

In Vitro studies of sugarcane variety co-91017 through micropopagation of shoot tip culture

Abstract

Saccharum officinarum is an important sugarcane variety in Tamil Nadu. In our present study to optimization and standardization of protocol for induction of callus and regeneration of whole plantlets was established through in vitro culture of Sugarcane (*Saccharum officinarum* L.) by using shoot tip as an explant. The multiple shoot regeneration at various frequencies was observed by using different concentration and combination of growth regulators containing MS medium. The highest percentage of callus induction was observed in MS medium supplemented with 2.5mg/L, 2-4 D. The best response in terms of multiple shoot induction was observed on MS medium supplemented with BAP 2.0mg/L+IBA 0.5mg/L. When in vitro shoot lets were inoculated on to the half-strength semi solid MS medium supplemented with 2.5mg/L NAA, rooting was more profuse. Rooted shoots were transplanted in the green house for hardening and their survival rate was 85% in the field condition

Keywords: saccharum officinarum, micro propagation, liquid ms medium, auxin, cytokinin

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Introduction

Plant tissue culture is a practice used to propagate plants from cells or tissues under sterile conditions to produce clones of a plant. When disease-free material is used as the source of explants or the explants are heat-treated to eliminate the diseases, the resultant micro propagated plants are disease-free and healthy. Sugarcane, a versatile crop cultivated by the farmers in Tamil Nadu and being a rich source of sugar, ethanol, biogas, manure, production of electricity, paper etc. During the cultivation, sugarcane leaves were used as a cow feed which require less amount of water compared with other crops and will take at least 10 months to mature. Realizing the advantages of sugarcane, people often preferred it for planting, even though the plantation period is more. In the cultivation period, lot of seedlings was unable to grow into a plantlet and hence, many researchers focused to produce a healthy, disease free plant by biotechnological approaches like micropopagation. Numerous reports revealed that micropopagation techniques were suitable for commercial seed production in sugarcane. To obtain sugarcane mosaic virus free plants, more emphasis was focused on apical meristem explants.^{1,2} With the help of *in vitro* techniques, lot of interest has been generated in the recent year for rapid multiplication of virus free materials through apical meristem culture³ and callus culture.⁴ From the published data available, it is evident that every part of the sugarcane plant is capable of inducing callus; however, only immature leaves⁵ and the inflorescence are capable of producing morphogenic callus to an appreciable level.

In sugarcane variety, Co-91017 is an important crop in Tamil Nadu which was cultivated by maximum number of farmers. During the process of plantation, most of the plants will not grow into plantlets and hence the present study was delineated to evaluate the optimum concentration of growth hormones required by the plantlets micro propagated under *in vitro* conditions, which will further be supplied to farmer for plantations.

Materials and Methods

Culture medium

Media often need to be varied for different plant genera and species according to the kind of culture to be undertaken. MS media as reported by Murashige et al.,⁶ or modified MS media as supplemented with various plant growth regulators have proved to be suitable for callus growth, direct and indirect morphogenesis and shoot culture of sugarcane as reported by several authors.

The medium was prepared by adding required amounts of stock solutions and final volume was made up with deionized water. The pH of the medium was adjusted to 5.8 by using 1N KOH or 1N HCl and were autoclaved at 121°C for 15 minutes. The sterilized medium was then transferred to media storage room under aseptic conditions.

Callus induction medium

MS medium supplemented with the different range of concentration of auxins were tried individually for callus induction such as 2,4-D (0.5, 1.0, 2.0, 2.5 and 3.0mg/L) and NAA (0.5, 1.0, 2.0, 2.5 and 3.0mg/L) with liquid MS basal medium.

Shoot regeneration medium

MS medium supplemented with the different concentrations and combinations of Kinetin, BAP (0.5-3.0mg/l) and IBA (0.5 & 1.0mg/l) for multiple shoot regeneration. It was sub-cultured every 7 days of incubation.

