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*Full Length Research Paper*

# The effect of in-vitro environmental conditions on some sugarcane varieties for micropropagation

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**The present paper deals with the effect of light intensity, photoperiod and growth room temperature on *in-vitro* morphogenetic responses of leaf sheath explants of sugarcane varieties, CoS 96258 and CoS 99259. High frequency callus initiation was recorded in leaf sheath explants incubated in dark for 10 to 15 days and then, transferred in light. Maximum shoot regeneration and number of shoots per culture could be recorded under 16 h photoperiod of 4000 lux light intensity at a growth room temperature of 25 ± 2°C in both varieties of sugarcane.**

**Key words:** Callus culture, shoot regeneration, *in-vitro* growth condition, sugarcane.

## INTRODUCTION

Commercial sugarcane belonging to the genus Saccharum (Poaceae) is an important industrial crop accounting for nearly 70% of sugar produced worldwide. Compared to other major crops, the efforts to improve sugarcane are limited and relatively recent, with the first induction of interspecific hybrids about 80 years ago. Production of sufficient quantity of seed material of a new variety of sugarcane for planting in a vast area generally takes over 10 years if multiplied through conventional methods of seed multiplication. There are also chances of perpetuation of sett-borne diseases. *In vitro* micropropogation technique is emerging as a powerful tool for rapid and large scale production of disease free planting material in a number of crops. Several agro industries and research institutes are now engaged in the micropropogation activities for faster multiplication of newly released and commercially important varieties of sugarcane (Yadav et al., 2004).

Callus induction and subsequent shoot regeneration in sugarcane was first demonstrated (Heinz and Mee, 1969; Barba and Nickell, 1969). Later, Heinz and Mee (1971) and Liu and Chen (1976) demonstrated that sugarcane plants regenerated from callus showed wide variation in chromosome number. Callus cultures of sugarcane have successfully been established by several investigators also (Nadar et al., 1978; Bhansali and Singht, 1984; Visessuwan et al., 1999; Lal, 2003) using shoot apices, leaf sheath and young inflorescence as explants on Murashige and skoog (MS) medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and coconut milk. Among these explants, young leaf sheath has been widely used as the explants for callus induction and subsequent shoot regeneration. Maximum frequency of callus formation from leaf sheath explants was demonstrated on MS medium containing various concentration of 2,4-D (Mannan and Amin, 1999; Lal, 2003; Ramamand et al., 2006). The effect of tissue culture explant sources on sugarcane yield component (Hoy et al., 2003) was also studied. Development of sugarcane Saccharum species commercial cross is based on maximum likelihood approach for estimation of linkage and linkage phase (Garcial et al., 2006).

Plant tissue culture offers a methodology for crop improvement through direct and indirect regeneration of plants in sugarcane. The potential benefit of somaclonal variation for improved cane yield and sugar quality along with increased resistance against disease like Fiji disease, Downy Mildew and those caused by sugarcane mosaic virus have been documented (Krishnamurthi and Tlaskal, 1974; Naik and babu, 1989; Wagih and Adkins, 1998). An assessment of somaclonal variation in

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**Table 1.** Effect of light intensities on callus formation and shoot regeneration from leaf sheath callus culture of sugarcane varieties CoS 96258 and CoS 99259. Medium: MS salts + BAP + Kn + NAA (0.5 mg/L each) + sucrose (30 g/L) + agar (8 g/L).

