planning, facilitation, engagement, and building social capital with the farmer groups as well as provide training in agricultural technologies.

7. Community literacy.

Consultation with farmers revealed their desire to learn to write, read, and count. Some farmers went to school for a few years, but a few never did. Buyers took advantage of farmers' illiterateness. Therefore, the facilitators along with the LFPI mentors, conducted a community literacy program. This program tapped into the Department of Education-Maguindanao Alternative Learning System (ALS). Twenty-eight farmers from Bisang took the ALS Accreditation and Equivalency Assessment and Certification for elementary and high school.

Improvement in livelihood of farmers

The 2018 baseline survey showed that most farmers practiced corn monocropping which yielded insufficient income. Only a few planted vegetables and root crops. Farmers earned more from their individual and communal vegetable farms. LATIFA farmers earned much more than TFPA as the latter needed to re-establish their farm due to drought (Table 3). Seventeen of 27 TFPA farmers also relied heavily on their vegetable plots, resulting in a 64% increase in income compared to the LATIFA whose corn crop was felled by the typhoon.

Table 3. Average vegetable farm to agricultural income (May-December 2019).

Farmer group	Average monthly vegetable farm income (PHP)	Average monthly corn farm income (PHP)	Average agricultural income (PHP)	No. of farmers with >50% of income from vegetable farm	Average vegetable to agricultural income of farmers with >50% income from vegetables[a] (%)	Total vegetable income to total agricultural income[b] (%)
LATIFA (*n*=20)	4,314.89	20,614.58	24,928.47	7	44	17.31
TFPA (*n*=27)	1,035.85	2,892.31	3,928.28	17	64	26.37

LATIFA – Lambukon Amligan T'boli Integrated Farmers Association, Lambukon, Canahay, Surallah in South Cotabato.
TFPA – Teduray Farmers and Producers Association, Bisang, Talisawa, Datu Abdullah Sangki in Maguindanao.
Average currency conversion for May-June 2019: PHP 51.448 to USD 1.

[a] $\frac{1}{N}\sum_{i=1}^{N}\left(\frac{vegincome_i}{agincome_i}x100\right)$.

[b] $\frac{total\ \ vegetable\ \ income}{total\ \ agri\ \ income}\ \ x100$.

With the increase in income comes better food security. Maguindanao is classified as one of the "food poor" provinces in Mindanao (NEDA, 2012). A Maguindanao farmer-partner stated that before, they only ate once a day, but because of their vegetables, their families now have regular meals. Vegetables are good source of micronutrients (Ali and Tsou, 1997), which enable farmers to have healthier meals. Facilitators also report that farmers were not able to give a counterpart food during activities. After receiving training and support they are now able to contribute toward providing food as counterpart contribution. The tilapia farm of LATIFA and the chickens of TFPA provided them a source of protein and additional cash. The Canahay Local Government Unit (LGU) brought visitors to the demonstration farm who purchased their produce. The Bisang LGU plans to give the TFPA group a market stall. Farmers from both sites, sell produce during farmers' days. Even their children bring vegetables to their school and sell their vegetables to the teachers. Cross-visits were conducted to successful farms to learn agricultural best practices. Facilitators find farmers are more inspired to work on their farms after cross visits. Table 4 shows the technologies taught to the farmers and the 17 vegetable crops they grew including yard long beans, eggplant, okra and squash, the most common. Other crops grown were cassava, peanuts and yam.

Through the assistance from the Department of Agriculture - Surallah, farmers accessed insurance from the Philippine Crop Insurance Corporation (PCIC). When typhoon "Chedeng" (Tropical Depression 03W) struck in March 2019 and destroyed their crops, they filed insurance claims. They used the insurance money to have electricity and satellite cable connected to their community. Maguindanao is classified as the second lowest in length of paved roads to road length in Mindanao (NEDA, 2012). The Datu Abdullah Sangki LGU bulldozed the rough roads and laid limestone, after visiting the community during an activity. This will reduce costs of transaction and acquiring inputs, while increasing output prices (Dercon et al., 2009), making the community more accessible.

The farmers and site facilitators both reported an increase in self-confidence in dealing with one another and with institutional partners. Before, they found approaching agencies too intimidating. Since they have an established partnership with the agencies, they are now more confident in accessing training and livelihood programs offered.

Challenges encountered by the program and how they were addressed

A typhoon and drought damaged the demonstration farms of LATIFA and TFPA, respectively. Facilitators and farmers worked overtime to re-establish the farms. In 2019, the Autonomous region in Muslim Mindanao (ARMM) transitioned into Bangsamoro Autonomous region in Muslim Mindanao (BARMM), to which Maguindanao now belongs. Early established partnerships were dissolved, so once again the team presented the LIFE Model to the new ministries before forging a new memorandum of agreement.

Table 4. Technologies taught to farmers and vegetable crops grown.

Technologies	Vegetable crops
Setting up a plastic house	Yard long beans
Soil sterilization	Eggplant
Mulching	Okra
Cover cropping	Squash
Seed sowing, hardening and transplanting of seedlings	Malabar spinach
Agroforestry and nursery establishment	Chinese cabbage
Pruning, ratooning	Tomato
Use of insect attractants	Bitter gourd
Container gardening of vegetables	Bottle gourd
Preparation and application of vermicompost,	Radish
insect repellant, organic fertilizer, and ferticide	Cucumber
Planting distances, staggered planting	Luffa
Crop rotation, companion cropping	Sweet pepper
Maturity indices, sorting and grading	Ginger
Produce postharvest quality maintenance	Spring onions
Seed selection and seed storage; breaking of seed	Hot pepper
dormancy (seed stratification)	Mung beans
Selection of resistant cultivar	Water convolvulus

In addition, facilitators encountered some challenges in procuring farm supplies. This extension model implemented by a public university required compliance to government procedures. Training on basic guidelines in government procurement was conducted accordingly. The Program team applied for cash advances to provide immediate funds for field activities and regularly consulted with the facilitators. Communication with facilitators was exacerbated by poor cellular connection in the field, which delayed communications. Meetings were scheduled in advance to enable facilitators to find places with good signal.

All facilitators indicated that they needed more training in facilitation, contextualization, data gathering, report writing, as well as MS Office before starting project implementation. UP Mindanao addressed these needs through short training sessions during mid- and end of year meetings in conjunction with LFPI's mentoring sessions. The LIFE training manual is currently being prepared.

CONCLUSIONS

The facilitators served as the main agricultural extension agents to the farmer groups who linked them with institutional partners. Identifying and defining the functions and tasks they play in the implementation of the Livelihood Improvement through Facilitated Extension (LIFE) model, aided in preparing them to successfully serve as facilitators for change. Likewise, it identifies the training and other physical support mechanisms, that need to be provided. Building a strong rapport with farmers led to improved active participation in the training and activities organized by the program. Up to seven local institutional partners also co-assisted with the program. Facilitators visited the farmers often. They spent 44.78 and 11.56% of their time, respectively, in engaging with farmers and partners and helped the farmer groups succeed in their vegetable farming. The farmers increased their income by 44 to 64% through selling vegetables and other crops from their communal demonstration and individual farms. Hence, this study validates the importance of an extension agent being available, responsive and sensitive to the needs of the farmers. They establish mutual trust and rapport with the target beneficiaries and other partners and serve as bridges between research findings and practice so farmers may improve their lives.

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Factors affecting the adoption of GAP by growers in producing crops in Phetchaburi province, Thailand

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Abstract

In Phetchaburi province, Thailand, the growers have been trained to adopt GAP because of concerns about food safety, environmental pollution, and consumer health. This study aimed to investigate GAP adoption by growers and examine the factors influencing the adoption of GAP in producing a crop. The data were collected using semi-structured questionnaires. Fifty-one growers from the Amphur Khao Yoi, Tha Yang, Ban Lat, Ban Laem, and Nong Ya Plong regions of Phetchaburi province were selected in the study which ran from April to June, 2019. Descriptive statistics such as percentage, mean and standard deviation were used to analyze growers' socio-economic characteristics. In addition, correlation analysis was used to identify factors influencing GAP implementation. The results of the correlation analysis indicated that farming experience and cultivated area significantly influenced the adoption of GAP for producing a crop.

Keyword: good agricultural practice, Phetchaburi, crop production

INTRODUCTION

In Thailand, Thai Agricultural Standard (TAS 9001-2009) is the standard that relates to good agricultural practice for producing fruits and vegetables. It specifically addresses the requirement to address the impacts of improper use of agrochemicals on food safety, environment and health. The demand from consumers for safe food also highlights the urgency to implement GAP (Suwanmaneepong et al., 2016). This study aimed to investigate GAP implementation and to examine factors influencing the implementation of GAP on crop production in Phetchaburi. The findings from this study may be helpful to better understand those factors that influence GAP implementation on crop production, and how to encourage farmers to participate GAP implementation.

MATERIALS AND METHODS

Study area, sampling and sample size

A total of 51 growers in Phetchaburi, Thailand, who were registered with DOAE and who practiced GAP in 2019-2020, were subjected to the questionnaire used in this study. A purposive sampling technique was employed to select these 51 growers in the Amphur Khao Yoi, Tha Yang, Ban Lat, Ban Laem, and Nong Ya Plong of Phetchaburi. The survey was conducted using semi-structured questionnaires from April to June 2019.

Data analysis

The primary data collection was used to identify those factors that influenced the implementation of GAP and the main variables that were involved. Descriptive statistics, including frequency distribution, percentages, means, standard deviations, multiple regressions and correlation coefficients, were used for statistical analysis.

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RESULTS AND DISCUSSION

Socio-economic characteristics of the respondents

The socio-economics characteristics of the respondents are shown in Table 1. The data included gender, education level, the number of family members, the number of family laborers, farming experience, membership of farming organizations, cultivated area, land owner status, financial support, income year^{-1}, and GAP training.

Table 1. Socio-economic characteristics of respondents (*n*=51).

Attributes	Characteristics	Frequency	Percentage
Gender	Male	32	69.6
	Female	14	30.4
Age of farmer	20-30 years	0	0.0
	31-40 years	3	6.1
	41-50 years	8	16.3
	51-60 years	24	49.0
	>60 years	14	28.6
Education level	Lower than primary school	1	2.0
	Primary school	13	26.0
	Junior secondary school	12	24.0
	Senior secondary school	18	36.0
	Bachelor's degree	5	10.0
	Master's degree	0	0.00
	Doctoral degree	1	2.0
The number of family members	1-3 persons	15	35.7
	4-6 persons	22	52.4
	>6 persons	5	11.9
The number of family laborers	1 person	6	17.6
	2 persons	16	47.1
	3 persons	8	23.5
	>3 persons	4	11.8
Farming experience	<10 years	7	16.3
	10-20 years	8	18.6
	>20 years	28	65.1
Membership of farming organization	Yes	33	91.7
	No	3	8.3
Cultivated area	<10 rai	15	33.3
	10-20 rai	11	24.4
	>20 rai	19	42.2
Land ownership status	Owner	28	59.6
	Rent	19	40.4
Financial support	Government project	5	12.8
	Bank	11	28.2
	Own funds	23	59.0
Income/year	<100,000 Baht	18	36.7
	100,001-200,000 Baht	20	40.8
	200,001-300,000 Baht	6	12.2
	300,001-400,000 Baht	1	2.0
	400,001-500,000 Baht	1	2.0
	>500,000 Baht	3	6.1
GAP training	At least 1 time	18	38.3
	(>2 times)	23	48.9
	Never	6	12.8

Most of the respondents were male (69.6%) who were also head of the family. Most (77.6%) of the respondents were of old age (51 years to over 60 years old). This finding was

consistent with a report of Attavanich et al. (2019) that indicated that the age of the head of households in Thailand was similar.

Education level is an important factor that contributes to the rates of learning and adoption of improved technologies which, in turn, lead to increased rates of food production (Fakkhong and Suwanmaneepong, 2017). About 86.0% of respondents had an education at the primary school to senior secondary school level. The family was characterized as medium-sized (4-6 persons) (52.4%) with about two persons (47.1%) involved in farming activities. About 65.1% had farming experience of more than 20 years which indicated that they had adopted agriculture as their profession.

The long experience in farming by these growers might influence and strengthen their perceptions about certain farming practices (Farouque and Takeya, 2007), such as applications of fertilizers and pest control measures. About 91.7% were members of organizations which helped them to manage their farm with modern technologies and integrated financial services. Most respondents owned less than 10 rai to more than 20 rai of land. The percentage of the farmers, who rented the land (59.6%) was higher than that who owned their land (40.4%). About 59% of the respondents used their own funds to manage their farm and about 77.5% had income less than 200,000 Baht $year^{-1}$.

Most respondents (87.2%) indicated that they attended GAP training programs organized by DOAE at least once a year. The farmers in the eastern region of Bangkok also participated in an agricultural training program from one to five times per year (Fakkhong and Suwanmaneepong, 2017).

Most of the growers producing a crop in Phetchaburi obtained their knowledge about GAP from agricultural extension officers (45.3%), followed by friends (15.6%), TV (15.6%), newspaper (9.4%), radio (7.8%) and social media (6.3%). This indicated that agricultural extension officers played an important role in disseminating information about GAP to the growers. The growers adopted the GAP system in producing their crops due to several reasons, such as concern about health (31.1%), care of the environment (28.4%), product price (20.3%), customer preference (17.6%), and agreement among members of the community (2.7%). They explained that GAP, compared to a conventional system, was beneficial to the environment (28.6%) and consumer's health (17.5%) and was responsive to high demands (22.2%) and high prices (19.0%). The constraints recorded for growers adopting GAP included the issues associated with water source and availability (29.0%), usage of chemical substances (19.4%), farm location (16.1%), farming practices before harvest (16.1%), data collection (9.7%), personal hygiene (6.5%), practices during harvest and postharvest (1.6%), and storage and transportation of produce (1.6%). Source and availability of water for irrigation was the major constraint because the farms in Phetchaburi obtained water from irrigation canals that may be contaminated from hazardous or prohibited substances.

GAP implementation level of the farmers

Most growers in the study area implemented GAP on their farms at a moderate level (Table 2). Most (67%) of the respondents were farmers who owned a large farm (cultivated area >20 rai). Some growers had found it very difficult to comply with GAP rules and standards, which contributed to the low proportion of growers practicing GAP. In addition, about 48.9% of the respondents had participated in GAP training only twice and this might have impacted on their capability to implement GAP.

Factors influencing the implementation of GAP among growers in the study area

Multiple regression was employed to investigate factors influencing the implementation of GAP practices among growers. The results revealed an F-ratio of 32.874 which was not significant. However, an r^2 value of 0.997 indicated that the ten variables explained 99.7% of the implementation of GAP by growers. These ten variables included gender, age, education level, the number of family members, the number of family laborers, farming experience, membership of a farmer organization, cultivated area, land ownership status, and GAP training, were not significant to the implementation of GAP.

Table 2. GAP implementation level of growers.

GAP items	Minimum	Maximum	Mean	S.D.	Practical level[a]
Water source	1	4	2.75	0.86	Moderate
Cultivation site	1	4	2.88	0.88	Moderate
Use of hazardous agricultural substances	1	4	3.08	0.97	Moderate
Product storage and on-site transportation	1	4	3.00	0.93	Moderate
Disease and pest-free production	1	4	3.00	0.82	Moderate
Management of quality production	1	4	3.31	0.75	Moderate
Harvesting and post-harvest handling	1	4	3.03	0.86	Moderate
Data recording	1	4	3.13	0.80	Moderate

[a]Level of GAP implementation was justified into 4 levels (none = 0-1.5, low = 1.6-2.5, moderate = 2.6-3.5 and high = 3.6-4.0).

However, the result of Pearson correlation coefficient showed that farming experience and the cultivated area were highly and positively significant to GAP implementation (Table 3). The positive and significant correlation between farming experience and GAP implementation indicated that growers producing a crop in Phetchaburi would increase their level of implementation of GAP if they had more or longer experience and cultivated area. This finding is consistent with the report of Ganpat et al. (2014) who indicated that the level of compliance with GAP was directly related to farming experience and with that of Suwanmaneepong et al. (2016) who demonstrated that farming experience had a positive relationship to GAP implementation by fruit farmers in Rayong, Thailand. Pongvinyoo et al. (2014), in contrast, reported that farming experience had negative and significant impact on the perception of GAP understanding among coffee growers in Chumphon, Thailand. However, Pongvinyoo et al. (2014) reported that cultivated area had the positive impact to GAP implementation which is corresponded to our study. It can be concluded that different attributes will play a role to drive the GAP implementation by Thai growers in different parts of Thailand. It is thus recommended that to promote GAP it is very important to understand the socio-economic characteristics of the growers in each specific area. This insight should make the DOAE officials in certain area to prepare to work more closely with other agencies, such as school under the Ministry of Education, in both planning and promoting GAP. The collaboration with other agencies should increase the number of growers who will adopt GAP for crop production nationwide.

Table 3. Multiple regression and Pearson correlation coefficient results.

	Multiple regression[a]					Pearson corr.	Sig.
	B	S.E.	Beta	t	Sig		
(Constant)	1.162	0.420		2.766	0.221		
Gender	-1.515	0.653	-0.915	-2.321	0.259	-0.285	0.078
Age	0.016	0.239	0.015	0.069	0.956	0.265	0.094
Education level	-1.021	0.364	-1.398	-2.805	0.218	0.037	0.818
Number of family members	-0.225	0.130	-0.164	-1.737	0.333	-0.061	0.730
Nnumber of family laborers	0.643	0.253	0.796	2.540	0.239	0.183	0.343
Farming experience	-0.305	0.284	-0.345	-1.072	0.478	0.563**	0.000
Membership of a farmer organization	0.468	0.297	0.214	1.576	0.360	0.056	0.757
Cultivated area	1.200	0.393	1.319	3.057	0.201	0.475**	0.003
Land ownership status	1.431	0.535	0.864	2.675	0.228	0.073	0.707
GAP training	0.716	0.313	0.669	2.286	0.263	-0.21	0.899
F ratio	32.874						
R squared	0.997						
Adjusted R squared	0.967						

[a]Dependent variable: total GAP implementation score.
**Correlation is significant at 0.01 level.

CONCLUSIONS

Growers implementing GAP in Phetchaburi will produce good quality and more valued crops than those who do not implement these standards. Farming experience and cultivated area played a major role in determining the implementation of GAP with longer experience leading to higher adoption.

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Dynamics of pesticide usage by vegetable growers in the highlands of Northern Mindanao, Philippines

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Abstract

Vegetable growers in the Philippines are becoming dependent on the use of pesticides as a convenient approach to control pest and diseases. The highlands of Claveria, Misamis Oriental is the vegetable bowl of the region in Northern Mindanao, Philippines. A survey was conducted in October, 2018 to April, 2019 to assess the dynamics of pesticide usage among vegetable growers in the region. A total of 100 growers who have engaged in vegetable production for at least a decade were selected to be surveyed. Moreover, growers producing solanaceous vegetables, cabbage and snap beans were identified for the study as these vegetable types dominated the present vegetable production in this area. To gather data from the growers, personal interviews using a pre-tested structured survey questionnaire were conducted. Results showed that farmers are aware of the harmful effects of pesticides for both human and animal health. However, they consider pesticides as an effective method in protecting and controlling pest and diseases (74%). Therefore, they still continually used them. Most of the growers have no training in integrated crop management (ICM). Technicians from chemical companies and neighboring growers are their primary source of information on pest control measures. Generally, broad spectrum and highly toxic chemicals with methomyl (29.6%) and Lambda-cyhalothrin (18.5%) based insecticides and S-metolachlor (22.2%) based herbicides are the most popular chemicals used. Furthermore, they are cheaper compared to specific and less toxic pesticide chemistry available in the market. The results of this study provide a greater understanding on the usage of pesticides by vegetable growers in this area. Creating awareness and suitable information on integrated crop management is necessary the extension service support network will help improve decisions making about pesticides usage.

Keywords: pesticide application, dynamics, perception, vegetable production

INTRODUCTION

Vegetable production has become an important source of income for both growers and field laborers. Furthermore, it serves as a vehicle for reducing poverty in rural areas. On the other hand, the cost of vegetable production is high due to the need to purchase increased inputs, such as pesticides and fertilizers, to sustain production levels. If used improperly, many of these purchased inputs have deleterious effects on human health and the environment (Yilmaz, 2014). Fresh vegetables are one of the key sources of vitamins and minerals for human beings. Consumption of vegetables helps to meet the recommended daily intake of these essential nutrients for life (Bakırcı et al., 2014; Chiu et al., 2018).

In the Philippines, agriculture is considered the most common form of employment. It comprises 41% of the total labor force (Perez, 2015). Economically, the agriculture sector is about 20% of the gross domestic product (GDP). Crop production contributes about 510 billion pesos to the national income (Lu, 2009). The spreading concern about global food security, has led to various approaches to improve food production systems and increase productivity. Toxic chemicals are purposely introduced to the environment to reduce

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agricultural production losses incurred due to the presence of pests (Singh and Kaur, 2012). Despite their substantial contribution, the use of pesticides posts serious concerns. These are occupational hazards, arising from the exposure of growers to pesticides, residence living adjacent to heavily treated agricultural land, and the people who apply the pesticides.

Observations by agriculturalists feel growers in the major vegetable producing barangays of Claveria have a stereotype-knowledge on pest management. This is dependent on their financial capacity to take advantage of readily accessible control measures. Generally, their immediate reliance is always dependent on the use of chemical despite their claims of being aware of the consequences of pesticide use in vegetable cultivation. Growers perceive, that over the years the severity of pests is increasing, and greater quantities of pesticides are required to control pests.

This study was conducted to understand the dynamics of pesticide usage in selected vegetable growers in Claveria, Misamis Oriental. Specifically, the study aimed to: 1) determine the demographic and socio-economic profile of vegetable growers, 2) evaluate the pest management practices followed by the growers, and 3) assess the perceptions of growers about pesticides usage from selected vegetable producing localities, respectively.

MATERIALS AND METHODS

The study was conducted from October, 2018 to April, 2019 in eight identified vegetable growing areas of Claveria, Misamis Oriental. This included barangays of Aposkahoy, Cabacungan, Hinaplanan, Lanise, Luna, Panampawan, Rizal and Tamboboan, where a total of 100 growers were evenly selected among the identified barangays.