Rooting medium

Micro shoots were measuring about 5-6cm in length and transferred to half-strength MS medium supplemented with different concentrations of IBA and NAA (0.5-3.0mg/l) either individually or in combinations.

Choice of explants

Before micropropagation commences, careful attention should be taken for the selection of the stock plant or plants. They must be a typical of the variety or species and free from any symptoms of disease. The kind of explants chosen, its size, age and the manner in which it is cultured can all affect whether tissue culture can be successfully initiated, and whether morphogenesis can be induced. Choice of the correct explants is important, where propagation is based on direct or indirect shoot initiation.^{7,8}

The part of the plant from which explants are obtained depends on:

- i. The kind of the culture to be initiated.
- ii. The plant species to be used.

Collection of explants

Healthy and young shoot tip were collected from the field grown plants of sugarcane (*Saccharum officinarum* L.) maintained by Research & Development (R&D) farm of E.I.D parry garden in Pettavaithalai, Trichy.⁹

Preparation of tops for shoot tip culture

Shoot tip explants can be obtained from three sources: actively growing canes, elongating axillary shoots and dormant axillary buds. In our experiment, shoot tip is an explants collected from actively growing sugarcane tops. The leaf sheaths of sugarcane were carefully removed one by one until the inner white sheaths were exposed. The explants were sized to 10cm length by cutting off two ends, locating the growing point in the middle of the top.

Surface sterilization of explant

Actively growing points of sugarcane top, taken as explants from 8-12months old sugarcane cultivar. The outer mature leaves were removed till a spindle of about 1cm was obtained. The spindle (3.0-4.0cm) was excised and thoroughly washed 30minutes under tap water. The explants were washed thoroughly for 20-30minutes under running tap water, 0.1% mercuric chloride for 10minutes. Finally, the young shoot tip was washed thoroughly with sterile distilled water for 3 to 5times.

Inoculation

The explants were picked up carefully with sterilized forceps and kept in a sterile Petri dish. Using a fine scalpel and forceps, which are heated and cooled every time before and after use, the leaf sheaths were removed one by one till the suitable size were obtain (2 to 2.5cm). Initially, 3 to 4 longitudinal slits was given superficially through the scalpel. By charitable superficial transverse cuts on the base, the leaf whorl was removed carefully without exert pressure on the internal tissues. The repeated process is done until the apical dome with 2 to 3 leaf primordia was exposed. This process should be done very carefully without damaging the apical dome. The young shoot tip explants was sterilized and inoculated on sterilized MS medium⁶ supplemented by various concentration and combination of plant hormones.

Incubation

In medium the pH was adjusted in the direction of 5.8 and autoclave for 15minutes at 121 °C. The cultures were incubated with

16hour of light and 8 hour of dark (fluorescent tubes are used as a light source), with artificial illumination of 2000-3000 lux by placing the cultures at 25-30cm below the fluorescent light and maintain the temperature at 25±2 °C.

Statistical analysis

Randomized Block Design (RBD) was set up in an experiments and each experiment had 10 replicates and was repeated 3 times. In each replication, 10 explants were used per treatment. Observation was recorded in terms of percentage response of callus formation, number of shoots, shoot length, number of roots and root length respectively. Duncan's Multiple Range Test (DMRT) was used to compare means at 5% probability level according to Gomez & Gomez.^{9,10}

Results and discussion

Callus induction

The Callus induction was observed in 2 weeks after inoculation of the explants in MS medium amended with different concentrations of 2,4-D and NAA (0.5, 1.0, 2.0, 2.5 and 3.0mg/L). Callus induction was triggered in all concentrations of 2,4-D, but increased callus induction and regeneration of explants was observed in the concentration of 2.5mg/L 2, 4-D. On this hormone concentration, explants produced creamy white callus which showed 90% of callus induction (Table 1) (Figure 1) and many other reports revealed such types of calli.^{11,12} In contrast to present study, MS media containing 3.5mg/L of 2,4-D¹³ and 3.0-4.0mg/L 2,4-D¹⁴ showed higher percentage of callus induction (Figure 2). The highest mean rate of callus induction was produced on MS medium supplemented with 3% sucrose, 3mg/L 2,4-D and 0.5 mg benzyl adenine/l. On this medium, callus induction from spindle explants was highest in the genotypes CoS 767 and CoJ 64.¹⁵

