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Evaluation of antifungal activity of essential oils against aflatoxigenic *Aspergillus flavus* and their allelopathic activity from fumigation to protect maize seeds during storage

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ABSTRACT

Four essential oils were evaluated for antifungal activities against ten isolates of *Aspergillus flavus*. All strains, except *A. flavus* PSRDC-2, were strongly inhibited by clove (*Syzygium aromaticum* (L.) Merr. & L.M. Perry) and vatica (*Vatica diospyroides* Symington) oil. The oils contained 14 and 24 compounds, respectively, with eugenol (62.4%) as the main component of clove oil and benzyl acetate (48.8%) as the main component of vatica oil. Clove oil showed 84.7% inhibition on conidial germination of *A. flavus* PSRDC-2 at 100 $\mu L\,L^{-1}$, while complete inhibition of disease infection on maize seeds occurred at 10 $\mu L\,L^{-1}$. Vatica oil at 50 $\mu L\,L^{-1}$ exhibited strong antifungal activity as it completely inhibited growth, sporulation, conidial germination, and disease infection of *A. flavus* PSRDC-2 both *in vitro* and on maize seeds. Fumigation of vatica oil for 6 h could protect and cure the infected maize seed. Both clove and vatica oils exhibited dose-dependent allelopathic activity on maize germination, stem length, and root length, with vatica oil being more phytotoxic than clove oil.

1. Introduction

Maize (*Zea mays* L.) is the most produced cereal in the world. Deterioration of stored seeds, including maize, caused by fungi is a common problem in Thailand warehouse systems due to the warm and humid climate. Various microorganisms, especially *Aspergillus flavus*, were frequently found to contaminate maize seeds during storage (Ellis et al., 1991). Infections by *A. flavus* and others reduce the quality and economic value of stored products (Ng'ang'a et al., 2016). The Phitsanulok Seed Research and Development Center in the Department of Agriculture of Thailand is responsible for quarantining the maize seeds before export.

The antimicrobial properties of plant products have been well known and used for food preservation worldwide (Tiwari et al., 2009; Zahed et al., 2010; Li et al., 2016; Nerilo et al., 2016). Among the different groups of plant products, essential oils are especially recommended as one of the most promising for the formulation of safer antifungal agents (Varma and Dubey, 2001). Food spoilage fungi are usually controlled by chemical fungicides; however, these agents often have adverse effects on human health and environment (Vicente et al., 2004). Using chemical fungicides is even more harmful when applied during the post-harvest period, as there is only a short break between treatment and consumption.

Repeated usage can also cause some fungi to resist broad spectrum fungicides such as benzimidazoles, prochloraz, and imazalil. Therefore, research on using essential oils, which are natural antimicrobial and antifungal substances, has received much attention. Application of essential oils to control *Aspergillus* spp. on food and agricultural products have been reported: thyme essential oils in bakery products (Guynot et al., 2003); bay, clove, and cinnamon oil in wheat grain (Aldred et al., 2008); ergosterol oil in stored shelled groundnuts (Dhingra et al., 2009), Mexican oregano (*Lippia berlandieri* Schauer); cinnamon (*Cinnamomum verum*) or lemongrass (*Cymbopogon citratus*) essential oils in edible films (Avila-Sosa et al., 2012); cinnamon essential oil in food packaging, *Origanum vulgare* L. and *Rosmarinus officinalis* L. essential oils in grapes (de Sousa et al., 2013); *Ageratum conyzoides* (mentrasto) and *Origanum vulgare* (oregano) essential oils in corn and soybeans; *Chenopodium ambrosioides* and *Clausena pentaphylla* essential oils in storage of pigeon pea seeds (Pandey et al., 2014); and *Litsea cubeba* essential oil in licorice (Li et al., 2016).

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Essential or volatile oils are complex mixtures of volatile constituents biosynthesized by plants, which mainly include terpenes, terpenoids, aromatic, and aliphatic constituents, all characterized by low molecular weight (Bassole and Juliani, 2012). Essential oils can exhibit antimicrobial and allelopathic properties (de Feo et al., 2002). The allelopathic potential of some essential oils has been previously reported, including *Ruta graveolens* essential oils (de Feo et al., 2002), *Schinus areira* essential oils (Scrivanti et al., 2003), *Artemisia princeps* essential oils (Liu et al., 2006), *Juneperus ashei* essential oils (Young and Bush, 2009), and *Schinus molle* essential oils (Zahed et al., 2010). It was reported that the essential oils from two *Achillea* species have inhibitory effects on the germination and seeding growth of *Amaranthus retroflexus*, *Cirsium arvense*, and *Lactuca serriola* (Kordali et al., 2009).

Control of pathogenic fungi in food and agricultural products by direct contact with essential oils from various plants has been reported. Examples of control of *A. flavus* by using essential oils include *Cinnamomum jensenianum* Hand.-Mazz and *Zanthoxylum molle* Rehd. (Tian et al., 2012, 2014), *Zingiber officinale* (Nerilo et al., 2016), and *Litsea cubeba* (Li et al., 2016). Clove oil (Passone et al., 2012), and thyme oil (Passone et al., 2012; Stevic et al., 2014; Bernardos et al., 2015) were used to control *Aspergillus* spp. In addition, control of different *Penicillium* species by applying essential oil of *Citrus reticulata* Blanco (Tao et al., 2014) and rosemary (Teodoro et al., 2014), and control of *Fusarium verticillioides* by *Rosmarinus officinalis* L. essential oil (da Silva Bomfim et al., 2015) were also reported. Vatica (*Vatica diospyroides* Symington), an endemic medicinal and fragrant dipterocarp of Thailand, has strong anticancer activity (Kinghorn et al., 2011; Srisawat et al., 2013), but its effect against *A. flavus* has not been studied.

