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Development of an efficient protocol for production of healthy sugarcane seed cane through Meristem culture

Shubhangi Salokhe

Symbiosis Foundation, Pune, India

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ABSTRACT

Healthy seeds are a prerequisite for improving the productivity of sugarcane crop. The best way to ensure the production of disease-free sugarcane seeds is via a technique called 'Meristem Culture' (Tissue Culture). An efficient protocol for meristem culture of sugarcane has been developed in this study. The potting mix for the survival of meristem cultured plantlets in a greenhouse was standardized, and agronomy for growing tissue culture plantlets in the field was developed. Multiple shoots were observed on Murashige and Skoog's (MS) medium supplemented with BAP 0.2 mg/l and Kinetin 0.1 mg/l. Best rooting was observed on MS media supplemented with IAA 1 mg/l and NAA 1 mg/l. Soil mixture containing [Soil + Vermiculite + Sand in 4: 1: 1 proportion (by volume)] + Vermicompost @ 25% by volume of medium + NPK is best suited for hardening tissue cultured plants in a greenhouse. Two feet distance between 2 plants and 3 feet distance between 2 rows was found as an appropriate method for planting tissue cultured plantlets in the field. Setts obtained from tissue cultured plants planted at 15 cm spacing gave higher yield than the other spacing. Setts obtained from micro-propagated plants (M) gave the highest yield as compared to setts from conventional sugarcane raising. The protocol developed in this study, shows potential for development of sugarcane meristem cultured plantlets and use of these plants for planting in field to obtain healthy sugarcane seed.

1. Introduction

Sugarcane (*Saccharum officinarum* L.) is a major commercial crop of India, cultivated on 51.14 lakh hectares of land (Cooperative Sugar 2020). It is an industrial crop and one of the main crops of earning foreign exchange in India [31]. Brazil, India, Pakistan, China and Indonesia are the largest producers of sugarcane. The biggest producer of sugarcane is Brazil, and as it grows over 40% of the worlds crop [28]. Because of the high production potential of sugarcane and its ability to be cultivated in countries with low economic and social development, it could become a key source of income and improve quality of life in many countries [9].

The cane is supplied to sugar industries, where various products are prepared from its juice (sugar, jaggery, alcohol, etc.). *Sugarcane* is the best example of *renewable natural agricultural resource* since it *provides sugar*, *besides biofuel*, *fibre*, *fertilizer* and various by-products [7]. It is recognized as an important energy crop because of the possibility to produce ethanol from molasses and directly from cellulose. It is also one of the most efficient crops in the world for converting solar energy into chemical energy.

For commercial cultivation, sugarcane is propagated vegetatively and requires a considerable amount of seeds. Good planting material (seed) contributes to higher yields; therefore, the supply of healthy seed cane is the main prerequisite for improving the productivity of the sugarcane crop [13]. Availability of disease-free and genuine to type planting material is essential for achieving a high yield of sugarcane cultivation. Unfortunately, since the system of scientific seed production, i.e. breeder seed (stage 1), foundation seed (stage2) and certified seed production (stage 3) is not being followed, the sugarcane farmers are facing a problem of a lack of good quality seed material for planting [2,24]. As sugarcane is propagated vegetatively, it favours the accumulation of pathogens (Vishwanathan, 2016). Hence along with seed canes, disease-causing pathogens are also being introduced into the new areas.

The slow accumulation of different pathogens over a period of time makes minor diseases into major ones [26]. Several epidemics due to red rot, smut, wilt, grassy shoot, ratoon stunting, yellow leaf and leaf scald occurred in the past indicated that disease infected seed can play a significant role in their creation and further spread [17]. Affected planting material is a major problem in propagation, exchange of germplasm and distribution of superior genotypes of sugarcane [12]. Methods used for the elimination of viruses and the development of good quality seeds in sugarcane are thermotherapy (heat therapy) and tissue culture technology [21]. Heat therapy can be used to eliminate many viruses from a

E-mail addresses: salokhes2002@yahoo.co.in, salokhes2002@gmail.com, salokhes2002@yahoo.co.in, salokhes2002@gmail.com.

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variety of sugarcane and provides a proven approach to remove pathogens from seed canes. There are, however, some disadvantages of this therapy as sugarcane seed is treated with heat; it gives very low germination percentage. The media compositions were equally important for in-vitro regeneration of sugarcane [16].

Thermotherapy or meristem culture (tissue culture), or frequently a combination of both techniques has been successfully used to eradicate viruses from infected sugarcane plants [30,32]. If explants are taken from disease-free plant material or if explants are heat-treated to eliminate diseases, then the resultant plants are healthy and disease-free [15]. Meristem culture demonstrated to be an efficient tool for eliminating virus from plants, giving the possibility to produce disease free propagation material [22]. In order to achieve the demand for sugar by 2020 in India, sugarcane production has to be increased. For increasing sugarcane production, availability of good quality seed material of high yielding varieties is essential. Tissue culture technique can be applied in breeding, propagation, disease elimination, rejuvenation of older varieties, and development of clones suitable for abiotic and biotic stresses [18]. [5] suggested the in-vitro protocol for rapid micro propagation of sugarcane which can be adopted for producing genetically-homogenous and WLD-free planting material in establishing sugarcane nurseries. Four sugarcane varieties and three explants derived seedlings were utilized to assess the yield with respect to single bud setts. In this experiment in vitro grown plants showed better response for certain characters [20]. Sugarcane micro propagation enables the massive multiplication of sugarcane plants to obtain certified vitro plants and increase the sugarcane productivity per unit area (Bello-Bello et al., 2017).