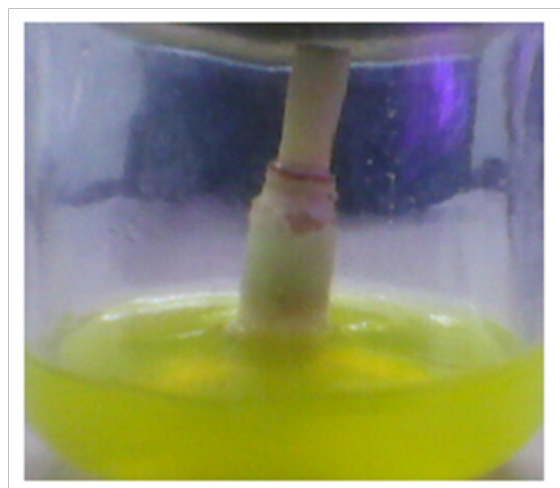


Figure 1 Initiation.

Sterilized explants of Co 740 showed significant callus induction on MS medium containing 100mg of myo-inositol and 3mg/L of 2,4-D and 10% v/v coconut milk, and callus induce from leaf explant supplemented with 2,4-D at the concentration of 5.0mg/L showed.¹⁶ The concentrations of NAA (2.0 and 3.0mg/L) induced calli (20 to 30%) which was globular, grayish and hardy in nature. All these study resembles that sugarcane plant requires higher concentration of 2,4-D for callus induction (Table 1).

Regeneration of micro shoots

Various concentrations and combinations of cytokinin (BAP and Kinetin) and auxins, (IBA) were used for shoot regeneration. Shoot multiplication (Figure 3) from Co-91017 explant was observed on MS medium supplemented with BAP (2.0mg/L)+IBA (0.5mg/l) (Table 2). On this combination 92% of explants showed shoots and the number of functional shoots was 13.4±0.90 with average length of 6.8±0.37cm. The combination BAP+IBA showed positive effects on shoot formation which showed more significant regeneration and the combination of BAP+NAA showed better result, but not effective than the combination of BAP+IBA.¹⁷

At lower concentration of BAP (0.5mg/L) and Kinetin (0.5mg/L), there was loss of proliferation and vigour.¹⁸ MS medium containing 3 or 4mg/L BAP and 0.2mg/L of IAA was studied and percentage induction of multiple shoots, mean multiple shoot number, were higher (90% and 16.5, respectively) with 3mg/l BAP+1mg/L IAA combination.¹⁹ The higher shoot multiplication was recorded on the medium containing 1.5mg/L Kinetin. The treatment with 1.5mg/L kinetin+1.0mg/L BAP+20% CW showed the higher values for length of main shoot in all tested sugar cane cultivars.²⁰

However, the combinations of low level of auxin and high level of cytokinin were necessary for differentiation of adventitious shoot in sugarcane variety Nayana than individual concentration of cytokines. All these studies concluded that the regeneration ability of callus was specific and genotype dependent phenomenon and it is parallel with the hormonal concentration and combinations.^{21,22}

In vitro rooting

Different concentrations and combinations of auxin used to regenerate adventitious roots. Among those combinations, IBA and NAA showed better response with profused rooting (Figure 4). In half strength MS medium supplemented with 2.5mg/l NAA showed best rooting response (Table 3) and the maximum number of roots per micro shoots were 13.9±0.56, which taken only 8-10days for root initiation with average root length 4.3±0.94cm for the variety Co-91017 (Table 3). Optimal growth was observed in half strength MS medium supplemented with or without NAA.²³ In half strength MS medium, stronger rooting response was observed in the concentration of 5.0mg/L NAA.²⁴