A survey method was used for this study. This survey consisted of personal interviews with the growers using a pre-tested structured survey questionnaire. Questionnaires were originally prepared in English but translated in *Bisayan* (local dialect) during the interview to obtain a greater understanding of the interviewee's responses. Farmer respondents were pre-identified after consultation with the Municipal Agriculture Office of Claveria. The number of potential respondents distributed proportionately based on the identified number of farmers per barangay. Furthermore, pre-determined farmers known to engaged in vegetable production for quite a long time were purposively selected to expediate the gathering of information as to the dynamics of pesticide usage.

The content of the survey questionnaire was categorized into three discrete areas: 1) demographic and socio-economic profile, 2) pest management, and 3) their perception about pesticides usage. Growers producing tomato, eggplant, cabbage, sweet pepper, and snap bean were purposively identified as respondents of the study. These vegetable types dominated the present vegetable producing barangays of Claveria. The collected data tabulated and analyzed by means of Frequency and Percentages. MS Excel was used to fast track data tabulation and processing.

RESULTS AND DISCUSSION

Socio-economic characteristics of growers

The socio-economic characteristics of vegetable growers in selected barangays of Claveria, Misamis Oriental are presented in Table 1. The largest proportion of growers (44.19%) were in the age category of 30 to 49 years of age. Only 7.75% belong to young age category ranging 20 to 29 years old, and a few (13.18%) belonged to age category of 60 to 79 years old. In Northern Mindanao age and experience are important variables in describing the potential responsiveness to change of vegetable growers. Previous estimates from the Bureau of Agriculture Statistics (BAS) in 2003 was for an average age of 67 years old for all growers in the Philippines. Considering that majority of the age group of the respondents is ranging from 30 to 59 years old, this implies that they could still be trained to enhance their potential in a holistic vegetable farming system. The major category of growers (33.93%) has at least 10 to 20 years of farming experience (Table 1). The least experienced growers, 18.75% having at least 10 years of experienced or less. Results show that growers are generally more experienced in cultivating vegetables.

Table 1. Demographic and socio-economic profile of vegetable growers in Claveria, Misamis Oriental, Philippines.

Particulars	Percentage (%)	Particulars	Percentage (%)
Age of the farmer		Farming experience	
20-29 years old	7.75	<10 years	18.75
30-49 years old	44.19	10 to 20 years	33.93
50-59 years old	34.88	21 to 30 years	27.68
60-79 years old	13.18	>31 years	19.64

The present vegetable production in Claveria survey region, showed most of the growers cultivated less than one ha, regardless of vegetable type. The range was from 50 to 100% (Table 2). Only a few growers cultivated an area between one to four ha. This is mainly for pepper (33.30%) production and 2.80% grew snap beans within this farm size. The Claveria, Misamis Oriental region was previously known as the "Tomato Country". It still holds true, as shown by the results of this study. About 50% of growers interviewed, cultivated 5 ha or more of tomato, while 33.30% of the respondents also cultivated eggplant on the same farm size.

Table 2. Average farm size (ha) per vegetable type cultivated by growers in Claveria, Misamis Oriental, Philippines.

Farm size (ha)	Percentage of growers per vegetable category				
	Cabbage	Pepper	Snap bean	Tomato	Eggplant
<1	100.00	66.70	97.20	50.00	66.70
1-<4	-	33.30	2.80	-	-
5-10	-	-	-	50.00	33.30

Claveria vegetable growers do not grow their vegetables in plots. They grow vegetables on per ha basis. Based on the survey results, each cropping season the major vegetables growers plant tomato and eggplants in blocks of up to 10 ha. Most other vegetables (cabbage, pepper and snap beans) are produced in blocks less than one ha. Growers produce these vegetables based on market demand. Tomatoes they produce tons per cropping season as there is already ensured buyer, who supplies tomatoes to Luzon.

Integrated crop management (ICM) is a sustainable development program included in a sustainable agriculture system. By managing crop profitably without damaging the environment or depleting natural resources for future generations (Kern, 2000). However, based on the results for this survey, the majority (82%) of the vegetable growers declared they have not attended training pertaining to ICM or another related fields (Figure 1). This reflects the current situation of vegetable production in the locality, where excessive input is rampant. According to Abang et al. (2013), a lack of famer training creates an implication of increase danger of pesticide misuse, where farmer relying on their own practices. Furthermore, the risk of using highly toxic and hazardous chemicals is relatively high. These concerns are not only a serious health threat but also impacts the environment through continued mishandling of chemicals.

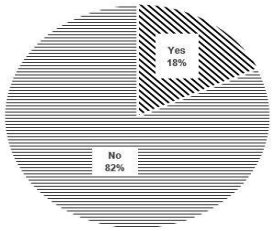

Figure 1. Respondents who have attended training on pest management.

Perception on deleterious effect of pesticides

A considerable percentage (9.15) of the farmer respondents mentioned the mortality of beneficial insects (Table 3). Growers are still continuously using pesticides, because it is cheaper and easy to implement compared to other methods such as sanitation and manual picking to controlling pest in the field. Contrary to the reports by Wesseling et al. (1997), Ngowi et al. (2007), and Matthews (2008), this study found growers used pesticides without full understanding of the impact on human health and the environment. This study reports most of the respondents (60.59%) are already aware of the harmful effects of pesticides for both human and animal health. In addition, 26.76% of the vegetable growers are aware of the offsite effects of pesticide application. They relate it with pollution of both air (14.08%) and water (12.68%).

Table 3. Deleterious effect of pesticides as perceived by vegetable growers in Claveria, Misamis Oriental, Philippines.

Perceived pesticide effects	Percentage (%)
Mortality of natural enemies	9.15
Water pollution	12.68
Air pollution	14.08
Harmful to human health and animal	60.59
Harmful to crops	3.50

Growers administered the spraying of pesticides by themselves (57.73%). This is carried out so they can save money and they are ensured of the quantity of pesticides applied. In some instances, the availability of labor is scarce. On the other hand, considerable number of growers (42.27%) hired labor to perform pesticide application (Table 4). Most of the respondents indicated they wear protective cloth when spraying or applying chemicals. However, most do not have complete personal protection equipment (PPE). For instance, raincoats or other protective clothing including an oro-nasal mask, sunglasses, gloves, hat, or boots (Nguyen et al., 2018).

Table 4. People who performed spraying and the protective measures used as practiced by vegetable growers' in Claveria, Misamis Oriental, Philippines.

Labour description	Percentage (%)	Description of measures used	Percentage (%)
Source of labour in spraying		Protective measures used	
Farmer	57.73	Cover face with cloth	13.70
Hired labor	42.27	Cover body and face with cloth	86.30

Sources of information on pest management

Growers (40.75%) source chemical information from chemical company technicians and from neighboring growers (32.59%). These are the major sources of information for pest control and management by most of the vegetable's growers in the area (Table 5). In addition, regarding the types of pesticides growers used, some growers are partly active on social media, getting pest control advice from the internet (9.63%). Furthermore, other growers obtain advice from TV, radio, and relatives while others rely on their own experiences (4.44%).

Findings from this study shows that pesticide usage appears to be influenced by authorized dealers who are motivated by pesticide sales. Hoi et al. (2009) also reported the influence of suppliers on growers and the application of pesticides and their usage. Furthermore, other growers obtained pesticides information from the open market, and from small, unauthorized shops as reported by Nguyen et al. (2018). Findings also show pesticide information is provided to small scale growers, which is relayed from pesticide technician who influences neighboring farmers. These farmers are usually large-scale famers who are highly considered in terms on the decision-making processes and indicate

what pesticide material should be purchased.

Table 5. Sources of information in terms of pest management by vegetable growers in Claveria, Misamis Oriental, Philippines.

Sources	Percentage (%)	Sources	Percentage (%)
Neighboring farmer	32.59	Experienced	4.44
Pesticides technician	40.75	TV	2.96
Relatives	7.41	Others (Internet)	9.63
Radio	2.22		

Crop stage and frequency of pesticide application

For vegetable crops, attacking pests cause most damage during the first vegetative stage of crop development. Based on the results (Table 6), about 52.1% of growers confirmed the need for intensive spraying at early development stage of crop. Heong and Escalada (1997) reported that insecticides are frequently used in vegetables during the early stages of development. Most insect pests target the young developed or developing leaves. This leaf damage triggers the use of highly toxic compounds to control these pests (Heong and Escalada, 1997). Even at harvest, application of pesticides is still considered necessary by 20.8% of growers.

Table 6. Crop stages and chemicals sprayed, frequency of application practiced by vegetable growers in Claveria, Misamis Oriental, Philippines.

Particulars	Percentage (%)	Particulars	Percentage (%)
Crop stage		Frequency of application	
Vegetative	52.1	Once a week	54.1
Fruiting stage	27.1	Twice a week	29.2
Harvesting	20.8	Thrice a week	16.7

As to the frequency of spraying, more than half (54.1%) of the respondents declared they sprayed once a week. In addition, a considerable number of growers frequently sprayed twice to three times a week, 29.2 and 16.7%, respectively. During rainy days, growers indicated they see the need for frequent spraying of pesticides. Growers observed pesticides are easily washed of the plants by the rain and insect pest become active, tending to attack the plants with higher frequency.

Integrated crop management versus chemical pesticides

Growers perception toward ICM compared to traditional chemical pesticide application methods revealed 64.7% of the growers considered the use of chemical pesticides a must, while 35.3% answered No. Most of the responding growers answered that they rely heavily on pesticide usage to protect crops from insect pests and diseases. Considering the effects on human health and consumption, vegetables sprayed with chemicals, 50% of growers considered it. The remaining respondents indicated it is not injurious to humans (Figure 2). Growers explained that it is not injurious to human health if consumers properly wash the vegetables before cooking. On the other hand, 38.5% believed that there will be a market demand of pesticides-free commodities in their respective area or locality. However, most of the growers (61.85%) do not believe that the use of pesticides will be a must to protect and prevent rampant pests and diseases.

Types of pesticides used

The different types of pesticides used by growers in their vegetable production is presented in Table 7. For four decades, growers have been using highly toxic insecticides with the following active ingredients: Methomyl (Lannate), 0,0-dimethryl phophorodithioate of diethyl mercaptosuccinate (Malathion 50% EC) and Lambda-cyhalothrin (Karate).

Generally, these broad-spectrum pesticides are cheaper and high toxicity compared to any other chemicals that are more specific and less toxic. However, these alternatives command higher prices in the market. For herbicides, the most popular chemical used by vegetable growers is S-metholachlor (Dual Magnum). Methomyl, S-methyl (EZ)-N-(methylcarbamoyloxy) thioacetimidate is an oxime carbamate insecticide. It is effective as a contact insecticide and systemic insecticide that causes overall systemic poisoning in target insects (Aktar et al., 2010).

Table 7. Pesticides commonly used by vegetable growers for the last four decades in Claveria, Misamis Oriental, Philippines.

Commercial name	Level of toxicity	Active ingredient	1980s (%)	1990s (%)	2000s (%)	2010 to present
Insecticide						
Thiodan	Extreme	Endosulfan	7.5	N/A	N/A	N/A
Furadan 3G	Extreme	Carbofuran	-	13.8	6.7	3.7
Lannate	High	Methomyl	17.5	13.8	26.7	29.6
Karate 5CS	High	Lambda-cyhalothrin	37.5	10.3	13.3	18.5
Kotetsu	Moderate	Chlorfenapyr	6.3	17.2	0.0	0.0
Prevathon	Moderate	Chlorantraniliprole	-	20.7	3.3	3.7
Malathion 50% EC	Slight	0,0-dimethyl phosporodithiate of diethyl1 mercaptosucciante	18.8	3.5	20.0	14.8
Fungicide						
Mancozeb	High	Manganese ethylene bisdithiocarbamate	-	13.8	6.7	7.4
Zebra Blue	High	Mn-Zn-ethylene bisdithiocarbamate	-	-	10.0	0.0
Herbicide:						
Dual Magnum	High	S-metolachlor	-	-	13.3	22.2
Astron	High	2-(4-mesyl1-2-nitrobenzoyl1) cyclohexane-1,3-dione	12.5	6.9	0.0	0.0

N/A - no longer available.

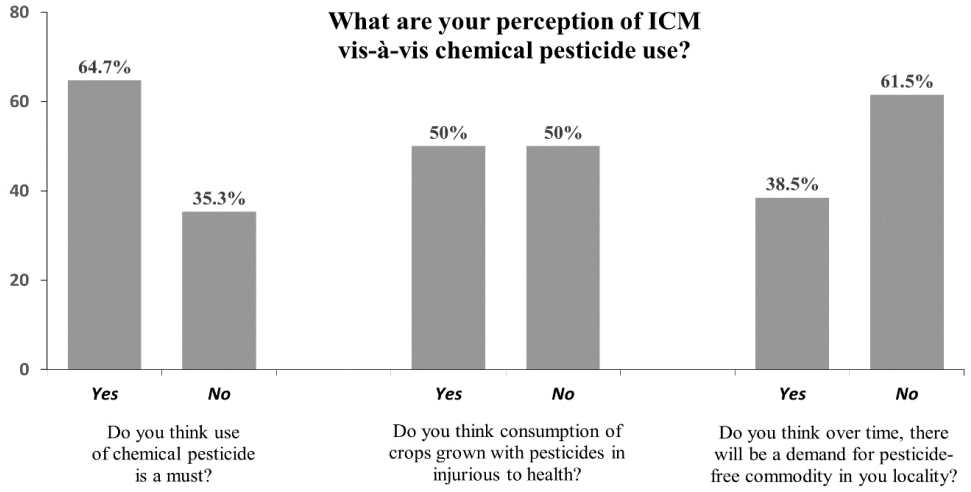

Figure 2. Farmer's perception of ICM vis-á-vis some chemical pesticides.

For herbicides, the most popular is the S-metolachlor (Dual Magnum). It has a favorable soil behavior and low risk for developing weed resistance. Metolachlor integrates well into sustainable weed-management systems and conservation tillage (Wesseling et al., 1997). Growers perceived the effectiveness of pesticides in terms of reducing pest infestation and increased production. These findings support the claims of Wilson (2000). Despite the high costs for agricultural, environmental and health concerns arising from

pesticide use, chemical inputs increased food production.

Pesticides application management practices

Respondents survey results on spray scheduling relative to harvest time, revealed some alarming results. Growers would spray just a few days before their next harvest (Table 8). Although some preferred to spray pesticides at least once a week before the day of harvest. Others spray four or six days before the harvest. Grower decisions to determine the number of days and intervals between spraying and harvest is greatly affected by climatic factors. On rainy days growers spray one day before harvest or the next day after the harvest. This is carried out to stop insect pests destroying the crop and stop insects penetrating the vegetables. Colmenárez et al. (2016) reported on the adoption of ICM improved agricultural production systems, aside from being convenient and helping reduce the negative effect of pesticides on consumer health and the environment. According to Tisdell et al. (1984), pesticides are an integral part of commercial production of crops, without the use of pesticides, high yields may not be sustained.

Table 8. Days pesticides were applied before and after harvest by vegetable growers in Claveria, Misamis Oriental, Philippines.

Spraying schedule	Eggplant (%)	Tomato (%)	Pepper (%)	Cabbage (%)	Snap bean (%)
The next day	6.2	9.1	26.7	20	-
2 days after	37.5	-	26.7	33.3	-
3 days after	6.3	18.2	20	40	-
1 week after	50	72.7	26.6	6.7	20.69
Twice a week	-	-	-	-	79.31

CONCLUSIONS

About 82% of growers have not participated any training on integrated crop management. They are not aware of beneficial insects and are highly dependent on the use of pesticides for production of solanaceous vegetables e.g. tomato, sweet pepper and eggplant. Respondents are aware as to the harmful effects of pesticides for both human and animal health. However, growers (74%) considered pesticides an essential tool in protecting and controlling pest and diseases. Therefore, they will continually use them in their vegetable production systems.

Although most of the growers declared spraying once a week prior harvest, however, in the case of snap beans, grower's spray twice to three times a week despite a weekly harvesting schedule. The frequency of spraying is highly influenced by weather conditions. The frequency of rainfall, the increased need to spray chemicals on their crops. Growers also mixing more than is prescribed dosage. They considered this practice to provide a stronger and more effective cultural practice against pests and diseases. Growers generally use knapsack sprayers for pesticides application.

The key sources of pesticide information are technicians from chemical companies and neighboring growers. Generally, growers use broad spectrum and highly toxic chemicals. The results of this study increase understanding of vegetable grower cultural practices in Claveria. Creating awareness and suitable information on integrated crop management through extension service support and other services to improve decisions making process about pesticides management and usage would be highly desirable.

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Organic farming decisions: case study of golden banana in Thailand

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Abstract

This study examined the essential factors that determine how farmers make decisions for adopting organic farming practices, especially with the case of golden banana in Thailand. A survey of 150 farmers was carried out of farmers who used either organic or traditional (synthetic chemical) farming practices. The methods of analysis included statistical, qualitative, and the use of a binary logistic regression. The binary logistic regression analysis showed that the factors that were statistically significant in determining the change from traditional to organic farming, when considering the marginal effect, were the availability of distribution channels for organic golden bananas, evidence of noticeable economic benefits, and perceived health benefits. The results also identified some other characteristics of farmers that differ between those involved with organic golden banana farming versus those involved with traditional farming. These results overall provide some insights for stakeholders and indicate some policy implications for those wishing to support organic production of golden banana. Furthermore, each of these aspects might be useful for policy makers and authorities, as well as entrepreneurs, who wish to encourage organic farming of other commodities.

Keywords: organic, chemical, farming, golden banana

INTRODUCTION

With the developing trends of concerns about the health risks of some food products and the need for greater environmental conservation, many consumers around the world have an increased interest in food quality of food. Many people indicate that they wish to consume organic products, especially in countries in Europe, in the United States, and in Japan. This provides an incentive for many food and agricultural producers to be interested in changing to organic production. The overall concept of organic farming is to protect the whole agricultural agro-ecosystem, to promote agricultural practices that capitalize on natural soil fertility, to enhance environmental biodiversity, and to limit or exclude the use of synthetic chemical products (Mäder et al, 2002; Teizzi, 1999).

In 2020, according to the International Federation of Agriculture Movement, the adoption of organic farming methods continues to grow. The latest world data (2018) show that the regions with the largest areas of organic agriculture land are Oceania with 36 million ha (half the world's organic agriculture land) and Europe with 15.6 million ha (22%). Latin America has 8 million ha followed by Asia with 6.5 million ha (9%), North America with 3.3 million ha (5%) and Africa with 2 million ha (3%).

In many countries, organic agriculture is not widely practiced and needs more producers to adopt organic farming methods. Typically, the value of organic-agricultural exports is less than the value of manufacturing exports. However, growth in the adoption of organic methods may expand quickly and shift this relationship. Fruits are important to the economy in Thailand and they bring in a significant amount of income for the country. Golden banana is one of these exported fruits which continues to have further potential. It has a lot of vitamins, as well as carbohydrates and calcium, and can be produced all-year-round. Data from the Ministry of Agriculture show that the production of bananas in

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2018 was 37,914,319 kg with a value of 724,845,257 baht. According to the Food and Agriculture Organization of the United Nations, global exports of bananas, excluding plantain, reached a record high of 19.2 million t in 2018, an increase of 5.7% compared to 2017.

Artukoglu et al. (2009) examined those factors that influenced organic cotton production in Salihil, Turkey. A sample of 25 organic and 25 traditional cotton farmers, each with similar total areas of production, was subjected to voluntary interviews. The analysis of the data used a Probit model and factors that were shown to be important for organic cotton production were the farmer's age, education level, cotton plantation experience, and the membership of a cooperative. Kafle (2011) studied factors that influenced the acceptance of organic methods in vegetable farming in Jitawan, Nepal. A sample of 65 organic farming households, selected randomly, were interviewed during February-April, 2011. Multiple regression analysis showed that the factors that were related to the acceptance of organic vegetable production were farm size, the participation rate of farmers in organic-farming training and site-visiting, as well as a close match of organic farming practices with traditional practices. Karki et al. (2011) studied 181 farmers involved with organic tea production in Ilam and Panchthar in the East of Nepal. Their study surveyed 86 organic farmers and 95 traditional farmers, selected randomly, during February-April, 2010. Data were analyzed by SPSS. The significant factors that were identified included environmental concerns by the farmers, good market trends for organic products, economic benefits, and concerns for improved health. Rana et al. (2012) studied factors impacting organic pepper farming in the Idukki district, India. A sample of 100 organic and 100 original pepper farmers was surveyed. Using logistic regression analysis, the important factors that were identified for the acceptance for organic pepper farming were age, agriculture experience, plantation area, and government monetary support.

The objectives of this research were to study the factors that caused farmers to undertake organic farming of golden banana in Thailand. The study aimed to define the characteristics of farmers who carry out organic farming versus those involved with traditional, synthetic chemical-based farming. The data were from primary sources and from interviews with farmers involved with either organic or traditional farming. The study, involving 150 farmers, was carried out in the provinces that are the most intensive areas involved with golden banana production (covering 82.96% of the producing volume and area), with 5,979 rai being in Phetchaburi Province and 9,760 rai in Patumthani Province. The study used binary logistic regression analysis and the variables that were studied included personal factors, economic factors, social factors, and the attitudes of farmers to organic farming.

MATERIALS AND METHODS

Logistic regression

The model used to specify those factors that were critical in making decisions by farmers to change from traditional or synthetic chemical-based farming to organic farming was a logistic regression with 12 independent variables. These variables included: personal information status, educational status, banana farming experience, the number of plant varieties in the plantation area, the type of neighborhood farming, membership of an agricultural cooperative, the presence of an organic golden banana distributional channel, comparative economic benefits, consciousness of health impacts, difficulties associated with changing to organic farming, matching or coincidental qualities between organic and original traditional farming, and investment required to convert to organic farming.

Logistic regression analysis is used when the dependent variable in a study of interest is dichotomous (in other words, organic vs non organic). The logistic regression is more appropriate than either multiple regression or discriminant analysis in such circumstances (Hosmer and Lemeshow, 1989). Like multiple regression, logistic regression analysis can be used to determine which independent variables and their interactions are required to satisfactorily describe attrition or retention. Logistic regression analysis also provides predicted probabilities of retention for combinations of the independent variables.