Therefore, in this research, we aimed at development of an efficient protocol for production of healthy and disease free sugarcane tissue cultured plants (micro propagated plants) on a large scale in the laboratory and for hardening of tissue cultured plantlets in the green-house. Development of agronomy for growing tissue culture plants in the field, as well as for growing seeds obtained from tissue cultured plantlets has also been performed. Development of protocol for tissue culture of sugarcane and development of agronomy for tissue cultured plants was essential to make use of tissue culture plantlets in three-tier system of sugarcane seed production.

2. Materials and methods

2.1. Plant material

The present study was conducted at Vasantdada Sugar Institute (VSI), Pune, India. Laboratory experiments were conducted to standardize a protocol for large scale and low-cost production of meristem cultured (tissue culture) plants of sugarcane. During this study, explants (meristems) from sugarcane variety 'Co 86032' were used from 6 months old sugarcane seed nursery of VSI. Field experiments were conducted on the farms of Vasantdada Sugar Institute.

2.2. Explants source and surface sterilization

Meristems are the centres of plant growth located in the tips of the new shoots and leaves. Tops of Sugarcane variety 'Co 86032' of 6 months old from field were brought to the laboratory. The upper leaves were removed, and sugarcane meristems along with outer cover were washed with distilled water, then surface sterilized with 0.1% mercuric chloride for 5 min and then washed 3 times with sterile distilled water to remove the traces of chemicals. Leaf-sheaths surrounding the meristem were removed aseptically with the help of surgical blades, and then meristem explants were obtained. The explants were then inoculated by proper dissecting and sizing the meristem (0.5–1.0 cm).

2.3. Culture media and conditions

[19] medium was used as a basal medium in laboratory experiments for the development of a protocol for meristem culture of sugarcane. MS media was enriched with 3% sucrose and 10% coconut water. This medium was supplemented with different combinations of cytokinins and auxin; pH of the medium was maintained at 5.8. The culture tubes/-bottles were incubated on illuminated racks at 16 h light and 8 h dark period in 24 h at the room temperature of $25 \pm 1\ ^{\circ}C$ in a growth chamber in the tissue culture laboratory.

2.4. In vitro shoot multiplication

Different combinations of cytokinins and auxins were tested to select the best media for shoot formation and root formation in meristem culture of sugarcane. MS [19] media of HiMedia supplemented with different concentration (0, 0.1, 0.25, 0.5, 1, 1.5 and 2 mg/l) of "6- Benzyl aminopurine (BAP)" and "Kinetin (KIN)" from MERCK was tested on shoot multiplication on 10 explants. Each experiment was repeated 3 times to confirm the results. Subculture was carried out every 2–3 weeks. After subculture, the cultures were transferred into new glass bottles and kept for shaking on 120 rpm in the illuminated shakers (Steelmet Novatech) in the laboratory for about 20 days.

After 5 weeks of shoot growth actively growing shoots were transferred to fresh medium (MS + 0.25 mg/l BAP + 0.1 mg/l Kin) in glass bottles and kept in the illuminated shakers for shaking (120 rpm) and for further growth. Multiple shoots were developed on this media after 20 days. Then these culture bottles were transferred to illuminated racks in a laboratory at 25 °C with 16 h light and an 8 h dark period in 24 h. First sub culturing was done after 30 days of growth. The culture was subdivided in bunches containing 4–5 shoots/bunch, and then these bunches were transferred into a fresh medium in glass bottles and again kept on shakers for 15 days, finally kept on illuminated racks. In this way sub culturing was done for 5 to 6 times by transferring into fresh media bottles to obtain multiple shoots.

2.5. In vitro rooting of plantlets

Shoots obtained from the above culture bottles were transferred to rooting medium for inducing rooting to the shoots. Rooting medium was prepared by supplementing MS basal medium with IAA or NAA. Various concentrations (0.1, 0.25, 0.5, 1, 1.5 and 2 mg/l) of IAA and NAA were tested. Each experiment was repeated 3 times. 10 plants per treatment were tested for each repetition.

2.6. Acclimatization of the plantlets in the greenhouse

Experiments were conducted to standardize the potting mix for the survival of meristem cultured plantlets. Well rooted plantlets about 6–8 cm height were taken from the culture bottles and washed with tap water to remove the traces of medium. These plantlets were taken to the greenhouse to transfer them to a suitable potting mix. The plantlets were transplanted into poly bags of 15×10 cm size containing a mixture of soil, sand, vermiculite and Vermicompost in different proportions as indicated below:

1) C (Control) - Soil: Vermiculite: Sand in 4: 1: 1 proportion (by volume).
2) F1 – C + Vermicompost @ 25% by volume of medium and NPK (55 mg urea, 360 mg single super phosphate and 95 mg muriates of potash/kg of the medium).
3) F2 – C + Vermicompost @ 50% by volume of and NPK @ double dose of fertilizer as in F1.
4) FU1– F1 + 0.5% solution of urea spray at 15 days after transplanting @ 200 ml solution on 160 plantlets.
5) FU2 – F1 + 0.5% solution of urea spray two times i.e. at 15 days and 30 days after transplanting @ 200 ml solution each time on 160 plantlets.
6) V1– C + Vermicompost @ 25% by volume of medium.
7) V2 – C + Vermicompost @ 50% by volume of medium.

