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A competent protocol for large scale production of sugarcane (*Saccharum officinarum* L.) through meristem culture

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Abstract: A rapid micro propagation and acclimatization response of two different varieties of sugarcane Co86032 and CoN 04131(*Saccharum officinarum* L.) was obtained in this study. The shoot apical meristem of different sizes wascultured on Murashige and Skoog medium supplemented with different concentrations and combinations of benzylaminopurine and kinetin either alone or in combination with each other alongwith GA_3. Best shoot formation response in Co 86032 was obtained on MS medium containing 1.5mg/l BAP while in CoN 04131 the combination of 0.5 mg/l BAP with 0.25 mg/l Kinetin showed best shoot formation response from apical meristem. Meristem of 3.0 mm size proved to be the best size for micropropagation of sugarcane. Excellent multiplication response of *In vitro* formed shoots was obtained when the concentration of BAP was decreased to 1.0 mg/l in Co 86032and 0.25 mg/l BAP and Kin in CoN 04131 (*i.e*. 0.25 mg/lBAP + 0.25 mg/l Kinetin. MS medium containing 1.0 mg/l NAA and 2.0 mg/l IBA showed 100% rooting response of *In vitro* regenerated shoots of both the varieties of sugarcane within eight days of inoculation. Best hardening response was obtained in sand+ soil + pressmud (1:1:1) media.

Keywords: Benzylaminopurine, Gibberellin, Kinetin, Meristem culture and Naphthalene acetic acid

INTRODUCTION

Virus infection is the major problem in commercial sugarcane crop. Viruses cause heavy loss of sugarcane plants by reducing quality and yield. Present study is focus on meristem culture for develop a competent protocol, virus eradication and rapid production of sugarcane plants. Plant tissue culture techniques have become a powerful tool for studying and solving basic and applied problems in plant biotechnology. During the last thirty years, micropropagation and other *in vitro* techniques have become more widely used in commercial horticulture and agriculture for the mass propagation of crop plants (Das *et al.,* 1996; Khattak *et al.,* 2014). Sugarcane (*Saccharumofficinarum* L.) is an important industrial cash crop of India. It is an economically important, polysomatic, highly heterozygous, clonally propagated crop that accounts for more than 60% of the world's sugar production (Guimarces and Sobral, 1998; Khan *et al.,* 2008). Lack of rapid multiplication has been a serious problem in sugarcane breeding (Ali and Afghan, 2001). Time required and continuous contaminations by systemic diseases are the serious problems to multiply an elite genotype of sugarcane in the open field (Nand and Singh, 1994). Micropropagation is currently the only realistic means of achieving rapid, large-scale production of disease-free seed canes of newly developed varieties in order to speed up the breeding and commercialization process in Sugarcane (Lorenzo *et al.,* 2001). Barba *et al.,* (1978) reported that within 9 months callus culture of apical meristem produce planting material from a single spindle which was sufficient to plant a hectare of land. Sauvaire and Glozy, (1978) used auxiliary buds for micropropagation of sugarcane. Cheema and Hussain, (2004) also reported shoot tip culture and meristem culture for mass propagation of sugarcane. This study was carried out to develop protocol for multiplication on large scale production of elite cultivars grown in India.

MATERIALS AND METHODS

Apical portion from the shoot of sugarcane were excised from field growing plants. As sugarcane apical meristem had covering of rolled leaves, therefore, apical meristem was wrapped deep in leaf sheaths and is naturally sterilized. Therefore, it was not necessary todisinfect them. However, to prevent any contamination from outer covering of leaves, surface sterilization was carried out as follows. For sterilization, explant was first washed with running tap water. Then treated with house hold detergent for five minutes. This was followed by second washing with tapwater to remove all the traces of detergent. The explant was then treated with 0.1% Bavistin and mancozeb fungicide solution for 15 minutes. After this explant treated with 0.2%