Controlling post-harvest pathogens can be achieved by applying essential oils and plant extracts using direct contact or fumigation methods. When essential oils are applied directly on a food surface by dipping, powdering, or spraying, their highly hydrophobic and volatile active substances are bound by food components, while other components of the essential oils are partitioned through the product according to their affinity with water (Avila-Sosa et al., 2012). Fumigation of essential oils is applied when direct application cannot be used. However, to our best knowledge, there are no reports on the control of *A. flavus* by fumigation with essential oils. Thus, the objectives of this study are (i) to evaluate the effect of four essential oils on ten isolates of *A. flavus*, (ii) to study the effects of concentration of the two most effective essential oils on the most recalcitrant strain of *A. flavus* both *in vitro* with potato dextrose agar (PDA) and *in vivo* with maize seed, and (iii) to assess the effects of the two selected essential oils on the germination of maize seed.

2. Materials and methods

2.1. Pathogenic fungi and inoculum production

Ten strains of *Aspergillus flavus*, namely PSRDC-1, PSRDC-2, PSRDC-3, PSRDC-4, PSRDC-5, PSRDC-6, PSRDC-7, PSRDC-8, PSRDC-9, and PSRDC-10, were used in this study. They were isolated from contaminated maize seed and provided by Phitsanulok Seed Research and Development Center, Department of Agriculture, Thailand. All strains were maintained on PDA (39 g L^{-1}; Difco Laboratory) at 4 °C. Spore inocula of pathogenic fungi were collected in 5 mL of water on 7-day-old cultures. The spore suspensions were vortexed with glass beads (2.5-mm-dia.) to separate the spores and then filtered through 30 μm mesh filters to remove mycelial fragments. The spore count was performed using a hemacytometer before being diluted with sterilized water to achieve a concentration of 10^6 spores mL^{-1}.

2.2. Screening of cinnamon, clove, capsicum and vatica oil against ten isolates of A. flavus

The four essential oils used in this study are of cinnamon (*Cinnamomum bejolghota*), clove (*Syzygium aromaticum* (L.) Merr. & L.M. Perry), capsicum (*Capsicum annuum* var. acuminatum Fingerh), and vatica (*Vatica diospyroides* Symington), purchased from Saiburi Samoonprai Ordinary Partnerships Company Hat Yai, Thailand. Their effects on radial growth of the ten strains of *A. flavus* on PDA were evaluated using an antifungal bioassay (Li et al., 2012). Large Petri dishes (140 mm-dia. × 20 mm height with 0.5 L inner volume) contained four small Petri dishes (50 mm dia. × 15 mm height). Three of the smaller dishes each contained 5 mL of PDA inoculated with a 0.5-cm-dia. fungal plug from the periphery of an actively grown culture of pathogenic fungi, while the fourth dish contained a piece of autoclaved filter paper (Whatman®), to which one of the essential oils was added at 50 μL L^{-1} (droplet to filter paper in an enclosed Petri dish system). Equivalent amounts of sterile distilled water were used as a control. The Petri dishes were covered with a lid, sealed with parafilm M®, and incubated at room temperature (28 ± 2 °C). After 2 days of incubation, the diameters of the colonies in the smaller plates were recorded. For each treatment, there were three replicates, and the experiment was repeated three times. The two most effective essential oils were selected for further studies along with the most recalcitrant fungal isolate.

To assess whether the growth suppression activity of the essential oils inhibited or killed the pathogen, a plug (0.5-cm-dia.) of the pathogenic fungi from the no-growth PDA agar plate was transferred to a new PDA plate and incubated at room temperature (28 ± 2 °C) for 5 days. If there was still no growth, then the pathogen was killed.

2.3. Chemical composition of the selected essential oils

The selected essential oils were analyzed for their chemical composition by a gas chromatograph mass spectrometer (GC–MS) (Trace GC Ultra/ISQMS, Thermo Scientific Inc., USA) equipped with a HP-5MS UI column (30 m × 250 μm, film thickness 0.25 μm, Agilent, Santa Clara, CA, USA) according to the method by Wang et al. (2009).

2.4. Effects of antifungal activity of the selected essential oils at various concentrations on inhibition of growth, sporulation, and conidial germination of the selected strain of A. flavus on PDA plates

An antifungal bioassay was performed to study the effects of the concentrations of clove and vatica oil on radial growth and sporulation of the selected strain of *A. flavus* on PDA plates (as described above). Concentrations of essential oil varying from 0, 1, 10, 50, and 100 μL L^{-1} were added (droplet to filter paper in an enclosed Petri dish system). Similarly, the Petri plate without essential oil treatment was set as a control. After 5 days incubation at room temperature (28 ± 2 °C), the diameters of the colonies in the smaller plates were recorded, and the total number of the conidia per plate was assessed. There were three replicates, and the experiment was repeated three times.

To study the effect of the concentration of the selected essential oils on conidial germination, 50 μL aliquots at a high concentration of suspended conidia (10^4 conidia mL^{-1}) were spread onto PDA in smaller plates. Each concentration of the essential oil was placed alongside the conidial suspensions inside larger plates and sealed as above. Conidial germination was examined after 24 h of incubation. To know whether the essential oil fumigation could kill conidia or just delay the germination, conidia exposed to the essential oils were tested according to the method as described by Li et al. (2010).