8) M − C + Multinutrients sprays.

(The multi-nutrient was made up of N, P, K, Fe, Mn and Zn in 9:9:5:2:2:2 proportions. Multinutrient spray was sprayed 3 times on plantlets. The first spraying was done on the 2nd day after planting @30 ml/L of water. The second spray was done at 15 days after planting @ 50 ml/L of water, and the third spray was done 45 days after planting @ 75 ml/L of water.)

In the greenhouse study, the meristem cultured plantlets were transplanted on poly bags in the greenhouse. Each treatment consisted of 20 plantlets. All poly bags were labelled and were placed in a completely randomized design in the greenhouse. Observations were recorded at 2 months age before transplanting in the field.

2.7. Breeder's seed production from sugarcane meristem cultured sugarcane plants

Breeder's seed nursery is the first step of a three-tier system of seed production. Well-hardened meristem cultured plantlets from greenhouse were used for planting sugarcane nursery for breeders seeds. A procedure was developed for planting meristem cultured plantlets in the field and spacing required (row to row and plant to plant).

2.8. Foundation seed production from seed obtained from meristem cultured sugarcane plants

The present field study of meristem cultured plantlet's seeds was taken with a view to develop agronomy so that this technology can be used more efficiently. Seeds obtained from meristem cultured sugarcane plantlets at the age of 10 months were used for the production of foundation seeds of sugarcane. Performance evaluation was done for the seeds obtained from meristem cultured plantlets with appropriate cultural practices specific to meristem cultured plantlets.

This experiment was conducted in the field in a split-plot design with 3 replications. The spacing between 2 rows (RS) served as the main plot and spacing between 2 meristem cultured (tissue cultured) setts (), and conventional sugarcane setts (C) was the sub-plot (Table 1) (see Table 2).

Setts obtained from 10 months old crop grown by using meristem cultured plants (TC), and conventional sugarcane (C) from sugarcane Co 86032 variety were used. The agronomical practices recommended by Vasantdada Sugars Institute for sugarcane cultivation were followed. The seed crop was harvested at 10 months age. The data related to the number of canes/clumps, number of canes/ha, number of three eye bud setts/ha., millable height/cane, the girth of cane, number of internodes per cane and length of internodes was recorded.

2.9. Statistical analysis

The results of experiments carried in the laboratory were analysed statistically using Completely Randomized Design (CRD) with 10 replications and significance level (P, 0.05) and the results of experiments carried in the greenhouse were analysed statistically using Completely Randomized Design (CRD) with 20 replications and significance level (P, 0.05). Split Plot Design with three replications was used in the field study of meristem cultured plantlets. The result is indicated by placing an asterisk on the –*F*- value in the analysis of variance. If the computed -*F*- value is smaller than or equal to the tabular -*F*- value at 5% level of significance, the variation due to treatments is said to be non-significant.

Table 1
Main treatments (Spacing between two rows).

Main treatment	The spacing between 2 rows
RS 90	Row Spacing 90 cm
RS 120	Row Spacing 120 cm
RS 150	Row Spacing 150 cm

Table 2
Sub treatments (Spacing between two setts of sugarcane when planted in the field).

Sub treatment	The spacing between two setts (cm)
TC15	Two eye bud setts (obtained from micro-propagated plantlets) planted at 15 cm spacing.
TC30	Two eye bud setts (obtained from micro-propagated plantlets) planted at 30 cm spacing.
TC45	Two eye bud setts (obtained from micro-propagated plantlets) planted at 45 cm spacing.
C15	Two eye bud setts (obtained from conventional sugarcane) planted at 15 cm spacing
C30	Two eye bud setts (obtained from conventional sugarcane) planted at 30 Cm spacing.
C45	Two eye bud setts (obtained from conventional sugarcane) planted at 45 cm spacing

Such a result is indicated by placing -*ns*- on the computed -*F*- value in the analysis of variance, significance level (P, 0.05).

3. Results and discussions

3.1. Development of appropriate media for sugarcane meristem culture

Suitable nutrient media, its chemical composition and physical form, are essential for the success of in vitro culture of plants. An efficient and low-cost protocol for meristem culture of sugarcane on a large scale using meristem explants has been developed. Nutrient media based on [19] was used as a basal medium in all experiments. Multiple shoots and roots were developed by using various combinations and concentrations of growth regulators. The effect of the different combinations of BAP and Kinetin on shoot multiplication and the effect of auxins (IAA or NAA) on rooting was tested.

3.2. In vitro shoot multiplication

In the present investigation, sugarcane apical meristem of 5 mm size was used as explants. Liquid media was used for this study. Initially, meristems were inoculated on filter paper support in test tubes. 10 explants in 10 test tubes were inoculated for each treatment. These test tubes were incubated on illuminated racks at 16 h light and 8 h dark period in 24 h at the room temperature of 25 ± 1 °C in a growth chamber in the tissue culture laboratory. Polyphenols were observed around meristem within 10 days, and then these meristems were transferred to fresh medium in new test tubes. Observations were recorded after 30 days from the first inoculation for growth of meristem ex. number explants showing multiple shoots, number of shoots per explants etc. These observations on growth of explants are presented in Table 3.