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citric acid and ascorbic acid, the explants were washed three times with sterilized distilled water to remove all the traces of fungicides and antioxidants. Explants brought in the laminar hood and treated with 0.1% $HgCl_2$.The sterilized explants were then inoculated by proper dissecting and sizing the meristem (0.5-1.0 cm) on MS (Murashige and Skoog, 1962) medium supplemented with different concentrations of BAP either alone or in combination with Kinetin or GA_3. For multiplication of induced shoots hormonal concentration was decreased and shoots multiplication was observed after 24 days of shoot induction. For *In vitro*rooting MS medium containing different concentrations of NAA and IBA was used either alone or in combination with each other. Sucrose 3% was used in all the media. The pH of the medium was adjusted to 5.76 with 0.1 N solution of NaOH or HCl. MS medium was used both in solid and liquid forms. For solidification 0.7% agar was used. In case of liquid medium autoclaved cotton was used to support the plant tissues. Themedium was autoclaved at 121ºC and 15 Ibs/inch^2 pressure for 15 minutes. Cultures were maintained under fluorescent light having 2500 lux light intensity. The incubation temperature was 26◦C ± 1◦C with 16 hour light and 8 hour dark period in every 24 hour cycle. First sub-culturing was done after five weeks and rest sub-culturing after two weeks. During each sub-culturing all dead or dis-coloured or vitrified shoots were removed. Hardening was carried out in glass house under natural light conditions.

RESULTS AND DISCUSSION

Shoot formation from apical meristem: The criteria on of good growth for newly formed shoots from apical meristem was based on the production of broad and dark green colored leaves, healthy stems and number of small germinating buds at the base of stem. From table 1 it is observed that in Co 86032best results for shoot formation were obtained in MS medium containing 1.5 mg/l of BAP. In this medium all explants showed shoot proliferation response within 18 days with maximum number of 1.8 shoots per explant. By increasing the concentration of BAP, frequency of shoot proliferation was decreased and time taken for shoot formation was also delayed, In case of CoN 04131, shoot formation response varied from 9 to 13.4 days. Best response was obtained in medium BAP 0.5 mg/l + Kinetin 0.25 mg/l. All explants showed 100% shoot formation within 10 days with 1.6 shoots per explant (Table 2). MS+ BAP 1mg/l and BAP+Kin 1mg/l media also showed hundred percent shoot formation but time taken was more and the number of shoots per explants were less. All other combination did not prove good for shoot formation in CoN 04131 (Table 2). In the present investigations shoot apical meristem of different sizes ranging from 0.5- 5 mm was used. As shown in table 3, time for shoot formation was increased by decreasing the size of meristem. Maximum rate of survival was achieved when meristem of 3 mm size was used. This size exhibited 100% survival with 90% regeneration potential within 20 days of inoculation. For shoot formation both solid and liquid media were used. Best results were obtained on media solidified with agar at 7 gm/l.Producing large number of identical clones by *In vitro* culture technique is being routinely used for wide range of plant species (Biondi, 1986). The results of present study demonstrate the regeneration potential of shoot apex of different sizes into plants. Siddiqui (1993) also reported the role of size of meristem shoot formation and proliferation. In the present investigation, best results for shoot formation and proliferation were obtained when meristem of size 3.0 mm was used. The present study also demonstrates the effect of phytohormones for shoot formation and multiplication. Among various phytohormones mainly two cytokinins *i.e.,* BAP and Kinetin were used in MS medium either alone or in combination with each other or with GA_3. The effect of cytokinin was different in both the varieties. In case of Co 86032, BAP alone provided good results for shoot formation. Among different concentrations of BAP used, 1.5 mg/l provided best shoot formation response (Table 1). In case of CoN 04131, 0.5 mg/l of BAP with 0.25 mg/l Kinetin provided good shoot formation response (Table 2). Similarly other scientist also reported the use of kinetin with BAP for shoot formation in sugarcane (Ali *et al.,* 2008; Goel *et al.,* 2010 and Tolera *et al.,* 2014).

Multiple shoot formation: After 5–6 weeks of shoot growth, actively growing shoots were transferred to fresh medium in bottles for further growth and proliferation. Both solid and liquid media were tested. Best results for shoot multiplication were obtained in liquid medium. Proliferation of shoot started and during secondary proliferation stage, lateral shoots developed from the base of newly initiated shoot. As a result a dense mass of shoots (25-30) was developed in each culture bottles. After 15 days these bunches were further sub-divided in bunches containing 4-5 shoots and were transferred into fresh medium in bottles. In this way shoot multiplication was maintained for several passages by regular transfer to fresh medium. The best shoot multiplication response in Co 86032was obtained in MS+BAP medium *i.e.* MS medium containing 1.0 mg/l BAP (Table 4). In this medium 28.6 shoots were obtained after five weeks of sub-culturing. Addition of kinetin and GA3 did not show any support to shoot multiplication in Co 86032. In case of CoN 04131, best shoot multiplication was achieved in SM5 medium *i.e.,* MS media containing 0.25 mg/l BAP and Kin each (Table 4). From table 4 it was noted that in both the varieties rate of shoot multiplication increased by decreasing the concentration of BAP. It was also observed that shoot multiplication response was enhanced in liquid medium while solid medium delayed shoot multiplication response.

