SUGAR TECH (2001), 3 (1&2) : 27 - 33.

Plantlet Regeneration of Sugarcane Varieties and Transient GUS Expression in Calli by Electroporation

G. Seema, H.P. Pande, J. Lal and V.K. Madan
Indian Institute of Sugarcane Research, P.O. Dilkusha, Lucknow – 226002, India

Electroporation of intact calli of six sugarcane varieties Co 1148, CoLk 8102, CoLk 8001, CoPant 84212, CoS 767 and CoJ 64 was carried out with a view to generate a transformation protocol employing embryogenic callus as target tissue in electroporation. The apical dome explants of varieties were cultured on Murashige and Skoog medium containing various growth regulators for callusing. Plantlet regeneration protocol in all the varieties was established from embroygenic callus for carrying out electroporation. Electroporation was successfully carried out using 20 mg plasmid DNA (pMOG 410) carrying GUS-int reporter gene with a single square pulse of 20 mm pulse width at electric fields of 5-12 KV/cm. After four days, transient GUS expression could be observed in intact calli of all the varieties.

KEY WORDS : Sugarcane, somatic embryogenesis, callus, electroporation, reporter GUS-int, transient GUS expression

INTRODUCTION

Sugarcane is an important agro-industrial crop of tropical and subtropical world. Improvement of the crop by transformation has been attempted in sugarcane chiefly by particle bombardment (Fitch *et at.,* 1995; Bower *et al.,* 1996; Zhang *et al.,* 1999; Smith *et al,* 1999), *Agrobacterium* (Arencibia *et al.,* 1998; Enriquez Obregon *et al.,* 1998) and direct DNA uptake by protoplasts (Chen *et al.,* 1988). Reports on intact tissue electroporation in many crops are available such as *Hordeum scutella* (Hansch *et al.,* 1996), wheat embryos (Kloti *et al.,* 1993), cassava somatic embryos (Luong *et al.,* 1995), wheat callus (Zaghmout and Trollinder, 1993), maize microspores (Jardinaud *et al.,* 1995), rich tissues (Deykeser *et al.,* 1990) and maize embryogenic callus (Halluin *et al.,* 1992). These reports on intact tissue electroporation have mentioned pretreatment with enzyme or woundings as a prerequisite for transgene expression (Luong *et al.,* 1995; Hansch *et al.,* 1996). There are only limited reports on the use of electroporation as a means of gene transfer in sugarcane such as electroporation of protoplasts (Hauptmann, 1987), intact cells (Arencibia *et al.,* 1992). However, no attempt has been made on intact calli electroporation in sugarcane. This necessitated a reliable plant regeneration protocol to be established for improvement of varieties. In this context the present study was undertaken to establish a reliable plant regeneration protocol in sugarcane and study its feasibility for direct gene transfer to embryogenic callus by electroporation. We report callus induction and regeneration in six commercial sugarcane varieties viz. Co 1148, CoLk 8102, CoLk 8001, CoJ 64, CoPant 84212 and CoS 767 through somatic embryogenesis and transient gene expression of b-Glucuronidase reporter gene without any pretreatment of callus.

Corresponding author : G. Seema
Fax : +91-522-480738; e-mail : iisrin@lw1.vsnl.net.in

MATERIALS AND METHODS

Callus induction : Six months old field grown plants of Co 1148, CoJ 64, CoPant 84212, CoLk 8102, CoLk 8001 and CoS 767 were used for excision of apical dome explant. The apical dome with two spindle leaves were surface sterilized with 70% alcohol for 2 minutes followed by chlorinated water for 20 minutes and washed six times in sterile water. They were then transferred to MS (Murashige and Skoog, 1962) basal medium containing different concentrations of growth hormones (Table 1) containing 30g/*l* sucrose at pH 5.7 solidified with 0.7% agar. For callus induction the cultures were incubated for one month in dark at 25 ± 1°C.

