Electronic Journal of Plant Breeding, 9 (3) : 1077 - 1082 (Sep 2018) DOI: 10.5958/0975-928X.2018.00134.5
ISSN 0975-928X

Research Article

Development of a simple and genotype independent *in vitro* regeneration system in Sugarcane [*Saccharum* spp] using shoot apex explants

M. Shankar[1], V. Thiruvengadam[1], M. Shanmuganathan[2], S. Ganesh Ram[1] and PL. Viswanathan[3]*
[1]Department of Plant Genetic Resources, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore – 641 003.
[2]Sugarcane Research Station, Tamil Nadu Agricultural University, Sirugamani – 639115.
[3] Department of Oilseeds, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore – 641 003.
***E-Mail**: palavisu@gmail.com

(Received:16 Aug 2018; Revised: 05 Sep 2018; Accepted: 09 Sep 2018)

Abstract
Sugarcane (*Saccharum* spp) is commercially cultivated in Tamil Nadu for industrial purposes. Germplasm of this crop is mainly conserved in the form of clonal gene bank. For long term conservation of vegetatively propagated crops, cryo-preservation offers a promising approach which could be realized through a reliable and genotype independent tissue culture system. In the present study, two sugarcane genotypes namely Co 86032 and TN SC Si 7 were evaluated for their *in vitro* regeneration response through optimization of various factors influencing organogenesis. The results indicated that shoot apices explants inoculated on medium supplemented with 0.25 mg/l BAP + 5.0 mg/l kinetin + 0.05 mg/l NAA recorded highest shoot induction frequencies of 92.2% and 84.4% for Co 86032 and TN SC Si 7 respectively. On an average, 6 to 8 shoots were formed per explant. After 60 days of culture, single shoots were separated from the multiple shoots and transferred to the rooting medium. Among the different media tested, highest regeneration frequencies of 63.3% (Co 86032) and 56.6% (TN Sc Si 7) were found on medium supplemented with 5 mg/l NAA. Thus, a simple and genotype independent *in vitro* regeneration system was developed which could be well adopted for conservation of sugarcane germplasm in the cryo gene bank.

Key words
Sugarcane, shoot apex, multiple shoots, *in vitro* regeneration.

Introduction

Sugarcane (*Saccharum* spp), a major source of world sugar, is grown throughout the tropics and subtropics. Major sugarcane industries are found in Brazil, India and China in which India ranks second in global sugarcane production (Annual report, 2016-2017). Most of the sugarcane varieties currently cultivated are derived from the interspecific hybridization of *Saccharum officinarum* and *Saccharum spontaneum* through nobalization process (Snyman *et al.,* 2011). *Ex situ* conservation of vegetatively propagated crop species such as sugarcane is usually carried out in the field gene bank. Field gene bank consists of large germplasm collections that are kept in living condition that requires continuous growth and maintenance (Reed *et al.,* 2004). However, under such conditions, it is extremely difficult to maintain clonal gene bank free from viruses and also requires large inputs of labour and land (Maxted *et al.,* 2000).

To overcome this situation, *in vitro* conservation techniques for the preservation of healthy sugarcane plantlets are routinely followed through various procedures and manipulation of *in vitro* conditions favouring slow growth (Baksha *et al.,* 2002). The advantages of *in vitro* conservation include maintenance of material in pathogen-free condition and facilitate safer distribution of genetic stocks. *In vitro* conservation approaches provide short to medium (slow growth) and long-term (cryo-preservation) conservation of genetic resources. For cryo-preservation, an *in vitro* regeneration system for the target species should be available for long-term conservation of vegetatively propagated crop species. As sugarcane is a very important commercial crop, breeding research demand continuous supply of sugarcane germplasm for long period of time. Therefore, the present investigation has been undertaken with a view to establish a genotype independent *in vitro* plant regeneration protocol in sugarcane using shoot apices as explants that could support the establishment of a functional cryo gene bank facility for sugarcane germplasm on a large scale.