| Light intensity (Lux) | CoS 96258 | | | CoS 99259 | | |
|---|---|---|---|---|---|---|
| | % explants showing callus initiations | % callus cultures showing shoot regeneration | Number of shoots per culture | % explants showing callus initiations | % callus cultures showing shoot regeneration | Number of shoots per culture |
| Darkness | 55.3 ± 4.7 | - | - | 59.4 ± 5.3 | - | - |
| 1000 | 43.9 ± 5.1 | 9.7 ± 1.3 | 3.4 ± 0.6 | 47.9 ± 5.7 | 6.8 ± 1.3 | 3.9 ± 0.8 |
| 2000 | 36.4 ± 4.2 | 26.4 ± 3.4 | 4.3 ± 0.7 | 39.8 ± 4.9 | 29.3 ± 3.2 | 5.7 ± 0.7 |
| 3000 | 21.7 ± 2.4 | 34.7 ± 4.1 | 6.3 ± 0.8 | 23.3 ± 2.6 | 36.6 ± 3.9 | 8.3 ± 0.9 |
| 4000 | 10.6 ± 1.6 | 51.1 ± 6.3 | 12.3 ± 1.5 | 12.7 ± 1.7 | 58.7 ± 6.1 | 13.1 ± 1.7 |
| 5000 | 7.3 ± 0.6 | 47.2 ± 5.2 | 11.2 ± 1.7 | 9.3 ± 0.8 | 53.4 ± 5.4 | 10.7 ± 1.2 |

micropropagation plant of sugarcane by random amplified polymorphic DNA (RAPD) marker has been done (Tawar, 2008). Genetic diversity associated with *in vitro* and bud propagation of Saccharum varieties using RAPD analysis (Da Silva et al., 2008; Patel et al., 2008) also has been done. New varieties through somaclonal variation (Jalaja NC et al., 2006) also have been developed. Rapid micro propagation of sugarcane (*Saccharum officinarium L.*) varieties is done by shoot tip culture (Shabaz et al., 2008). Several reports on induction of callus and shoot regeneration in sugarcane are available. However, phenotypic instability has often been reported among *in vitro* raised plants of sugarcane (Irvine et al., 1991; Naggi et al., 1991; Buner and Grisham, 1995; Taylor et al., 1995). This tissue culture generated variation termed as somaclonal variation (Larkin and Scowcraft, 1981) largely depends on various modes of plant regeneration. Among various factors, the role of growth regulators on *in vitro* morphogenesis has been extensively studied, however, other factors like light intensity, photoperiod and growth room temperature have not been thoroughly worked out in sugarcane.

The present investigation was therefore proposed to study the effect of light intensity, photoperiod and growth room temperature on morphogenetic responses of leaf sheath explants of sugarcane varieties CoS 96258 and CoS 99259.

## MATERIALS AND METHODS

Fresh tops of sugarcane varieties CoS 96258 and CoS 99259 grown at the research farm of Sardar Vallabh Bhai Patel University of Agriculture and Technology, Meerut were collected. After peeling out the outer leaf sheath, the material was wiped with 70% ethanol. 6 to 8 cm long spindle segment having growing tips and furled young leaf sheath were then excised out from the tops. For preparation of leaf sheath explants, the segments were washed thoroughly under running tap water for 25 to 30 min to remove the dust particles. After washing with tap water, the segments were soaked in 1% (v/v) Cetavlon solution for 10 to 15 min followed by thorough washing with clean water. The material was then rinsed with 70% alcohol for 30 to 40 s followed by washing with sterile distilled water. The material was then surface sterilized with 0.1% (w/v) aqueous solution of mercuric chloride ($HgCl_2$) containing a few drop of Triton X-100 for 8 to 10 min with continuous shaking. Finally the segments were washed 4 to 5 times with sterile double distilled water to remove the traces of chemicals.

The surface sterilized spindle segments were aseptically dissected out into 1 cm long pieces and spit longitudinally into equal halves with the help of sterile surgical blades and forceps. The leaf sheath explants, thus prepared were immediately inoculated in agar (8.0 $L^{-1}$) solidified MS medium (Murashige and Skoog, 1962) containing 2,4-D (3.0 mg/L) and incubated under varying conditions of light intensities, photoperiods and temperatures.

## RESULTS AND DISCUSSION

### Effect of light intensity on callus initiation

After inoculation, the explants were incubated under different light intensities. One set of cultures was also maintained under complete darkness for 3 weeks and then transferred to the light condition (3000 lux). The responses regarding callus formation were recorded 4 weeks after inoculation.

