International Letters of Natural Sciences *Online: 2015-11-03*
ISSN: 2300-9675, Vol. 48, pp 14-22
doi:10.56431/p-9f9s5x

Callus induction and Organogenesis in Sugarcane (*Saccharum officinarum* L.) var 93v297

Arjun and Srinath Rao*

Plant tissue Culture and Genetic Engineering Laboratory,
Department of P. G. Studies and Research in Botany,
Gulbarga University, Kalaburagi- 585 106

* Corresponding author: srinathraomm@gmail.com, Cell: +919986110109

Keywords: *Saccharum*, callus, immature leaf explants, organogenesis, rhizogenesis.

ABSTRACT. An efficient protocol for induction of callus and regeneration of a sugar cane var 93v297 has been developed and reported here. Callus induction from immature young leaf explants derived from 2-3-month-old plants was achieved on Murashige and Skoog's (MS) medium supplemented with different auxins viz, 2,4-D, NAA and IAA. Among different auxins, 2, 4-D at 3.5mg/l + 0.5mg/l BAP was found favourable in inducing callus. Addition of coconut milk and BAP further enhanced the growth of callus maximum being on MS medium supplemented with 0.5mg/l BAP (3602.33±0.88mg). Calli were further evaluated for regeneration. MS medium supplemented with 1.0 mg/l BAP was found suitable where 100% calli regenerated with maximum number of multiple shoots per callus mass (41.40±0.89). Highest number of root emergence (28.33±1.16) and maximum root length (3.40±0.67cm) was achieved on MS medium supplemented with 3mgl/l NAA. The *in vitro* grown plants were transferred to polycups containing a mixture of sterilized sand, soil and cocopeet (1:1:1) for hardening. The hardened plants were transferred to green-house conditions where they survived with 90% frequency.

1. INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is known in India from time immemorial. It belongs to the family of grasses (Poaceae). It occupies a distinct position as an agro-industrial crop of India, covering around 5.06 million hectares area (over 3% of the total cultivated area) with an annual production of 341.20 million tons and annual yield of 6.74lakh Hg/Ha (FAOSTAT, 2014), contributing about 9.5% to the gross value of the agricultural production in the country. Sugarcane accounts for nearly 70% of the worlds' sugar and is an economically important cash crop in tropical and sub-tropical climatic regions (Chatenet et al., 2001). Apart from use in sugar production, it is gaining importance for production of ethanol and some by-products like molasses, stock feed, alcoholic drinks, bagasses and cane wax. Sugarcane is a monoculture, clonally propagated crop and is prone to variety of pests and diseases and propagation from cuttings facilitates the spread of the pathogens and may result in epidemics (Schenck and Lehrer, 2000). Due to its importance globally, constant efforts are being made world over for its improvement, through tissue culture techniques (Heinz and Mee, 1969; Ahloowalia and Meretzki, 1983; Liu, 1993). Barba and Nickel (1969) who first independently demonstrated that plantlets could be developed from sugarcane callus cultures, and from the published results, it is evident that every part of the sugarcane plant is capable of producing callus (Liu, 1993). Callus induction is a very important for inducing genetic modifications in this crop (Matsuoka et al., 2001). Immature leaf rolls (Rao and Jabeen 2013; Soares et al., 2014), apical meristem (Kaur and Gosal, 2009; Ramgareeb et al., 2010) and the young leaves (Chengalrayan and Gallo-Meagher 2001) are capable of producing morphogenic callus to any appreciable level. Callus based regeneration will be very important as *in vitro* mutations and somaclonal variants could be induced and subsequently used for crop improvement. Keeping in view of the importance of callus induction and callus mediated regeneration, the present investigations is carried out for optimizing a complete plant tissue culture protocol (callus induction, regeneration and acclimatization).