Plant regeneration : The calli were transferred to basal MS media containing various hormones for regeneration (Table 2). The cultures were incubated at 25 ± 1°C with 16/8 h light/dark photoperiod. The shoots with emerging roots were later transferred to liquid MS medium for further growth and development

Table 1 : Concentration of hormones in MS media used for callus induction

Medium	Hormone (mg/l)		
	BAP	2,4-D	Kinetin
CMS 1	0.0	0.0	0.0
CMS 2	0.0	3.0	0.0
CMS 3	0.5	3.0	0.0
CMS 4	1.0	3.0	0.0
CMS 5	0	3.0	0.5
CMS 6	0	3.0	1.0

Table 2 : Concentration of hormones used for plant regeneration

Medium	Hormone (mg/l)		
	IAA	NAA	KIN
SMS 1	1	1	2
SMS 2	2	1	2
SMS 3	3	1	2
SMS 4	4	1	2
SMS 5	5	1	2
SMS 6	0	0	0

Hardening of plants : The plantlets were transferred to pots filled with sterile soil and kept under polythene sheet cover under controlled conditions for 10 days inside the culture room till the plantlets reached 2-3 cm in height. They were subsequently transferred to glasshouse for further growth and shifted to field after hardening.

Intact calli electroporation : The callus was cut into small pieces of 1g each for electroporation with GUS-int reporter gene. *Agrobacterium* strain EHA105K (pMOG 410) was used for plasmid DNA isolation (Hood *et al.*, 1993). Plasmid DNA was isolated using Qiagen plasmid DNA isolation kit. The callus was incubated with 20 mg plasmid DNA and 800 ml electroporation buffer [HEPES 10mM, Mannitol 0.425 M, KCl 80 mM, $CaCl_2$ 5mM], pH 7.2, on ice for 1 hour. Electroporation was carried out with BTX 200 Electro Cell Manipulator. A single square pulse of 20 mm pulse width at electric fields of 6.5, 7.8, 10.4 KV/cm was used for electroporation. Then the electroporated calli were transferred to callus induction medium. GUS assay was done after 4 days as per Jefferson, (1987) and modified by Arencibia *et al.* (1995).

RESULTS AND DISCUSSION

Callus induction : All the varieties gave rise to nodular, globular, friable, pale yellow calli on callus induction medium (Fig. 1). Callus initiation could be seen from the cut surfaces of explants in all the media after one week of culture. None of the varieties developed callus on medium lacking growth hormones. Although the addition of coconut milk has been reported essential for callusing but in our studies no such supplements were added for inducing 100% callusing in all the varieties tested. The callus growth rate differed on different media (Table 3). Although the effect of variety (V) and medium (M) on callus growth rate was not significant, their interaction (M x V) was found to be significant at 1% level. The varieties Co 1148 and CoS 767 recorded highest callus growth rate on medium containing 0.5 mg/*l* BAP and 3mg/*l* 2,4-D. Varieties CoJ 64 and CoPant 84212 recorded very significant callus growth at 1% level with 3 mg/*l* 2,4-D alone and also in combination with 1 mg/l BAP. Variety CoLk 8102 recorded very significant growth with 3mg/*l* 2,4-D with combinations of 0.5 mg/*l* BAP, 1

Table 3 : Growth rate of calli on callus induction media

Variety	CMS 1	CMS 2	CMS 3	CMS 4	CMS 5	CMS 6
Co 1148	0.018	0.021	0.041	0.007	0.008	0.008
CoJ 64	0.009	0.061**	0.019	0.014	0.015	0.033
CoPant 84212	0.024	0.044**	0.013	0.025	0.011	0.010
CoLk 8102	0.009	0.016	0.068**	0.070**	0.087**	0.015
CoLk 8001	0.013	0.049**	0.015	0.041**	0.034	0.011
CoS 767	0.015	0.017	0.037	0.020	0.017	0.028