The data presented in Table 1 showed that the highest frequency of callus initiation from leaf sheath explants took place in cultures receiving dark treatment as compared to those maintained under different light intensities in both varieties. Maximum of 59.4 ± 5.3% explants induced callus formation in variety CoS 99259 and 55.3 ± 4.7% in CoS 96258. The frequency of callus formation gradually decreased with the increasing intensity of light in both the varieties. The explants initially incubated under complete dark, retained their original cream colour for a longer period while those incubated under light turned green with time. The callus initiation occurred at the cut margins of explants incubated in dark within 10 and 15 days, whereas in explants incubated directly under light condition (1000 to 2000 lux), the callus

**Table 2.** Effects of photoperiod on shoot regeneration from leaf sheath callus cultures of sugarcane. Medium: MS salts + BAP + Kn + NAA (0.5 mg/L each) + sucrose (30 g/L) + agar (8 g/L).

| Photoperiod at 4000 Lux (h) | CoS 96258 | | CoS 99259 | |
|---|---|---|---|---|
| | % cultures showing shoot regeneration | Number of shoots per culture | % cultures showing shoot regeneration | Number of shoots per culture |
| 8 | 32.7 ± 4.3 | 3.3 ± 0.8 (++) | 36.3 ± 3.9 | 4.7 ± 0.7 (++) |
| 12 | 45.7 ± 5.1 | 10.7 ± 1.5 (++) | 48.6 ± 5.7 | 12.3 ± 1.3 (++) |
| 16 | 67.9 ± 7.2 | 13.6 ± 1.6 (+++) | 71.4 ± 6.9 | 15.6 ± 1.7 (+++) |
| 20 | 41.3 ± 3.5 | 6.7 ± 0.9 (++) | 43.4 ± 5.3 | 11.3 ± 1.6 (++) |
| Continuous light | 23.6 ± 3.2 | 3.7 ± 0.6 (+) | 28.6 ± 3.2 | 5.3 ± 0.6 (+) |

Culture growth: (+++) good; (++) moderate; (+) poor.

initiation took more than 20 days. Under 4000 to 5000 lux light intensity, the explants turned green within a week and become non-response.

Better growth of callus was observed in the explants that were initially incubated under dark than in those incubated under low light intensities (1000 to 2000 lux). However, high light conditions (3000 to 5000 lux) did not favour the growth of initiated calli. The calli initiated from explants receiving dark treatment grew rapidly and gave rise to compact, pale yellow and morphogenic callus. At 5000 lux, callus initiation could be observed in > 10% explants, and also the callus growth was very poor. The results reveal that the frequency of callus formation reduced with the increasing intensity of light. This might be due to increased morphogenetic potential caused by low chlorophyll contents in the cells. A loss of callusing in presence of light occurred possibly due to synthesis of chlorophyll which would reduce the callusing potential of leaf sheath explants.

## Effect of light intensity on shoot regeneration from leaf sheath callus

In order to study the effect of light intensity on shoot regeneration from leaf sheath callus, the actively growing calli of both varieties were inoculated on agar gelled (8.0 g/L) MS medium supplemented with 6-benzyl amino purine (BAP), kinetin (Kn) and α- naphthalene acetic acid (NAA) (0.5 mg/L each). The callus culture was incubated under different light intensities under 16 h photoperiod at 25 ± 2°C and also, under complete darkness. Data enumerated in Table 1 showed that the regeneration frequency increased in both varieties with the increasing light intensity. Maximum of 58.7 ± 6.1% shoot regeneration was recorded in variety CoS 99259, whereas it was 51.1 ± 6.3% in variety CoS 96258 at a light intensity of 4000 lux. No shoot regeneration took place from the callus in both varieties in cultures incubated in dark.