Following Hosmer and Lemeshow (1989), procedures were utilized for selection of significant independent variables and their interactions: beginning with the stepwise selection of main effects; then, forced entry of the main effects significant to that step, followed by stepwise selection of interaction terms given the main effects variables in the model; finally, assessment of the final model through examination of goodness-of-fit statistics.

Binary logistic regression

Binary logistic regression estimates the probability that a characteristic is present (e.g., estimate probability of "success") given the values of explanatory variables, in this case a single categorical variable; $\pi=Pr(Y=1|X=x)$.

Model:

$$\Pi i = \Pr(Yi=1|Xi=xi) = \exp(\beta 0+\beta 1xi) \,/\, 1+\exp(\beta 0+\beta 1xi)$$

$$\text{or, } \operatorname{logit}(\pi i) = \log(\pi i/(1-\pi i))$$

The model for testing the hypothesis is:

$$Y_i = \alpha_i + \beta_{it}X_{ij} + \varepsilon_i \qquad i=1,\ldots 150, \text{ and } j=1,\ldots 12$$

Y=f(AGE, EDU, EXPER, TYPE, SIDE, MEM, CHAN, ECO, HEALTH, COMPLEX, COMPAT, INVEST)

where the dependent variable (Y) is the decision of farmers for golden banana farming which can be either chemical golden banana farming or organic golden banana farming.

Independent variables are as follows: AGE is the age of the farmer interviewed; EDU is the education level of the farmer (years attending schools); EXPER is the experience in golden banana farming; TYPE is the number of varieties of fruits in the farming enterprise; SIDE is the format of the neighborhood farm as a dummy variable; MEM is the membership status of an agricultural cooperative - has the farmer joined a cooperative or not?; CHAN is the sale distribution channel for organic golden banana, which can be either some organizations or parties or cooperatives available to buy the product, or there is not a commitment for the product to be bought; ECO is the comparative economic benefit; HEALTH is the consciousness about health impacts; COMPLEX is the identification of the difficulties involved in changing to organic farming; COMPAT is the compatibility with the original assets or the original farming practices; INVEST is the investment commitment required to change to organic farming.

RESULTS AND DISCUSSION

Most of the farmers were 41-50 years old (37.1%) or 51-60 years old (27.5%). Those between 31 and 40 years old or more than 60 years old comprised about 16.4%. Most farmers were primary school graduates (54.3%), or high-school graduates (18.3%). The organic farmers were mostly primary graduates. Surprisingly, all of the organic farmers had some previous training about organic farming. In comparison, about 31.7% of the traditional farmers had had some training.

Goodness-of-fit statistics

The Proper test of the regression variables used the Hosmer and Lemeshow method. It required χ^2 (0.05,8) to equal 15.5073 and χ^2H-L to equal 12.8192 with a significance value of 0.1182 for a difference to be greater than the significance level of 0.05. If those assumptions are met, we can then test our hypothesis by the regression specification. If the χ^2 (0.05,12) is equal to 21.0261 and the Model Chi-Square is equal to 128.85 and the significance level is 0.00, then, we can accept the assumptions for the relationship among variables. In the test, the Pseudo R^2 equals 0.802.

The significant test of the values of ßi, Exp(β) and marginal effect

The results of the binary logistic regression analysis of the survey data recorded from 150 farmers with either organic or with traditional production practices are shown in Table 1.

Table 1. Results of significance test of Exp(β) and the marginal effect. Source: calculations.

Variable	B	SE	Z	Significance	Exp (β)	Marginal effect
AGE	0.096	0.090	1.057	0.290	1.100	-
EDU	0.272	0.204	1.334	0.182	1.312	-
EXPER	0.139	0.158	0.879	0.380	1.149	-
TYPE	-0.306	0.681	-0.449	0.653	0.736	-
SIDE	-0.889	1.453	-0.612	0.541	0.411	-
MEM	2.592	1.694	1.530	0.126	13.361	-
CHAN	4.648	2.162	2.150	0.032**	104.330	0.0437
ECO	5.945	3.435	1.731	0.084*	381.897	0.0155
HEALTH	7.227	4.386	1.648	0.099*	1,376.765	0.0052
COMPLEX	-1.730	2.590	-0.668	0.504	0.177	-
COMPAT	0.051	3.359	0.015	0.988	1.052	-
INVEST	-0.197	2.954	-0.067	0.947	0.822	-
CONSTANT	-21.152	8.652	-2.445	0.015	0.000	-

** 95% level of statistical significance; * 90% level of statistical significance.

According to the results relating to ßi, Exp(β), and the marginal effect, the interpretations are as follows:

The ages of farmers (AGE), with an Exp(β) equal to 1.100, were positively related to the commitment to become organic golden banana producers. Hence, as the age of the farmers increased, the chance of a decision to become involved with organic golden banana farming increased.

The education level of farmers (EDU), with an Exp(β) equal to 1.312, and with the independent and dependent variables also having a positive relationship, the data indicate that as the coefficient representing the education of the farmers increases, the chance of a decision to become involved with organic golden banana farming also increases.

The golden banana farming experience of the farmers (EXPER) had an Exp(β) equal to 1.149, so this showed a positive relationship. The coefficient indicates that if golden banana farming experience increased, the chance of a decision to become involved with organic golden banana farming would increase.

The number of varieties of fruits planted in the farmer's farm (TYPE) had an Exp(β) equal to 0.736, indicating that if the numbers of varieties of fruits planted in the farm increased, the chance of a decision to become involved with organic golden banana farming would decrease.

The type of neighborhood farming (SIDE) had an Exp(β) equal to 0.411, indicating that if the type of neighborhood farming was more diverse, then the chance of a decision to become involved with organic golden banana farming would decrease.

The cooperative membership status of the farmer (MEM), with an Exp(β) equal to 13.361, indicated that if a farmer had cooperative membership, then the chance of a decision to become involved with organic golden banana farming would increase.

The marketing distribution channel (CHAN), with an Exp(β) equal to 104.330, indicated strongly that if the farmer had a marketing distribution channel for their organic golden bananas, the chance of a decision to become involved with organic golden banana farming would increase. This factor had a high significance level. The marginal effect was equal to 0.0437 and this showed if the organic golden banana farmer has the choice of additional distribution channels, this will increase the chance that the farmer will change to organic farming.

Comparative or relative economic benefits (ECO) with an Exp (β) equal to 381.897

indicates that if farmers consider that there is a high comparative or relative economic benefit from organic golden bananas, the chance of a decision to become involved with organic golden banana farming would increase. The marginal effect was equal to 0.0155 and this implies that when farmers compare organic golden banana farming and the economic benefit, this will increase the incentive to undertake organic farming.

Health consciousness (HEALTH) with an Exp(β) equal to 1,376.765 indicates that if farmers are concerned about health quality issues, then the chance of a decision to become involved with organic golden banana farming would increase. The marginal effect was equal to 0.0052, indicating that where farmers compare and understand that traditional banana plantation might affect their health, and if this understanding is with more farmers, then the opportunity for more farmers to change to organic production will increase.

Awareness of the difficulties associated with organic farming (COMPLEX) had an Exp(β) equal to 0.177, which indicates that if awareness of difficulties by the farmer to become involved with organic farming increases, then the chance of a decision to become involved with organic golden banana farming would decrease.

Compatibility to the previous assets and production practices in the original farm (COMPAT) had an Exp(β) equal to 1.052, which indicates that if compatibility to the previous assets in the original farm is increased, then the chance of a decision to become involved with organic golden banana farming would increase.

Investment decision for organic farming (INVEST) had an Exp(β) equal to 0.822, which indicates that as farmers tended to invest more in farming, the chance of a decision to become involved with organic golden banana farming would also tend to increase.

Overall, the main factors involved in the decision making of golden banana farmers to convert to organic production were firstly, access to a marketing distribution channel (CHAN) (a marginal effect of 0.0437), secondly, identification of a comparative economic benefit (ECO) (a marginal effect of 0.0155) and thirdly, consciousness of health benefits (HEALTH) (with a marginal effect of 0.0052).

CONCLUSIONS

In general, organic farming adopts environmentally-friendly production processes. It aims at reducing environmental impacts, as well as increasing the use of renewable sources in comparison with those used in conventional farming practices (Colantoni et al., 2014; Fedele et al., 2014; Trydeman-Knudsen et al., 2014). In this study, it seemed likely that profitability would be an important, but not necessarily the most important factor, for a farmer to consider (Sgroi et al., 2014; De Gennaro et al., 2012). In this study, the most important factors, in ranked order, were access to a distribution channel, profitability of the enterprise, and awareness/understanding of health benefits. A peer group effect and relevant training, as well as availability of investment capital (due to the fact that organic farming in Thailand has some establishment costs) are also factors that need to be considered. This study also involved a detailed comparison between the economics of organic and conventional production (data not presented). If the government was to provide more opportunities for farmer training or assist with aspects such as the establishment of marketing chains, then the organic golden banana farming targets would be more reachable.

Considering the results obtained in this study, we can postulate that in the longer term, if the distribution channels are more robust and the profitability of the organic golden banana sector is higher in comparison with the conventional production sector, then there will be a more widespread adoption of organic production practices for golden banana. More sustainable development of the organic golden banana supply chain will have advantages from both economic and environmental standpoints. This example of the opportunity to improve organic golden banana production can be applied to other areas of organic farming for the development of safe, healthy agricultural production in Thailand. The participation in the golden banana supply chain by producer associations or cooperatives also appears to have useful implications. Overall, a stronger sector will enhance generational continuity in rural areas. The findings of this study should be useful to policy makers and to authorities involved with the rural sector as well as to entrepreneurs who are committed to

understanding and promoting organic farming in Thailand.

ACKNOWLEDGEMENTS

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Comparison of wind loads on rectangular net house with numerical simulation and wind tunnel tests

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Abstract

This study clarified the adaptability of numerical experiments using a canopy model while estimating wind load on net houses. A salient characteristic of the model is its capability to reduce the computational load. Net houses, which are used widely in subtropical regions of Japan, have effective wind resistance because nets allow some air permeability, in contrast to greenhouses covered with film. However, the wind loads imparted to net houses cannot be incorporated into design accurately because the wind forces on the net houses have not been refined. This experiment examined wind loads of rectangular net houses through numerical experiment and wind tunnel tests to improve the wind-resistant design of net houses. Solidity ratios of the nets using wind tunnel test were 29, 34 and 38%. The net mesh sizes were 0.4, 0.6 and 1.0 mm, respectively. When there is a normal wind direction to the plane of a net house, the wind force coefficients for the windward side were approximately 0.5 for 29 and 34%, and 0.6 for 38% (solidity ratios). Numerical experiments can calculate the equivalent wind load which was confirmed by wind tunnel testing.

Keywords: canopy model, mesh size, net house, solidity ratio, wind resistance

INTRODUCTION

Countermeasures against strong winds in greenhouses have become an important issue for Japanese horticulture. According to the Ministry of Agriculture, Forestry and Fisheries (2020), typhoon Hagibis in September 2019, had the highest recorded instantaneous wind speed of 43.8 m s^{-1} in the Kanto region. It caused in excess of 343 billion yen financial damage to agriculture, of which 8.3 billion yen was greenhouse damage. Greenhouse damage caused by the typhoon extended to 34 of 47 prefectures in Japan.

Despite such threats, in the Okinawa prefecture, Japan, an average seven typhoons strike each year. In the Okinawa prefecture, rectangular, 2.3 m high net houses, covered entirely by netting are used to cultivate chrysanthemums. Typhoon damage to net houses and crops is generally lower compared to open cultivation systems. The reduction in wind load can be anticipated for greenhouses covered with net. However, rectangular net houses are constructed to ensure strength, but the accuracy of that strength remains unknown. Farmers require large net houses, capable of accommodating the cultivation of many different crops. Therefore, to improved net house strength, especially when they collapse during a typhoon, carefully calculation of wind load is critical. Conducting calculations of wind load, necessitates the use of wind force coefficients for the net houses. As a study of the aerodynamic characteristics of nets, the relation between porosity and wind force coefficients have been reported (Bailey et al., 2003; Valera et al., 2005). Mistriotis and Castellano (2012) reported aerodynamic characteristics of a full-scale model covered by net. Robertson et al. (2002) reported wind force coefficients obtained from wind tunnel tests of a scale model with a flat roof covered with a net. Nevertheless, few reports describe studies through which wind force coefficients of net houses have been obtained by numerical experiment.

For this study, the wind force coefficients necessary for the wind resistant design of net-houses were obtained using wind tunnel tests. Then the wind tunnel test results were

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reproduced through numerical experiment using a canopy model, which produced a calculation load. This does not need an accurate reproduction of the configurations of net houses but numerical calculations can deliver the same results. Future net house improvement and design are expected to be achieved through this numerical experiment.

MATERIALS AND METHODS

Numerical experiment

1. Numerical method.

When unsteady turbulent flow fields including perforated bodies such as nets are solved numerically, a method to reproduce the appearance of nets in the analysis region, and to solve the flow fields around them, can be considered a direct method. However, it is difficult to calculate the net configurations, with sufficient detailed of resolution. Nevertheless, it can be reproduced in the calculation region. Simplification by modeling must be conducted to reduce the computational load. As described herein, the unsteady turbulent flow field around the net is solved numerically. This is achieved by using large eddy simulation, based on a turbulence model derived by performing a spatial averaging including the object. Whereas, the spatial averaging is conducted to the governing equation of the fluid. A system of zero equations using the Smagorinsky model were used as the turbulent flow model. The equation used for calculation is shown in 1-6.

An overview of the governing equations is provided below. The equation of continuity is presented as Equation 1 and the equation of motion is shown as Equation 2. Therein, u_i, u_j is the x_i filtered component of velocity. Wind velocity of x_i direction (x_1=x, x_2=y, x_3=z), ρ stands for density, p denotes the pressure, and the values are given per fluid volume. Also, G represents the proportion of fluid occupied in the control volume. The effective viscosity ν_e denotes the sum of air viscosity ν and the sub-grid eddy viscosity VSGS in Equation 3. VSGS follows the Smugolinsky model. It is given by the strain rate tensor in Equation 4 and the scale determined by x_i directional grid width Δx_i in Equation 5 and the model constant C_S=0.1. External force term f_{xi} representing the influence of the object in Equation 2 is modeled as Equation 6 using x_i-directional drag on net F_{xi} (N) in averaging volume, V_f is the fluid volume within the average volume V_o (m^3), that is the volume of computational grid, net quoting area A_{xi} (m^2), drag parameters C_{xi}, and air density ρ (kg m^{-3}). Maruyama (2008) presents additional information related to the derivation of canopy model.

$$\frac{\partial G\bar{u}_j}{\partial x_j} = 0 \tag{1}$$

$$\frac{\partial G\ \bar{u}_i}{\partial t} + \frac{\partial G\bar{u}_i\bar{u}_j}{\partial x_j} = -\frac{1}{\rho}\frac{\partial G\bar{p}}{\partial x_i} + \frac{\partial 2G\nu_e\bar{D}_{ij}}{\partial x_j} - G\bar{f}_{xi} \tag{2}$$

$$\nu_{SGS} \equiv (C_S L)^2 |D|,\ |D| \equiv \sqrt{2\bar{D}_{ij}\bar{D}_{ij}} \tag{3}$$

$$\bar{D}_{ij} \equiv \frac{1}{2}\left(\frac{\partial \bar{u}_i}{\partial x_j} + \frac{\partial \bar{u}_j}{\partial x_i}\right) \tag{4}$$

$$L \cong \sqrt[3]{V_f} = \sqrt[3]{GV_0} = \sqrt[3]{G\Delta x_1 \Delta x_2 \Delta x_3} \tag{5}$$

$$f_{xi} = \frac{F_{xi}}{V_0} = \frac{1}{2}\rho A_{xi} C_{xi} \sqrt{u_{x1}^2 + u_{x2}^2 + u_{x3}^2} \times u_{xi} \tag{6}$$

u_{xi} represents the filtered wind speed in the x_i direction (x_1=x, x_2=y, x_3=z).

Because the ratio of the fluid volume in the computational grid and the area are determined according to the net configuration, the values of the corresponding aerodynamic parameters were optimized by comparison with the wind tunnel tests and calculations. In the optimization calculation, the computational grid was an unequal spaced grid of the right-angled coordinate system as presented in Figure 1. The computational grid in the normal direction were spaced equally, but for the upward model, it progressively increased. The computational grids in the horizontal planes were spaced equally. The grid number was 161 in the *x* and y directions, 55 in *z* direction.

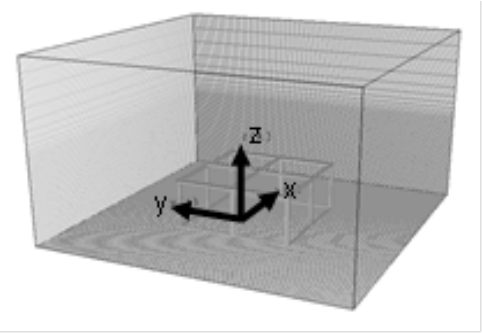

Figure 1. Calculation area and grid system for numerical experiment.

2. Boundary conditions.

A turbulent boundary layer corresponding to a wind tunnel test was generated numerically for an inflow boundary condition. A fluctuating wind velocity field, of a turbulent boundary layer developed on the wind tunnel floor was assigned to an inflow boundary. As shown in Figure 2, *U* represents the mean wind speed at each height; σ^2 denotes the variance of *U*. The wind velocity profile generated in the calculation becomes almost equivalent to that in the experiment. The turbulence was slightly less than the experimental value. However, the minimum width of the LES calculation grid was 10 mm. Results suggest that small fluctuations below the scale were not captured. The drag measurement model was 230 mm high, below the turbulent boundary layer thickness. The inlet and the outlet boundary condition were the same as detailed calculation described in Maruyama (2008). For the side and upper boundary surfaces, where the wind on the boundary blows into the calculation region the Dirichlet boundary condition was used, that is the generated velocity was given. Where the wind on the boundary blows out from the calculation region, the convective boundary condition was used. On the floor surface, the no-slip condition by which the wind velocity becomes zero.

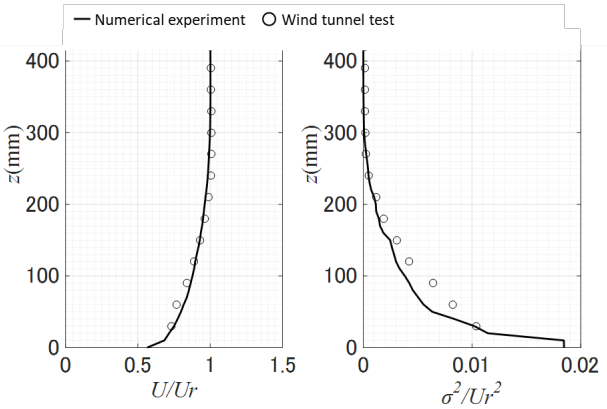

Figure 2. Inflow profile.

Wind tunnel tests

1. Airflow properties of the approaching flow and Reynolds number effects.

Measurements of wind force were carried out in the Boundary layer wind tunnel at the Disaster Prevention Institute, Kyoto University. The tunnel length is 21 m. The cross-section measurement is 2.5 m and height is 2.0 m. Wind tunnel test indicates that the change in wind force coefficients were small, at a wind speed of 12 m s^{-1} or higher. Results suggest the Reynolds number had no effect on the wind speed of 12 m s^{-1} or higher. The wind speed in the wind tunnel test was set as 12 m s^{-1}. Results also show the scale model height was within the boundary layer. The approaching flow was extremely flat, with weak turbulence and no obstacles.

Measurements nets of three types with the following parameters were used: f=38%, s=0.4 mm; f=34%, s=0.6 mm; and f=29%, s=1.0 mm. There in, s represents the mesh width; F denotes the net solidity ratio. The net was installed on a square-shaped frame, constructed by bending a round, 4-mm-diameter stainless steel rod. It was attached to a six-component force balance installed under the floor through a support rod. Detailed measurements were taken in accordance with a study reported by Tomisaka and Maruyama (2007). The following Equations 7 and 8 were used to obtain C_f from the measured results. F_n (N) signifies the normal wind load to the measurement plane, A (m^2) stands for the measurement area, q (N m^{-2}) denotes the dynamic pressure, α (kg m^{-3}) the air density, and U_r (m s^{-1}) the average wind speed at the reference location, 375 mm upwind of the net house at a height of H=230 mm.

$$C_f = \frac{F_n}{q \times A} \tag{7}$$

$$q = \frac{1}{2} \alpha \, Ur^2 \tag{8}$$

2. Wind load measurement.

The scale model for wind load measurements was made to height H, with width and depth of 2.61 H. Three types of netting were used for the wind load measurements. As shown on the right of Figure 3, four types of surface plane measurements were set up; <1/3 edge> was a separated lateral scale model, and touched the side post; <1/3 center> was central plane model; <1/2 edge> was a separated lateral in the scale model; <1/4 roof> was one plane separated into roof of the scale model. The value of C_f is presented as positive, when acting from the outside of the net house to the inside. When acting from inside of the net house to the outside, it was presented as a negative.

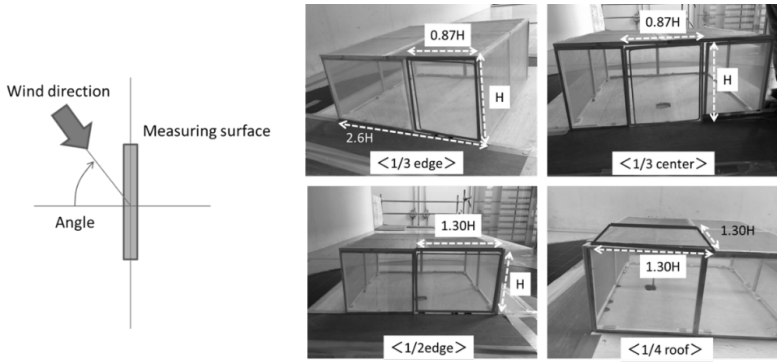

Figure 3. Angle of the wind direction (left) and each measurement plane (right).