CD (M x V) = 0.03467; SE (M) = 4.258; SE (V) = 3.887; SE (M x V) = 9.521; [HH]Significant at 1% level

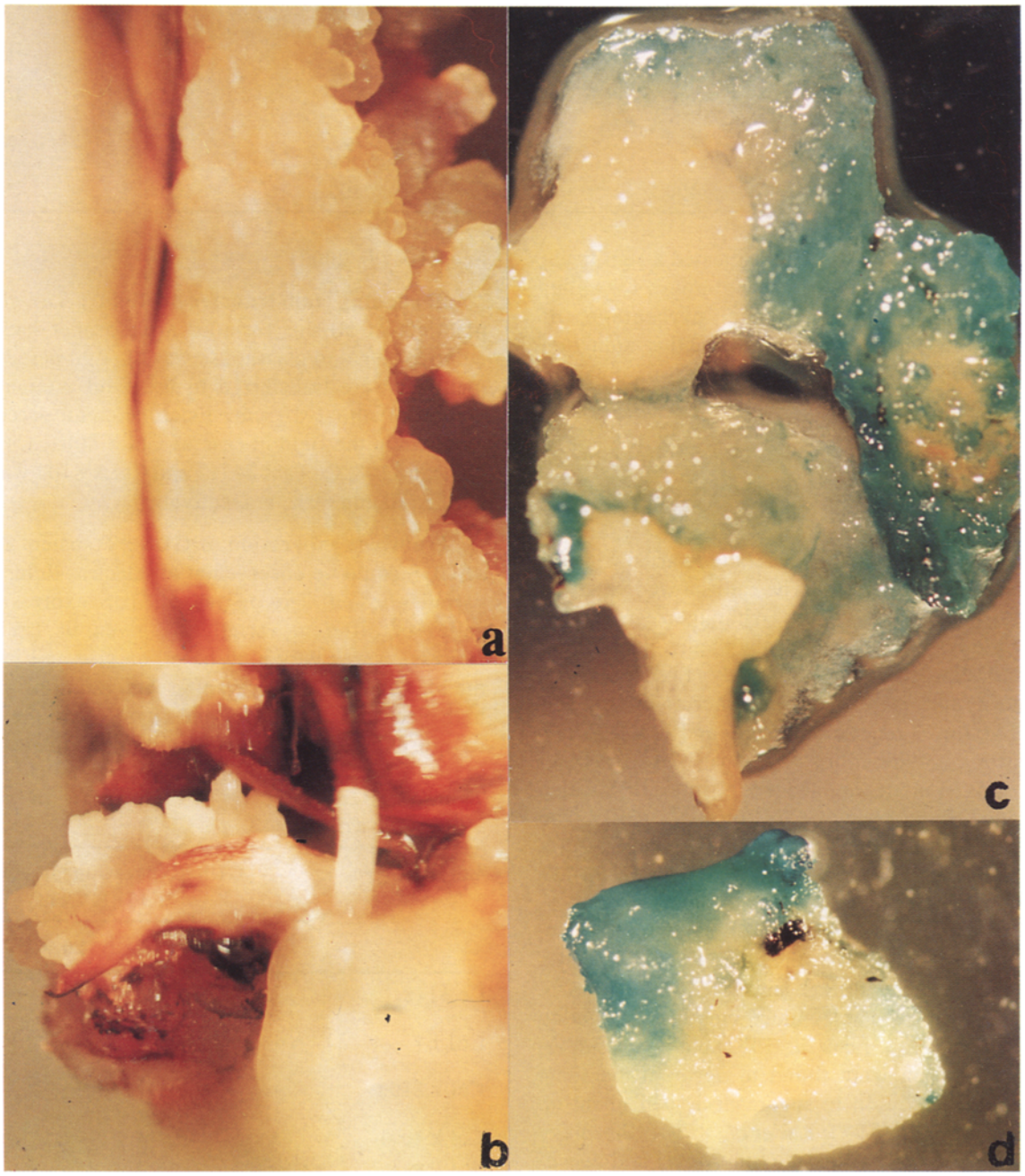

Fig. 1. : Embryogenic callus and transient GUS expression in sugarcane varieties (a) Embryogenic callus of Co 1148 on CMS 3; (b) Embryogenic callus of CoS 767 on CMS 3; (c) Transient GUS expression on CoLk 8001 callus at 7.8 KV/cm; (d) Transient GUS expression in CoJ 64 at 6.5 KV/cm