The growth regulators (BAP and Kinetin) had a marked effect on the frequency of shoot initiation and establishment of shoot cultures. The results showed that the number of explants which showed multiple shoots were higher in T3 medium (MS medium supplemented with BAP 0.25 mg/l and Kinetin 0.1 mg/l); higher number of multiple shoots (3.7) was observed on this media (Table 3). Healthy shoots were developed on this medium. Increasing the concentration of BAP and Kinetin reduced the number of explants with multiple shoots, as well as the number of shoots per explant. This may be related to the fact that higher level of cytokinins inhibits cell division.

Result of this experiment showed that balance between cytokinins and auxin is essential for the production of shoots and its further multiplication. Many workers have reported the use of BAP with Kinetin for shoot formation in sugarcane [10,27]. The current result obtained was not in agreement with the earlier results reported by Ref. [2], they obtained maximum shoot multiplication on MS medium with 1.0 mg/l BAP, and 0.25 mg/l Kin. This might be related to the fact that higher levels of Cytokinin inhibit cell division and hence organogenesis [33]. Best

Table 3
In vitro shoot multiplication from meristem of sugarcane.

Treatment	Media Combination	Number of explants inoculated	No. of explants showed multiple shoots	Number of shoots per explant
T1	MS (Control)	10	0	0.0 ± 0.00
T2	MS + 0.1 mg/l BAP + 0.1 mg/l Kin	10	3	1.6 ± 0.09
T3	MS + 0.2 mg/l BAP + 0.1 mg/l Kin	10	8	3.7 + 0.56
T4	MS + 0.1 mg/l BAP + 0.25 mg/ Kin	10	4	1.8 ± 0.06
T5	MS +0.2 mg/l BAP + 0.25 mg/l Kin	10	5	2.8 ± 0.45
T6	MS + 0.5 mg/l BAP + 0.25 mg/l Kin	10	4	1.7 ± 0.47
T7	MS + 0.2 mg/l BAP + 0.5 mg/l Kin	10	4	1.5 ± 0.42
T8	MS + 0.5 mg/l BAP + 0.5 mg/l Kin	10	3	1.6 ± 0.06
T9	MS + 1 mg/l BAP + 1 mg/l Kin	10	2	1.5 ± 0.09

Table 4
In vitro rooting of meristem cultured plantlets of sugarcane.

Treatment	Media Combinations	Number of plants used for rooting	Number of Plants showed rooting	Number of roots per plant
T1	MS	10	0	0.00 ± 0.00
T2	MS + IAA 0.25 mg/l	10	2	5.8 ± 0.17
T3	MS + IAA 0.5 mg/l	10	3	6.3 ± 0.24
T4	MS + IAA 1 mg/l	10	10	14.70 ± 0.28
T5	MS + IAA 1.5 mg/l	10	7	11.40 ± 1.22
T6	MS + IAA 2 mg/l	10	8	11.10 ± 0.48
T7	MS + NAA 0.25 mg/l	10	2	4.6 ± 0.57
T8	MS + NAA 0.5 mg/l	10	3	8.6 ± 0.10
T9	MS + NAA 1 mg/l	10	9	15.00 ± 0.36
T10	MS + NAA 1.5 mg/l	10	7	11.90 ± 0.75
T11	MS + NAA 2 mg/l	10	8	11.70 ± 0.64

treatment for in vitro shoot multiplication of sugarcane was MS + 0.2 mg/l BAP +0.1 mg/l Kin. In this treatment 8 out of 10 explants showed multiple shoots and number of shoots per explant was higher (3.7).

3.3. Subculture

Shoot multiplication was done on the same medium (MS + 0.2 mg/l BAP + 0.1 mg/l Kin) by sub culturing on fresh medium in glass bottles and kept for shaking on 120 rpm in the illuminated shakers in the laboratory for about 15 days. After shaking bottles were transferred to illuminated racks for incubation.

3.4. In vitro rooting of plantlets

The study was conducted for in vitro rooting of plantlets. For this study, micro shoots developed after 4–5 subcultures were used for inoculation on different media. For rooting studies, the regenerated micro shoots were used.

The results showed that an increase in the concentration of NAA and IAA from 0.25 mg/l to 1 mg/l increased the number of planlets showing roots and number of roots per plant. Best rooting was observed on T4 (MS media supplemented with IAA 1 mg/l and NAA 1 mg/l). At this concentration, 100% shoots formed roots and number of roots per plants was higher (14.70) in this treatment. The current result obtained was in agreement with the earlier results reported by Ref. [3]. Further increase in the concentration of NAA or IAA reduced the number of roots noticeably [18]. also reported that NAA is the ideal growth regulator for induction of rooting in the sugarcane. Many researchers reported that 5 mg/l of NAA was good for rooting, but increasing NAA beyond 5 mg/l inhibits rooting [25]. used 1.0 mg/l IBA as the best root initiating growth hormone. Conversely, further increasing the concentration of NAA and reduced the shoot length and number of roots noticeably. Best treatment for inducing roots to in vitro grown meristem culture plants of sugarcane is MS + NAA 1 mg/l.