RESULTS AND DISCUSSION

Wind force coefficients

Result from this wind tunnel test was reported by Tamaki et al. (2018). The scale model was set at the center of the turntable position 16.5 m downwind from the inlet of test section. The turntable was rotated 1.3° s^{-1}. The turntable was stopped turning for 10 min on each angle when wind direction angle on the measurement plane are 0°, ±45°, ±90°, ±135°, ±180°. Wind load on the measurement plane was measured at a sampling frequency of 100 Hz, during the turntable is stopping. There is C_f of the net of f=38% s=0.4 mm for each wind direction against the measurement plane (Figure 4). C_f is an average value calculated from the measured values for 60 s, when the scale model was stopped at 45° intervals. This is shown in Figure 4 as a white circle. The C_f in wind tunnel test of the rectangular net house showed approximately the same values when the wind directions were equal, even if the measured planes differed for <1/3 edge>, <1/2 edge>, and <1/3 center>.

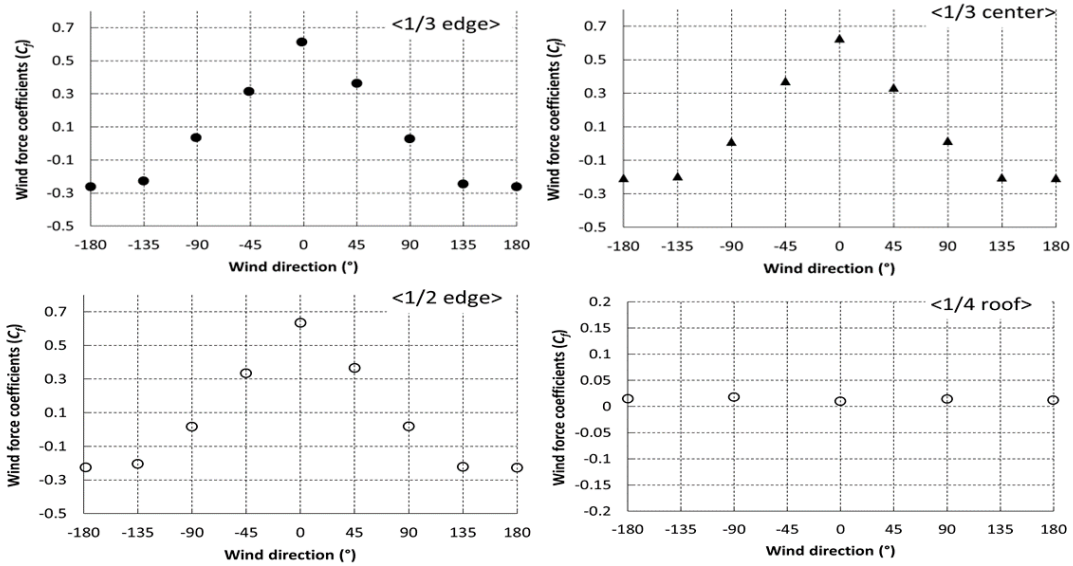

Figure 4. Wind force coefficients (f=38% s=0.4 mm).

In all the measurement plane, C_f was maximized at the wind angle of 0°. It became a negative value near 180° in which the measurement plane was the leeward side. In the case of the measurement plane <1/3 edge>, the C_f at the wind angle of 0° was about 0.6 at f=38% s=0.4 mm; it was about -0.2 at 180°, when the measurement plane was located at leeward side the scale model had rotated 180°. At the net of f=34% s=0.6 mm and f=29% s=1.0 mm, the maximum value of C_f became approximately 0.5 at the wind angle of 0°. The value of C_f was smaller than the C_f of f=38% s=0.4 mm. At 180° the measurement plane was located on the leeward side when the scale model was rotated by 180°, the C_f was about -0.3, which was approximately equivalent to the C_f of f=38%. In addition, C_f at the wind direction angle of 45° decreased to about one-half of the wind direction angle of 0°. Several studies have reported that the C_f when wind load of normal wind direction to net was applied to net it is decided by the solidity ratio and mesh size (Tamaki et al., 2009). From the results of this test and using the three-dimensional model, C_f at the wind direction angle of 0°, also varied with the solidity ratio and mesh size. Wind pressure on the upwind side front of a building without ventilation generally tends to be large at the central area and small at the edge. Regarding these test results, C_f at the central area and the edge showed almost equal values: each for C_f of <1/3 center>, <1/3 edge>, and <1/2 edge> at the wind angle of 0°, were almost identical. Results show C_f at the side plane of approximately zero, the value of C_f of the roofs were almost constant, irrespective of the wind direction angle when f=29% s=1.0 mm, as shown in

Figure 4 in the lower right, plane; it was 0.02 at most. The tendency was C_f of f=34% and f=38% were similar, f=29%.

Wind force coefficients by numerical experiments

By numerical experiments, wind forces were calculated and the wind force coefficient C_n was obtained; C_n was obtained by optimizing the drag parameter (C_x) in Equation 6. Figure 5 shows C_n of s=38% by simulated <1/2 edge> scale model of a wind tunnel test. When C_x was given 2.0, C_n the average windward plane was 0.61. C_n of the leeward plane, when the scale model was rotated by 180° it was -0.19. C_n and the roof was -0.029. The windward and leeward values of C_n resemble results from the wind tunnel test. The Cn and C_f for roof are almost zero.

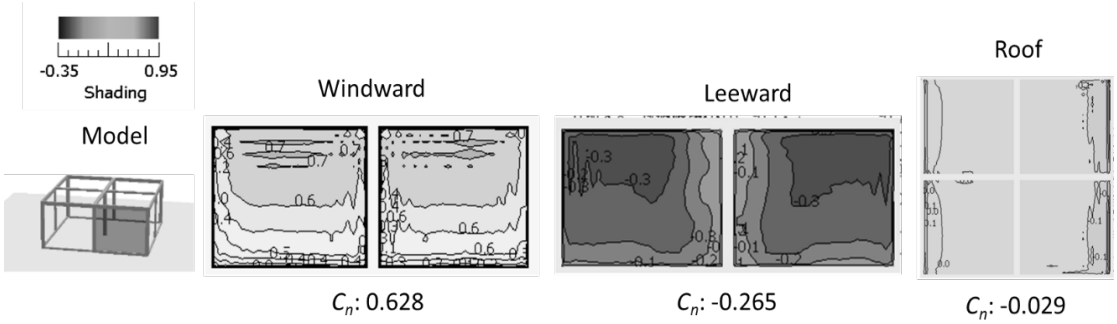

Figure 5. Wind force coefficients (C_n) for f=38%, s=0.4 mm.

Figure 6 shows the change of the C_x on windward plane, this is the same wind force coefficients as obtained in the wind tunnel test. The results, range of the wind force coefficients were the same as those obtained from the wind tunnel test. The drag parameter, corresponding to the solidity ratio of the net was obtained. The optimized drag parameters were 0.85 for s=29%, 0.9 for s=34%, and 1.4 for s=38%. The leeward and roof C_n values were almost equal, even if the solidity ratio was different. As inferred from these results, numerical calculation using the canopy model is useful for wind resistant design of the net house as long as it is within the range of the net solidity ratio and the mesh size used in this experiment.

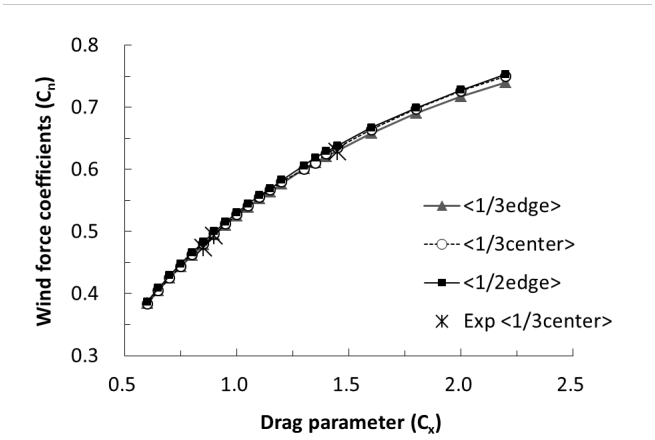

Figure 6. Change of wind force coefficients (C_n) of windward with drag parameter (C_x).

CONCLUSIONS

To verify adaptability of the canopy model for the numerical simulation when considering wind loads of a rectangular net house in this study, results obtained from

numerical experiment and wind tunnel tests were compared. Wind tunnel tests were conducted to clarify the wind velocity around the net house and the prevailing wind direction on the nets, and its inward force for the windward side plane. The maximum value reached was about 0.5 the mean wind force coefficient, with solidity rate *f*=34% (mesh size 0.6 mm) and about 0.6 with a solidity rate *f*=29% (mesh size 1.0 mm) and a solidity rate *f*=38% (mesh size 0.4 mm). On the leeward side, the outward force on the leeward plane was recorded. It was approximately -0.2 wind force coefficient. The wind force coefficients obtained by numerical experiment was like the wind tunnel test. The value of the mean wind force coefficients on the roof plane during the wind tunnel test was about 0.02 irrespective of the wind direction angle. This value is almost zero and the same as the numerical experiment result. The results from this study confirmed the numerical calculations obtained for the canopy model tests are a useful practical analysis method. It is difficult to conduct wind tunnel tests for all net types. Furthermore, it will be necessary to reproduce the object configuration in the calculation region, even with a numerical experiment. Therefore, as in the numerical calculation with the technique using the canopy model, the analysis time can be shortened. Our purpose is to obtain a technique that will calculate wind loads on each plane of a net house, even for many different types of nets and net houses. As a result of this research, net houses can be safety maintained, without collapse when typhoons occur, because the wind force coefficients on net houses can be calculated. The results of the numerical calculations have determined that canopy models is a useful practical analysis method. Further studies will be developed and numerical experiments conducted using the result of field test of wind load measurement on net houses. Presently field test on wind load measurements on net house are being conducted.

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Identification of PID parameters for system-specific nutrient mixing control for ISE-based hydroponic nutrient management

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Abstract

Hydroponic crop cultivation systems are becoming more popular, but automatic target nutrient solution preparation is still a critical issue. Nutrient mixing control parameters need to be identified for specific systems, as they are not universal for different systems with different components and design specifications. In this study, parameters (Kp, Ki and Kd) of the proportional-integral-derivative (PID) control method were identified for nutrient mixing of a small-scale ion-selective electrode (ISE)-based hydroponic nutrient management system through simulation and validation tests. The nutrient mixing test bench consisted of tanks for nutrients A, B, distilled water, and a nutrient mixing tank, K^+, NO_3^-, and Ca^{2+} ISEs, pipes, nutrient mixing pumps, and a controller. A PID control method was implemented, and the parameters were identified through mathematical simulations using the Ziegler-Nichols method, and laboratory validation tests. The effects of different pump flow rates on the characteristics of the unit step response were also analyzed. The nutrient mixing performance was evaluated based on the control accuracy and response time. For the experimental test bench, PID control parameters were adjusted. The best performance was observed, when the Kp, Ki, and Kd were 5.85, 4.35, and 1.56, respectively. The settling time, oscillation duration, overshoot (%), and steady-state error for those values were 177 s, 64 s, 13.38%, and -0.95%, respectively. Low pump flow rate (0.03 L min^{-1}) showed the minimum steady-state error (-0.40%) but took a long time to reach steady (364 s). In contrast, the rising time for the high flow rate (0.1 L min^{-1}) was short (89 s), but the steady-state error was high (-1.36%). The result indicates that the pump flow rate needs to be adjusted system-specifically along with the PID parameters. The PID-based nutrient mixing model and identification process of the parameters would be feasible for preparing the target hydroponic solution with minimum error. However, the flow rates of the input solutions need to be adjusted properly for large scale applications.

Keywords: hydroponics, nutrient mixing, PID controller, parameter identification, flow rate

INTRODUCTION

Hydroponics is becoming more popular in vertical farming and controlled environment agriculture production facilities, as it enables efficient use of nutrient and water, the high growth rate of crops, and is low labor intensive (Pignata et al., 2017; Lira et al., 2018; Cho et al., 2018). All essential macro- and micro-nutrients are supplied by mixing with water in hydroponic systems. However, automatic target nutrient solution preparation is always a critical issue as the excessive supply of stock nutrients may result in toxicity, and inadequate supply can cause nutrient deficiency, leading to retardation of crop growth,

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development, and reproduction (Chowdhury, 2020). For this reason, the selection of a proper nutrient mixing control method is essential, which should be able to supply the stock nutrients more precisely and prepare the target nutrient solution with minimum error.

Several control methods have been used for nutrient mixing, such as an on/off control method (Sun et al., 2015; Kim et al., 2015), a fuzzy control method (Fuangthong and Pramokchon, 2018), a proportional-integral-derivative (PID) control method (Li et al., 2016; Wu et al., 2019), a matrix-based control method (Jung et al., 2015), and many other hybrid methods. Among these, the PID control method is preferred for its continuously modulated output capability. It is usually used in feedback control systems, where actions are taken by continuous calculating of past, present, and predicted control errors (Ang et al., 2005). However, system-specific identification of the PID parameters (Kp, Ki, Kd) is necessary for this control method. In different studies, the PID algorithm has been applied and parameters were determined according to their input parameters and system specification for the preparation of the target hydroponic nutrient solution (Wang et al., 2014; Li et al., 2016; Wu et al., 2019).

Accuracy and precision of nutrient ion monitoring are also mandatory for the preparation of the target nutrient solution. Growers use EC and pH methods, but EC can only provide information about nutrient ion concentration in the solution (Son et al., 2020). EC cannot identify the quantity of individual ions in the solution, which is essential for plants, as plants uptake some specific nutrients selectively at different growth stages, such as nitrogen during physical development, and phosphorus, and potassium for flower formation (Jones, 2016). Sometimes, the EC and pH methods can include measures of the non-nutrient ions too (Bamsey et al., 2012). An ion-specific nutrient monitoring technique could be an alternative to these limitations. Ion-selective electrodes (ISEs) are used widely in recent studies due to their robustness, real-time measurement capability, hazardless, and problem-solving capability for individual ion monitoring (Cho et al., 2018).

A nutrient solution containing the optimum concentration of each required nutrient ion is essential for maximum plant growth. For the automatic preparation of such a nutrient solution, the selection of a proper nutrient mixing control method, together with ion-specific nutrient sensing are very important. As very few studies have been conducted focusing on the application of the ion-based PID control method for nutrient solution preparation, the objective of this study was to identify the parameters of the PID control method for target nutrient solution preparation on a small-scale ISE-based hydroponic nutrient management system through simulation and validation tests.

MATERIALS AND METHODS

Identification of PID parameters through simulation

1. PID control method.

The PID control method is a feedback mechanism, widely used in natural and artificial systems, where a continuous constant output is required. It controls the error (*e(t)*) based on proportional (P), integral (I), and derivative (D) terms by continuously calculating the difference of set-point and measured variables (Ang et al., 2005). The basic formula for the PID control method is shown in Equation 1.

$$u(t) = K_p e(t) + \ K_i \ \int_0^t e(\tau)\ d(\tau) \ + \ K_d \ \frac{d_e}{d_t} \qquad (1)$$

where u is the control signal, and e is the control error. The control parameters, Kp, Ki, and Kd are the proportional, integral, and derivative gains, respectively. The PID parameters always need to be tuned system-specifically for the desired results (Wang et al., 2014).

2. PID based nutrient mixing control system.

A control system is an interconnection of the system components which provides the

desired output. In the feedback control system, the output is modulated based on its feedback signal.

The PID method is based on a nutrient mixing control system having four major components (controller, actuator, plants, and sensor) (Figure 1). The overall response of this system can be determined from its mathematical representation. The basic mathematical formulae for a dynamic control system is a differential equation, which follows the balance law or conservation principle (Haugen, 2010). In the nutrient mixing process, high concentration stock solutions (A and B) were mixed with distilled water, and the target concentration nutrient solution was prepared. Considering the mass balance law, the differential equation for nutrient mixing is expressed in Equation 2.

$$\begin{aligned} \frac{d\ M_T(t)}{d(t)} &= M_i(t) - M_W(t) \\ \frac{d\ M_T(t)}{d(t)} &= [M_A(t) + M_B(t)] - M_W(t) \\ \frac{d\ V_T C_T(t)}{d(t)} &= [V_A C_A(t) + V_B C_B(t)] - [V_W(t) C_T(t)] \\ \frac{d\ C_T(t)}{d(t)} &= \dot{C}_T(t) = \frac{1}{V_T}[V_A C_A(t) + V_B C_B(t) - V_W(t) C_T(t)] \end{aligned} \quad (2)$$

where M_T, M_i, M_w are the mass of the target solution, the input stock nutrients, and distilled water. M_A, M_B, V_A, V_B, C_A, C_B are the mass, volume, and concentration of stock nutrient solutions A, B, respectively. V_TC_T is the volume and concentration of the target nutrient solution. V_W is the volume of distilled water.

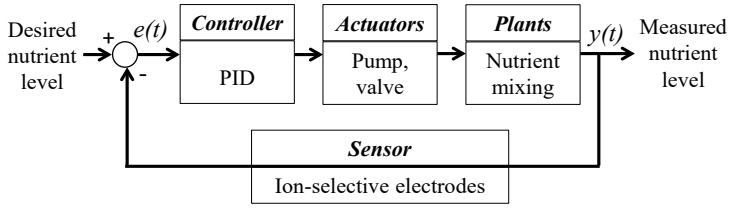

Figure 1. Block diagram of the PID method based nutrient mixing control system.

The output for each possible input of any control system can be predicted by its transfer function. Considering the input and output variables of Equation 2 and using the Laplace transform, the transfer function for nutrient mixing control was derived (Equation 3) which was used during the simulation of the PID parameters.

$$\begin{aligned} L\{\dot{C}_T(t)\} &= \frac{1}{V_T}\mathcal{L}\ [V_A C_A(t) + V_B C_B(t) - V_W(t) C_T(t)] \\ sC_T(s) - C_{T_0} &= \frac{1}{V_T}\ [V_A C_A(s) + V_B C_B(s) - V_W(s) C_T(s) \\ sC_T(s) + V_W(s) C_T(s) &= \frac{1}{V_T}\ [V_A C_A(s) + V_B C_B(s) \\ C_T(s) &= \frac{[V_A C_A(s) + V_B C_B(s)}{V_T(s + V_W(s))} = \frac{2}{s+1} \end{aligned} \quad (3)$$

3. Simulation of PID parameters.

A PID control model for the nutrient mixing system was developed in MATLAB-Simulink interferences (ver.: R2015a, MathWorks, USA). As the concentration of stock nutrients changes only during the nutrient mixing (equal volumes of nutrient A and B

were supplied according to the manufacture's guidelines), the first-order open-loop Ziegler-Nichols (Z-N) method was selected for tuning the PID parameters (Ogata, 2010). Equation 4 shows the Z-N tuning rules based on the step response of the nutrient mixing process.

$$K_p = 1.2\ \frac{T}{L} \qquad K_i = \frac{K_p}{2L} \qquad K_d = K_p \times 0.5L \tag{4}$$

where L is the delay time (s), and T is the time constant (s). In this method, delay time (L) and time constant (T) are determined based on the tangent line drawn at an inflection point on the step response curve (Figure 2a).

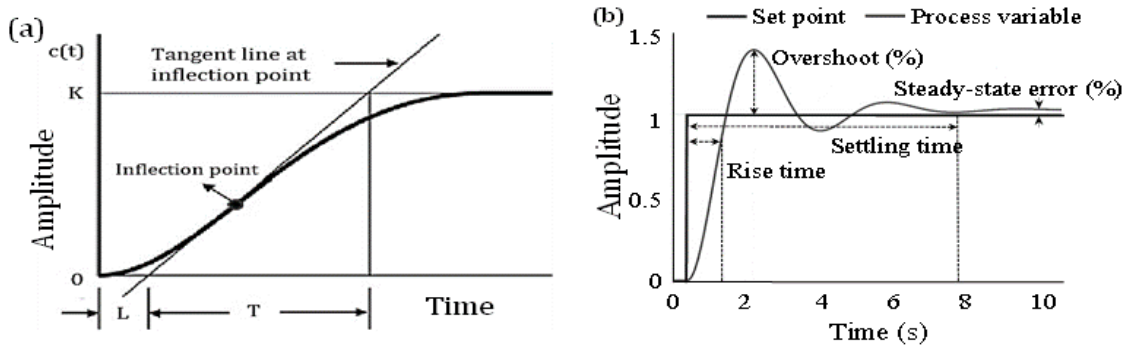

Figure 2. Open loop Z-N tuning method for PID controller (a), and characteristic response curve for the unit step input of the PID control method (b) (Ogata, 2010).

In the simulation, PID parameters were identified at the rated pump flow rate (0.05 L min^{-1}). A range of 0 to 10 was applied for each parameter (Kp, Ki and Kd). From the results, five sets of parameters were selected for validation tests based on the rising time, minimum settling time, overshoot, and steady-state error (Figure 2b).

Validation tests using a test bench

The selected sets of parameters were validated using an automated nutrient solution preparation test bench (Figure 3). Each set of parameters was put into the nutrient mixing program code and the response characteristics were recorded for determining the best performance. The nutrient mixing performance was evaluated based on the rising time, duration of oscillation, time to reach steady, percentage of maximum overshoot, and percentage of steady-state error. A ±2% dead-band of the target value was considered as a perturbation. Moreover, the effects of different pump flow rates (0.03, 0.05, and 0.1 L min^{-1}) on the selected parameters were also evaluated.

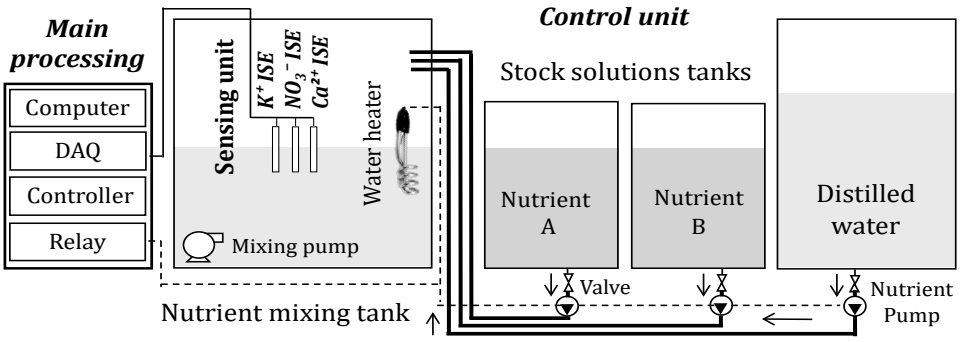

Figure 3. Schematic diagram of the automatic nutrient mixing test bench.