Table 4 : Percentage embryogenesis on callus induction media

Variety	Media					
	CMS 1	CMS 2	CMS 3	CMS 4	CMS 5	CMS 6
Co 1148	0	100	87.5	87.5	87.5	100
CoJ 64	0	100	100	87.5	87.5	100
CoPant 84212	0	100	100	100	87.5	100
CoLk 8102	0	100	62.5	75	87.5	100
CoLk 8001	0	62.5	100	50	75	50
CoS 767	0	100	100	100	100	75

mg/*l* BAP or 0.5 mg/*l* kinetin. This indicated a significant variation in requirement of growth hormones for callus induction in each variety. The percentage of embryogenesis in each variety on the callus induction media is given in Table 4. The kind of callus obtained corresponded to a type II kind which is regenerable (Gupta *et al.*, 1995). The optimum concentration of 2,4-D was found to be 3 mg/*l* while Wagih *et al.* (1999) had used 2,4-D upto 6 mg/*l* and found that the callus fresh mass reduced considerably.

Plant regeneration : The regeneration varied with the varieties and the culture media. The calli showed initiation of shoot regeneration in 4-5 days. Although regeneration of all the varieties occurred in all the media, the number and length of shoots varied enormously (Fig. 2 and Fig. 3). Previous report mentions use of hormone free MS media for plantlet regeneration (Aftab and Iqbal, 1999). On the contrary, combinations of auxins (IAA and NAA) and cytokinins (kinetin and coconut water) have also been reported (Heinz *et al.*, 1977; Liu *et al.*, 1981). The maximum number of shoots of Co 1148, CoJ 64 and CoLk 8102 was on SMS 5 while that of Co Pant 84212, CoS 767 and CoLk 8001 was on SMS 2, SMS 4 and SMS 5, respectively. The maximum length of shoots of CoJ 64, CoPant 84212 and CoLk 8102 was found on SMS 4, CoLk 8001 and CoS 767 on SMS 3 and Co1148 on SMS 5. Earlier worker have been able induce shooting and rooting on separate media (Cheema *et al.*, 1992) but in our studies shooting and rooting were found to occur in the same media. Plant regeneration in sugarcane is dependent on genotype (Heinz *et al.*, 1977; Lal *et al.*, 1994; Cheema *et al.*, 1992 and Liu, 1981), tissue (Liu, 1984) and age of explant (Manickavasagam and Ganapati, 1988). Sugarcane plant regeneration has been reported to occur via organogenesis (Heinz *et al.*, 1977; Liu, 1981). However, some reports of somatic embryogenesis are also known (Lal *et al.*, 1994 Manickavasagam and Ganapati 1998; Seema *et al.*, 2000).

Hardening and transfer to field : The survival of plantlets of all the varieties after proper hardening in culture room and glasshouse was 80 – 90 percent. All the plantlets were transferred to the field after attaining a height of about 10 cm. Establishment percentage of plantlets in the field in case of CoLk 8001, CoJ 64, CoS 767, Co 1148, Co Pant 84212 and CoLk 8102 was 100, 90, 90, 85, 70 and 60%, respectively.