There were three replicates, and the experiments were repeated three times.

2.5. Effect of fumigation with the selected essential oils on infection and sporulation of A. flavus on maize seeds at different concentrations

Maize (*Zea mays* L.) seeds were soaked in 100 mL of distilled water for 5 h, and then autoclaved at 121 °C for 15 min (Yang and Chang, 2010) before being used. The clove and vatica oil were tested for infection and sporulation of the selected strain of *A. flavus*. Five maize seeds were transferred to three of the smaller dishes, and then 50 μL of spore inocula of the selected strain of *A. flavus* at 10^5 spore mL^{-1} were spread on each maize seed. For the fourth dish, various concentrations of essential oils (0, 1, 10, 50, 100 μL L^{-1}) were added to each Petri dish and the same amounts of sterile distilled water were used as a control. All Petri dishes were covered with a lid, sealed with parafilm M®, and incubated at room temperature (28 ± 2 °C) for 5 days. Then, the maize seeds in the dishes were individually analyzed for the percentage of seeds infected and percentage reduction of infected seeds under stereo-binocular microscope (Sumalan et al., 2013). They were calculated using the following formula: Percentage of seeds infected = [(Number of infected maize seeds/Number of maize seeds) × 100%] (Doolotkeldieva, 2010), and percentage reduction of infected seeds = [{(Control – Treatment)/Control} × 100%]. The total number of conidia per seed was assessed by mixing the infected maize seeds with sterile water to dilute before counting the number of conidia under compound microscope. There were three replicates (five maize seeds each), and the experiment was conducted three times.

2.6. Preventive and curative effects of the selected essential oils against A. flavus on maize seeds

Experiments on the preventive effects of clove and vatica oil against *A. flavus* on maize seeds were conducted. Fifteen maize seeds were fumigated with 50 μL L^{-1} of each essential oil at 0, 6, 12, and 24 h and transferred to three of the smaller dishes, and then 50 μL of spore suspension of *A. flavus* at 10^5 spore mL^{-1} were spread on each maize seed and incubated at room temperature (28 ± 2 °C) for 5 days. Similarly, the Petri dish without essential oil treatment was set as a control.

The curative effects of the selected essential oils against *A. flavus* on maize were studied. Fifteen maize seeds were inoculated with 50 μL of a spore suspension of the selected strain of *A. flavus* at 10^5 spore mL^{-1} and transferred to three of the smaller dishes, followed by fumigation with 50 μL L^{-1} of each essential oil at 0, 6, 12, and 24 h after being inoculated with *A. flavus*. All treatments were incubated at room temperature (28 ± 2 °C) for 5 days. Similarly, the Petri dish without essential oil treatment was set as a control. After 5 days of incubation the maize seeds in the dishes were individually tested for fungal growth under stereo-binocular microscope (Sumalan et al., 2013). The percentage of both preventive and curative protection was determined using the following formula: Protection percentage = [{(Control – treatment)/Control} × 100%]. There were three replicates, and the experiment was conducted three times.

2.7. Evaluation of the efficacy of the fumigation protective period of the selected essential oils to control A. flavus on maize seeds

Five treatments were established to test the efficacy of the fumigation protective period of vatica oil to control *A. flavus*. A dose of 50 μL L^{-1} of the vatica oil (the minimum dose that completely controlled maize seeds infection) was chosen for all treatments. The maize seeds were fumigated with the selected essential oil for 0, 1, 2, 4, and 6 h, after which the fumigant was removed. A 50-μL spore suspension of *A. flavus* (10^5 spore mL^{-1}) was inoculated on maize seeds and incubated at room temperature (28 ± 2 °C). The infected maize seeds from each experiment were monitored every 2 days for 14 days under stereo-binocular microscope (using the method as previously described in section 2.5). The protection percentage was calculated using the following formula: Protection percentage = [{(Control – treatment)/Control} × 100%]. There were three replicates, and the experiment was conducted three times.

2.8. In vitro phytotoxicity of fumigation with the selected essential oils on maize seed germination

Maize seeds were surface sterilized with 15% sodium hypochlorite for 10 min before being washed with abundant distilled water (Zahed et al., 2010). One hundred seeds were fumigated by using the selected essential oils at concentrations of 0, 5, 10, 50, and 100 μL L^{-1} for 24 h and placed on moist paper at the bottom of a plastic box (120 mm width × 170 mm length × 68 mm height with 1 L inner volume), then incubated at room temperature (28 ± 2 °C) for 5 days. Distilled water was used as a control treatment. The toxicity of seed germination was reported as percentages of seed germination, stem length, and root length. For each treatment, there were four replicates.

2.9. Statistical analysis

Statistical Package for the Social Sciences (SPSS) version 15 for Windows was used to analyze the data using the ANOVA module of Statistica software. Duncan's Multiple Range Test (DMRT) was employed for evaluation of statistical significance ($p < 0.05$).

3. Results

3.1. Screening of cinnamon, clove, capsicum and vatica oil against ten isolates of A. flavus

The influence of capsicum, cinnamon, clove, and vatica oil fumigation on growth inhibition of ten isolates of *A. flavus* is illustrated in Table 1A. The four essential oils significantly inhibited the mycelial growth of all isolates of *A. flavus* on PDA plates ($p < 0.05$). Clove and vatica oil fumigation proved to be the most effective as they completely inhibited (100%) nine isolates of *A. flavus*. Cinnamon oil and capsicum oil were less active (27.8–76.9% and 14.9–34.3% inhibition, respectively). *A. flavus* PSRDC-2 was the only strain that showed resistance to all four essential oils with inhibition percentages of 14.9%, 27.8%, 58.7%, and 71.5%, respectively.