Materials and Methods

Setts of two sugarcane genotypes Co 86032 and TN SC Si 7 were received from Sugarcane Research station, TNAU, Sirugamani and planted in 'F'Block of new area at Department of Forage

crops, TNAU, Coimbatore during *Kharif* 2017. Sugarcane setts were planted in six rows by adopting the spacing of 90 cm between rows. The recommended agronomic practices and need based plant protection measures were adopted as per the crop production manual.

Healthy sugarcane tops from four to five months old field grown plants were collected and wiped with rectified spirit. After removing the surrounding two to three leaf sheaths, swabbing with cotton dipped in rectified spirit was done each time. This was followed by cutting into 7 to 8 centimetres long bits for ensuring the presence of growing points with few whorls of leaves. The cut bits were washed with detergent (surf) in 500 ml flask by shaking for 5 min to remove the debris. After draining the detergent and rinsing with water, the cut bits were treated with 70% ethyl alcohol for 1 min and again rinsed with sterile water 4-5 times till alcohol was completely washed away.

Under the laminar air flow cabinet, a superficial transverse cuts at the base of the apical shoot was given and the leaf whorls were removed until the apical dome with two to three leaf primordial was exposed. The shoot apex of 2 mm size were excised and used as explants for *in vitro* regeneration studies.

The shoot apices were inoculated in the shoot induction medium supplemented with different hormonal combinations with or without the addition of activated charcoal. The cultures were maintained initially in the dark for 7 days. Details of different media compositions tested for shoot induction is given in Table 1.

The explants after shoot initiation were transferred to the semi-solid shoot induction medium (SMSI) for shoot elongation/multiple shoots formation. The shoot induction percentage was calculated by using the formula given below.

$$\text{shoot induction } (\%) = \frac{\text{No.of explants with shoots}}{\text{Total no.of explants cultured}} \times 100$$

After two months of shoot elongation and proliferation, the individual shoots were removed and transferred to rooting medium supplemented with different concentrations of growth hormones. The media compositions tried for successful rooting of shoots are given in the Table 2. The regeneration frequency was calculated by using the formula given below.

$$\text{Regeneration frequency } (\%) = \frac{\text{No.of explants with shoots \& roots}}{\text{Total no.of explants cultured}} \times 100$$

The *in vitro* experiments were carried out in the Tissue culture unit of Department of Plant Genetic Resources, Tamil Nadu Agricultural University, Coimbatore. All the cultures were maintained at a temperature of 25±2˚C with continuous illumination supported by Light Emitting Diodes (LED) lights providing a light intensity of 2500 lux with a photoperiod cycle of 16 h light and 8 h dark.

The experiments were performed following Completely Randomized Design (CRD) and the data were subjected to statistical analysis as per the method suggested by Panse and Sukhatme (1978). Each treatment was replicated thrice. An analysis of variance (ANOVA) was computed using statistical software AGRES.

Results and Discussion

While benefits of *in vitro* slow growth methods are recognized for germplasm conservation of elite sugarcane cultivars, the full positive potential of this technique and that of cryopreservation are yet to be realized. For any vegetatively propagated crops, such as sugarcane, to be made amenable for cryo-conservation, the first and foremost requirement is that the *in vitro* regeneration system should be sufficiently operational for the target species (Engelmann, 1997). Upon culture of appropriate explants *in vitro*, the tissue culture protocol should facilitate the induction of multiple shoots and subsequent normal plantlet regeneration. Ali *et al*., 2008 emphasized the importance of meristem shoot size for successful storage under liquid nitrogen and subsequent recovery through an efficient regeneration system. This could be well achieved through direct organogenesis process which involves no callusing phase thus enabling the regeneration of true to type plants. Various factors such as genotype, explants, and nutrient composition of the culture medium and culture conditions greatly influence the regeneration of plantlets during *in vitro* culture (Khanna and Raina, 1998).