Table 1. Effect of different hormones on shoot formation from apical meristem in sugarcane cvCO 86032.

Media	Conc. (mg/l)	No. of explant culture	Days for shoot formation	Frequency of shoot formation (%)	Number of Shoot/ Explant	Shoot Length (cm)
MS+ BAP	1.0	10	22	89	1.8	1.45
	1.5	10	18	100	1.8	1.45
	2.0	10	27	71	1.4	1.63
	2.5	10	26	82	1.3	1.50
BAP+Kin	0.25	10	23	90	1.4	1.44
	0.50	10	18	100	1.5	1.35
	0.75	10	25	89	1.1	1.42
	1.0	10	29	71	1.1	1.40
$BAP+GA_3$	0.25	10	32	61	0.8	1.61
	0.50	10	28	81	0.9	1.62
	0.75	10	18.7	100	1.0	1.50
	1.0	10	24	78	1.3	1.61
Mean			24.22	84.33	1.37	1.49

MS- Murashige and Skoog; BAP- Benzylanmino purine; Kin- Kinetin and GA_3- Gibberellic acid

Table 2. Effect of different hormones on shoot formation from apical meristem on shoot formation on in sugarcane cvCON 04131.

Media	Conc. (mg/l)	No. Of Explant culture	Days for shoot formation	Frequency of shoot formation (%)	Number of Shoot/ Explant	Shoot Length (cm)
MS+ BAP	1.0	10	21	100	1.6	1.59
	1.5	10	27	90	1.5	2.0
	2.0	10	26	90	1.2	2.04
	2.5	10	31	80	1.3	1.08
BAP+Kin	0.25	10	24	90	1.4	1.80
	0.50	10	22	100	1.6	1.50
	0.75	10	30	81	1.2	2.07
	1.0	10	20	100	1.4	1.24
$BAP+GA_3$	0.25	10	34	70	0.9	2.17
	0.50	10	28	82	0.9	2.07
	0.75	10	26	81	1.3	2.01
	1.0	10	24	82	1.1	2.18
Mean			26.08	87.16	1.28	1.93

MS- Murashige and Skoog; BAP- Benzylanmino purine; Kin- Kinetin and GA_3- Gibberellic acid

Table 3. Effect of size of shoot apical meristem on shoot formation in sugarcane.

Size of Meristem (mm)	No. of Meristem culture	Days for shoot induction	Meristem survived	% age of Survival	Meristem showing shoot induction	%e age of shoot induction
0.5	10	28	7	70	5	50
1.0	10	29	8	80	6	60
2.0	10	30	9	90	8	80
3.0	10	20	10	100	9	90
4.0	10	22	10	100	8	80
5.0	10	23	9	90	9	90

MS- Murashige and Skoog; BAP- Benzylanmino purine; Kin- Kinetin and GA_3- Gibberellic acid

The regenerated shoots obtained were further multiplied by sub-culturing on fresh medium and maintained for one year. Maintenance and multiplication of germplasm stock in reduced space for several months was also reported by Engelman (1995). The present study also highlights the role of cytokinin particularly BAP for shoot formation. The primary mode of action of plant growth regulators involves binding of active substances to a specific receptor molecule which bind either on cell surface or within the cytoplasm. The concentration of the receptors to target tissue determines the response potential (Flores and Tobin, 1988). Bud formation begins with an asymmetric division of target cell, several cells back from the tip. This step is initiated by cytokinin binding to an unidentified receptor within the target cell and its further development requires the continuous presence of cytokinins (Saunders and Hepler, 1982). The initial response of cytokinin may be mediated by an increase in the cytosolic calcium concentration by promoting calcium uptake from the medium. Calcium ions have been shown to act through the regulating protein calmodulin. Each calmodulin have four high affinity calcium binding sites. Calmodulin alone is inactive as a regulator but

Table 4. Effect of different hormones on shoot multiplication in sugarcane.

Media	Concentration Mg/l	No. of shoot per culture Co 86032	CON 04131
MS+ BAP	1.0	28.6	25.6
	1.5	24.5	23.4
	2.0	17.6	17.7
	2.5	4.1	17.2
MS+BAP+Kin	0.25	15.7	29.6
	0.50	17.8	27.0
	0.75	17.4	22.6
	1.0	19.5	23.0
MS+BAP+GA_3	1.0	16.1	26.5
	1.25	7.6	21.8
	1.50	11.5	25.2
	1.75	9.5	23.4
Mean		15.82	23.58

MS- Murashige and Skoog; BAP- Benzylanmino purine; Kin- Kinetin and GA_3- Gibberellic acid

Table 5. Rooting of regenerated shoot of sugarcane cv86032.