3.5. Hardening of meristem cultured plantlets in a greenhouse

Transfer of *in-vitro* sugarcane Plantlets from their sterile culture vessels to the soil requires a careful and stepwise procedure and suitable potting mixes; as this is one of the important criteria for the survival of meristem cultured plants. The in vitro raised plantlets are tender and fragile, and therefore their hardening requires special attention. The present study was undertaken using meristem cultured plantlets of sugarcane variety 'Co 86032' to develop a procedure for hardening of meristem cultured sugarcane plantlets. In vitro rooted plantlets were taken out of glass bottles and then washed under tap water to remove traces of media then excess leaves are trimmed out. Then these plants are used for transplanting in greenhouse.

The survival and growth rates of meristem cultured plantlets of sugarcane are given in Table 5 (see Table 4). The survival of plantlets which were grown in soil mixtures with chemical fertilizers and/or Vermicompost (F1, FU1, FU2, V1 and V2) was higher (from 85 to 95%), compared to the survival of plantlets (70%) in control conditions (Table 5). The survival of plantlets was only 45% in soil mixture in which multi nutrients were sprayed on plants.

An effect of soil mixtures on tillering to sugarcane plantlets has been observed (Table 5). The use of fertilizers along with urea spraying two times, i.e. at 15 days and 30 (FU2) gave tillering in 80% plants. Whereas FU1 gave tillering in 50% plants. In all other treatments, only 20–30% plants showed tillering at 2 months age. Sugarcane plants height was measured at 60 days of plants age. Plant height was significantly higher in other treatments as compared to the control. It was observed between 8.73 in control to 12.31 cm in F1 treatment, followed by 11.15 cm in FU2 treatment.

The number of leaves per shoot ranged between 5.28 and 7.15. It is higher in FU 2 treatment (7.15) where urea is sprayed twice followed by V1 treatment. The leaf width was in between 0.64 and 0.81 cm. It is higher in FU 2 followed by FU 1. However, there was no significant difference in number and width of leaves due to different soil mixtures. In case of the length of the 4th leaf, there was no significant difference. The shoot weight per plant was between 1.48 g and 3.07 g (Table 5). Shoot weight differed significantly due to the difference in soil mixture (F1, F2, FU1, FU2, V1, V2 and M). Use of fertilizers (F1) and Vermicompost at higher dose, i.e. 50 % by volume (V2) gave higher shoot weight of 2.16 gm to 3.07 gm as compared to that of 1.80 g in control. A lower dose of Vermicompost i.e. 25 % by volume of soil mixture had no effect on

Table 5
The survival and growth rates of meristem cultured plantlets of sugarcane on different soil mixtures.

Treatments (Different Soil Mixtures)	Survival (%)	% Plants Showing Tillers	The Height of Plant (cm)	Number of leaves per shoot	Length of the 4th leaf (cm)	The Width of 4th leaf (cm)	The Weight of Shoot (gm)	The Weight of Roots (gm)
C	70	20	8.73	5.28	39.11	0.64	1.80	0.36
F1	95	25	12.31	5.31	42.55	0.70	3.07	1.42
F2	85	25	10.81	6.13	44.90	0.81	2.67	1.10
FU1	90	50	10.91	5.64	35.86	0.73	2.40	0.97
FU2	95	80	11.15	7.15	41.10	0.74	2.82	1.41
V1	85	30	10.14	6.70	31.41	0.65	1.48	0.63
V2	85	30	10.19	6.33	36.35	0.67	2.16	0.90
M	45	5	10.38	5.44	39.44	0.68	1.52	0.47
CD (P = 0.05)	–	–	0.46[a]	NS.	NS.	NS.	0.12[a]	0.070[a]

NS - Non-significant.
[a] Significant.

shoot weight.

No additional benefits on shoot growth when fertilizers were used at double the rate of spraying of urea along with fertilizers have been observed. Similarly, there was no additional benefit on shoot weight when fertilizers were used at double the rate or spraying along with fertilizers. The weight of shoots in multi nutrient spray was 1.52 gm, and this value was lower than 1.80 gm in control (Table 5). The trend observed for the weight of roots was similar for the weight of shoots. Root weight was observed significantly higher in soil mixture F1 and FU2 (1.42 g and 1.41 g), which was higher as compared to that of control 0.36 g (Table 5). In case of root weight also, there was no additional benefit of increased fertilizer dose or urea spraying. There is a positive relation between shoot weight and root weight. Root weight increased with increase in shoot weight, except in the treatment of multi nutrients spray.

It has been observed that survival rate of plants was higher, as well as shoot weight and root weight of meristem cultured sugarcane plantlets were also higher in a soil mixture C + Vermicompost @25% by volume of medium and NPK as 55 mg urea, 360 mg single super phosphate and 95 mg muriate of potash/kg of medium. This is the best medium for hardening of meristem cultured plantlets in a greenhouse. There was no additional benefit of chemical fertilizer when it was used at double the rate. An increase in number of plants showing tillers was observed in treatment of urea spraying along with fertilizer application. There was a positive relation between root growth and shoot growth as indicated by their weight (Table 5). The result obtained in the present study was not in agreement with the earlier finding of [33].