The nutrient mixing test bench (Figure 3) was a combination of three units: sensing, control, and main processing. The specifications of the components that were used in each unit are presented in Table 1. The sensing unit consisted of K^+, NO_3^-, and Ca^{2+} ISE sensors. The control unit consisted of tanks, pumps, and valves for supplying the distilled water, and nutrients A and B. Four nutrient mixing pumps and a water heater were placed into the nutrient mixing tank for quick mixing and maintaining the proper solution temperature, respectively. The main processing unit was a combination of a microcontroller, relays, and a data acquisition device. A LABVIEW coded program was used for the automated operation of this test bench. Target nutrient solution was prepared using commercial nutrient solutions, Nutrient A and Nutrient B (Daeyu Co., LTD, Republic of Korea), which contained all of the essential macro- and micro-nutrients at a specific ratio. For ease of understanding of the performance of the PID control method, the K^+ ion concentration only of the target nutrient solution is presented in the graphs.

Table 1. Specification of the components used in the nutrient mixing test bench.

Subsystem	Component	Model	Specification
Sensing	K^+ ISE sensor	K-BTA, Vernier, OR, USA	Range: 1-39,000 ppm pH range: 2-12 Opt. temp. range: 0-40°C
	NO_3^- ISE sensor	NO_3-BTA, Vernier, OR, USA	Range: 1 to 10,000 ppm pH range: 2.5-11 Opt. temp. range: 0-40°C
	Ca^{2+} ISE sensor	Ca-BTA, Vernier, OR, USA	Range: 1 to 40,000 ppm pH range: 2-8 Opt. temp. range: 0-40°C
Control	Pump for distilled water	8095-902-260, Shurflo, CA, USA	Type: Positive displacement Max. flow rate: 2.3-5.9 L min^{-1} Max. pressure: 0-800 kPa
	Pump for stock nutrients	AB11, Goso, China	Flow rate: 0-0.01 L min^{-1} Pressure: 100 kPa
	Electric valve	CR02, HK, Hong Kong	Brass G1/2", 2 way DN15, 12 V DC, 3 wire
Main processing	LabVIEW	Ver.: 2017, National Instrument, TX, USA	-
	NI-DAQ device	NI USB-6343, National Instrument, TX, USA	No. of input channels: 16 Sample rate: 500 kHz
	Computer	-	Intel core-i3, RAM: 8GB

RESULTS AND DISCUSSION

Simulated PID parameters

The results of the PID parameters simulation, where performance curves of five sets of PID parameters are presented based on minimum settling time, overshoot, and steady-state error, are shown in Figure 4.

The best performance was observed when the Kp, Ki and Kd values were 5.85, 4.35, and 1.56, respectively. However, the rising and settling time for all of the selected sets of parameters were almost the same (around 50 and 200 s, respectively). A difference in the overshoot volume (%) was observed, but no variation was found in the steady-state error.

Validated PID parameters

The sets of selected parameters with a rated pump flow rate (0.05 L min^{-1}) and stock solution concentration were validated through nutrient mixing tests (Figure 5). The best performance of the PID control method was found when the Kp, Ki and Kd values were 5.85, 4.35, and 1.56, respectively, as also observed in the simulation.

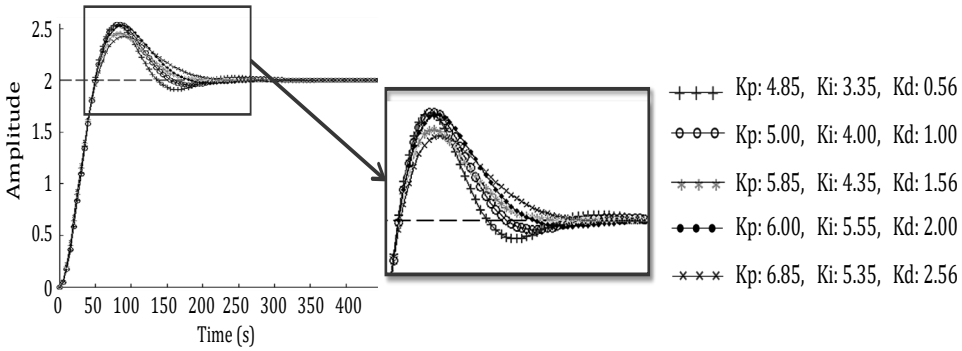

Figure 4. Determination of the Kp, Ki, and Kd values through simulation for nutrient mixing control.

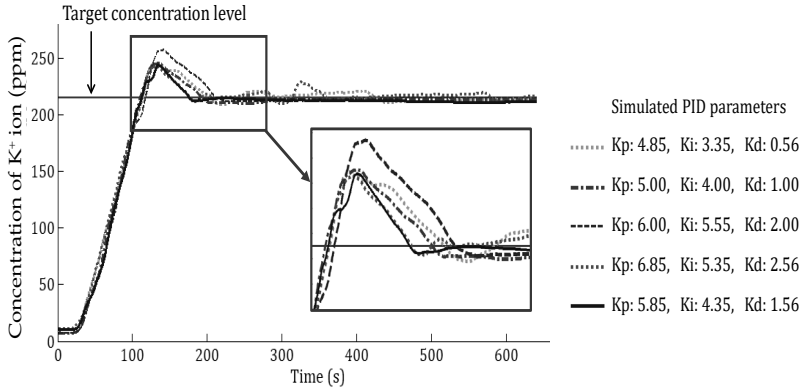

Figure 5. Validation tests of the selected sets of the PID parameters for the target nutrient solution preparation (K^+ ion mixing performance have shown here).

The rising time, oscillation duration, time to reach steady, overshoot (%), and steady-state error for that set were 113 s, 64 s, 177 s, 13.38%, and -0.95%, respectively (Table 2). Li et al. (2016) applied the PID algorithm, where the pump flow rate and stock nutrients concentrations were 6 m^3 h^{-1} and 200 g L^{-1}, and after adjusting the PID parameters, overshoot (%), oscillation and settling time were 3%, 25 s and 100 s, respectively. Similarly, Wu et al. (2019) tested the PID method for maintaining EC level (2.0 mS cm^{-1}), and showed that the response time and maximum overshoot were 148 s and 0.64 mS cm^{-1}, respectively. Wang et al. (2014) observed 3.4% overshoot also during target EC level maintenance through the PID control method.

Table 2. Performance evaluation of the selected sets of the PID parameters based on the rising time, oscillation duration, settling time, overshoot, and steady-state error for the targeted K^+ ion concentration, volume, and stock solution supply rate.

Target K^+ ion level (ppm)	Target volume (L)	Flow rate (L min^{-1})	Kp	Ki	Kd	Rising time (s)	Oscillation duration (s)	Time to reach steady state (s)	Over-shoot volume (%)	Steady state error (%)
215	20	0.05	4.85	3.35	0.56	114	178	292	13.7	-0.08
			5.00	4.00	1.00	111	83	194	14.1	-1.24
			5.85	4.35	1.56	113	62	175	13.4	-0.40
			6.00	5.50	2.00	118	94	212	19.6	-0.64
			6.85	5.35	2.56	114	62	178	12.9	0.69

The effects of the pump flow rate on the PID control method are shown in Figure 6, and detailed data are given in Table 3. A low pump flow rate (0.03 L min^{-1}) had the lowest steady-state error (-0.20%) but it took the longest time to reach a steady state (364 s). The rising time for the high flow rate (0.1 L min^{-1}) was short (89 s) relative to other flow rates but the steady-state error was high (-1.36%). Li et al. (2016) also observed a similar result, where the duration of oscillation was changed from 15 to 125 s when the output liquid flow rate was changed from 10 to 4 m^3 h^{-1}.

Table 3. Effects of pump flow rate on the PID control method based on the settling time, overshoot, and steady-state error for the targeted K^+ ion concentration and volume.

Target K^+ ion level (ppm)	Target volume (L)	Flow rate (L min^{-1})	Rising time (s)	Duration of oscillation (s)	Time to reach steady state (s)	Overshoot volume (%)	Steady state error (%)
215	20	0.03	172	192	364	11.16	-0.20
		0.05	113	62	175	13.38	-0.40
		0.10	89	117	206	14.16	-1.36

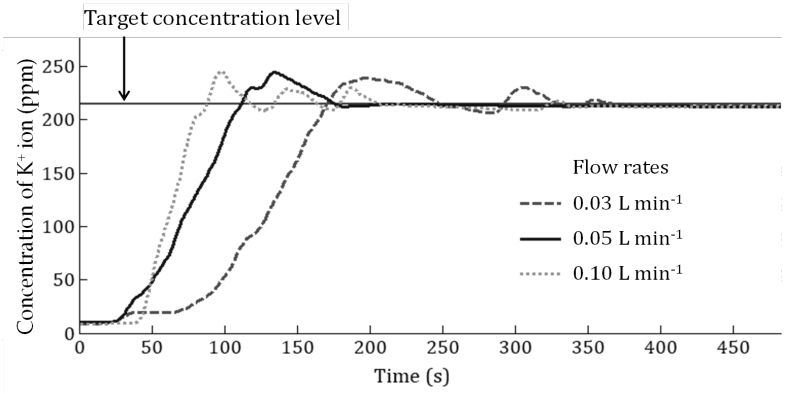

Figure 6. Effects of pump flow rate on PID control method during the K^+ ion mixing.

CONCLUSIONS

This study was conducted to improve the nutrient mixing control efficiency, stability, and accuracy in hydroponic nutrient management systems. For this purpose, the PID control method was applied, and parameters were identified. During the mathematical simulation, a range of 0 to 10 was applied for Kp, Ki and Kd parameter determination, and five sets of coefficients were selected for validation based on the settling time, overshoot, and steady-state error. The effects of pump flow rate (0.03, 0.05, and 0.1 L min^{-1}) on the PID control method were also evaluated. The PID control method showed ±1% steady-state error for all sets of parameters. However, the best performance was observed when Kp, Ki, and Kd were 5.85, 4.35, and 1.56, respectively. An inverse relation was observed between pump flow rate and the steady-state error. The results indicate that the PID method and identified parameters would be feasible to prepare the target nutrient solution with minimum error, but PID parameters, pump flow rate, and capacity of the nutrient mixing pump need to be adjusted properly for large scale applications.

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Simplification of the enhancement of visibility of leaf veins in longan using digital mammography

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Abstract

The leaf blades in the canopies of higher plants play an important role in intercepting light energy, CO_2 assimilation and water transpiration. They are, therefore, critical for plant growth and development in the overall plant life cycle. The leaf vein network is important for the distribution of water (the xylem route) and for sugar transport (the phloem route). In this study, apical shoots with 3-5 fully-expanded leaves from 3-year-old longan (*Dimoparpus longan* L. 'Puang-thong') trees at Lopburi Province, Thailand were collected. The apical shoots (20 cm in length) were dipped into 4% KI (w/v) under either controlled temperature at 40.2 to 41.9°C (KI-40) or at room temperature (KI-RT). Control shoots were dipped in water. Shoots were then kept overnight before being examined by digital mammography. In the KI-40 treatment, leaf vein networks with high resolution were observed. Minor leaf veins were able to be distinguished in the KI-40 but not in the KI-RT treatment. A histogram distribution in the KI-40 leaves clearly showed the intensity enhancement (intensity 118.67) when compared with KI-RT leaves (intensity 65.45). In addition, the minor vein length in the KI-40 leaves was shown to be higher in the zone close to the leaf margin, compared with the zone adjacent to the mid rib (the main leaf vein). The KI-40 treatment showed that it is possible to explore some leaf vein characteristics, as a result of the improved contrast resolution, when using digital mammography.

Keywords: X-ray, digital mammography, leaf vein, leaf image intensity

INTRODUCTION

Based on leaf structure, the architecture of vein networking within a leaf has a crucial role in controlling water/nutrient transport, as well as the transport of photosynthates in higher plants (Gan et al., 2019). Vein networks can also play an important role within the main skeleton of the leaf in terms of its expansion for light harvesting (Wen et al., 2018). Aside from the biological relevance of venation, electrode networks have been developed to resemble leaf veins in the structure of solar cells (Han et al., 2016). In regard to the structure of leaves, the assessment of leaf vein length per unit area using imagery is one of the emerging issues that limits phenotyping assays (Price et al., 2014). Recently, Du et al. (2019) reported on the benefits that a synchrotron and computed tomography (CT) can have for application to plant science and to various aspects of agriculture. The X-ray images provided by a synchrotron and by a CT scan have been shown to be useful in providing high resolution scans of leaf vein networks (Blonder et al., 2012). However, a synchrotron may not be suitable for routine evaluation because of the difficulty of using the light source (Schneider et al., 2018). In addition, operation of the CT is more costly than a digital 2-dimension (2D) X-ray (Nass et al., 2001).

Recently, however, medical digital 2D X-ray tools have been developed that could provide a rapid, simple and reliable method for use on plant samples as well as being user-friendly to investigators in the plant sciences who are studying plant architecture with

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non-destructive methods. Nonetheless, sufficient resolution of the captured images needs to be developed (Schneider et al., 2018). In order to prepare clear images, iodine can be used as a chemical agent to label the transport system, including that in the leaf veins of higher plants (Blonder et al., 2012). In this study, therefore, a 2D X-ray method, such as that used in mammography imagery, was investigated together with the possibility of magnifying the image obtained using potassium iodine to label the transport system. The aim was to develop an effective routine practice for the non-destructive investigation of longan leaf vein structure.

MATERIALS AND METHODS

Sample preparation

A leaf sample of longan was used as a model for examining leaf vein structure. Cuttings of shoots were provided by Suan Pin Wong, Lopburi Province, central region of Thailand. The cut ends of the shoots were dipped in 4% potassium iodine solution (Blonder et al., 2012) and separated to: 1) treatment in the range of 40.2-41.9°C with hot air (LiQi, Ningbo, P.R. China) in a box for 30 min; or 2) kept at room temperature. Shoots from both treatments were then kept at room temperature overnight. These two treatments were coded as KI-40 and KI-RT, respectively. For a control, the cuttings were dipped in water at room temperature. All shoot samples were scanned by digital mammography. The cuttings were positioned for an upright view and were suspended with duct tape using a paddle. The samples were compressed using the standard mammogram compression force.

Mammography condition

A digital mammography system (Amulet Innovality, Fujifilm, Japan) was used to acquire a series of images. All images were craniocaudal projections. The kV and mAs of each image were controlled by an automatic exposure system which ranged from 22 to 49 kV and from 2 to 300 mAs.

Image analysis

The manipulation of the original digital 2D mammography images, in DICOM format, from the usual medical images to an image with color distribution was managed using a medical analysis tool according to Dückelmann et al. (2010). After the image was manipulated, a grayscale was created for image data analysis, ImageJ (Centeno et al., 2015; Schneider et al., 2018). A histogram of intensity distribution on the leaf blade in the region of interested (ROI) was then calculated. The length of a minor vein was tracked and determined in areas either close to the midrib or close to the leaf margin in an area of about 8.2 mm^2 (Figure 1).

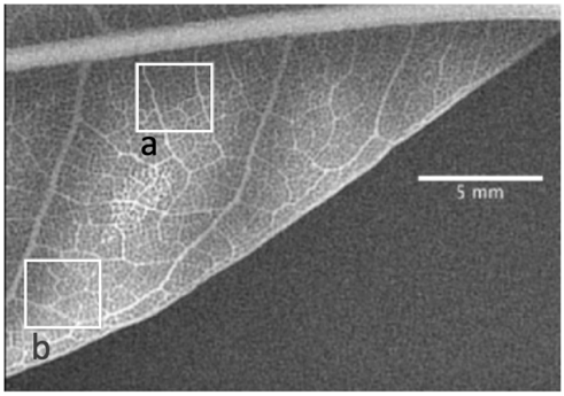

Figure 1. Zone of analysis of the length of a minor vein in the midrib zone (a, MR) and leaf margin zone (b, LM).

RESULTS

A longan leaf treated with KI under high temperature conditions (40°C; KI-40) was able to display the two-dimensional structure of minor leaf veins using ImageJ software. In comparison, the minor leaf veins in the KI-RT treatment were not clear (Figure 2). The converted intensity of the KI-40 leaf image had a high value, especially in the mid and margin zones of the leaf blade. When using an 8-bit grayscale for the histogram, the 8-bit grayscale image creates a value from 0 to 255 on gray scale for each pixel, where 0 is completely back and 255 is completely white. When the mean intensity was high, it indicates that a whiter pixel was found. In the experiment, a wide range of intensity distribution in the KI-40 treatment was observed (mean intensity 157). Shorter ranges of intensity distribution in the KI-RT and control histogram were demonstrated (mean intensities of 88 and 74, respectively). Similarly, the distribution intensity values in the leaf blade zone were 118.7±36.4, 65.5±36.4 and 73.9±7.9 in the KI-40, KI-RT and control treatments, respectively (Figure 3).

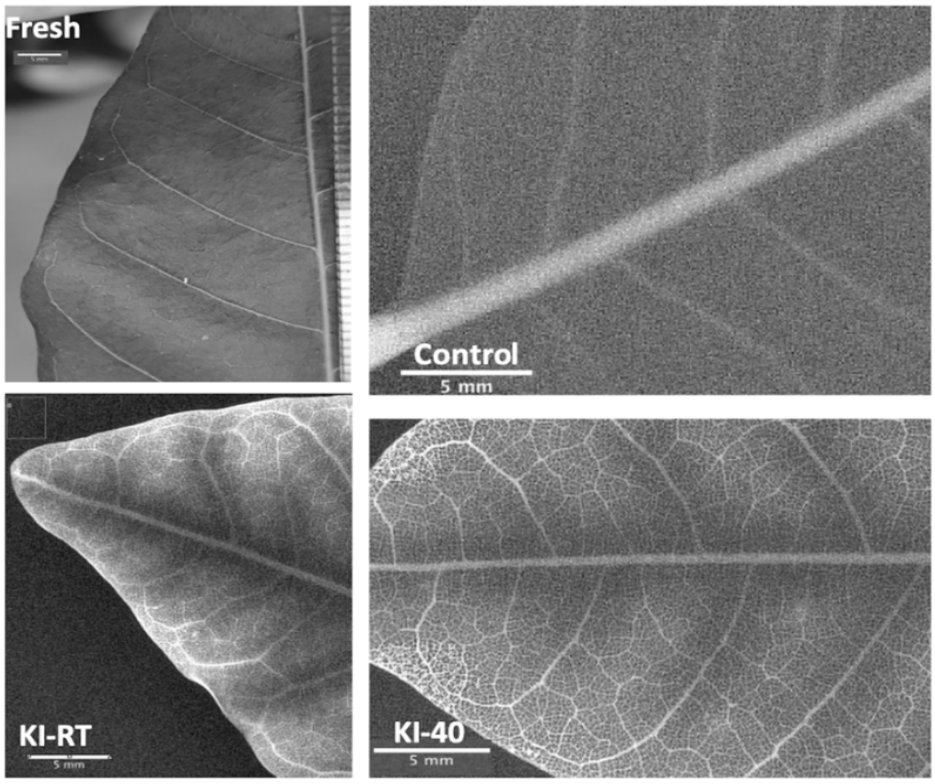

Figure 2. Fresh leaf and enlarged image of a mammogram X-ray image of a leaf sample in control (water), leaf dipped in potassium iodine at room temperature (KI-RT), and leaf dipped in potassium iodine at 40°C incubation temperature (KI-40).

In the contrast image of the KI-40 leaves, the minor veins were enabled to be tracked within the equal areas of the leaf blade. The results showed that the minor vein length per area in the margin leaf zone was longer than that in the mid rib (middle leaf vein) zone (Figure 4).

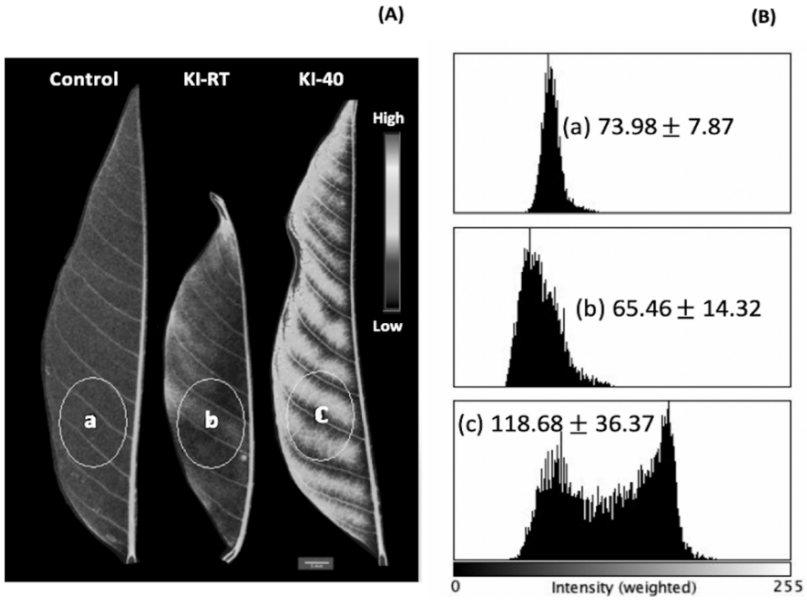

Figure 3. Converted intensity within a longan leaf using mammogram X-ray imaging (A), Histograms of intensity distribution and mean intensity in 8 bits of gray scale of leaf image (B) in the control (water), leaf treated with potassium iodine at room temperature (KI-RT) and leaf treated with potassium iodine plus 40°C incubation temperature (KI-40) under equally selected areas shown as a, b and c, respectively.