Transient expression of GUS : Transient expression of GUS could be observed at all electric fields in all the varieties tested. The degree of GUS expression in the callus of different varieties is given in Table 5. The expression was very high based on size and intensity of the blue spots which prevented their counting (Fig.1). At high electric fields of 11.7 and 10.4 KV/cm the callus lost its morphological properties although blue colour was

Table 5 : Degree of GUS expression in sugarcane varieties

Variety	GUS Expression at electric field (KV/cm)			
	6.5	7.8	10.4	11.7
Co 1148	+	++	+++	+++
CoJ 64	+	++	+++	+++
CoLk 8102	++	++	+++	+++
CoLk 8001	+	++	++	+++
CoPant 84212	+	++	+++	+++
CoS 767	++	++	+++	+++

+ : Average expression; ++ : Good expression; +++ : Very high expression

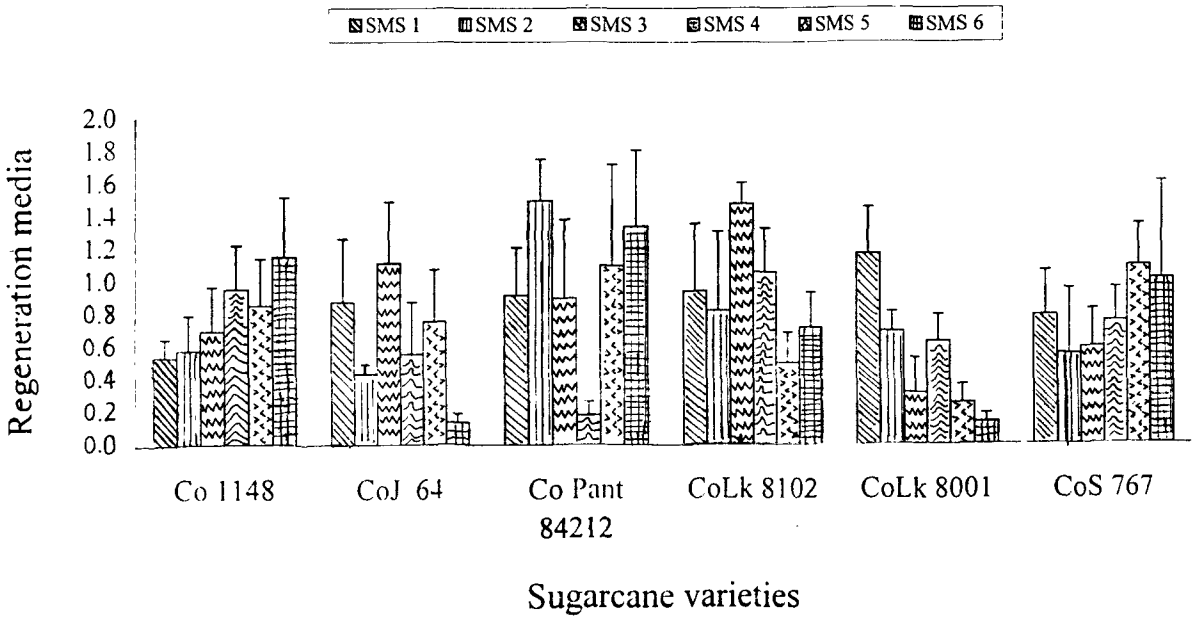

Fig. 2. : Response of sugarcane varieties for shoot length on regeneration media

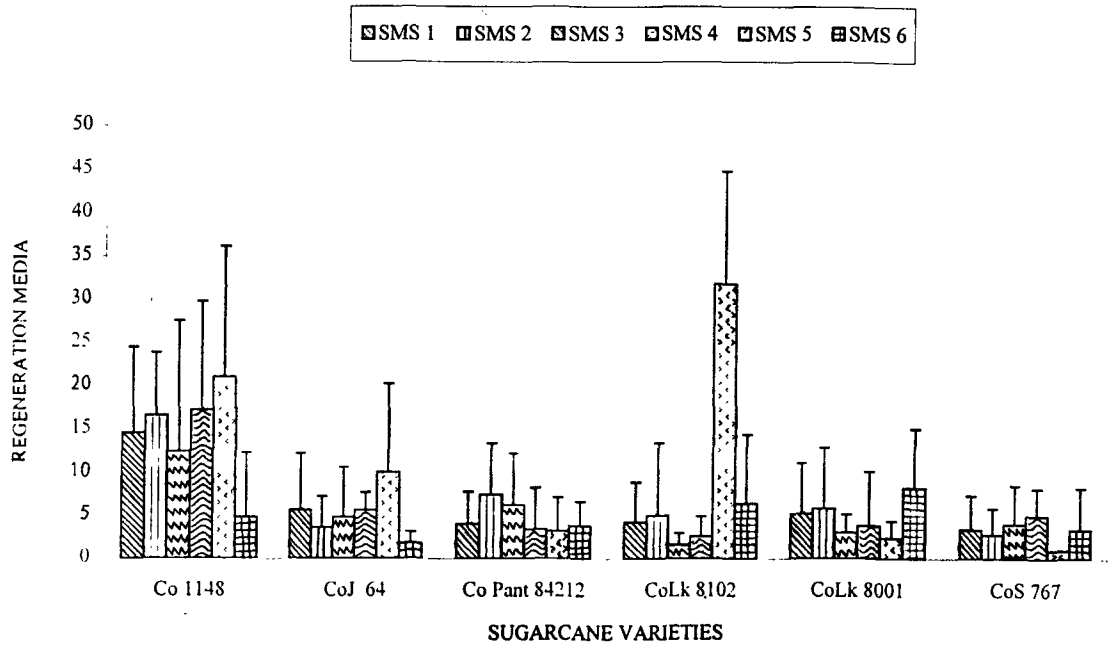

Fig. 3. : Response of sugarcane varieties for number of shoots on regeneration media

apparent. At lower electric fields callus not only retained its morphological properties but also showed further proliferation. The blue coloration due to the intron containing GUS gene allows discrimination between Agrobacterial and plant expression of GUS gene because bacteria cannot splice the intron. GUS expression was restricted to the surface of the callus. The transient expression at all electric field tested in all varieties may occur because in the cuvette all cells are in close contact with excess foreign DNA and transformation force is every where in the cuvette, thus facilitating target cells to take up foreign DNA. Previous reports in other crops indicate pretreatment of intact tissues with macerozyme or wounding as essential for GUS expression (Luong *et al.*, 1995; Jardinaud *et al.*, 1995) but in the present experiment transient expression of GUS occurred without any pretreatment of the intact callus.