3.6. Sugarcane breeder's seed production from sugarcane meristem cultured plants

Experiments were carried out to develop the procedure for planting meristem cultured plantlets in the field. Special attention was given for watering of plants, withholding watering, planting procedure, row to row distance, plant to plant distance, intercultural operations etc. As a result of conducted experiments the procedure of planting meristem cultured plantlets in the field has been standardized:

- The watering of plantlets should be withheld the day before planting in the field so that the soil in the plastic bag becomes hard and compact. This is necessary to avoid damage to the root system of the plantlets.
- The plot should be ready with the application of green and organic manures for improving soil fertility.
- Furrows should be opened at a distance of 3 feet and recommended basal doses of chemical fertilizers should be applied and mixed in the ground. Because meristem cultured plants also require nutrients like conventional sugarcane.
- The plantlets should be kept in the field on the ridges of furrows along with poly bags.
- The pits of the size of the plastic bags in the center of the furrow should be kept at a distance of 2 feet.
- The plantlet should be planted along with whole soil balls in the pits and subsequently covered it with sufficient amount of soil. Immediately after transplanting, irrigation needs to be given.
- All recommended intercultural operations (weeding, harrowing, irrigation, pest control, etc.) should be performed within time.
- Canes obtained from meristem cultured plantlets should be harvested within 10 months.
- One eye buds obtained from meristem cultured sugarcane crop should be used to grow foundation seed.

The agronomy for growing meristem cultured plants was also experimented with. 2 feet distance between 2 meristem culture plantlets and 3 feet distance between 2 rows of sugarcane proved to be suitable for planting of meristem cultured plantlets in the field for the development of breeder seed. The current result obtained was in agreement with the earlier report of [23]. Best quality seed can be obtained at the age of 10 months of the meristem cultured sugarcane grown in the field.

Performance of seeds obtained from meristem cultured sugarcane was better as compared to conventional seeds, as the yield obtained from meristem cultured cane was 26% higher than conventional sugarcane. Seeds obtained from meristem cultured cane were healthy and free from diseases (Fig. 1).

3.7. Foundation seed production from meristem cultured sugarcane seeds

Performance of seeds obtained from meristem cultured plantlets was compared with seeds obtained from conventional sugarcane. This experiment was conducted in a split-plot design with 3 replications. Setts obtained from 10 months old crop grown by using meristem cultured plantlets (TC) and conventional sugarcane (C) from sugarcane 'Co 86032" variety was used. The recommended agronomical practices (recommended by Vasantdada Sugarcane Institute) were followed, and the seed crop was harvested at ten months age of the crop.

Statistical tests were done to analyse the influence of row and set spacing and interaction of both on sugarcane seeds (for meristem cultured plants as well as from conventional canes) for some agronomic characteristics (Milliable height, girth of cane, number of internodes/cane, number of canes per clump, number of canes/ha and yield of three eye budded setts/ha).

Influence of row spacing on agronomic traits of meristem cultured sugarcane seed.

Analysis of variance (ANOVA) revealed that the number of canes/clump and number of internodes/cane were significantly affected by row spacing (Table 6). Results showed that an increase in row spacing from 90 cm to 150 cm resulted in an increased number of internodes/cane and number of canes per clump. The reason for the increased cane population, may be less competition between plants because of higher spacing [23]. also reported a higher population of stalks in TC plots. Increase in