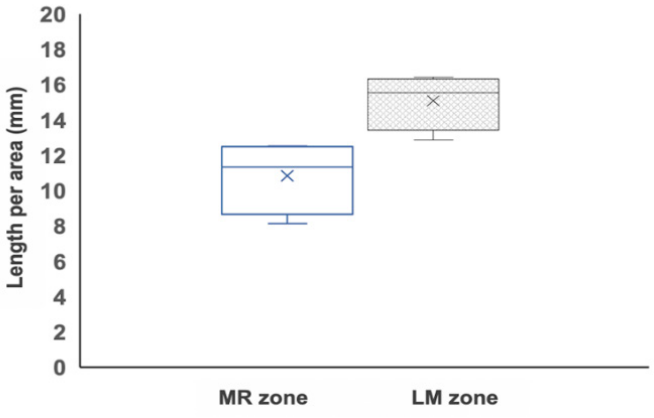

Figure 4. Minor vein length and length tracking images of minor veins in KI-40 leaves in the mid rib zone (middle leaf vein) (MR) and in the leaf margin zone (LM) under equal areas (8.2 mm^2). The MR and LM means (n=4) were significant different at $p \leq 0.05$.

DISCUSSION

The non-destructive X-ray technique has been shown to be useful in studies of plant structure and function (Le et al., 2019). The commercial 2D X-ray mammography has the capability of providing routine and/or replicate images rapidly (Nass et al., 2001). However, optimization of the X-ray images, that are stimulated by radioactive energy in the X-ray proton mammography, is required (Bick, 2000). An X-ray image with improved contrast, when using iodine solution in wheat spikes, was previously demonstrated (Karunakaran et al., 2015). In the current study, the iodine treatment enhanced the contrast in the leaf veins of the KI-RT leaves which were more reflective than those in the control leaves (Figure 2). It

might be considered that the amount of iodine agent that was delivered, in the KI-RT samples of longan shoots to enhance the quality of the images in the leaf vein structure under the mammography system, was sufficient.

However, the image intensity of the minor vein structure in the KI-40 treated leaves showed exceedingly high contrast, compared to that in the KI-RT leaves (Figure 3). In the KI-40 treatment, the high temperature of 40°C would have enhanced the absorption of iodine into the leaves. In a transgenic *Arabidopsis* line (NIS plant), a temperature of 30°C also promoted iodine uptake and translocation into the *Arabidopsis* plants (Landini et al., 2012). As a result of this effective staining, therefore, the minor vein network in the KI-40 leaves was able to be used as a phenotypic character.

Furthermore, the length of minor veins per unit area in the margin zone of the KI-40 leaves was able to be measured and was found to be greater than that of veins in the mid rib zone (Figure 4). In an image of a *Protium wanningianum* leaf, the network of minor veins also appeared to be enriched in the area of the leaf margin (Ronellenfitsch and Katifori, 2016) However, the structural distribution of veins in the vein network depends on plant species, position in the plant canopy, overall leaf area and the leaf developmental stage (Sack et al., 2012).

CONCLUSIONS

The results indicated that 2D X-ray mammography on leaves stained with iodine under high temperature conditions was able to define longan leaf structure, especially the minor leaf vein network. Furthermore, the non-destructive analysis of plant tissue for routine/replicate measurement at a reasonable cost was shown to be possible with a 2D X-ray mammography system.

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Evaluation of the deposition of ^{137}Cs in Japanese persimmon trees and yuzu trees from rainfall by collecting raindrops with sphagnum pads

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Abstract

The radiocaesium emitted due to the Tokyo Electric Power Fukushima Daiichi Nuclear Power Plant accident, initially migrated into fruit trees via the above-ground part of the trees. The behavior of the intercepted ^{137}Cs was evaluated for 6 persimmon and 22 yuzu trees. Radiocaesium can be deposited as dry or wet sediment. To measure the amount of ^{137}Cs in stemflow and raindrops, a collection pad comprised of sphagnum was used. This was attached to trees in a persimmon orchard and several yuzu orchards over 3 years, starting in 2016. Additionally, the effect of bark-washing on persimmon was examined by measuring the amount of ^{137}Cs trapped by the sphagnum pads on the leaf, calyx and main trunk following rainfall. The amount of ^{137}Cs mm^{-1} of precipitation retained by leaves was highest in 2016 in both types of fruit tree and decreased with time. The highest ^{137}Cs interception was detected on the same individual trees in 2016 and again in 2017. In the yuzu orchard in 2018, a higher concentration of ^{137}Cs in fruit than that in previous year were detected in the tree with more ^{137}Cs collected on the leaves than that in the previous year. This indicates the increase in the concentration of ^{137}Cs in fruit also depended on ^{137}Cs contamination on the leaves. These findings demonstrated that it is possible to identify trees which intercepted higher levels of ^{137}Cs during rainfall using sphagnum pads. Therefore those trees are most likely to have more contaminated leaves or fruits.

Keywords: calyx, leaves, raindrop, stemflow, Fukushima Daiichi nuclear power plant accident

INTRODUCTION

The Fukushima Daiichi Nuclear Power Plant (FDNPP) accident, caused by the Great Eastern Japanese earthquake occurred on March 11, 2011. A large quantity of radioactive material was released into the environment from March 12 to 14, 2011 (International Atomic Energy Agency, 2015). The radioactive deposition occurred primarily via rainfall and snowfall. The highest radioactive levels were recorded on March 15, 2011 (Chino et al., 2011). The Fukushima Prefecture is one of the major deciduous fruit production areas in Japan (Ministry of Agriculture, Forestry and Fisheries, 2018). Fallout and contamination occurred over a wide range of different fruit tree orchards in the Fukushima Prefecture. Therefore, a focus was on researching the environmental pathway for radioactive particles to enter fruit in these orchards. Both ^{137}Cs and ^{134}Cs were deposited with ^{137}Cs constituting the major concern for orchards due to its long physical half-life (30.1 years), so only this radionuclide will be considered here. The ^{137}Cs activity concentration in most deciduous fruit decreased with time, and by 2020 was below the detection limit for most fruits, with the exception of Ampo-gaki persimmon and yuzu (*Citrus junos* Siebold ex Tanaka). Ampo-gaki is a local specialty in the northern region of the prefecture and produced by drying Japanese persimmon (*Diospyros kaki* Thumb.) which increases the ^{137}Cs concentration compared with the fresh fruit. Yuzu fruit is also a local specialty in Fukushima

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city, and used mainly as a flavoring material in various processed foods. In the initial period after the FDNPP accident, the interception of ^{137}Cs by the leaves and fruit of the evergreen yuzu trees resulted in 10-folds increase of ^{137}Cs concentrations compared to other deciduous fruit (Hamada et al., 2012; Sato, 2020). The concentration of ^{137}Cs in fruit for persimmon and yuzu trees decreased within a few months to values that were below the provisional activity limits of 100 Bq kg^{-1} FW, applied to general foods from April, 2012. However, a small number of fruit still exceeded the limits.

One aspect considered, was the potential for secondary contamination. Migration of intercepted ^{137}Cs due to rainfall onto the fruit tree canopy. This may come from the surrounding environment, such as the forest surrounding the orchards (Sato et al., 2015). As the FDNPP accident occurred during the dormancy period for deciduous orchards in Fukushima, ^{137}Cs was deposited directly onto the bark of the fruit trees and directly onto the topsoil. ^{137}Cs was also transferred to the soil via throughflow (wet leaves shed water onto the ground surface) and water flowing over bark, termed "stemflow" (Schimmack et al., 1993; Kato et al., 2012; Loffredo et al., 2014). The dynamics of throughfall and stemflow in forests has been widely reported (e.g., Loustau et al., 1992; Steinbuck, 2002; Mattaji et al., 2012). However, there have been few studies of these processes in fruit tree orchards.

There was some concern that ^{137}Cs may be transferred from the fruit tree surfaces exposed during the dormant period to leaves and fruits due to rainfall during the growing season. In response, a research study was initiated to specify and quantity the routes for ^{137}Cs contamination in fruit orchards. Initial studies showed that sphagnum pads effectively collected ^{137}Cs in stemflow and raindrops on fruit trees (Sato et al., 2017a, b). In this study, we investigated the amount of ^{137}Cs in the stemflow from the canopy and in raindrops transferred to the calyx or leaves during rainfall.

MATERIALS AND METHODS

Study sites and trees

A Japanese persimmon orchard in the Date city (37°47'43.1"N; 140°34'14.3"E) and a yuzu orchard in Fukushjma city (37°46'25.5"N; 140°28'24.8"E), approximately 60 and 65 km northwest of the FDNPP were selected for investigation in 2011 to 2019. Six Japanese persimmon 'Hachiya' trees (Figure 1A) and 22 yuzu trees (Figure 1B) were used in this study. The yuzu orchard was located in a mountainous area, so the yuzu trees were planted on a steep sloping site, and some trees were directly below a road. Three of the persimmon trees were washed ("washed tree", WT) with a high-pressure washer on December 21, 2011 (Sato et al., 2015). The other trees, including 22 yuzu trees, were not washed ("unwashed tree", UWT). Five cm of topsoil in the yuzu orchard was removed as part of a decontamination procedure from March to August in 2015.

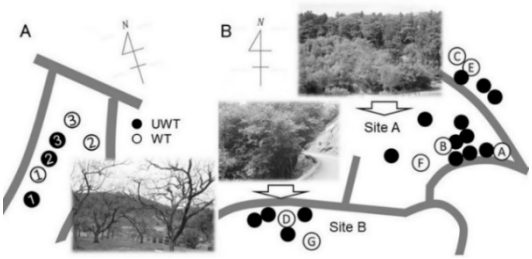

Figure 1. Diagrametic representation of the survey orchards and trees. Japanese persimmon orchard. WT means "washed tree". These were washed with a high-pressure washer and UWT means "unwashed tree" (A). Yuzu orchards. Six trees at A to F were used to measure the amounts of deposited ^{137}Cs onto the leaves of the canopy and surrounding forest during rainfall from 2016 to 2018 (B). Twenty-two trees including trees for A to F were used for the study to collect ^{137}Cs in stemflow through the trunk during the spring growth flush in 2019.

Preparing the ^{137}Cs collection pads

Two size of the sphagnum collection pads were made by encapsulating approximately 2.5 g (for calyx and leaf) and 6 g (for trunk) of sphagnum (Besuguro-Supagumosu, New Zealand, NZ) into a tea pack (polyester polyethylene composite fiber). Sphagnum from NZ was used because it was expected to contain a negligible amount of ^{137}Cs from past nuclear accidents and weapons testing (Sato et al., 2017a).

Studies in the Japanese persimmon orchard

The different aspects of this study are outline in Table 1. To quantify concentrations of ^{137}Cs in the stemflow due to bark washing, sphagnum pads were tied onto the trunk of UWT and WT in 2015. The sphagnum pads were covered with polyethylene film to shield the pads from throughfall (Figure 2). In 2016, four locations in each of 3 UWTs were selected for a study that aimed to quantify the relationship between the amounts of deposited ^{137}Cs on the calyx and the concentration of ^{137}Cs in fruit due transfer by raindrops from the canopy. Sphagnum pads were attached onto the calyx of the fruit. Another fruit on the same branch was also bagged as a control. The concentration of ^{137}Cs from bagged mature fruit and non-bagged fruit with attached sphagnum pads on the same branch were compared. The deposited ^{137}Cs on the leaf of the UWT and WT were compared in both 2017 and 2018. To monitor the annual contamination status in the study trees, the bark of the main trunk of UWT and WT were initially sampled to measure the concentration of ^{137}Cs on October 18, 2011 prior to bark washing. Subsequent, measurements were taken on October 28, 2013, June 4, 2015, June 2, 2017 and April 9, 2018.

Figure 2. Images of the methods used to collect ^{137}Cs in stemflow and raindrop using a sphagnum pad. To collect the ^{137}Cs in stemflow, sphagnum pads were attached onto the trunk (A) of UWT and WT in the Japanese persimmon orchard and of UWT in the yuzu orchard. To collect the deposited ^{137}Cs, sphagnum pads were attached onto the calyx (B). To study the effect of deposited ^{137}Cs on the calyx and the concentration of ^{137}Cs in mature fruit, a fruit on a lateral branch was selected and a sphagnum pad attached and bagged, control treatment (C). To collect the intercepted ^{137}Cs by leaves, sphagnum pads were attached onto leaves of the persimmon and the yuzu trees (D).

Studies in the yuzu orchard

An outline of the study is also shown in Table 1. Sphagnum pads were attached onto leaves at the marginal section of the canopy of the yuzu trees. This was to confirm the amounts of deposited ^{137}Cs on the leaf during rainfall (Figure 2). Four trees for A to D shown in Figure 1 were surveyed for a period of three years from 2016 to 2018. The concentration of ^{137}Cs in mature fruit was measured by collecting five fruits from each tree to explore the relationship between the amount of ^{137}Cs deposited on leaves and the concentration of ^{137}Cs in the fruit. In 2019, sphagnum pads were attached onto the trunk of 22 selected trees to trap the ^{137}Cs in stemflow during the spring flush of rapid growth (Figure 2). Leaves of the spring shoots were collected on June 26 to measure the ^{137}Cs concentration. The ratio of the concentration of ^{137}Cs in leaves of the spring high growth period, June 26, 2019 was compared to that on June 13, 2018. A regression analysis was carried out between the amounts of the ^{137}Cs in stemflow and the annual ratio of the concentration of ^{137}Cs in leaves of the spring shoot.

Table 1. Study outline for collection of ^{137}Cs in raindrops and stemflow using a sphagnum pad from two experimental orchards.

Orchard	Study year	No. of trees	Collection period	Location of attached pad	No. of pads tree^{-1}	Sampling days of fruit or leaf
Japanese persimmon	2015	3UWT&3WT	Jul. 8-23	Trunk	1	
	2016	3 UWT	Jun. 15-Oct. 19	Calyx	4	Oct. 19 (fruit)
	2017	3UWT&3WT	Jun. 15-Sep. 20	Leaf	3(WT), 5(UWT)	
	2018	3UWT&3WT	Jun. 27-Oct. 24	Leaf	3(WT), 4(UWT)	
Yuzu	2015	13 UWT	-	-	-	Nov. 6 (fruit)
	2016	4 UWT	Jun. 25-Oct. 28	Leaf	6	Nov. 14 (fruit)
	2017	6 UWT	Jun. 12-Oct. 18	Leaf	4	Oct. 25 (fruit)
	2018	5 UWT	Jun. 13-Oct. 25	Leaf	4	Jun. 13 (leaf), Oct. 25 (fruit)
	2019	22 UWT	Jun. 14-Jun. 26	Trunk	2	Jun. 26 (leaf)

Sample treatments and radiocaesium measurements

The sphagnum samples attached to the trunk of the Japanese persimmon and the yuzu trees were removed from the tea pack and placed in a 100 mL polypropylene container (U-8 pots). Whereas the sphagnum pads and tea packs attached onto the calyx and leaves were directly measured for ^{137}Cs. Bark samples were collected from five trees prior to washing on October 18, 2011. These were combined into one sample for ^{137}Cs concentration measurement. Bark samples from each tree were collected after 2013. The Japanese persimmon fruit were shredded using a food processer, after peeling, removing seeds and calyxes. Yuzu fruits were separated into the pericarp (flavedo with albedo) and pulp with seeds. Leaves and fruit were placed in U-8 pots after freeze-drying for at least 72 h. The concentration of ^{137}Cs in all samples measured using a germanium detector (GEM40-76 germanium detector, Seiko EG&G ORTEC, Tokyo, Japan) at the Fukushima University. Gamma-ray emission at energies of 662 keV measured for 3,600 to 80,000 s. The concentration of ^{137}Cs in bark was converted to a DW basis using a dry-to-wet ratio after drying for one day at 105°C. After freeze-drying, the concentration of ^{137}Cs in the fruit of yuzu trees was converted to a wet weight basis using a dry-to-wet ratio. The means of the measured values from the pericarp and pulp with seeds was assumed to represent the concentration of ^{137}Cs in yuzu fruit.

Calculation of the effective half-life of the concentration of ^{137}Cs in bark of Japanese persimmon and of the retention of ^{137}Cs during rainfall

A single negative exponential model expressed as a predictor variable of the temporal changes in the concentration of ^{137}Cs in the bark during the first year after the nuclear accident (Antonopoulos-Domis et al., 1991). This was obtained by the least-squares method adopted for the quasi-Newton method:

$$y = K\exp(-\lambda x) \quad (1)$$

$$T_{eff} = \ln 2\, \lambda^{-1} \quad (2)$$

where y is the concentration of ^{137}Cs in the barks collected in the xth year after the nuclear accident; K is the concentration in the year of the nuclear accident; x is the number of years after the nuclear accident; and λ is the decay constant; T_{eff} is the effective half-life.

The retention of ^{137}Cs due to rainfall was represented as the following.

$$R = 1000A\, P^{-1}\ (\text{mBq g}^{-1}\ \text{mm}^{-1}) \quad (3)$$

where R is the retention of ^{137}Cs, A is the amount of ^{137}Cs intercepted per 1 g of sphagnum (Bq g^{-1}) and P is the amount of precipitation during the collection period at each orchard

(mm).

RESULTS AND DISCUSSION

Studies in the Japanese persimmon orchard

The concentration of ^{137}Cs in the bark of both the UWT and WT decreased with time. The concentration of ^{137}Cs in the bark of WT was significantly lower than that of UWT by approximately one order of magnitude for each year of sampling (Figure 3A). The measured values of UWT and WT were fitted to a single negative exponential model. However, the concentrations of ^{137}Cs in the bark for both treatments in the last year of measurement (2018) were lower than the values predicted by the relevant model. The T_{eff} values for the UWT and WT were 2.1 and 3.2 years. The amount of ^{137}Cs g^{-1} DW sphagnum (mean ± SD) encapsulated in the tea pack collected from stemflow on the main trunk of UWT and WT was 1.08±0.15 and 0.37±0.12 Bq g^{-1}, respectively. There was significantly lower amounts of ^{137}Cs from the trees that had been bark-washed (at p=0.0003 by t-test). Similarly, the retention of ^{137}Cs on leaves via raindrop significantly lower in bark-washed trees (Table 2). These results indicate that the concentration of ^{137}Cs in stemflow and raindrops in the canopy reflected the contamination status of the bark during six years after FDNPP accident, especially if there is a 10 times difference in pollution levels. The amount of ^{137}Cs in the sphagnum pad attached onto the calyx in UWT was 0.04-1.6 Bq g^{-1} DW per 1g of sphagnum. The higher the value of ^{137}Cs intercepted within one tree, the larger was the range of variation, as shown by tree UWT1. Figure 3C shows the concentration of ^{137}Cs in the fruit which was bagged, non-bagged or equipped with sphagnum pad on the same lateral branch, expressed as Bq kg^{-1} DW. The amounts of ^{137}Cs (Bq g^{-1} DW) of sphagnum pads attached on the calyx in four locations of the branch are reported on the *x*-axis. There was no significant correlation in the concentration of ^{137}Cs in fruit due to bagging or to the amount of intercepted ^{137}Cs by sphagnum pads, whereas there were significant differences in the concentration of ^{137}Cs in fruit within a branch, which varied by up to three folds.

Table 2. The retention of ^{137}Cs on sphagnum pad attached onto calyx or leaves of Japanese persimmon. Arithmetic mean and standard deviation of the values per year and ANOVA results are reported.

Bark washing	Replicate tree label (Figure 1)	^{137}Cs retained (mBq g^{-1} mm^{-1})[a]		
		Year[b]		
		2016 (Calyx)	2017 (Leaves)	2018 (Leaves)
UWT	1	0.79	0.60	0.08
	2	0.27	0.16	0.17
	3	0.67	0.18	0.12
		0.58±0.27	0.31±0.25	0.12±0.05
WT	1		0.10	0.08
	2		0.09	0.04
	3		0.19	0.02
			0.13±0.06	0.05±0.03
p value by ANOVA[c]	Year		0.013*	
	Bark washing		0.014*	

[a]Accumulated precipitation (mm) during the collection period in 2016, 2017 and 2018 were 680, 449 and 408.
[b]Sphagnum pads were attached on calyx in 2016 and on leaves in 2017 and 2018.
[c]The measured values in 2017 and 2018 by logarithmic transformation. Significance: *p<0.05.

Based on the effective half-life of ^{137}Cs, there was a considerable reduction in the concentration of ^{137}Cs in the bark due to bark washing for up to the first three years after the accident. High pressure bark washing conducted six months after the FDNPP accident on Japanese persimmon trees reduced the concentrations of ^{137}Cs in fruit by about 30% (Sato et al., 2015). Previous studies have indicated that there may be secondary contamination of

fruit from the bark. Sekizawa et al. (2016a, b) and Sato (2020) demonstrated that ^{137}Cs migrated to fruit via the calyx. In this study, the bagged fruits showed higher concentrations than that of the non-bagged fruit on the same branch. This is in contrast to the prior studies. The source of ^{137}Cs migration was unlikely to be on the calyx. Which is consistent with the view of Sekizawa et al. (2016b) that stemflow and throughflow was unlikely to influence the concentration of ^{137}Cs in the fruit via the calyx. An alternative potentially important pathway was suggested by Sato (2020) who demonstrated that the transfer rate of ^{137}Cs via leaves at the young fruit stage or the fruit growing stage was 10.2±3.3 or 16.4±5.0% in the Japanese persimmon 'Hachiya'. This previous study indicated the transfer rate of ^{137}Cs via leaves depended on the distance between fruit and the contaminated leaf. If the deposition onto leaves is assumed to be similar to that on the calyx, differences in the orders of two magnitudes were found by this study. In addition, contamination of the leaves may contribute to the differences in the concentrations of ^{137}Cs in fruit for the year 2016 (Figure 3).