The frequency of transformation is reported to be 8-100 times higher using cell electroporation compared to particle bombardment (Arencibia *et al.*, 1995). Moreover it can be performed with a simple electroporation device and does not require the construction of DNA binding tungsten particles. Electropration also has high penetration power (Dillen *et al.*, 1995). Altough cell suspension cultures raised from callus of sugarcane have been electroporated (Arencibia *et al.*, 1995) this is the first report of intact calli electroporation in sugarcane. Further studies are underway for optimizing the conditions for electroporation and development of transformed plants.

ACKNOWLEDGEMENT

The authors are thankful to the Director, Indian Institute of Sugarcane Research, Lucknow for his support and encouragement. We also thank Dr Srinivasan, of Indian Agricultural Research Institute, New Delhi for providing the strain EHA105K (pMOG410).

REFERENCES

Aftab, F. and Iqbal, J. (1999). Plant regeneration from protoplasts derived from cell suspension of adventive somatic embryos in sugarcane (*Saccharum* spp.hybrid cv. CoL-54 and cv-CP-43/33). Plant Cell Tissue & Organ Culture, **56** : 155 - 162.

Arencibia, A., Molina, P., Gutierrez, C., Fuentes, A., Greenidge, V., Menendez, E., De. La. Riva. G. and Selman, G. (1992). Regeneraton of transgenic sugarcane (*Saccharum officinarum* L.) plants from intact meristematic tissues transformed by electroporation. Biotecnologia Aplicada, **9** : 156 - 165.