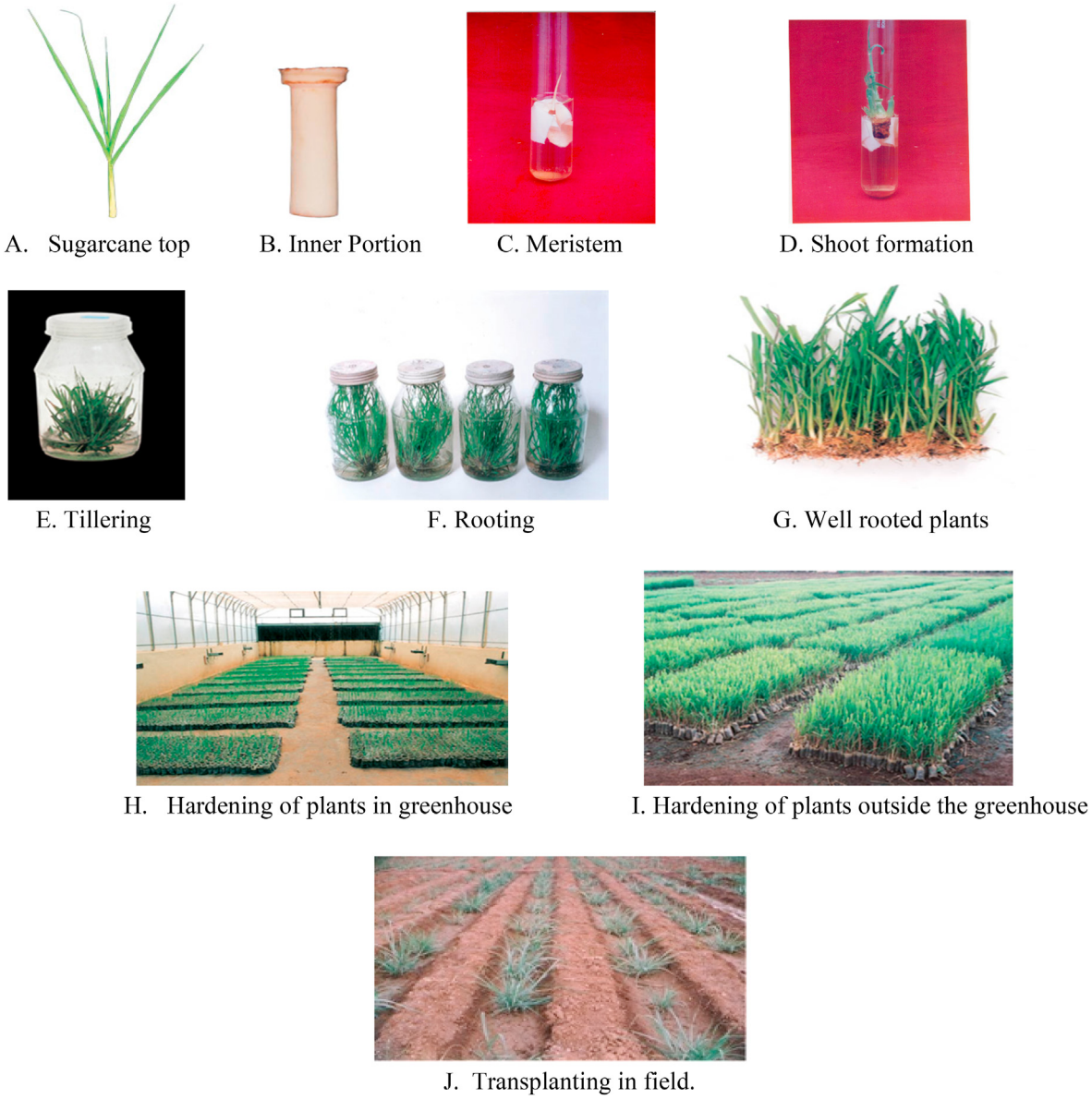

Fig. 1. Different stages of sugarcane meristem culture Following are the photographs of the stages of sugarcane meristem culture from experiments in laboratory and greenhouses.

row spacing from 90 cm to 150 cm did not make any significant change in the number of canes/ha and the number of three eye bud setts/ha. However, row spacing of 90 and 120 cm found equally effective in the production of the eye budded setts, and it is superior over 150 cm spacing (Table 6). There are references for research on the spacing of meristem cultured plantlets, but no one has done this type of research on spacing for seed obtained from meristem cultured plantlets.

Increase in the number of canes per meristem culture raised cane, and increased vigour is a phenomenon reported by many scientists. The current result is in contrast with the findings of [4] who reported increased stalk population and reduced cane and sugar yield in meristem culture seed over conventional seed sources.

Influence of spacing between two setts on some agronomic traits of sugarcane.

The effect of different spacing between setts at the time of planting on some agronomic traits (Milliable height, girth of cane, number of internodes/canes, number of canes per clump, number of canes/ha and yield of three eye budded setts/ha) is presented in Table 7.

The higher cane yield could be achieved either through higher seed rate or through maintaining population to an optimum level. A number of canes/clump, number of canes/ha, number of internodes/ha and yield of 3 eye bud setts, were all highly influenced by setts spacing (Table 7). Results showed that an increase in row spacing from 90 cm to 150 cm resulted in an increased number of internodes/cane and number of canes per clump. Setts obtained from meristem culture plants (M) gave the highest yield 116 thousand canes per hectare as compared to setts from conventional sugarcane (c) 95 thousands canes per hectare. Setts produced from meristem cultured sugarcane plantlets at 15 cm spacing gave higher yield (116 thousands canes per hectare) than any other spacing. A significant decrease in the number of canes/clump, number of canes/ha, number of 3 eye buds setts/ha and number of internodes/ha was observed, where setts spacing was increased from 15 to 45 cm in both meristem cultured as well as conventional sugarcane setts. There are references for research on the spacing of meristem cultured plants, but no one has done this type of research on spacing for seed obtained from meristem cultured plants.

Number of canes per clump, no. of canes per hectare and yield of 3 eye bud setts per hectare are the three important factors on which sugarcane

Table 6
Influence of row spacing on agronomic traits of meristem cultured sugarcane seed.

SN.	Agronomic characters and yield	Row Spacing 90 cm	Row Spacing 120 cm	Row Spacing 150 cm	S E + -	C D at 5%
1	Milliable height (cm)/cane	224.32	229.65	230.18	3.84	NS.
2	Girth of cane (cm).	11.14	11.24	11.63	0.05	NS.
3	No. of internodes/ cane.	16.08	16.39	16.99	0.06	0.18[a]
4	Length of internodes.	15.26	16.32	15.33	0.33	NS.
5	No. of canes/clump.	12.05	12.67	14.78	0.78	2.24[a]
6	No. of canes/ha (thousand).	103	103	98	0.80	NS.
7	Yield of three eye bud setts/ha (thousand).	72.8	72.7	69.2	5.9	NS.

NS - Not significant.
[a] - Significant.

yields are judged. All these three are significantly higher in meristem cultured sugarcane seed cane as compared to conventional sugarcane seed cane. 116 thousands canes per hectare were observed in meristem cultured sugarcane which were higher than conventional sugarcane (96 thousands canes per hectare). Number of canes per clump was also observed higher in TC (15.43) as compared to conventional (13.10). In TC sugarcane, yield of 3 eye bud setts per hectare was higher (81.9 thousands) as compared to conventional sugarcane, (67.1 thousands). It shows that performance of meristem cultured sugarcane seed is excellent as compared to conventional sugarcane seed.