Studies on standardization of all these parameters were undertaken to develop a comprehensive protocol to achieve successful *in vitro* regeneration in sugarcane. Shoot apices were preferred as they contain pre-formed meristematic regions providing genetic stability potential and also possess ability to recover a plant (Ashmore, 2000). Furthermore, since regenerants produced through organogenesis should be identical to the mother plant, a small piece of material such as shoot apex isolated from the mother plant could be considered as germplasm as a suitable storage material (Kaviani, 2011).

Previously various authors have reported *in vitro* regeneration through shoot apices in sugarcane (Coleman 1970; Shukla *et al*., 1994; Jalaja *et al.*,

2008). Nevertheless most of these protocols could not be adopted for large number of genotypes as they are genotype and cultivar dependent (Tiel *et al.,* 2006). Therefore, the present study mainly focused on the development of genotype independent *in vitro* regeneration protocol for sugarcane using shoot apices as explants though manipulation of nutrient composition of the culture medium.

In this study, the effect of growth regulators on direct organogenesis of two sugarcane genotypes namely Co 86032 and TN SC Si 7 was studied using shoot apices as explants on MS medium supplemented with different concentrations of BAP, NAA and kinetin. The freshly isolated shoot apices were first cultured onto different media containing activated charcoal for shoot induction and the cultures were kept in the dark for 7 days (Fig. 1A). The presence of activated charcoal in the regeneration medium has a significant influence on the development of shoots (Fridborg *et al.,* 1978). The shoot apices were transferred to the same medium without activated charcoal and shoot initiation were observed after 20 days (Fig. 1B). Tabei *et al.,* 1998 reported that combination of phytohormones often determine the course of morphogenesis *i.e.,* shoot organogenesis. For multiple shoot regeneration, shoot tips were remarkably influenced by types and concentration of the auxin and cytokine used. The cytokinin BAP was more effective than kinetin and NAA. Skirvin (1984) elaborated on the effects of different hormone combinations in inducing shoot proliferation and elongation.

The results showed that the shoot induction frequency was ranged from 8.8% to 92.2% in Co 86032 and 6.6% to 84.4% in TN SC Si 7. Among the different media tested, highest shoot induction frequencies of 92.2% and 84.4% were noticed on medium supplemented with 0.25 mg/l BAP + 5.0 mg/l kinetin + 0.05 mg/l NAA (SMSI 2) for both Co 86032 and TN SC Si 7 genotypes (Table 3). Rapid shoot multiplication was noticed when the shoots were sub cultured on a semi-solid SMSI 2 medium. Overall, 70-80% of explants produced 5 to 7 multiple shoots from a single shoot apex within 45-60 days (Fig. 1C). An average of 6 shoots was formed per explant. During all stages of shoot induction and subsequent multiple shoot development, callus formation was not noticed on this medium.

After 60 days of culture, single shoots were separated from the multiple shoots and inoculated on rooting medium supplemented with different concentrations of NAA (Fig. 1D). Rooting of the shoots may be affected by pH, auxin level and nutrient concentration of the rooting media (Dannis and James, 1993). In the present study, root initiation was recorded after 20 days of culture in rooting medium supplemented with 5 mg/l NAA (Fig. 1E). The results indicated that regeneration frequency was ranged from 13.3% to 63.3% in genotype Co 86032 and 6.6% to 56.6% in genotype TN SC Si 7. Highest regeneration frequencies of 63.3% (Co 86032) and 56.6% (TN SC Si 7) were observed on medium supplemented with 5 mg/l NAA (SRM 1) (Table 4). These results are in agreement with previous research findings of Nadar and Heinz (1977), who reported the use of NAA during rooting from regenerated shoots. The next best response was found on medium one supplemented with 4 mg/l NAA (SRM 4) which recorded the regeneration frequencies of 36.6% and 26.6% in genotype Co 86032 and TN SC Si 7, respectively. After 30 days of culture on rooting medium, fully developed plantlets with normal shoots and roots were formed and transferred to screen house condition for further establishment.