The number of shoots per culture also increased with the increasing intensity of light. At 4000 lux, maximum of 13.1 ± 1.7 and 12.3 ± 1.5 shoots per culture were recorded in CoS 99259 and CoS 96258, respectively however, the number of shoots per culture was numerically lower beyond 3000 lux in both the varieties. High frequency shoot regeneration from leaf callus at 4000 lux of light intensity has also been reported in several other varieties of sugarcane by earlier workers (Pawar et al., 2002; Ramanand and Lal, 2004; Singh, 2005).

## Effect of photoperiod on shoot regeneration from leaf sheath callus

The actively growing callus cultures were incubated under different photoperiods (Table 2). The results show that the frequency of shoot regeneration increased with the increasing photoperiod in both varieties. About 36.3 ± 3.9% callus culture showed shoot regeneration in CoS 99259 and 32.7 ± 4.3% in CoS 96258 under 8 h photoperiod. Maximum of 71.4 ± 6.9% callus cultures showed shoot regeneration in CoS 99259 and 67.9 ± 7.2% in CoS 96258 under 16 h photoperiod. When the callus cultures of both varieties were incubated above 16 h photoperiod (up to 20 h or continuous light conditions), there was a significant decrease in shoot regeneration frequency.

The number of shoots per culture was also increased significantly with the increasing photoperiod up to 16 h, however, it was drastically reduced under photoperiods above 16 h. Maximum of 13.6 ± 1.6 and 15.6 ± 1.7 shoots per culture could be produced in variety CoS 96258 and CoS 99259, respectively under 16 h photoperiod, while it was minimum of 3.3 ± 0.8 and 4.7 ± 0.7 shoots per culture, respectively under 8 h photoperiod. Normal healthy plants with dark green leaves were observed under 16 h photoperiod. It is apparent from the data that a 16 h photoperiod was optimum both for shoot regeneration and production of maximum number of vigorous shoots per culture.

The results indicate that an appropriate photoperiod was essential for optimum proliferation of shoots. Under

**Table 3.** Effects of temperature on shoot regeneration from leaf sheath callus of sugarcane. Medium: MS salts + BAP + Kn + NAA (0.5 mg/L each) + sucrose (30 g/L) + agar (8 g/L).

| Temperature (°C) | CoS 96258 | | CoS 99259 | |
|---|---|---|---|---|
| | % cultures showing shoot regeneration | Number of shoots per culture | % cultures showing shoot regeneration | Number of shoots per culture |
| 20 ± 2 | 38.6 ± 3.6 | 8.7 ± 0.9 | 45.4 ± 4.9 | 9.8 ± 1.1 |
| 25 ± 2 | 54.7 ± 6.1 | 12.6 ± 1.6 | 63.3 ± 6.8 | 14.8 ± 1.5 |
| 30 ± 2 | 50.2 ± 5.9 | 9.7 ± 1.2 | 57.6 ± 6.1 | 11.6 ± 1.3 |
| 35 ± 2 | 31.4 ± 3.3 | 3.9 ± 0.5 | 34.1 ± 2.9 | 5.3 ± 0.7 |

continuous light, the morphogenetic responses were greatly reduced suggesting that a dark period was also essential and equally important for regeneration of shoots. These results are in conformity with the reports of earlier workers (Geetha et al., 2000; Lal, 2003; Wagih et al., 2004; Lal et al., 2008).

## Effects of temperature on shoot regeneration from leaf sheath derived callus

Data presented in Table 3 demonstrated that the temperature of growth room had a noticeable effect on shoot regeneration frequency and the number of shoots per culture in both experimental varieties. At a temperature of 20 ± 2°C, shoot inducted was observed in 45.4 ± 4.9% callus cultures in variety CoS 99259 and 38.6 ± 3.6% in variety CoS 96258. The regeneration frequency was significantly increased up to a maximum of 54.7 ± 6.1 and 63.3 ± 6.8% in CoS 96258 and CoS 99259, respectively by raising the temperature up to 25 ± 2°C. At 30 ± 2 and 35 ± 2°C, the frequency of shoot regeneration decreased in both varieties.