Arencibia, A., Molina, P.R., Riva, De. La. G. and Housein, G.S. (1995). Production of transgenic sugarcane (*Saccharum officinarum* L.) plants by intact cell electroporation. Plant Cell Reports, **14** : 305 - 309.

Arencibia, A.D., Carmona, E.R., Tellez, P. Chan, C.T., May, Y.S., Trujillo, L.E. and Oramas, P. (1998). An efficient protocol for sugarcane (Saccharum.spp.L) transformation mediated by *A. tumefaciens*. Transgenic Research, **7** : 213 - 222.

Bower, R., Elliot, A.R., Potier, B.A.M. and Birch, R.G. (1996). High efficiency, microprojectile mediated cotransformation of sugarcane, using visible or selectable markers. Molecular Breeding, **2** : 239 - 249.

Cheema, A.S., Shing, H. and Gosal, S.S. (1992). Response of different genotypes to callus induction and plant regeneration in sugarcane. Crop Improvement, **19(1)** : 6 - 13.

Chen, W.H., Gartland, K.M.A., Davey, M.R. Sotak, R., Gartland, J.S., Mulligan, B.J., Power, J.B. and Cocking, E.C. (1987) . Transformation of sugarcane protoplasts by direct uptake of a selectable chimeric gene. Plant Cell Reports, **6** : 297 - 301.

Chen, W.H., Davey, M.R., Power, J.B. and Cocking, E.C. (1998). Sugarcane protoplasts: factors affecting division and plant regeneration. Plant Cell Reports, **7** : 344 - 347.

Dekeyser, R.A., Claes, B., De, Rycke, R.M.R., Habets, M.B., V. Montagu, M.C. and Caplan, A.B. (1990). Transient gene expression in intact and organized rice tissues. Plant Cell, **2** : 591 - 602.

Dillen, W., Engler, G., Montau, M.V. and Angenon, G. (1995). Electroporation mediated DNA delivery to seedling tissues of *Phaseolus vulgaris* (common bean). Plant Cell Reports, **15** : 119 - 124.

Enriquez-Obregon, G.A., Vazquez-Padron, I., Prieto Samsonov, D.L., De La Riva, G.A. and Selman Housein, G. (1998). Herbicide resistant sugarcane (*S. officinarum*) plants by *Agrobacterium* mediated transformation. Planta, **206** : 20 - 27.

Fitch, M., Chang, V., Perlak, F., De. La. Druz, A. and Moore, P. (1995). Insect resistant transgenic sugarcane via particle bombardment. Proc Internl Soc Sugarcane Technol, **22** : 459 - 463.

Gallo Meagher, M. and Irvine, J.E. (1996). Herbicide resistant transgenic sugarcane plants containing the bar gene. Crop Science, **36** : 1367-1374.

Gupta, J.N., Kaur, R. and Cheema, G.S. (1995). Plants regenerated from protoplasts of sugarcane (*S. officinarum*), Current Science, **68** : 650 - 653.

Halluin, K.D., Bone, E., Bossut, M., Beuckeleer, M.D. and Leemans, J. (1992). Transgenic maize plants by tissue electroporation. Plant Cell, **4** : 1495 - 1505.

Hansch, R., Koprak, T., Heydemann, H., Mendel, R.R. and Schulze, J. (1996). Electroporation mediated transient gene expression in isolated scutella of *H. vulgare*. Physiologia Plantarum, **98** : 20 - 27.

Hauptmann, R.M., Ozias Akins, P., Vasil, V., Tabaeizadeh, Z., Rogers, S.G., Horsch, R.B., Vasil, I.K. and Fraley, R.T. (1987). Transient expression of electroporated DNA in monocotyledonous and dicotyledonous species. Plant Cell Reports, **6** : 265 - 270

Heinz, D.J., Krishnamurthy, M., Nickell, L.G. and Maretzki, A. (1977). Cell, Tissue and Organ Culture in sugarcane

improvement. In: Reinert J, Bajaj Y.P.S. (eds). Applied and Fundamental aspects of Plant, Cell Tissue and Organ Culture. Springer Verlag, Berlin pp 3-17.