The effect of fumigation with these four essential oils on the viability of the ten isolates of *A. flavus* on PDA was determined after fumigating for 5 days by transferring to a fresh PDA plate (Table 1B). All strains of *A. flavus* remained alive after fumigation with capsicum and cinnamon oil, and only *A. flavus* PSRDC-2 remained alive after fumigation with all four essential oils. The viability results were in agreement with the inhibition results. Therefore, clove and vatica oil were selected for further studies as they strongly inhibited the mycelial growth of *A. flavus*. In addition, *A. flavus* PSRDC-2 was selected as the strain to test against, as it was the only strain that demonstrated resistance against the four essential oils tested.

3.2. Chemical composition of the clove and vatica oil

A total of 14 and 24 different components were detected from the clove oil (Table 2) and vatica oil (Table 3), respectively. They represented 99.2% and 77.0% of the total components of clove and vatica oil, respectively, as identified by GC–MS analyses. The major

Table 1
Effect of fumigation with four essential oils (droplet to filter paper in an enclosed Petri dish system) on inhibition percentages (A) and viability (B) of *Aspergillus flavus* on PDA plate.

(A)

Essential oils	Percent inhibition of mycelia growth[a]									
	A. flavus PSRDC-1	*A. flavus* PSRDC-2	*A. flavus* PSRDC-3	*A. flavus* PSRDC-4	*A. flavus* PSRDC-5	*A. flavus* PSRDC-6	*A. flavus* PSRDC-7	*A. flavus* PSRDC-8	*A. flavus* PSRDC-9	*A. flavus* PSRDC-10
Capsicum oil	24.23 ± 0.26^{c}	14.98 ± 0.30^{d}	20.20 ± 0.30^{c}	21.08 ± 0.23^{c}	23.05 ± 0.19^{c}	21.99 ± 0.05^{c}	20.00 ± 0.16^{c}	30.69 ± 0.14^{c}	31.63 ± 0.44^{c}	34.36 ± 0.13^{c}
Cinnamon oil	37.31 ± 0.16^{b}	27.84 ± 0.53^{c}	30.05 ± 0.43^{b}	66.49 ± 0.43^{b}	76.95 ± 0.67^{b}	63.07 ± 0.98^{b}	53.16 ± 0.57^{b}	65.35 ± 0.56^{b}	65.58 ± 0.75^{b}	59.91 ± 0.24^{b}
Clove oil	100 ± 0^{a}	58.76 ± 0.44^{b}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}
Vatica oil	100 ± 0^{a}	71.56 ± 0.45^{a}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}

(B)

Essential oils	Viability of *A. flavus* after fumigation with essential oils[b]									
	A. flavus PSRDC-1	*A. flavus* PSRDC-2	*A. flavus* PSRDC-3	*A. flavus* PSRDC-4	*A. flavus* PSRDC-5	*A. flavus* PSRDC-6	*A. flavus* PSRDC-7	*A. flavus* PSRDC-8	*A. flavus* PSRDC-9	*A. flavus* PSRDC-10
Capsicum oil	A	A	A	A	A	A	A	A	A	A
Cinnamon oil	A	A	A	A	A	A	A	A	A	A
Clove oil	D	A	D	D	D	D	D	D	D	D
Vatica oil	D	A	D	D	D	D	D	D	D	D

[a] Mycelial growth was measured after inoculation for two days at room temperature (28 ± 2 °C). Data are the mean of six replicates ± standard errors (SE). Data followed by same letter within each column are not significantly different using ANOVA after Duncan's Multiple Range Test.
[b] Viability of *Aspergillus flavus* was determined five days after exposure to essential oils and transferred to a fresh PDA plate, A = alive and D = dead.

components detected from clove oil were eugenol (62.4%) and benzenemethanol (21.3%), while those detected from vatica oil were benzyl acetate (48.8%), benzyl benzoate (5.2%), 3-cyclohexene-1-methanol, α,α,4-trimethyl (5.1%), and isoeugenol (4.6%).

3.3. Effects of antifungal activity of clove and vatica oil at various concentrations on inhibition of growth, sporulation, and conidial germination of A. flavus PSRDC-2 on PDA plates

The inhibition of mycelial growth of *A. flavus* PSRDC-2 increased with increasing concentrations of clove and vatica oil during 5 days of incubation (Fig. 1A). At low concentrations (1 and 10 $\mu L\,L^{-1}$), clove oil showed stronger growth inhibition (85.7% and 94.0%, respectively) than vatica oil (64.0% and 74.8%, respectively). However, at high concentrations (50 and 100 $\mu L\,L^{-1}$), both clove and vatica oil yielded complete inhibition (100%) of growth.

The inhibition of conidial germination of *A. flavus* PSRDC-2 increased with increasing concentrations of clove and vatica oil after 24 h of incubation at room temperature (28 ± 2 °C) (Fig. 1B). At lower concentrations (1 $\mu L\,L^{-1}$), clove oil showed stronger conidial germination inhibition (29.6%) than vatica oil (7.6%). However, at high concentration (50 $\mu L\,L^{-1}$), only vatica oil yielded complete inhibition (100%) of conidial germination on *A. flavus* PSRDC-2. At the highest concentration (100 $\mu L\,L^{-1}$), clove oil still showed lower inhibition (84.7%) than vatica oil (100%).