As regards the number of shoots per culture, maximum of 12.6 ± 1.6 and 14.8 ± 1.5 shoots per culture were recorded in varieties CoS 96258 and CoS 99259, respectively at 25 ± 2°C. At 20 ± 2°C, the multiplication rate was however lowered down in both varieties. At 35 ± 2°C, a marked reduction in number of shoots per culture was noticed (Table 3). At 25 ± 2°C, healthy and highly green shoots were formed, however at the temperatures beyond 25 ± 2°C, thin, light green shoots could be regenerated. These results indicate that metabolic activities of growing tissues are directly/indirectly controlled by the growth room temperature.

Although, some species were grown successfully at much lower temperatures, that is, *Galanthus* and potato tubes at 15°C and some cultivars of *Narcissus* and *Allium* at 18°C. Some tropical species usually required higher temperature, example, palm tree grew at 27°C (Tisserat, 1981) and *Manastera diliciosa* at 30°C (Fonnesbech and Fonnesbech, 1980). An optimum temperature of 25 ± 2°C is commonly reported for several other crops under *in vitro* condition and was also observed in the case of sugarcane in the present investigation.

Based on the aforementioned observations, it can be concluded that *in vitro* morphogenetic responses of leaf sheath explants of sugarcane are highly influenced by the environmental conditions of growth room. Maximum shoot regeneration with more number of shoots per culture could be obtained under 16 h photoperiod of 4000 lux light intensity at a growth room temperature of 25 ± 2°C in both varieties of sugarcane.

## REFERENCES


Barba R, Nickell LG (1969). Nutrition and organ differentiation in tissue culture of sugarcane-a monocotylendon. Planta. 89 : 299-302.

Bhansali RR, Singh K (1984). Callus and shoot formation from leaf of sugarcane in tissue culture. Phytomorphol. 167-170.

Burner DM, Grisham MP (1995). Induction and stability of phenotypic Variation in sugarcane as affected by propagation procedure. Crop Sci. 35 : 875:880.

Da Silva CM, Mangolin CA, Mott AS, Machado MFPS (2008). Genetic diversity associated with in vitro and conventional bud propagation of Saccharum varieties using RAPD analysis. Plant Breeding .127: 160-165

Fonnesbech A, Fonnesbech M (1980). In vitro propagation of Monstera deliciosa. Hort. Sci. 15 : 740-741.

Geetha S, Padmanabhan W, Manuel WW, Ayyamperumal A (2000). In vitro production of sugarcane plants. Sugar Tech. 2 (3) : 47-48.

Garcia AA, kido EA, Meza AN, Souza HM, Pinto LR, Pastina MM, Leite CS, Silva JA, Ulian EC, Figuiera A (2006). Development of sugarcane Saccharum spp. Commercial cross, based on maximum likelihood approach for estimation of linkage and linkage phases. Theor. Appl. Gent. 112: 298-314.

Heinz DJ, Mee GWP (1969). Tissue culture differentiation and regeneration of plants in Saccharrum spp. Argon. Abstr. p. 10.

Irvine JE, Benda GTA, Legendre BL, Machado GR Jr (1991). The frequency of marker changes in sugarcane plants regeneratedfrom callus culture, II. Evidence for vegetative and genetic transmission, epigenetic effects and chemical disruption. Plant Cell Tissue .Org. Cult. 26: 115-125.

Jalaja NC, Sreenivasan TV, Pawar SM, Bhoi PG, Garker RM (2006). A New Sugarcane variety through somaclonal variation. Sugar Tech. 8 (283) : 132-136.

Krishnmaurthi M, Tlaskal J (1974). Fiji disease resistant Saccharum officinarum varieties. Pindar subclines from tissue culture. Proc. Int. Soc. Sugarcane Tech. 15 : 130-137.

Lal N (2003). High frequency of plant regeneration from sugarcane callus. Sugar Tech. 5(1&2) : 89-91.