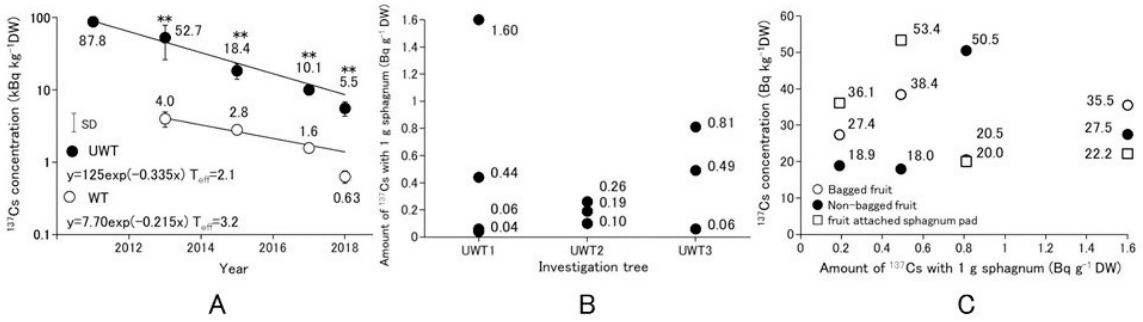

Figure 3. Comparison of change with time in the concentration of ^{137}Cs in bark of Japanese persimmon between bark-washed tree and unwashed trees (A); the amount of ^{137}Cs intercepted by sphagnum pad attached on the calyx in the three UWT in 2016 (B) and the concentration of ^{137}Cs in the bagged, and non-bagged fruit and fruit with a sphagnum pad (non-bagged) on the same lateral branch (C). One sphagnum pad in each of UWT2 and UWT3 dropped in (B). Significance: **p<0.01 by t-test in (A).

Studies in the yuzu orchard

Over the two years from 2016, the amount of ^{137}Cs accumulated in the sphagnum pad attached to yuzu leaves decreased by one-third. However, there were large differences between individual trees (Figure 4A). Figure 4B, C show changes with time in the retention of ^{137}Cs by the sphagnum pads and the concentration of ^{137}Cs in fruit in four trees for A to D over 3 years. There was no significant difference in the retention of ^{137}Cs among trees in 2016 or 2017. This was due of large differences in the amount of intercepted ^{137}Cs within one tree which is consistent with the observations on Japanese persimmon. Furthermore, the retention of ^{137}Cs in tree D was significantly higher than any of other two trees ($p<0.05$ by Tukey's test) sampled. From 2017 to 2018, an increase in the retention of ^{137}Cs was observed (Figure 4B). The concentration of ^{137}Cs in fruit of tree A and C decreased each year, whereas that of tree B increased markedly in the two years after 2015 and then decreased in 2018. The roots of B tree were exposed at the time of soil removal and subsequent heavy rain may enhanced the absorption of ^{137}Cs. Interestingly, the concentration of ^{137}Cs in fruit of tree D (Figure 4C) had the same trend with year as that of the retention of ^{137}Cs (Figure 4B). Furthermore, there was a significant positive correlation between the retention of ^{137}Cs on leaves in 2019 and the annual ratio of the concentration of ^{137}Cs in leaves of the spring shoot in 2019-2018 (Figure 5). In the yuzu orchard, four trees in site B, planted just below the road had higher ^{137}Cs retention (Figure 5).

The yuzu orchard area is located on a steep slope surrounded by the forest consisting of evergreen conifers, evergreen broad-leaved trees and deciduous broad-leaved trees.

Sphagnum pads were attached onto the leaves on the outer margin of the yuzu tree canopy, enabling collection of raindrops diffused from the surrounding environment. The highest retention of ^{137}Cs was detected in tree C in 2016 (Figure 4B) probably because it was directly under evergreen conifers. However, Figure 4A, B show the amount of ^{137}Cs deposition from the environment was reduced due to weathering (Kato et al., 2012 and 2017). Conversely, a similar time trend was found for the retention of ^{137}Cs. In 2018, the concentration of ^{137}Cs in fruit increased in tree D. This was caused by secondary contamination due to raindrop diffused from the environment. Shiraishi (1973) indicated ^{137}Cs migrates to pulp directly via the contaminated surface pericarp in Satsuma mandarin (*Citrus unshiu* Marc.). Sato (unpublished) has demonstrated that the transfer rate of ^{137}Cs via spring leaves in yuzu was a third higher than that of the deciduous trees. From these findings and the result of the study in 2019 (Figure 5), it seems that yuzu may be subjected to secondary contamination of leaves or fruits via raindrops/rainfall.

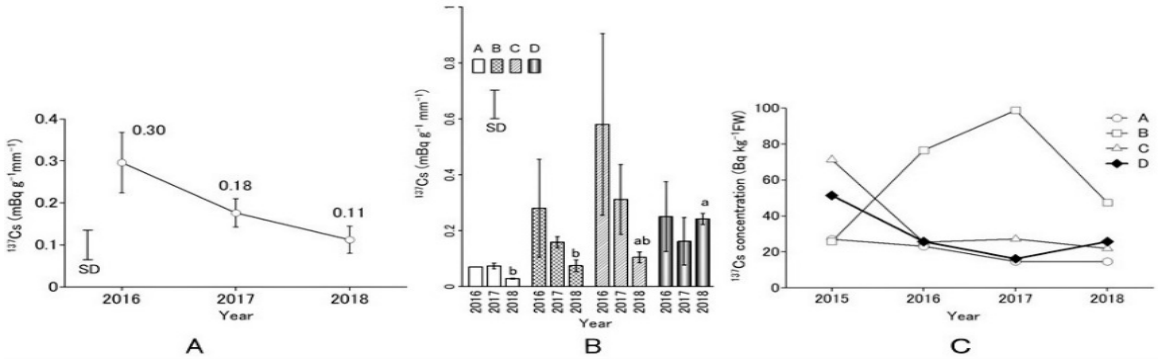

Figure 4. Changes in the retention of ^{137}Cs on the sphagnum pad attached on yuzu leaves over time (A, B), the concentration of ^{137}Cs in fruit (C). Different letters in graph B indicate a significant difference at $p<0.05$ by Tukey's test using the measured value in 2018.

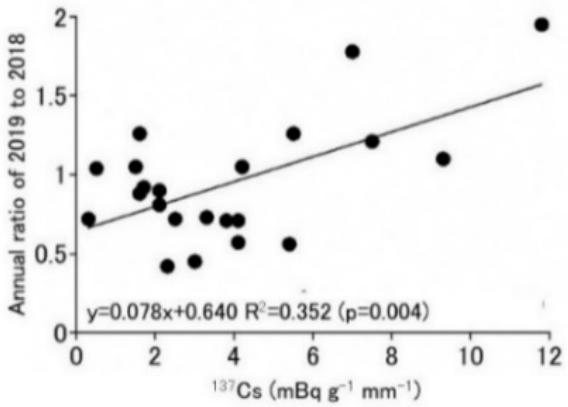

Figure 5. Relationship between the retention of ^{137}Cs in 2019 and the annual ratio of ^{137}Cs concentration in leaves for 2019 to 2018.

CONCLUSIONS

The following conclusions can be drawn from this study:

- The amount of ^{137}Cs in stemflow and raindrops in the canopy of Japanese persimmon reflected the contamination status of the bark for at least 6 years after the FDNPP accident.
- ^{137}Cs migration into fruit via calyx in Japanese persimmon was negligible. The main pathway of ^{137}Cs in stemflow and raindrop into fruit is more likely to be via leaves.

- The amount of collected ^{137} Cs per 1 mm of precipitation was higher in 2016 (the first year of measurement) in both Japanese persimmon and yuzu orchards and thereafter decreased with time.
- It was confirmed that secondary contamination of leaves or fruits in yuzu was due to radiocaesium in raindrops.
- Yuzu seems to be more susceptible to the secondary contamination of leaves or fruits than Japanese persimmon.
- Sphagnum pads were a useful tool to quantitatively evaluate secondary contamination from the canopy and the environment.

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Evaluation of eating quality and starch properties of sweet potato produced in northern Japan

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Abstract

Sweet potato (*Ipomoea batatas* L.) is one of the major root vegetables in Japan. It is eaten as a baked or steamed product and has also been used as a raw material for liquor and starch production. Sweet potato is produced mainly in temperate regions, such as occur in the central or southern parts of Japan. Currently, however, the production area is distributed as far as Hokkaido in the subarctic region in northern Japan. There have been few reports on the taste and quality of sweet potatoes that are grown in the subarctic regions. Consequently, in this study, some characteristics related to food processing properties were compared between the crop grown in the Kanto or Kyushu region, in central or southern Japan, with that from Hokkaido. Sweet potatoes grown in Hokkaido had the following features compared with those grown in Kanto or Kyushu: 1) significantly lower dry matter ratio (2.0 to 7.0%) and starch content (2.1 to 5.2%) in the tuberous roots; 2) lower firmness (6.5 to 20.4 kgf cm^{-2}) and higher Brix value (2.7 to 7.0 °Brix) in steamed roots; and 3) were moister and sweeter in a triangle sensory preference test. Furthermore, the pasting temperature of starch extracted from Hokkaido crops was significantly lower (2.5 to 8.7°C) than those from Kanto or Kyushu. Starch gel made from Hokkaido crops, with the lower pasting temperature, showed a significantly lower water separation ratio (8.3 to 9.4% after four weeks storage at 4°C) and fewer changes in firmness compared to those from Kanto and Kyushu. These results suggested that sweet potato starch from Hokkaido could be more resistant to retrogradation and more suitable for use in food processing.

Keywords: firmness, gel, pasting temperature, subarctic, taste, water separation

INTRODUCTION

Sweet potato (*Ipomoea batatas* L.) is a major root vegetable that is produced globally round 92 million t in 2018 (Food and Agricultural Organization, 2018). About 0.7 million t were produced in Japan in 2019 (Ministry of Agriculture, Forestry and Fisheries, Japan, 2019). Approximately half of the sweet potatoes consumed in Japan are eaten baked or steamed, 24% are used as raw material for liquor production, 13% for starch production, and 11% mainly for use in processed foods such as 'hoshi-imo', steamed, sliced, and dried sweet potatoes. Cooked sweet potatoes with a powder-texture were preferred previously in Japan. However, those that are more moist and sweeter have grown in popularity in the past 15 years (Kariya, 2016). Sweet potatoes with such characteristics, such as 'Beniharuka', have high consumer demand even in Southeast Asia, and 3,500 t were exported from Japan to countries such as Hong Kong, Singapore, Thailand and Taiwan in 2018 (Ministry of Finance, Japan, 2018). An increasing volume of sweet potatoes will be exported to meet increasing demands in the future.

In Japan, sweet potato is produced mainly in the temperate central (Kanto) and southern (Kyushu) regions. In recent years, however, the production area has spread out to

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include Hokkaido in the northern region, which has a subarctic climate (Kuranouchi et al., 2020). However, there have been few reports describing the taste and quality of sweet potato grown in these subarctic regions. In this study, some characteristics of sweet potatoes grown for food processing in Hokkaido were compared with those grown in Kanto or Kyushu regions.

MATERIALS AND METHODS

Plant materials and growing conditions

Four cultivars of sweet potatoes ('Beniazuma', 'Kokei No. 14', 'Koganesengan' and 'Kampachi') were used in this study. These cultivars were grown in the Donan Agricultural Experiment Station, Hokuto, Hokkaido, Japan (41°53.2'N, 140°39.2'E and 25 m a.s.l.), Saitama (Kanto; 35°52.5'N, 139°35.2'E and 8 m a.s.l.), or in Kagoshima (Kyushu; 31°33.3'N, 130°32.8'E and 4 m a.s.l.) in 2012 and 2013. The sweet potatoes were transferred to the Donan Agricultural Experiment Station immediately after harvest in Saitama and Kagoshima. Sweet potatoes were stored in the dark at 15°C with more than 95% relative humidity from late-October to mid-November.

Measurement of quality attributes

For analysis, 200-300 g of tuberous roots were used for each cultivar and production region. Each root was cut into thin pieces and mixed. About 50 g of cut roots were dried in a drying oven at 80°C to a constant weight for dry matter ratio determination. Starch was isolated by modification of the method of Ishiguro et al. (2003). A 40-g sample of cut root was homogenized with distilled water using an IFM-800DG food mixer (Iwatani, Tokyo, Japan) at 20,000 rpm for 90 s and then filtered through 200 mesh nylon with running water. On the following day, supernatant of the filtrate was decanted and the starch residue was dried at room temperature for two days and then at 105°C for 6 h. Dried starch was measured and the content was calculated. Starch pasting properties were investigated using a Rapid Visco Analyzer 3D (Newport Scientific, Narrabeen, Australia) with 6% (dry matter w/w) starch suspensions. The temperature was maintained at 30°C for one minute, raised from 30 to 95°C at the rate of 5°C min^{-1}, kept at 95°C for 6 min based on the methods of Katayama et al. (1999) and Tokimura et al. (2002).

Tuberous roots were cooked in a steamer for 40 minu and were sliced into 2 cm width pieces. The maximum penetration resistance of the sliced material was measured using a PSS-0.5K Mechanical Force Gauge (Imada, Aichi, Japan) with a 3-mm diameter cylinder probe as an indicator of firmness. Sliced pieces of steamed root were homogenized with distilled water equal to twice the weight of the potato pieces using a mixer at 10,000 rpm for 30 s. Each homogenate was filtered through No. 5C quantitative filter paper. The total soluble solids concentration (°Brix) of the filtrate was measured using a PAL-1 Pocket Refractometer (ATAGO, Tokyo, Japan). The differences of sensory texture and sweetness of steamed roots among production areas were evaluated, using a triangle preference test, by 21-22 panelists.

Starch gel was prepared based on a previously reported method (Tokimura et al., 2002). Starch was mixed with water to a final content of 8% (dry matter w/w) and heated to 90°C at the rate of 5°C min^{-1}. Hot starch solution was dispensed in about 20-mL aliquots into plastic cylindrical containers with 27-mm inner diameter. and sealed with plastic caps. The plastic containers were transferred to the refrigerator to cool them down to 4°C. The water separation ratio and penetration resistance of the starch gel were measured at 2 h, two weeks and four weeks after cooling the starch solution. The penetration resistance of starch gel was measured as an indicator of firmness using a TX-XT2i Texture Analyzer (Stable Micro Systems, Godalming, UK) with a P/5 Cylinder Probe (5 mm diameter) at 1 mm s^{-1} speed and calculated as the mean of stress values at 2 and 3 mm deformation.

Statistical analysis was performed with EZR (Easy R, Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria).

RESULTS AND DISCUSSION

Environmental conditions in the sweet potato production regions

The daily mean temperatures between May and November were highest in the southern Kyushu region and lowest in the northern Hokkaido region (Figure 1A). The sunshine durations in Hokkaido tended to be lower than in the other regions except for June (Figure 1B). The precipitation had greater annual range than daily mean temperature or sunshine duration with no obvious differences among the production regions (Figure 1C).

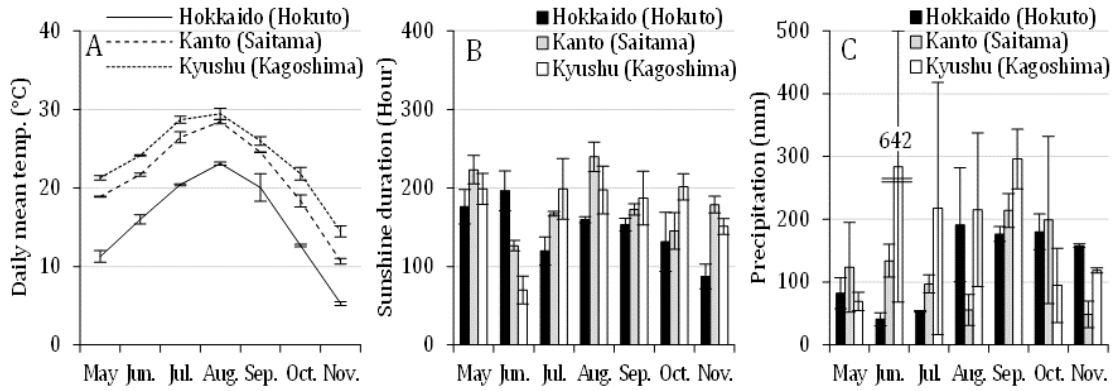

Figure 1. Transitions of daily mean temperature (A), sunshine duration (B), and precipitation (C) from May to November in Hokkaido, Kanto, and Kyushu regions. The data with bars are the averages with S.E. of 2012 and 2013 obtained from AMeDAS (Automated Meteorological Data Acquisition System).

Internal quality of raw root

The dry matter ratio of Hokkaido crops was lower than that in either Kanto or Kyushu by 2.9 ('Kampachi') and 4.7% ('Beniazuma'), which were shown to be significantly different in 2012, and significantly lower by 2.0 ('Kampachi') to 7.0% ('Koganesengan') in 2013 (Table 1). Hokkaido crops had significantly lower starch contents than any other ones with differences ranging from 2.1 ('Kokei No. 14') to 5.2% ('Koganesengan'). The dry matter ratio has been shown to have a high positive correlation with starch content (Tumwegamire et al., 2011) and has often been used as an indicator of starch content (Nakatani, 2010) and the texture of cooked root (Tomlins et al., 2012). Inukai et al. (2007) and Noda et al. (2001) showed that the dry matter ratio and starch content of sweet potato both decreased under low soil temperature conditions. Furthermore, Sumi and Koriyama (2013) reported a proportional relationship between total dry weight of sweet potato and solar radiation. It is likely, therefore, that the lower temperatures and reduced sunshine duration in Hokkaido compared with either Kanto or Kyushu, caused lower dry matter ratio and starch content of Hokkaido crops.

The pasting temperatures of the starch obtained from sweet potato crops grown in Hokkaido were significantly lower than those from either Kanto or Kyushu by 2.5 ('Kampachi' in 2012) to 8.7°C ('Koganesengan' in 2013) (Table 1). Higher soil temperatures during the development of sweet potato tuberous roots led to a clear enhancement of the starch pasting temperature (Noda et al., 2001). The starch pasting temperature of sweet potatoes grown in colder areas such as Hokkaido could, therefore, be decreased as compared to those in more temperate areas such as Kanto or Kyushu.

Table 1. Dry matter ratio, starch content, and starch pasting temperature of sweet potato grown in different production regions in 2012 and 2013.

Cultivar	Production region	Dry matter ratio (%)		Starch content (%)	Pasting temp. (°C)	
		2012	2013	2013	2012	2013
Beniazuma	Hokkaido	33.8*	33.1**	22.0**	69.8***	68.9***
	Kanto	38.5	36.3	24.4	75.1	73.0
Kokei No. 14	Hokkaido	33.7ns	30.7**	20.6*	69.9***	67.9***
	Kyushu	35.2	34.8	22.7	74.7	74.5
Koganesengan	Hokkaido	34.4ns	29.8***	20.3***	69.1***	67.6***
	Kyushu	39.1	36.8	25.5	75.8	76.3
Kampachi	Hokkaido	38.3**	36.2**	24.6**	73.1***	69.9***
	Kanto	41.2	38.2	27.3	75.6	74.3

Significance: *p<0.05, ** p<0.01, ***p<0.001, ns = not significant by t-test (*n*=5) within each cultivar in each year.

Internal quality and sensory evaluation of steamed root

Maximum penetration resistances of steamed roots produced in Hokkaido tended to be lower than those in either Kanto or Kyushu by 6.5 ('Kokei No. 14') to 20.4 kgf cm^{-2} ('Beniazuma') (Table 2). The total soluble solids concentration (°Brix) of steamed roots produced in Hokkaido was significantly higher than that in Kanto or Kyushu by 2.7 ('Kampachi') to 7.0 °Brix ('Koganesengan'). Steamed root grown in Hokkaido was evaluated moister and sweeter than that in Kanto or Kyushu for each cultivar in a triangle sensory preference test. Nakamura et al. (2010) reported that cultivars with a soggy or moderately soggy texture contained smaller amounts of starch and larger amounts of water (i.e., lower dry matter ratios). For this reason, steamed Hokkaido sweet potato, which had a lower dry matter ratio and a lower starch content, may have had a soft and moist texture compared with potatoes from either Kanto or Kyushu. On the other hand, Picha (1985) reported that the major sugars in baked roots were maltose, sucrose, glucose, and fructose and that maltose had the highest proportion of sugars due to beta-amylase activity on pasted gelatinized-starch. Nakamura et al. (2014) showed that maltose content in the heated root of cultivar 'Quick Sweet' that contained starch with a low pasting temperature was higher than that in 'Beniazuma' with a normal starch pasting temperature. The results in this study and previous reports suggest, therefore, that sweet potato grown in cold environment with a lower starch pasting temperature could change to have a sweeter taste by means of heat-cooking. Furthermore, the maximum sweetness intensity, and the time required to reach it, tended to be higher and shorter in soft gels, respectively (Bayarri et al., 2007; Mosca et al., 2012). Consequently, the softness of steamed Hokkaido sweet potatoes may also contribute to their higher perceived sweetness. Such eating qualities of Hokkaido sweet potatoes closely match latest trends in the consumption preferences for sweet potato (Kariya, 2016). Sweet potato production in Hokkaido will, therefore, likely increase with these changes in consumer preferences.

Physical properties of starch gel

The water separation ratios of starch gels two weeks after gelation ranged from 0.9 ('Kampachi' of Hokkaido) to 2.5% ('Kampachi' of Kanto) and there were only small differences among regions (Figure 2A). However, at 4 weeks the water separation ratio rose to 11.7 and 12.3% in the gel made from 'Koganesengan' at Kyushu and from 'Kampachi' at Kanto, respectively. In contrast, the increases in the water separation ratios were suppressed to 2.3 and 4.0% in 'Koganesengan' and 'Kampachi', respectively, at Hokkaido (Figure 2A). The penetration resistance of starch gels gradually increased two weeks after gelation in 'Koganesengan' and 'Kampachi' at Kyushu and Kanto, respectively (Figure 2B). However, those products from Hokkaido had a much lower penetration resistance level with both cultivars, but especially in 'Koganesengan'. Retrogradation, water separation from the starch gel, is an important property of starch that is used as a food ingredient in processing and in

various food products. Quality, as measured by food texture and physical properties, deteriorates due to retrogradation as time passes (Ishiguro et al., 2000; Qian et al., 1998). Noda et al. (2001) reported that pasting temperature of starch was negatively correlated with the molar percentage of short chain amylopectin with a degree of polymerization (DP) of 6-7. Furthermore, Ishiguro et al. (2000) indicated that the proportion of short chains of less than a DP of 10 of amylopectin was negatively correlated with hardness or water leakage of starch gel after one week to one month of storage. Ishiguro et al. (2003) also indicated that cultivation conditions such as planting or harvest period affected the retrogradation of sweet potato starch. These reports suggest that the lower starch pasting temperature of sweet potatoes grown in Hokkaido, which is different from that in the main production regions such as Kanto and Kyushu, could be more resistant to retrogradation and suitable for food processing such as with steamed sweet potato paste, hoshi-imo, as well as the production of starch dumplings.