Hood, E.E., Gelvin, S.B., Melcher, S. Leo and Heokema, A. (1993). New *Agrobacterium* helper plasmids for gene transfer. Transgenic Research, **2** : 208 - 218.

Jardinaud, M.F., Souvre, A., Beckert, M. and Alibert, G. (1995). Optimization of DNA transfer and transient b-glucuronidase expression in electroporated maize (*Zea mays* L.) microspores. Plant Cell Reports, **15** : 55 - 58.

Jefferson, R.A. (1987). Assaying chimeric genes in plants : the GUS gene fusion system. Plant Molecular Biology Reporter, **5** : 387 - 405.

Kloti, A., Iglesias, V.A., Winn, J., Burkhardt, P.K., Datta, S.K. and Potrykus, I. (1993). Gene transfer by electroporation into intact scutellum cells of wheat embryos. Plant Cell Reports, **12** : 671 - 675.

Lal, J., Pande, H.P. and Awasthi, S.K. (1994). Regeneration of multiple plantlets directly from leaf explants of sugarcane. Indian Journal of Sugarcane Technology, **2** : 127 - 131.

Liu, M.C. (1981). *In vitro* methods applied to sugarcane improvement. In : Thorpe, T.A. (ed) *Plant Tissue Culture : Methods and Applications in Agriculture.* Academic Press, N. York, pp 297 - 323.

Liu, M.C. (1984). In : Sharp, W.R., Evans, D.A., Emirate, P.V., Yamada, Y. (eds) *Handbook of Plant Cell Research.* MacMillan Publishing House, N. York, pp 572 - 605.

Luong, H.T., Shewry, P.R. and Lazzeri, P.A. (1995). Transient gene expression in cassava somatic embryos by tissue electroporation. Plant Science, **107** : 105 - 115.

Manickavasagam, M. and Ganapati, A. (1998). Direct somatic embryogenesis and plant regeneration from leaf explants of sugarcane. Indian Journal of Experimental Biology, **36** : 832 - 835.

Molina, P., Arencibia, A., Gutierrez, C., Fuentes, A., Menendez, E., Greenidge, V., De. La. Riva, G. and Selman, G. (1992). Advances in Gene technology: Feeding the world in the 21st century. *In* : Whelan, W.J., Ahmad, F., Bialy, H., Black, H., Black, S., King, M., Rabin, M.B., Solomonson, L.P., Vasil, I.K. (eds) Proc 1992, Miami Biotechnology Winter Symposium. Pp-60.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, **15** : 473 - 497.

Seema, G., Pande, H.P., Lal, J. and Madan, V.K. (2000). Plantlet regeneration of an Indian sugarcane variety by somatic embryogenesis. Indian Jour Sugarcane Technol, **15(1)** : 1 - 5.

Smith, G.R., Joyce, P.A., Nutt, K.A., Mc Qualter, B.R., Taylor, G.O. and Allsopp, P.G. (1999). Evaluation of transgenic sugarcane plants engineered for resistance to mosaic and cane crubs. Proc. Internl. Soc. Sugarcane Technol., **23** : 35 - 39.

Wagih, M.E. and Adkins, S.(1999). Transgenic sugarcane regenerated from protoplasts expressing the GUS reporter gene. Proc. Internl. Soc. Sugarcane Technol., **23** : 107 - 112.

Zaghmouth, O.M.F. and Trollinder, N.L. (1993). Simple and efficient method for directly electroporating *Agrobacterium* plasmid DNA into wheat callus cells. Nucleic Acids Research, **21** : 1048.

Zhang, L., Xu, J. and Birch, R.G. (1999). Engineered detoxification confers resistance against a pathogenic bacterium. Nature Biotechnology, **17** : 1021 - 1024.