The stronger root growth was influenced by IBA at the concentration of 1.0mg/L with maximum number of 41 roots per plant.²⁵ Stronger rooting response was also observed in MS liquid medium amended with NAA (5mg/L) and sucrose (70g/l).²⁶ MS medium containing 2.0mg/L IBA and 6% sucrose showed only 6-7 roots after 3weeks²⁷ and poor quality rooting response was observed at 0.1-0.5mg/L IBA along with 0.5-2.0mg/L BAP.²⁴ These findings also agree well with previous findings.²⁸ Alam et al.,²⁸ observed the rooting response at concentration of 2.5mg/L IBA and it shows 16 numbers of roots at the length of 1.1cm.²⁸ MS medium supplemented with auxins (NAA+IBA) 0.5mg/L for each one showed better result on rooting. It was also found that 0.5mg/L NAA+2.5mg/L IBA showed rooting response with 11.3±1.08 number of roots and 3.7±0.47cm of root length.²⁹

Table 1 Effect of different concentration of NAA and 2,4-D on callus induction on shoot tip culture of sugarcane in Co-91017 variety

Treatments	Hormones	Hormonal supplements (mg/L)	Number of explant showed callusing	% of Explant with callus induction
T1	NAA	0.5	0	0
T2	NAA	1.0	1	10
T3	NAA	1.5	2	20
T4	NAA	2.0	3	30
T5	NAA	2.5	4	40
T6	NAA	3.0	2	20
T7	2,4-D	0.5	2	20
T8	2,4-D	1.0	3	30
T9	2,4-D	1.5	4	40
T10	2,4-D	2.0	6	60
T11	2,4-D	2.5	9	90
T12	2,4-D	3.0	7	70

NAA: α-naphthalene Acetic Acid; 2,4-D: 2,4-Dichlorophenoxy Acetic Acid
 [No callusing: poor callusing =20-50%, considerable callusing =51-85%, Intensive callusing = 86-100%.]
 10 replicates/ treatment. Means are calculated by Duncan's multiple range test at the significance level of 5%.

Table 2 Effect of different concentration and combination of BAP, KN and IBA on shoot initiation on shoot tip of sugarcane variety Co-91017

Treatments	Hormones	Hormonal supplement (mg/L)	No of shoots/ explant	Average length of the shoots
T1	BAP	0.5	2.9±0.90	3.6±0.84
T2	BAP	1	3.2±0.51	3.9±0.77
T3	BAP	1.5	3.6±0.47	4.1±0.16
T4	BAP	2	4.5±0.94	5.2±0.54
T5	BAP	2.5	4.2±0.65	4.0±0.47
T6	BAP	3	4.1±0.51	4.4±0.75
T7	KN	0.5	2.0±0.47	2.9±0.04
T8	KN	1	2.1±0.44	3.0±0.47
T9	KN	1.5	3.0±0.81	3.1±0.89
T10	KN	2	3.2±0.82	3.5±0.40
T11	KN	2.5	4.0±0.70	3.9±0.28
T12	KN	3	3.5±0.56	3.6±0.47
T13	BAP+IBA	0.5+0.5	3.3±0.47	3.0±0.94
T14	BAP+IBA	0.5+1.0	3.6±0.62	2.8±0.89
T15	BAP+IBA	1.0+0.5	3.3±0.43	2.3±0.04
T16	BAP+IBA	1.0+1.0	2.9±0.47	2.1±0.23
T17	BAP+IBA	2.0+0.5	13.4±0.90	6.8±0.37
T18	BAP+IBA	2.0+1.0	10.9±0.31	4.2±0.61

BAP: 6-Benzyl Amino Purine; KN: Kinetin; IBA: Indole3-butyric acid
 10 replicates/ treatment

Table 3 Effect of different concentration of IBA and NAA on root induction on shoot tip of sugarcane variety Co-91017