Sporulation of the tested *A. flavus* PSRDC-2 (Fig. 1C) was significantly ($p < 0.05$) decreased with the increase of clove and vatica oil. The spore numbers of *A. flavus* PSRDC-2 formed on plates gradually reduced, and the inhibition rate increased with the increase of the oil concentration. Complete inhibition (100%) of sporulation of *A. flavus* PSRDC-2 was achieved at 10 $\mu L\,L^{-1}$ of clove oil and 50 $\mu L\,L^{-1}$ of vatica oil.

Table 2
Chemical composition (relative area percentage) of the clove oil detected by GC–MS analysis.

Number	Retention time (min)	Compound	Composition(%)
1	12.55	Benzenemethanol	21.33
2	16.51	Benzyl acetate	0.04
3	17.38	Methyl salicylate	0.02
4	19.03	chavicol	0.02
5	22.84	Eugenol	62.43
6	23.52	α-Copaene	0.11
7	25.42	trans-Caryophyllene	11.17
8	26.74	Humulen-(v1)	0.01
9	26.92	α-Humulene	1.31
10	30.45	Aceteugenol	1.79
11	32.41	Palustrol	0.08
12	33.06	Caryophyllene oxide	0.87
13	34.36	Humulene oxide	0.07
14	42.07	Benzyl benzoate	0.02
Total			99.27

3.4. Effect of clove and vatica oil concentrations on infection and sporulation of A. flavus PSRDC-2 on maize seeds

The effects of concentrations of clove and vatica oil fumigation on growth and sporulation of *A. flavus* PSRDC-2 on maize seeds were investigated (Fig. 2). The percentages of infected maize seeds were significantly ($p < 0.05$) reduced with increasing concentrations of clove and vatica oil, compared with the control. The most effective concentrations of clove and vatica oil were at 10 and 50 $\mu L\,L^{-1}$, respectively, as the fumigation could completely reduce (100%) the percentages of infected maize seeds by *A. flavus* PSRDC-2 after 5 days of incubation at room temperature (28 ± 2 °C). The sporulation of *A. flavus* PSRDC-2 on infected maize seeds was significantly ($p < 0.05$) inhibited by clove and vatica oil fumigation with the most effective (100% inhibition) at 10 $\mu L\,L^{-1}$ of clove oil and 50 $\mu L\,L^{-1}$ of vatica oil. Therefore, the effective dose of clove and vatica oil could inhibit growth as well as sporulation of aflatoxin-producing fungi.

3.5. Preventive and curative effects of clove and vatica oil against A. flavus PSRDC-2 infection on maize seeds

The minimum duration of clove and vatica oil fumigation on maize seeds required to protect against *A. flavus* PSRDC-2 infection was studied. The results (Fig. 3A) showed that fumigation with vatica oil for 6 h and clove oil for 24 h (before inoculation of the pathogen) gave the highest protection (100%) of maize seeds infected by the aflatoxigenic *A. flavus* PSRDC-2. Regarding the curative abilities (Fig. 3B), fumigation with vatica oil 6 h after

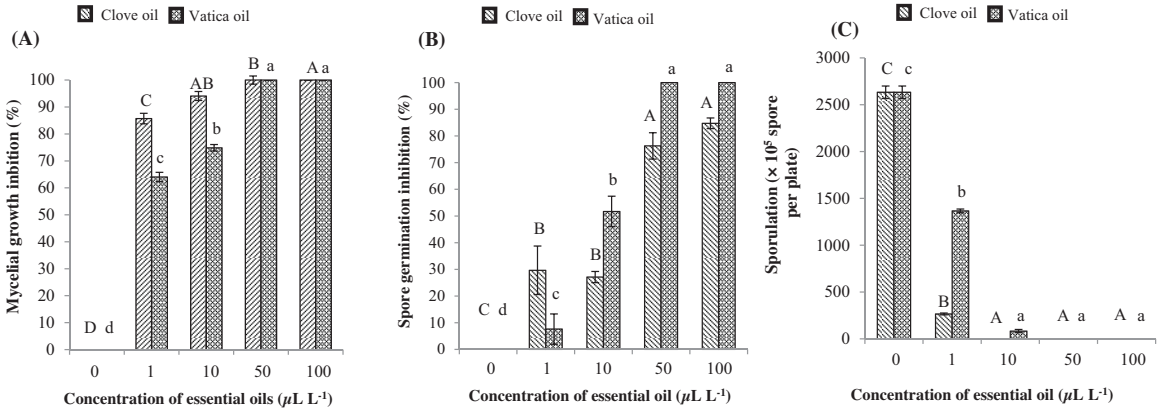

Fig. 1. Effects of different concentrations of clove and vatica oil on the mycelial growth inhibition (A), spore germination inhibition (B), and sporulation production (C) of *Aspergillus flavus* PSRDC-2 in PDA media. Different letters above bars indicate significant differences ($p < 0.05$) using ANOVA after the Duncan's Multiple Range Test in each group. Values are mean (n = 3) ± standard error.