Media	Con. (mg/l)	No. of Shoot cultured	Days to root initiation	Plant showing roots	Frequency of root formation (%)	Number of roots per plant
½ MS + NAA	1.0	10	9.4	9	90	1.2
	2.0	10	9.3	9	90	1.4
MS+ NAA	1.0	10	9.1	5	50	2.1
	2.0	10	9.1	4	40	2.3
½ MS + IBA	1.0	10	11.0	5	60	1.1
	2.0	10	1.9	8	80	1.2
MS +IBA	1.0	10	9.8	3	30	1.8
	2.0	10	9.7	4	40	2.1
MS+NAA+ IBA	1.0+1.0	10	8.3	6	60	2.7
	1.0+2.0	10	7.8	10	100	3.0
	2.0+1.0	10	8.0	8	80	2.9
Mean			8.49	6.45	65.45	1.98

MS- Murashige and Skoog; NAA- Naphthalene acetic acid and IBA- Indole buteric acid

the calmodulin-calcium complex can bind to and activate a number of enzymes including protein-kinase enzymes that add phosphorous to the serine or tyrosine hydroxyl group of proteins. The phosphorylation of enzyme can change their activity. So the calcium-calmodulin complex acts as a master switch, regulating alternative metabolic pathways within the cell. Therefore, the calcium ions may act as secondary messenger, transforming the hormonal signal into a biochemical switch regulating the initial stages of bud formation (Vervoodre and Grimes, 1994).

Rooting of regenerated shoots: The regenerated shoots were used for root induction in rooting media. Full and half strength MS medium supplemented with 12 different auxin concentrations was used. Frequency of root formation was different in all the media. Best root formation response was obtained in MS+NAA+ IBA medium *i.e.,* MS medium containing 1.0 mg/l NAA with 2.0 mg/l IBA. At this concentration 100% shoots formed roots within 7.8 days of inoculation with 3.0 roots per shoot in Co 86032 and 3.1 roots per shoot in CoN 04131 (Table 6). Half strength MS medium supplemented with the same auxins as used for full strength MS medium were also tested. No significant effect of half strength MS medium was found for root initiation and development (Tables 5 and 6).In the present study best rooting response was obtained in full strength MS medium supplemented with 1.0 mg/l NAA with 2.0 mg/l IBA (Tables 5 and 6). Nadgauda (2002) reported high concentration*i.e.,* 5.0 mg/l of NAA or combination of two auxins NAA and IBA for rooting in sugarcane. Yi *et al.*, (2004) also favoured the combination of NAA and IBA for rooting in *Phragmites communis*. Pruski *et al.* (2005) found the combination of IBA and NAA best for rooting. This inhibition of root initiation and elongation on higher concentration of auxins may be due to deposition of ethylene. As auxins of all types stimulate plant cell to produce ethylene, especially when high amount of synthetic auxins are used. Ethylene retard root elongation (Weiler, 1984). The study indicates that meristem-culture is not only feasible but it can be used as the helpful tool for rapid multiplication of disease free, high yielding and good quality planting material of highly adapted, genetically stable and newly released varieties of sugarcane.

Hardening of *In vitro* raised plants: For hardening *In vitro* raised plants were shifted in the fan and pad poly-house in three different medium compositions. Best hardening response was obtained in a mixture of sand + soil + pressmud at 1:1:1 after three week of trans-plantation in poly house.

Conclusion

The present study concluded thatMS medium containing 1.5 mg/l of BAP was best suitable media for shoot formationin variety Co 86032.In case of CoN 04131, best response was obtained in medium BAP 0.5 mg/l + Kinetin 0.25 mg/l. The best shoot multiplication responsewas obtained in MS+BAP mediumcontaining 1.0 mg/l BAP in variety Co 86032while, in variety CoN 04131, best shoot multiplication was achieved in SM5 medium *i.e.,* MS media containing 0.25 mg/l BAP and Kin each.Further, best rooting response was obtained in full strength MS medium supplemented with 1.0 mg/l NAA with 2.0 mg/l IBA. Therefore, this protocol has a potential to large scale production of sugarcane in both varieties through meristem culture.

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