In conclusion, a simple and genotype independent *in vitro* regeneration system using shoot apex explants was developed in the present study. The regeneration frequencies achieved in the two genotypes of sugarcane are comparable with other studies. This could provide an impetus for successful deployment of shoot apices as an appropriate storage tissue under liquid nitrogen as efficient recovery could be accomplished. Furthermore, this genotype independent tissue culture protocol offers significant scope towards its implementation across many research station/laboratories dealing with micropropagation as well as cryo-conservation of sugarcane germplasm.

References

Ali, A., Naz, S. H., Siddiqui, F. A., and Iqbal, J. 2008. Rapid clonal multiplication of sugarcane (*Saccharum officinarum*) through callogenesis and organogenesis. *Pak. J. Bot.*, **40**:123.

Ashmore, S. E., Azimi, M., and Drew, R. A. 2000. Cryopreservation trials in *Carica papaya*. In: *IV International Symposium on In Vitro Culture and Horticultural Breeding*. **560**: 117-120.

Baksha, R., Alam, R., Karim, M. Z., Paul, S. K., Hossain, M. A., Miah, M. A. S., and Rahman, A. B. M. M. 2002. *In vitro* shoot tip culture of sugarcane (*Saccharum officinarum*) variety LSD28. *Biotechnology*, **1***(*2-4): 67-72.

Coleman, R. E. 1970. New plants produced from callus tissue culture. *Surgarcane Res. Rep. USDA-ARS*, p. 38.

Dannis, P.S. and F.H. James, 1993. Growth of rooted "Galla" apple micro cutting *in vitro* as influenced by initial adventitious shoot count. *Horti. Sci*., **18**: 664-66.

Engelmann, F. 1997. *In vitro* germplasm conservation. In: *International Symposium on Biotechnology of Tropical and Subtropical Species*, Part 2. **46:** 41-48.

Fridborg, G., Pedersén, M., Landström, L. E. and Eriksson, T. 1978. The effect of activated charcoal on tissue cultures: adsorption of metabolites inhibiting morphogenesis. *Physiol. Plant.*, **43**(2): 104-106.

Jalaja, N. C., Neelamathi, D. and Sreenivasan, T. V. 2008. Micropropagation for quality seed production in sugarcane in Asia and the Pacific. Food and Agriculture Organization of the United Nations.

Kaviani, B. 2011. Conservation of plant genetic resources by cryopreservation. *Aust. J. Crop Sci.*, **5**(6): 778.

Khanna, H. K. and Raina, S. K. 1998. Genotype x culture media interaction effects on regeneration response of three *indica* rice cultivars. *Plant Cell Tissue and Organ Cult.* **52**(3): 145-153.

Maxted, N., Hawkes, J. G., Ford-Lloyd, B. V. and Williams, J. T. 2000. A practical model for *in situ* genetic conservation. In: *Plant Genetic Conservation*. p. 339-367.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. plant*. **15**(3): 473-497.

Nadar, H. M. and Heinz, D. J. 1977. Root and Shoot Development from Sugarcane Callus Tissue. *Crop Sci*., **17**(5): 814-816.

Panse, V. G. and Sukhatme, P. V. 1978. Statistical methods for agricultural workers. *Statistical methods for agricultural workers.*, (Ed. 3). Indian Council of Agricultural Research, New Delhi.

Reed, B. M., Engelmann, F., Dulloo, M. E., and Engels, J. M. M. 2004. Technical guidelines for the management of field and *in vitro* germplasm collections. *IPGRI Handbooks for Genebanks No. 7*. International Plant Genetic Resources Institute, Rome, Italy.p. 4

Shukla, R., Khan, A. Q., and Garg, G. K. 1994. *In vitro* clonal propagation of sugarcane: optimization of media and hardening of plants. *Sugar Cane.4*

Skirvin, R. M. 1984. Stone fruits. *Handbook of plant cell culture*, **3**: 402-452.