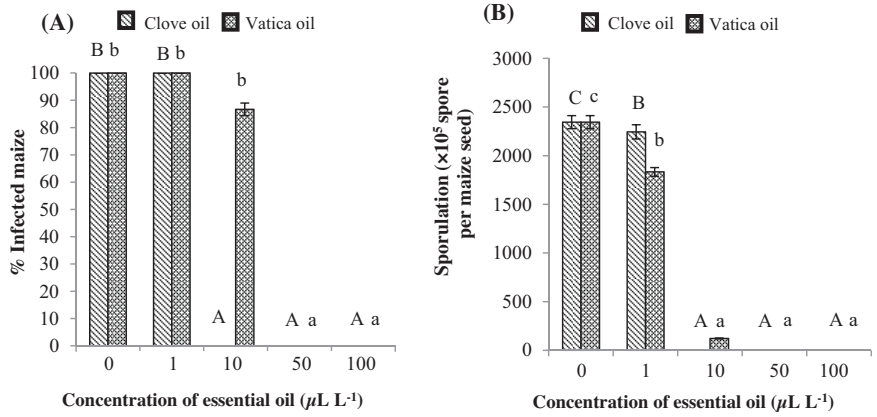

Fig. 2. Effect of different concentrations of clove and vatica oil on development in maize seeds inoculated (A) and sporulation production (B) of *Aspergillus flavus* PSRDC-2 after incubation for five days at room temperature (28 ± 2 °C). Different letters above bars indicate significant differences ($p < 0.05$) using ANOVA after the Duncan's Multiple Range Test in each group. Values are mean (n = 3) ± standard error.

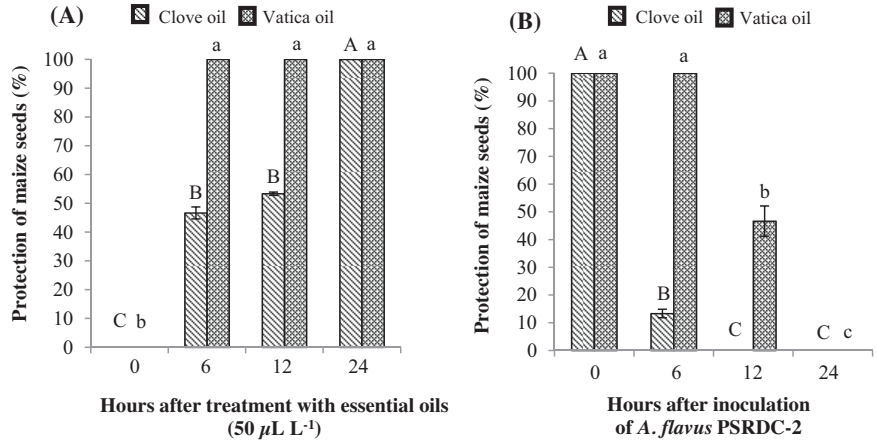

Fig. 3. Timing of preventive (A) or curative (B) measures by the clove and vatica oil fumigation on the protection of maize seed inoculated with *Aspergillus flavus* PSRDC-2 after incubation for five days at room temperature (28 ± 2 °C). Different letters above bars indicate significant differences ($p < 0.05$) using ANOVA after the Duncan's Multiple Range Test in each group. Values are mean (n = 3) ± standard error.

inoculating *A. flavus* PSRDC-2 showed 100% protection of infected maize seeds. Clove oil fumigation needed to be applied at the same time (0 h) as inoculation of *A. flavus* PSRDC-2 to obtain 100% protection of maize seeds. Therefore, only vatica oil possessed the curative effect when applied not more than 6 h after the infection of *A. flavus* PSRDC-2, while clove oil had no curative effect.

3.6. Evaluation of the efficacy of the fumigation protective period of vatica oil to control A. flavus PSRDC-2 on maize seeds

The fumigation protective period of vatica oil had an influence on the level of infection caused by *A. flavus* PSRDC-2 (Table 4). The inoculated maize seeds without fumigation showed signs of infec-

Table 3
Chemical composition (relative area percentage) of the vatica oil detected by GC–MS analysis.

Number	Retention time (min)	Compound	Composition (%)
1	11.57	Ethanol, 2-(2-ethoxyethoxy)-	0.42
2	12.41	1,8-Cineole	0.05
3	13.79	p-Cresol	2.35
4	14.55	l-Linalool	1.30
5	14.95	Benzeneethanol	0.17
6	15.59	Terpinene1-ol	0.15
7	15.89	Á-Terpineol	0.63
8	16.56	Benzyl acetate	48.83
9	16.63	Narceol	0.74
10	16.89	4-Terpineol	0.13
11	17.26	3-Cyclohexene-1-methanol, α,α,4-trimethyl-, (S)-	5.19
12	17.45	ç-Terpineol	1.84
13	19.09	Linalyl acetate	1.19
14	20.24	1H-Indole	0.11
15	21.65	Piperonal	0.72
16	22.66	Eugenol	0.38
17	26.32	1-Tridecanol	1.75
18	26.68	Isoeugenol	4.64
19	33.76	Ethyl phthalate	0.12
20	36.32	Jasmal	0.60
21	42.03	Benzyl benzoate	5.27
22	45.48	Isopropyl myristate	0.22
23	46.39	Cyclopenta[g]-2-benzopyran, 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl	0.18
24	47.03	Benzyl salicylate	0.09
Total			77.07

Table 4
Effect of protective period of vatica oil fumigation for controlling *A. flavus* PSRDC-2 on maize seeds.