Treatment	Hormones	Hormonal supplements (mg/L)	No of roots (28 days of incubation)	Average length of roots(cm)
T1	IBA	0.5	3.3±0.47	1.9±0.45
T2	IBA	1	3.6±0.61	2.3±0.41
T3	IBA	1.5	5.4±0.32	2.5± 0.32
T4	IBA	2	8.3±0.84	2.8±0.23
T5	IBA	2.5	11.5±0.70	3.6±0.65
T6	IBA	3	8.8±0.65	2.7±0.09
T7	NAA	0.5	3.3±0.65	1.1±0.45
T8	NAA	1	3.9±0.47	1.4±0.29
T9	NAA	1.5	5.3±0.74	1.6±0.23
T10	NAA	2	8.5±0.28	3.5±0.47
T11	NAA	2.5	13.9±0.56	4.3±0.94
T12	NAA	3	8.4±0.47	3.4±0.47
T13	NAA+IBA	0.5+0.5	0	0
T14	NAA+IBA	0.5+1.0	5.3±0.61	2.9±0.27
T15	NAA+IBA	0.5+1.5	5.4±0.74	1.8±0.73
T16	NAA+IBA	0.5+2.0	10.3±0.67	4.0±0.87
T17	NAA+IBA	0.5+2.5	11.5±0.88	4.6±0.57
T18	NAA+IBA	1.5+0.5	6.8±0.71	2.9±0.18
T19	NAA+IBA	2.0+0.5	6.3±0.92	2.6±0.34
T20	NAA+IBA	2.5+0.5	5.9±0.96	2.3±0.69

IBA, indole3-butyric acid; NAA, α-Naphthalene Acetic Acid
 10 replicates/ treatment

MS medium containing 1.0mg/L NAA and 2.0mg/L IBA showed 100% rooting response of *in vitro* regenerated shoots from sugarcane explants within eight days of inoculation.⁴ By transferring shoot clumps on 1/2 MS medium containing 2mg/L NAA and 1.0mg/L IBA showed 85-92% of rooting²⁰ was observed. Dhumale et al.,¹⁸ reported

that half strength MS medium containing 2mg/L IBA+1mg/L IAA was found to be optimum for root initiation and higher amount of rooting was recorded on MS medium supplemented with 7% sugar and 5mg/L NAA.^{30,31}



Figure 2 Callus induction.



Figure 3 Shoot multiplication.

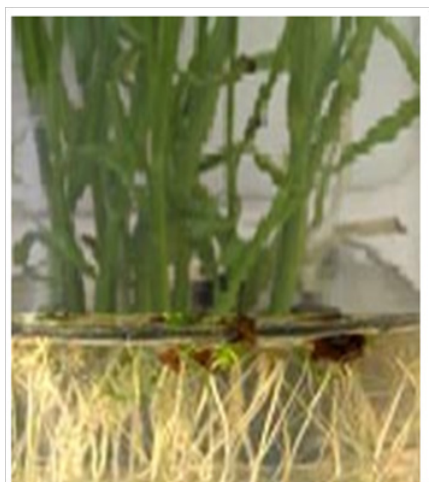


Figure 4 Root regeneration.

Conclusion

In the present study, protocol was developed for *in-vitro* propagation of sugarcane and was optimized by manipulating different concentration of growth regulators for rapid and efficient callus induction, shoot and root regeneration. Percentage of callus (90%) induction was found to be better in MS liquid medium amended with 2,4-D at a concentration of 2.5mg/l and 3mg/L. Cytokinin were found to play an important role in shoot initiation and was observed that half-strength liquid MS media at the combination of 2.0mg/L of BAP and 0.5mg/L IBA showed better result of bud break in Co-91017. NAA at the concentration of 2.5mg/l showed better response for rooting than IBA. Finally, the *in-vitro* propagated plants were then transferred into field and the survival rate was found to be 85%.

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None.

Conflict of interest

The author declares no conflict of interest.

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