Table 2. Firmness (maximum penetration resistance), sweetness (°Brix), and sensory evaluation (triangle preference test) of sweet potatoes grown in different production regions in 2012.

Cultivar	Production region	Maximum penetration resistance (kgf cm^{-2})	Brix value (°Brix)	Triangle preference test of Hokkaido compared to Kanto or Kyushu product		
				Correct/total	Texture	Sweetness
Beniazuma	Hokkaido	25.1**	28.9*	21/21	Moist***	Strong***
	Kanto	45.5	24.9			
Kokei No. 14	Hokkaido	8.7*	28.7**	22/22	Moist***	Strong***
	Kyushu	15.2	23.3			
Koganesengan	Hokkaido	25.8ns	26.8**	20/21	Moist***	Strong***
	Kyushu	28.1	19.9			
Kampachi	Hokkaido	23.3ns	27.1*	16/22	Moist**	Strong*
	Kanto	29.6	24.4			

Significance: *p<0.05, ** p<0.01, ***p<0.001, ns = not significant by t-test (*n*=5) or triangle performance test (*n*=21-22).

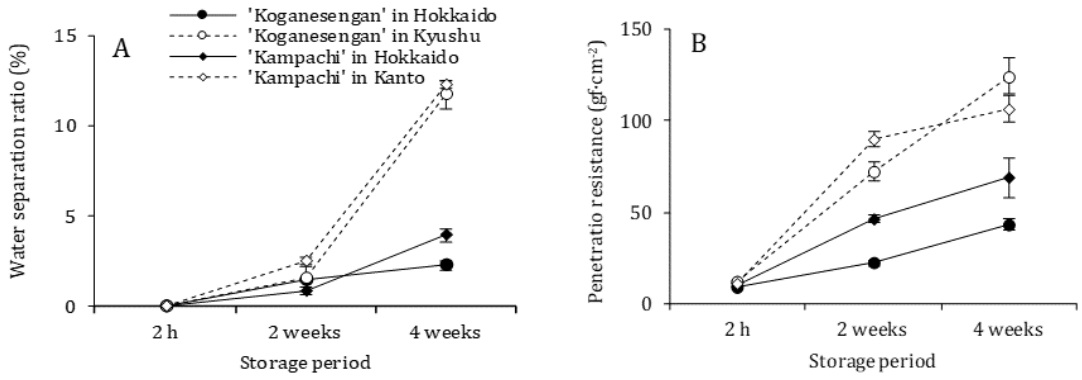

Figure 2. Changes of water separation ratio (A) and firmness (penetration resistance) (B) of starch gels from sweet potatoes grown in Hokkaido, Kanto, or Kyushu in 2013. Vertical bars indicate the standard error (*n*=5).

CONCLUSIONS

Compared with sweet potatoes grown in Kanto or Kyushu, in central or southern region Japan, the potatoes grown in northern Hokkaido showed the following features:

- Lower dry matter ratio and lower starch pasting temperature in the tuberous roots;

- Lower firmness and higher °Brix value in steamed roots;
- More moist texture and sweetness in the sensory evaluation of steamed roots;
- Lower firmness change and less water separation in starch gel stored for a long period.

Accordingly, Hokkaido sweet potatoes are closely matched with current consumer preference trends and are suitable for food processing.

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Production of mango seed butter for cosmetic use

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Abstract

Mango is one of the most important fruits of Thailand. Over 90% of production is for domestic consumption and for use in the processing industry. The processing waste is up to 40-50% by weight and 20-60% of that waste is mango seed. The flesh of the mango seed consists of fat with a fatty acid profile similar to the cocoa butter and shea butter used in cosmetic products. In this study, sliced mango seed was dried at 55°C followed by fat extraction using the Soxhlet technique with petroleum ether as the solvent. The fat content of mango seed from three cultivars, 'Kaew Kamin', 'Chok Anan', and 'Nam Dok Mai' was 7.25, 6.38 and 5.84% by weight, respectively. The main fatty acids in the mango seed fat, analyzed by GC method, were oleic acid, stearic acid, and palmitic acid, at 41.1-52.4, 20.1-32.5, and 10.5-12.9% by weight, respectively. The mango seed butters possessed anti-tyrosinase activity. Tyrosinase can cause melanin formation. The concentration of mango seed butter which could inhibit 50% of the tyrosinase activity (IC_{50}) was in the range of 0.47-1.54 mg mL^{-1}. The anti-oxidation capacity of mango seed butter was studied using the free radical scavenging method (2,2-diphenyl-1-picrylhydrozyl radical scavenging, DPPH). The anti-oxidation capacity was calculated as the concentration of sample required to scavenge 50% of the DPPH radical (SC_{50}). The SC_{50} of mango seed butter ranged from 1.02 to 45.7 mg mL^{-1}. The melting point of mango seed butter was 36.5-37.5°C which indicates that it could easily melt in the weather of Thailand. The mango seed butter was, therefore, mixed with 5-10% bee wax or carnauba wax as a stabilizer to increase the melting point and for ease of use as a cosmetic ingredient. It was shown that 5% carnauba wax was a more suitable stabilizer for the mango seed butter than bee wax due to its higher melting point.

Keywords: mango seed, mango butter, fat, anti-oxidation, anti-tyrosinase

INTRODUCTION

Mango (*Mangifera indica* L.) is ranked as the fifth most consumed fruit in the world, after citrus, banana, grape and apple. World production of mango is mainly concentrated in Asia which accounts for more than 75% of the total. The largest Asian producers are in South- and East-Asia, led by India, which produces 42% of the world's mangoes. China, Thailand, Indonesia, and Mexico are among the other major world producers (Fernandez-Stark et al., 2017). In Thailand, more than 21.06 million ha are used for agricultural production, in which mango occupied the largest fruit production area with 305,114 ha in 2008 that gradually increased to 334,029 ha in 2013 (Phavaphutanon, 2015). Over 90% of the production is for domestic consumption and for use in the processing industry. The processing waste is up to 40-50% by weight, and 20-60% of that waste is mango seed. The seed is currently discarded becoming a source of pollution and may cause outbreaks of mango seed weevil *Sternochetus olivieri* (Faust) which is a quarantine pest in many countries. It would be desirable, therefore, for the mango-seed waste from the processing industry to be utilized for the production of a value-added product.

The mango seed from different cultivars has been shown to range from 9 to 23% of the fruit weight (Palaniswamy et al., 1974). The mango seed kernel, which is 45-75% of the whole seed, consists of 7-12% fat (Gunstone, 2006). Mango seed butter could be made and

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used as an ingredient in cosmetic products, such as those used for moisturizing and restoring healthy skin. Moreover, the phenolic compounds in mango seed butter, which are antioxidants, could whiten the skin and be used for sun protection and for use in wrinkle reduction lotions (González et al., 2008). Free radical and reactive oxygen species from oxidation can stimulate skin wrinkling by increasing collagenase enzyme activity. The fat from the mango seeds also has the ability to inhibit the tyrosinase enzyme which can cause skin melanogenesis (Schieber et al., 2003).

The objectives of this research were to produce mango seed butter from mango seed waste, and to study its properties for use as a cosmetic ingredient. Such products could reduce mango processing waste and also create value to this mango waste component.

MATERIALS AND METHODS

Mango seed butter extraction

Mango seeds from 'Kaew Kamin', 'Chok Anan' and 'Nam Dok Mai' cultivars was collected from a mango processing plant. They were cleaned thoroughly and the seed coat was removed by peeling. The mango seed kernel was then sliced to about 0.1 mm thickness and dried at 55°C for 20 h. The dried mango seed kernel was ground and mango seed butter was extracted using the Soxhlet method with petroleum ether as the solvent. The ratio of dry mango seed: solvent was 1:3 and the extracting temperature was 70°C maintained for 14 h. The quality of the extracted mango seed butter was evaluated for fat content, fatty acid composition and other properties such as color, melting point, anti-tyrosinase activity and anti-oxidation activity.

Formation of mango seed butter flakes

The mango seed butter was processed into flake form by the addition of wax for convenient use in different cosmetic products. Bee wax and carnauba wax were used. The wax was mixed at 5.0, 7.5 and 10.0% (w/w) in melted mango seed butter. The mixture was then dropped onto a silicone plate (Silpat™) at 1 cm diameter and allowed to solidify at 25°C.

Quality evaluation

1. Fat content.

The extracted fat from dried mango seed was vacuum evaporated at 40°C for 2 h to eliminate the solvent and the fat was weighted and the fat content calculated.

2. Fatty acid composition.

A 25-mg sample of mango seed butter was weighted into a test tube then 2 mL of boron trifluoride-methanol solution and 250 µL of 2,2-dimethoxypropane was added. The sample was heated at 60°C for 10 min in a water bath. Subsequently, one mL of water and one mL of hexane were added. The clear solution on top was removed to a new test tube and anhydrous sodium sulfate was added for removing the water. The sample solution was filtered and fatty acid composition was analyzed by GC with FID detector (Buchanan et al., 2011).

3. Anti-tyrosinase activity.

The extracted mango seed butter was dissolved with 5% Tween-20 for concentrations of 0.001, 0.01, 0.1, 1 and 10 mg mL^{-1} and then tested for tyrosinase inhibition activity using the Dopachrome method in a microwell plate base following the technique of Manosroi et al. (2011). Anti-tyrosinase activity was calculated as the concentration of the sample which was able to inhibit 50% of tyrosinase activity (IC_{50}).

4. Anti-oxidation activity.

The extracted mango seed butter was dissolved in hexane for concentrations of 0.001, 0.01, 0.1, 1 and 10 mg mL^{-1}, then scavenging activity using 2,2-diphenyl-1-picryhydrazyl

(DPPH) radical, based on the method of Ranasinghe et al. (2015), was measured. A 50-µL aliquot from each sample concentration was mixed with 70 µL of methanol in a microwell plate and a pre-reaction absorbance reading was recorded at 517 nm. Then 80 µL of 0.5 mM DPPH in methanol was added and the mixture was incubated for 15 min at 25°C in the dark before a further absorbance reading at 517 nm. The anti-oxidation activity was calculated as the concentration of the sample which was able to scavenge 50% of the free radical (SC_{50}).

5. Color.

The color of mango seed butter from the three cultivars was measured as CIELAB which expressed color as three values: L* for lightness from black (0) to white (100), a* from green (−) to red (+), and b* from blue (−) to yellow (+) using a Konica Minolta Chroma meter: model: CR-400.

6. Melting point.

The melting point of mango seed butter was analyzed by the capillary melting point method based on O'Brien (2008). Mango seed butter was liquified at 60°C and kept at -18°C for 6 h. The solid fat was placed into one end of a closed-capillary tube about 0.5-1.0 cm from the tip of the tube, with the tube tied to a thermometer. The capillary tube with the thermometer was plugged into a cork stopper and attached to a clamp. It was then placed in a beaker of glycerol and gradually heated. The melting temperature of the sample was recorded as the melting point.

Statistical analysis

The experiment was conducted as a completely randomized design, and each treatment comprised four replicates. All data were analyzed by analysis of variance (ANOVA) and Duncan's multiple range test was applied and significant differences were recognized at $p \leq 0.05$.

RESULTS AND DISCUSSION

Fat content

The mango seed kernel from the three different cultivars had significantly different fat content ($p<0.05$). The mango seed from 'Keaw Kamin' had the highest fat content followed by 'Chok Anan' and 'Nam Dok Mai' which had 7.25±0.23, 6.38±0.22 and 5.84±0.18% on a dry weight basis, respectively. It has previously been reported that different mango cultivars contained different amounts of fat content varying from 3.7 to 12.6% (Lakshminarayana et al., 1983) and generally found between 7.7 and 10.6% (Bhatacharya, 1987). However, in some cultivars, the fat content in the seed kernel was greater than these values. For example, Mutua et al. (2017) found that mango seed in cultivars 'Apple' and 'Ngowe' from Kenya had fat contents of 13.04 and 13.08%, respectively.

Fatty acid composition

Unsaturated fatty acids were the major components in mango seed butter from the three cultivars (Table 1) being 62.11, 64.98 and 54.87% (w/w), respectively. Oleic acid and linoleic acid were the major unsaturated fatty acids whereas stearic acid and palmitic acid were the major saturated fatty acids (Table 1). The main fatty acids of mango seed butter in this study were, therefore, oleic acid and stearic acid which is consistent with the finding of Abdel-Razik et al. (2012) that the major fatty acids in the cultivar 'Zebda' were similarly oleic and stearic at concentrations of 37.19 and 45.58%, respectively. Jahurul et al. (2018), with the cultivar 'Waterlily' found oleic and stearic concentrations of 41.41 and 42.27%, respectively.

The fatty acid profile of mango seed butter was, therefore, similar to shea butter and cocoa butter, both of which melt at body temperature. Cocoa butter was found to contain stearic, palmitic and oleic acid as the major fatty acids at 33.7-40.2, 24.5-33.7 and 26.3-35.0% (w/w), respectively (Naik and Kumar, 2014). In comparison, shea butter contained stearic,

palmitic and oleic acid of 42, 4 and 45% (w/w), respectively (Oluwaseyi, 2015).

Table 1. Fatty acid composition of mango butter from three mango cultivars.

Fatty acid composition (%w/w)	Keaw Kamin	Chok Anan	Nam Dok Mai
Saturated fatty acid	37.89	35.08	45.14
Palmitic acid (C16:0)	10.91	12.97	10.46
Stearic acid (C18:0)	24.80	20.10	32.47
Arachidic acid (C20:0)	1.53	1.28	1.34
Behenic acid (C22:0)	0.31	0.30	0.42
Lingoceric acid (C24:0)	0.35	0.43	0.45
Unsaturated fatty acid	62.11	64.98	54.87
Cis-9-oleic acid (C18:1n9c)	45.53	52.36	41.06
Cis-11eicosenoic acid (C20:1n11)	0.47	0.36	ND
Linoleic acid (C18:2n6c)	13.11	9.83	8.41
Alpha-linolenic acid (C18:3n3)	2.08	1.93	1.18
Erucic acid (C22:1n9)	0.92	ND	ND
Eicosadienoic acid (C20:2n6)	ND	0.50	ND
Docasahexaenoic acid (C22:6n-3)	ND	ND	3.69
Nervonic acid (C24:1n-9)	ND	ND	0.53

Fatty acid content shown in this table came from an average of four replications.
ND = not detected.

Anti-tyrosinase activity

One of the important properties of butters used in cosmetic products is anti-tyrosinase activity. This enzyme can cause skin melanogenesis and hyperpigmentation by accelerating the oxidation of phenolic compounds (Panzella and Napolitano, 2019). The mango seed butters from the three cultivars were analyzed for anti-tyrosinase activity which was calculated as the concentration of mango seed butter which could inhibit 50% of tyrosinase activity (IC_{50}). The mango seed butter from 'Kaew Kamin' had the highest anti-tyrosinase activity (lowest IC_{50} of 0.47 mg mL^{-1}) following by 'Nam Dok Mai' and 'Chok Anan' of 0.89 and 1.54 mg mL^{-1}, respectively. In comparison, kojic acid which is used as a commercial whitening ingredient had a IC_{50} of 0.02 mg mL^{-1} (Table 2). While kojic acid has greater inhibition of tyrosinase and better stability in cosmetic products than the mango seed butter at high concentrations it is carcinogenic and damages the skin (Miyazawa and Tamura, 2007). Consequently, it is considered necessary to find alternative natural ingredients which can provide greater safety for long-term use.

Table 2. Anti-tyrosinase activity of mango seed butter from three cultivars, with kojic acid as a control.

Sample	Anti-tyrosinase activity (IC_{50}) (mg mL^{-1})[a]
Mango seed butter 'Kaew Kamin'	0.47±0.06c
Mango seed butter 'Chok Anan'	1.54±0.09a
Mango seed butter 'Nam Dok Mai'	0.89±0.07b
Kojic acid[b]	0.02±0.00

Averages in the same column followed by the same letters are not significantly different at 95% level by DMRT.
[a]IC_{50} – concentration in mg mL^{-1} required for inhibition of tyrosinase activity by 50%. Mean value, *n*=4.
[b]Kojic acid is the commercial cosmetic ingredient used as a positive control.

Anti-oxidation activity

Antioxidants in fruits can inhibit or delay the oxidative damage of proteins, nucleic acids and lipids caused by reactive oxygen species (ROS) during environmental stresses (Parrado et al., 2019). ROS will stimulate wrinkling of the skin by increasing the activity of

collagenase enzyme (Jenkins, 2002). Therefore, a high anti-oxidation activity is considered necessary for the ingredients used in cosmetic products. The mango seed butter was analyzed for anti-oxidation activity using the 2,2-diphenyl-1-picrylhydrozyl free radical scavenging method (DPPH method) and the anti-oxidation activity was calculated as the concentration of sample required to scavenge 50% of the DPPH radical (SC_{50}). Mango seed butter from 'Keaw Kamin' had the lowest SC_{50} of 1.02 mg mL^{-1} (consequently the highest anti-oxidation activity) followed by 'Nam Dok Mai' and 'Chok Anan' (Table 3). Soong and Barlow (2004) found that mango seed kernel had higher antioxidant activity than the seeds of tamarind, longan, avocado and jackfruit.

Table 3. Anti-oxidation activity of mango seed butter from three cultivars, with ascorbic acid as the positive control.

Sample	Anti-oxidation activity (SC_{50}) (mg mL^{-1})[a]
Mango seed butter 'Kaewkamin'	1.02±0.09c
Mango seed butter 'Chok Anan'	45.71±1.44a
Mango seed butter 'Nam Dok Mai'	12.72±0.56b
Ascorbic acid[b]	0.06±0.01

Averages in the same column followed by same letters are not significantly difference at 95% level by DMRT.
[a]SC_{50} – concentration in mg mL^{-1} required for scavenging the DPPH radical by 50%. Mean value, *n*=4.
[b]Ascorbic acid commercial cosmetic ingredient used as the positive control.

Color

Mango seed butter from 'Kaew Kamin' had the highest lightness (L*) value followed by 'Chok Anan' and 'Nam Dok Mai' (Table 4). The red-green (a*) value, which was low in all cultivars, was highest from 'Kaew Kamin' followed by 'Nam Dok Mai' and 'Chok Anan'. For the blue-yellow contrast (b*), 'Chok Anan' had the highest value followed by 'Kaew Kamin' and 'Nam Dok Mai'. The light pale-yellow color of the mango seed butter could be attributed to the content of carotenoids which have been found to be 1.02 mg 100 g^{-1} of dry mango kernel (Mostafa, 2013).

Melting point

The melting points of mango seed butters from 'Kaew Kamin', 'Chok Anan' and 'Nam Dok Mai' were all similar and were approximately 37°C (Table 4). This melting point is similar to the melting point of shea butter which is usually used as a cosmetic ingredient due to its meltable property at body temperature. The melting point of shea butter was found to vary between 25 and 45°C with an average of 35.9°C (Womeni et al., 2006).

Table 4. Color (CIELAB values) and melting point of mango seed butter from three mango cultivars.

Sample	Color score			Melting point (°C)
	Lightness (L*)	Green-red (a*)	Blue-yellow (b*)	
Mango seed butter 'Kaew Kamin'	72.23±0.55a	-3.27±0.18a	13.48±0.18b	36.75±0.29b
Mango seed butter 'Chok Anan'	59.62±0.59b	-0.64±0.04b	32.20±0.49a	36.50±0.41b
Mango seed butter 'Nam Dok Mai'	56.09±0.11c	-0.66±0.02b	12.35±0.34c	37.50±0.41a

Averages in the same column followed by same letters are not significantly difference at 95% level by DMRT.

Formation of mango seed butter flakes

According to its high cosmetic properties, especially its anti-tyrosinase and anti-oxidation activities, mango seed butter from 'Kaew Kamin' was chosen for the development of a dry flake form. This was done by mixing it with 5-10% (w/w) bee wax or carnauba wax as a stabilizer to increase the melting point for ease of use as an ingredient in cosmetic products along with convenience for distribution under hot weather conditions. The carnauba wax was a more suitable stabilizer for mango seed butter than bee wax (Table

5). The mango seed butter mixed with carnauba wax had higher melting point (6.58-8.92°C as compared to the control without stabilizer) than that of bee wax (1.92-2.08°C) (Table 5). The melting point of the mango seed butter mixed with 5% of carnauba wax made the mango seed butter more stable during storage with a melting point of 43.33°C. This would make it suitable for use as an ingredient in cosmetic products such as body lotions, moisturizer bars and body scrubs.

Table 5. Melting temperature of mango seed butter from 'Kaewkamin', with different concentrations of carnauba wax and bee wax.

Type of wax	Concentration (%w/w)	Melting point (°C)
Carnauba wax	5.0	43.33±0.58a
	7.5	45.33±0.58a
	10.0	45.67±1.53a
Bee wax	5.0	38.67±0.58b
	7.5	38.67±0.58b
	10	38.83±0.76b
Control	0	36.75±0.29

Averages in the same column followed by same letters are not significantly difference at 95% level by DMRT.

CONCLUSIONS

Mango seed butter from three cultivars, 'Kaew Kamin', 'Nam Dok Mai' and 'Chok Anan' extracted using the Soxhlet method, had both anti-tyrosinase and anti-oxidation activity indicating its suitability for use as an ingredient in cosmetic products. The mango seed butter from 'Kaew Kamin' had the highest tyrosinase inhibitory activity (IC_{50}) of 0.47 mg mL^{-1} and the highest anti-oxidation activity (SC_{50}) of 1.02 mg mL^{-1}. The mango seed butter from 'Kaew Kamin' was suitable when developed into flake form for ease of use as a cosmetic ingredient when it was mixed with 5% carnauba wax. This increased the melting point of mango seed butter to 43.33°C which would make it stable during storage and distribution.

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