Fumigation period (h)	Infection percentage (%)						
	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
0	93.30 ± 1.1^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
1	33.33 ± 2.3^{b}	40.00 ± 1.5^{b}	46.67 ± 2.0^{b}	66.67 ± 0.5^{b}	80.00 ± 5.1^{b}	100^{a}	100^{a}
2	26.67 ± 0.8^{b}	33.33 ± 1.2^{b}	40.00 ± 3.5^{b}	53.33 ± 2.7^{c}	66.67 ± 1.9^{c}	86.67 ± 2.9^{b}	96.67 ± 1.6^{a}
4	16.67 ± 0.7^{c}	23.33 ± 1.3^{c}	30.00 ± 2.7^{c}	43.33 ± 5.3^{d}	56.67 ± 2.1^{cd}	66.67 ± 3.9^{c}	74.50 ± 5.7^{b}
6	0^{d}	0^{d}	0^{d}	0^{e}	0^{e}	0^{d}	0^{c}

Data are the mean of three replicates ± standard errors (SE). Data followed by same letter within each column are not significantly different using ANOVA after Duncan's Multiple Range Test.

tion after 2 days with an infection level of 93.3%. Increasing the fumigation period from 1 to 6 h resulted in significant ($p < 0.05$) reduction of infected maize seeds (from 100 to 0%). Interestingly, the 6 h fumigation treatment completely controlled *A. flavus* PSRDC-2 infection. Therefore, the minimum fumigation period for controlling *A. flavus* PSRDC-2 infected on maize seeds was confirmed to be 6 h.

3.7. In vitro *phytotoxicity of clove and vatica oil fumigation on maize seed germination*

Fig. 4 shows the effect of the concentration of clove and vatica oil in the 24 h fumigation of maize seed on seed germination, stem length, and root length after five days of maize seed incubation. Clove oil and vatica oil strongly inhibited the germination, stem length, and root length in a dose-dependent manner with the effect being significantly pronounced with vatica oil. Indeed, at lower concentrations (from 0 to 10 $\mu L\,L^{-1}$ of clove oil and 0–5 $\mu L\,L^{-1}$ of vatica oil), the germination, stem length, and root length were not significantly ($p < 0.05$) reduced in response to both essential oils as compared to the control. A significant ($p < 0.05$) inhibitory effect of fumigation (100% inhibition) on germination, stem length, and root length was observed at 50 $\mu L\,L^{-1}$ vatica oil.

4. Discussion

The growth inhibition of *A. flavus* by essential oils using direct contact has been previously reported. Various sources of essential oils include *Eucalyptus globulus* (Tyagi and Malik, 2011); *Curcuma longa* L. (Sindhu et al., 2011); *Cicuta virosa* L. var. latisecta Celak. (Tian et al., 2011); *Cinnamomum jensenianum* Hand.-Mazz (Tian et al., 2012); orange peel (Velázquez-Nuñez et al., 2013); curcumin (Ferreira et al., 2013); thymol, eugenol, and menthol (Mishra et al., 2013); *Boswellia sacra* (El-Nagerabi et al., 2013); cinnamon (Manso et al., 2013); mentrasto and oregano (Esper et al., 2014); cinnamon, clove, eucalyptus, peppermint, and lemongrass (Ma-in et al., 2014); *Zanthoxylum molle* Rehd. (Tian et al., 2014); thyme (Khalili et al., 2015); *Zingiber officinale* (Nerilo et al., 2016); and *Litsea cubeba* (Li et al., 2016). However, this study has demonstrated the potential of applying essential oils to control aflatoxigenic *A. flavus* by fumigation. The inhibitory effects of essential oils were greater in fumigation than in a liquid state (Guynot et al., 2003; Matan et al., 2006; Tullio et al., 2006). In general, application by fumigation requires smaller concentrations of essential oils compared with those required for direct contact application to inhibit different types of microorganisms (Avila-Sosa et al., 2012). Four essential oils (cinnamon, clove, capsicum, and vatica oil) exhibited different efficiencies against the mycelial growth of 10 strains of *A. flavus*.

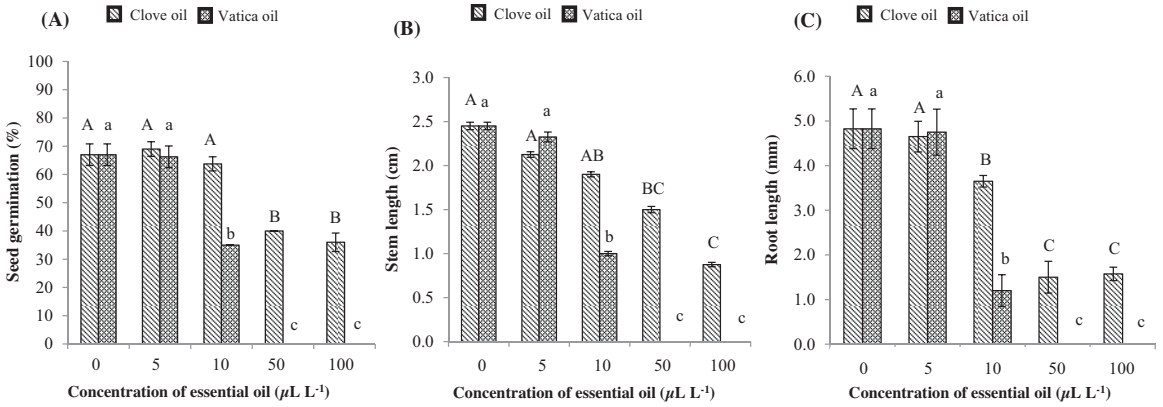

Fig. 4. Effect of the concentration of clove and vatica oil in the 24 h fumigation of maize seed on seed germination (A), stem length (B) and root length (C) after five days of seed incubation. Different letters above bars indicate significant differences ($p < 0.05$) using ANOVA after the Duncan's Multiple Range Test in each group. Values are mean (n = 4) ± standard error.