Liu MC, Chen W (1976). Tissue and cell cultures as aids to sugarcane breeding I. Creation of genetic variability through callus cultures. Euphytica. 25 : 393-403.

Larkin PJ, Scowcroft WR (1981). Somaclonai variation: a novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet. 60: 197-214.

Lal M, Singh RK, Srivastava shridha, Singh Neetu, Singh SP, Sharma ML (2008). RAPD worker used analysis of micropropogated plantlets of Sugarcane for early evaluation of genetic fidelity. Sugar Tech. 10: 99-103.

Mannan SKA, Amin MN (1999). Callus and shoot formation from leaf sheath explants of sugarcane (Saccharum officinarum) in vitro. Indian Sugar. 187-192.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15 : 473-497.

Nadar HM, Soerpratopo S, Heinz DJ, Ladd SL (1978). Fine structure of sugarcane (Saccharum sp.) callus and the role of auxin in embryogenesis. Crop Science. 18 : 210-216.

Naik GR, Babu KH (1989). Redifferentiation of NaCl tolerant sugarcane plant from callus derived resistant lines. Current Science. 57 : 432-434.

Nagai C, Ahloowalia BS, Tew TL (1991). Somaclonal variants from an intergeneric hybrid: Saccharum spp. x Erianthus arundinaceum. Euphytica. 53: 193-199.

Pawar SV, Patil SC, Jambhale VM., Naik RM, Mehetre SS (2002). Rapid multiplication of commercial sugarcane varieties through tissue culture. Indian Sugar. pp. 183-186.

Ramanand, Lal M (2004). An efficient protocol for in vitro micropropagation of sugarcane. Sugar Tech. 6 (1&2): 85-87.

Ramanand, Kureel N, Subhanand N, Lal M, Singh SB (2006). Plant regeneration through leaf callus culture in sugarcane. Sugar Tech. 8(1) : 85-87

Shabaz Ali Khan, Hamid Rashid, Fayyaz Chaudhary, Zubeda Chaudhary , Amber Afroz (2008). Rapid micropropagation of three elite Sugarcane (Saccharum officinarum L.) varieties by shoot tip culture. Afr. J. Biotechnol .7 (13) 2174-2180.

Singh AK (2005). Physiological and Biochemical basis of in vitro morphogenesis in sugarcane hybrids. Ph.D. Thesis, MJP Rohilkhand Univ, Bareilly. 2005.

Tisserat B (1981). Date Palm tissue culture, U.S. Deptt. of Agric. Agric. Research Service, Oakland, California.

Tawar PN, Sawant RA, Dalvi SG, Nikam AA, Kawar PG, Deverumat RM (2008). An assessment of somaclonal variation in micropropagated plant of sugarcane by RAPD markers. Sugar Tech. 10(2) : 124-127.

Taylor PWJ, Geijskes JR, Ko H-L, Fraser TA, Henry RJ, Birch RJ (1995). Sensitivity of random amplified polymorphic DNA analysis to detect genetic variation in sugarcane during tissue culture. Theor .Appl. Genet. 90 :1169-1173

Visessuwan R, Chiemsombat P, Naritoom K, Surija Chaijakorn M (1999). Role of growth regulators in meristem culture and production of virus free sugarcane germplasm. Sugar Tech. 1 (3) : 82-88.

Wagih ME, Adkins SW (1998). Embryogenic callus culture for Fiji disease virus elimination in sugarcane. 4th Breeding and Germplasm Workshop. ISSCT. Mauritius. 22nd May. pp. 1-15.

Wagih ME, Ala A, Musa Y (2004). Regeneration and evaluation of sugarcane somaclonal variants for drought tolerance. Sugar Tech. 6(1&2) : 35-40

Yadav S, Saini N, Jain RK (2004). Low cost multiplication and RAPD analysis of micropropagated plants in sugarcane. Physiol . Mol .Boil Plants. 10(2) : 269-276.