Snyman, S. J., Meyer, G. M., Koch, A. C., Banasiak, M., and Watt, M. P. 2011. Applications of *in vitro* culture systems for commercial sugarcane production and improvement. *In Vitro Cell. Dev. Biol. Plant*, **47**(2): 234-249.

Tabei, Y., Yamada, T., Morishita, T., and Osaki, T. 1998. Plant regeneration via shoot organogenesis from cotyledons in two wild Cucumis species, *C. figarei* and *C. metuliferus*. *JARQ*. **32**: 281-286.

Tiel, K., Enríquez, G. A., Ceballo, Y., Soto, N., Fuentes, A. D., Ferreira, A., and Pujol, M. 2006. Development of a system for rapid plant regeneration from in vitro sugarcane (*Saccharum officinarum* L.) meristematic tissue. *Biotecnol. Apl*. ***23***(1): 22-24.

Table 1. List of media compositions used for shoot induction and proliferation in sugarcane

S.No	Code	Media composition
1	SMSI 1	MS + 0.1 mg/l BAP + 0.015 mg/l kinetin + 30 g/l sucrose + 8 g/l agar + 0.5 mg/l activated charcoal
2	SMSI 2	MS + 0.25 mg/l BAP + 5.0 mg/l kinetin + 0.05 NAA mg/l + 30 g/l sucrose + 100 ml/l coconut water + 8 g/l agar + 0.5 mg/l activated charcoal
3	SMSI 3	MS + 2 mg/l BAP + 0.3 mg/l NAA + 30 g/l sucrose + 8 g/l agar
4	SMSI 4	MS + 0.2 mg/l BAP + 0.1mg/l kinetin + 30 g/l sucrose + 8 g/l agar + 0.5 mg/l activated Charcoal

*MS medium (Murashige and Skoog, 1962)

Table 2. List of media compositions used for rooting in sugarcane

S.No	Code	Media Composition
1	SRM 1	Half MS + 5 mg/l NAA + 30 g/l sucrose + 8 g/l agar
2	SRM 2	Full MS + 30 g/l sucrose + 8 g/l agar
3	SRM 3	Half MS + 2.5 mg/l NAA + 30 g/l sucrose + 8 g/l agar
4	SRM 4	Half MS + 4 mg/l NAA + 30 g/l sucrose + 8 g/l agar

Table 3. Effect of growth regulators on shoot induction and proliferation in the selected sugarcane genotypes

Media	Shoot induction frequency (%) (mean of three replicates)	
	Co 86032	TN SC Si 7
SMSI 1	32.2[b]	32.2[b]
SMSI 2	92.2[a]	84.4[a]
SMSI 3	8.8[c]	6.6[c]
SMSI 4	12.2[c]	12.2[c]
SE (d)	6.52	6.18
Cd (0.01)	19.06	18.05

Values followed by the same letter are not significantly different at $p > 0.01$

Table 4. Effect of growth regulators on regeneration frequency in the selected sugarcane genotypes

Media	Regeneration frequency (%) (mean of three replicates)	
	Co 86032	TN SC Si 7
SRM 1	63.3 [a]	56.6 [a]
SRM 2	13.3 [c]	6.6 [c]
SRM 3	23.3 [b]	16.6 [b]
SRM 4	36.6 [b]	26.6 [b]
SE (d)	5.09	7.26
Cd (0.01)	17.11	24.38