Among them, only *A. flavus* PSRDC-2 was the recalcitrant isolate to the fumigation of the four essential oils. Clove and vatica oil exhibited higher antifungal activities that may be attributed to the presence of eugenol (62.4%) and benzyl acetate (48.8%), respectively. The percentage of eugenol (62.4%) detected from clove oil in this study was similar to the report of Omidbeygi et al. (2007) (63.3%) while it differed from those reported in other investigations in the composition of clove essential oil, such as 88.5% (Chaieb et al., 2007) and 76.3% (Ma-in et al., 2014). The differences in composition might be caused by differences between growing regions, storage times, growing seasons, and analytical methods, as previously suggested (Sharma and Tripathi, 2008). Eugenol was reported to possess antifungal activity against white-rot fungi *Lenzites betulina* and brown-rot fungi such as *Laetiporus sulphureus* (Cheng et al., 2008), *Botrytis cinerea* (Wang et al., 2010), *Fusarium* spp. (Morcia et al., 2012), *Cladosporium* spp. and *Aspergillus* spp. (Abbaszadeh et al., 2014), *A. parasiticus* (Jahanshiri et al., 2015), *A. flavus* (Liang et al., 2015), and *Peronophythora litchi* (Gong et al., 2016). The higher antifungal activity of the integral essential oil may be attributable to some minor components resulting from a synergistic effect (Adegoke et al., 2000). Several researchers have reported that the antimicrobial activity of an essential oil is linked to its chemical composition (Cimanga et al., 2002). The functional groups (alcohol, phenols, terpenes, and ketones) of compounds found in plant materials (extracts/essential oil) are associated with their antimicrobial characteristics (Sartorelli et al., 2006).

Clove oil is normally reported to control pathogenic fungi such as *A. xavus* (Omidbeygi et al., 2007); *Candida*, *Aspergillus,* and dermatophyte species (Pinto et al., 2009); *Penicillium* sp., *A. niger,* and *A. versicolor* (Ma-in et al., 2014); *B. cinerea*, *P. expansum,* and *Neofabraea alba* (Daniel et al., 2015); and *P. digitatum* (Shao et al., 2015). However, vatica oil has never been reported with chemical composition and antifungal activity to control food spoilage fungi. The results from this study indicate that only vatica oil fumigation completely inhibited mycelial growth, sporulation, and conidial germination of *A. flavus* PSRDC-2 *in vitro* and on maize seeds at the tested concentration of 50 μL L^{-1}. Clove oil showed weak antifungal activity on spore germination at 50 μL L^{-1} concentration. The inhibition of spore germination of the tested fungi after exposure to the vatica oil fumigation is an interesting finding as spores are important structures for the survival and spread of fungi (Rabea et al., 2003). Therefore, application of these essential oils that strongly inhibit spore germination or cause the destruction of these structures has great potential for the control of spore populations and dispersal on various substrates or environments (Plascencia-Jatomea et al., 2003).

According to the *in vivo* assay, fumigation of vatica oil for at least 6 h showed complete (100%) protective and curative effects on maize seeds infected by *A. flavus* PSRDC-2, while clove oil needed 24 h for the same protective effect. For the curative effect, the results indicate that both essential oils could not control the fungi if spores of *A. flavus* PSRDC-2 were grown on maize seeds for more than 6 h. Therefore, based on this result, maize seeds must be fumigated by these essential oils before seed storage to allow vatica or clove oil to be effective. In addition, increasing the fumigation period of vatica oil from 1 to 6 h resulted in significant ($p < 0.05$) reduction of infected maize seeds. Therefore, fumigation with 50 μL L^{-1} of vatica oil for 24 h provided complete control of maize seed infection by *A. flavus* PSRDC-2 during the 14 days tested. The minimum period of fumigation of vatica oil for controlling infected maize seed was 6 h.

Using concentrations of clove and vatica oil higher than 50 and 10 μL L^{-1}, respectively, had a negative effect on seed germination, stem length, and root length. In general, these results agree with the literature on inhibitory activity exerted by essential oils on seed germination and radicle elongation (Abrahim et al., 2000; Turk and Tawaha, 2003; Qiming et al., 2006; Kordali et al., 2009; Young and Bush, 2009). Regarding seed germination, stem length, and root length of maize, vatica oil was more phytotoxic than clove oil. The essential oils and individual monoterpenes that strongly inhibit seed germination and plant growth of some agricultural crops and weeds include 1,8-cineole (Romagni et al., 2000; de Feo et al., 2002), α-pinene (de Feo et al., 2002; Scrivanti et al., 2003; Bulut et al., 2006; Singh et al., 2006), limonene (Scrivanti et al., 2003; Bulut et al., 2006), and terpinen-4-ol (Bulut et al., 2006).

In conclusion, the results of *in vitro* and *in vivo* studies indicate that using only vatica oil as a fumigant during maize seed storage is very promising. Both clove and vatica oils exhibited dose-dependent allelopathic activity on maize seed germination, stem length, and root length, with vatica oil being more phytotoxic than clove oil. Fumigation of clove and vatica oil of less than 5 μL L^{-1} per 100 maize seeds could be applied to protect *A. flavus* during maize storage.

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