Analysis of variance showed that there was no significant interaction between row spacing and spacing between sets (Table 8). However, independent effects were exerted on number of internode/cane, number of canes/clump, yield of canes/ha and number of 3 eye bud sets/ha. The analysis of variance showed that there was no significant variation in agronomic characters when compared with row spacing except for the number of canes/clump and number of internodes per cane (Table 8). There are references for research on the spacing of meristem cultured plantlets, but no one has done this type of research on spacing for seed obtained from meristem cultured plantlets.

All the above experiments helped to develop agronomy for seed production from meristem cultured sugarcane plants. We have taken demonstrations of meristem cultured plantlets on farms of sugar factories in Maharashtra for showing performance of meristem cultured plantlets to farmers and sugar factories employee (MD, AO and CDO). Now farmers and sugar factories have realized the benefit of meristem culture technology for improving sugarcane yield.

4. Conclusions

Meristem culture (tissue culture) of sugarcane is the most satisfactory and viable method for the production of healthy and pathogen-free seed material of sugarcane. An efficient protocol for in vitro rapid multiplication of sugarcane and hardening of meristem cultured plantlets in a greenhouse was developed. The agronomy for using meristem cultured sugarcane plants in the field as breeder's seeds and agronomy for using seeds obtained from meristem cultured plants, as the foundation for seeds was established.

Murashige and Skoog medium supplemented with BAP 0. 2 mg/l and Kinetin 0.1 mg/l was the best medium for shoot multiplication in vitro. Murashige and Skoog medium supplemented with IAA or NAA 1 mg/l was the ideal medium for root formation to the shoots. The survival percentage, shoot and root weight of meristem cultured sugarcane plantlets were higher in a soil mixture C + Vermicompost @ 25% y volume of medium and NPK as 55 mg urea, 360 mg single super phosphate and 95 mg muriates of potash/kg of the medium. This was the best soil mix for increasing survival of meristem cultured plants in the greenhouse.

Two feet distance between 2 meristem culture plants and 3 feet distance between 2 rows of sugarcane was found suitable for planting of meristem cultured plantlets in the field for the development of breeder seed. In foundation seed production an increase in row spacing from 90 cm to 150 cm resulted in an increased number of internodes/cane and number of canes per clump. Spacing between two setts highly influence the number of canes/clumps, number of canes/ha, number of internodes/ha and number of 3 eyes budded setts per hectare. Setts obtained from meristem cultured plants (M) gave the highest yield as compared to sets from conventional sugarcane (C). Setts produced from meristem cultured sugarcane plants at 15 cm spacing gave a higher yield than the other spacing. A significant decrease in the number of canes/clump, number of canes/ha, number of 3 eye bud setts/h and number of internodes/ha was observed where setts spacing was increased from 15 to 45 cm. Number of canes per clump, no. of canes per hectare and yield of 3 eye bud setts per hectare were significantly higher in meristem cultured sugarcane seed cane as compared to conventional sugarcane seed cane. Performance of seeds obtained from meristem culture was excellent as compared to conventional sugarcane seeds.

Thus, the protocol developed in this study shows potential for the development of sugarcane meristem cultured plants and the use of these

Table 8
Calculated F at 5% level of significance for agronomic characteristics as influenced by row and sett spacing.

SN.	Source of variation	Row spacing (A)	Setts spacing (B)	Interaction (A[a]B)
1	Milliable height/cane	1.52[N.S.]	1.56[N.S.]	0.84[N.S.]
2	Girth of cane	1.78[N.S.]	0.56[N.S.]	1.21[N.S.]
3	No. of internodes/cane	2.94[a]	3.48[a]	1.05[N.S.]
4	Length of internode	2.78[N.S.]	0.57[N.S.]	0.62[N.S.]
5	No. of canes/clump	6.65[a]	4.01[a]	0.15[N.S.]
6	Yield of canes/ha	1.02[N.S.]	6.40[a]	1.35[N.S.]
7	Yield. of three eye bud setts/ha	0.51[N.S.]	3.22[a]	0.68[N.S.]

NS. Not significant.
[a] Significant.

Table 7
Influence of spacing between 2 setts on agronomic traits of sugarcane.

S·N	Agronomic traits	TC15	TC30	TC45	C15	C30	C45	SE + -	CD at 5%
1	Milliable height (cm)/cane	222.98	233.44	231.83	221.13	224.23	223.71	5.22	NS.
2	Girth of cane (cm)	11.26	11.46	11.62	10.93	11.10	11.25	0.28	NS.
3	No. of internodes/cane	16.99	17.06	17.21	16.14	16.06	15.66	0.48	1.36[a]
4	Length of internodes	15.83	15.77	14.97	16.00	15.85	15.39	0.77	NS.
5	No. of canes/clump	15.43	14.17	13.73	13.10	12.43	11.95	0.88	2.53[a]
6	No. of canes/ha (thousand)	116	109	105	95	95	89	0.5	1.4[a]
7	Yield of 3 eye bud setts/ha (thousand)	81.9	76.9	74.1	67.1	66.8	62.6	4.0	11.4[a]

NS – Not significant.
[a] - Significant.

plants for quality sugarcane seed production. This will help to supply healthy seed cane to the sugarcane farmers, which is the main prerequisite for improving the productivity of the sugarcane crop. We can also make use of this technology for rapid multiplication of newly released varieties and for supplying seeds of the newly released varieties to the sugarcane farmers within a short period of time.

Declaration of competing interest

No Conflict of Interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jafr.2021.100126.

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