Values followed by the same letter are not significantly different at $p > 0.01$

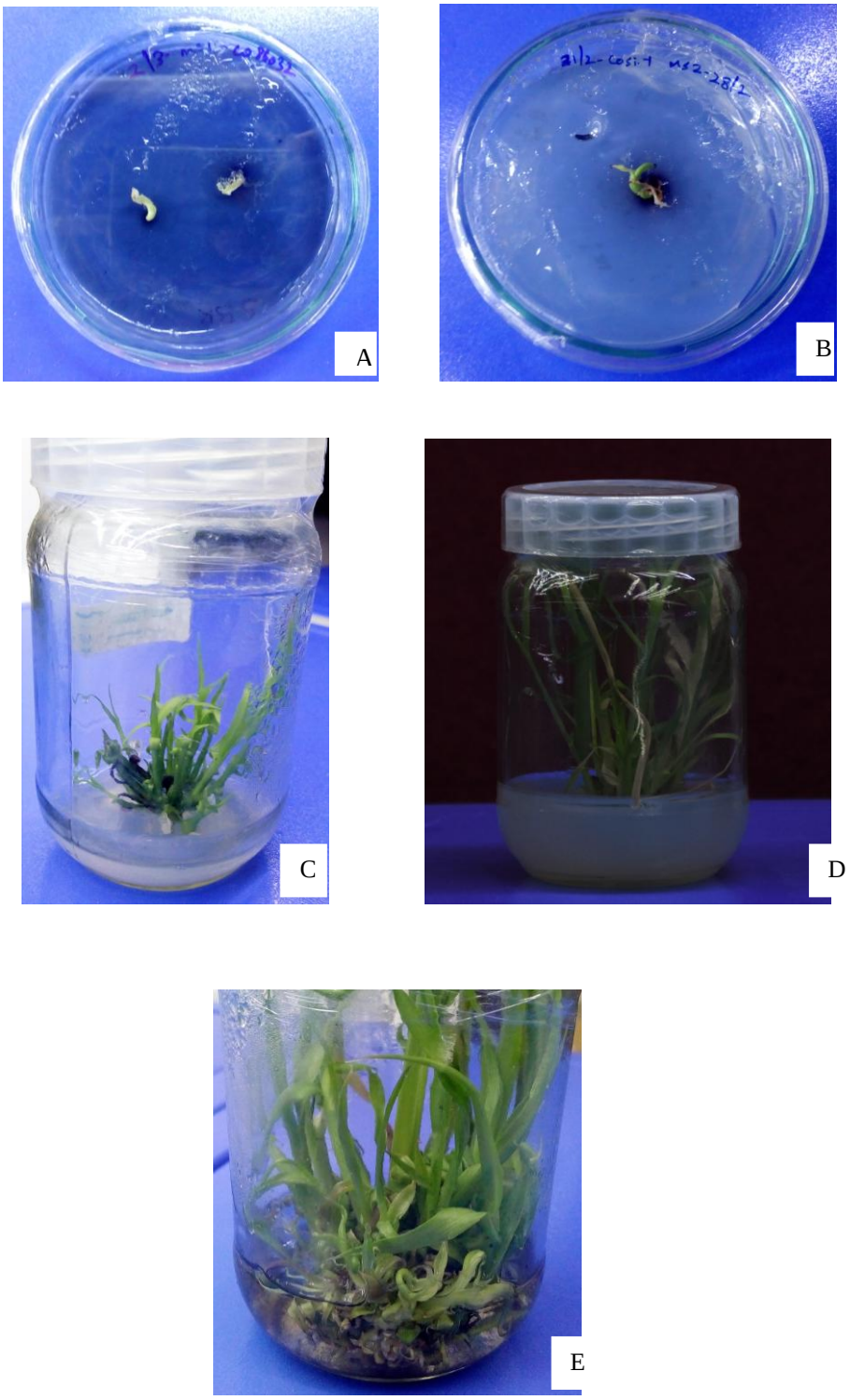

Fig.1. (A-E): *In vitro* regeneration of sugarcane genotypes using shoot apex explants

A: Shoot apices inoculated on SMSI 2 medium.
B: Shoot initiation after 20 days of culture in the SMSI 2 medium
C: Induction of multiple shoots after 40 days of inoculation on SMSI 2 semi solid medium.
D: *In vitro* regenerated multiple shoots after 60 days of culture on SMSI 2 medium
E: Plantlet establishment after 20 days of transfer to rooting medium SRM 1.