2. MATERIALS AND METHODS

The plant material sugarcane var 93v297 was obtained from Vyuru Agriculture Research Station, Andhra Pradesh and from fields nearby Kalaburagi (India). Young leaf sheath of 2-3-months-old plants were used as explants. The explants were thoroughly washed under running tap water to remove surface adhered particles and then washed with 1% (v/v) soap solution and Bavistin for 10 minute and further washed with distilled water till froth was completely removed, then they were surface sterilized for 3 min in 0.1% (w/v) mercuric chloride, rinsed with deionised water thrice to remove traces of mercuric chloride. Further the outer 2-3 layers of the explants were removed and inner leaves were cut into pieces measuring approximately 1cm and were then aseptically inoculated on Murashige and Skoog's (1963) medium supplemented with 3% sucrose and different types of auxins viz., 2, 4-dichlorophenoxyaceticacid (2, 4-D), α-naphthaleneacetic acid (NAA), and Indole acetic acid (IAA) at various concentrations and medium was gelled with 0.8% agar (w/v). pH of the medium was adjusted to 5.7 prior to autoclaving at a pressure of 15psi at 121 ºC for 15 minutes, then dispensed into pre-sterilized culture tubes and the cultures were maintained under cool-white florescent light at $60\mu M^{-2}S^{-1}$ (16h light/8h dark) at 26±1 ºC for four weeks. A complete randomized design with 30 explants per culture was maintained and all the experiments were repeated thrice. Relative growth rate of callus was determined after 4-weeks of culture. For regeneration, approximately 250±10 mg callus were placed on MS medium supplemented with 3% sucrose containing MS salts with or without cytokinins.

3. STATISTICAL ANALYSIS

All the data was subjected to Tukey-Kramer multiple comparison test (One way ANOVA) to see the standard error (SE) and level of significance using Instat graphpad prism software made for windows (La Jolla, USA).

4. RESULTS AND DISCUSSION

Induction of Callus: Immature young leaf sheaths of sugarcane were used as starting material to generate callus on medium containing different types of auxins at various concentrations (Table 1). Callus induction was observed within ten days after inoculation of the explants on the medium. All the growth regulators used viz., 2, 4- D, NAA and IAA used at various concentrations (1.0-4.0mg/l) induced callus with varying frequencies. 2, 4-D at 3.5mg/l induced maximum amount of callus with 100% frequency followed by NAA at 1.0mg/l with 70% and IAA with 50% frequency at 1.0mg/l. Growth in terms of fresh weight of callus was 2740±0.94mg on 3.5mg/l 2, 4-D, followed by 1.0mg/l NAA which was 712±0.54mg (Plate: 1a, b, c) and the least was recorded on 1.0mg/l IAA supplemented medium (320±0.33mg), further increase in the concentrations of the auxins resulted in decreased growth rate of callus (Table- 1). Supplementing coconut water at 5% to 2, 4-D containing medium further increased the growth of callus (3208±0.72mg). Highest growth of callus was observed on MS medium supplemented with 3.5mg/l 2, 4-D + 0.5 mg/l BAP (3602.33±0.88) (Table: 2, Plate: 1c, d), however further increase in the concentration of BAP resulted in the decreased growth of callus. Immature leaf as an explants along with auxin 2, 4-D has been reported as the best combination for callus induction in sugarcane (Ho and Wasil, 1983; Heinz et al., 1990; Naik et al., 1990; Nandlal, 2003; Gill et al., 2004; Dibax et al., 2011 and Alcantara et al., 2014). During the survey of literature pertaining to callus induction 2, 4-D was found obligatory in many reports whatever may be the explants (Kaur and Gosal, 2009; Ramgareeb et al., 2010; Alcantara et al., 2014). In the present investigations also, it was noticed that 2, 4-D is an important auxin for callus induction when compared to NAA and IAA. However, Gallo-Meagher et al., (2000) reported that picloram is better than 2, 4-D for callus initiation and proliferation in the sugarcane cultivars NIA98, NIA204 and BL4, such contradicting results may be attributed to different genotypes used in these studies. Sugarcane is known to be highly genotype dependent for its response to callus induction (Gandonou et al., 2005). Beneficial effect of supplementing cytokinin (Kn or BAP) (Kaur and Gosal 2009; Rao and Jabeen 2013) or coconut water (CW) with 2, 4-D resulted in better growth

of callus has been reported by many authors (Alam et al., 2003; Rahulbaksha et al., 2003; Gopitha et al., 2010).

Organogenesis: Callus was evaluated for multiple shoot induction on MS medium supplemented with different cytokinins viz., BAP, Kn and TDZ. Approximately 100-150mg of fresh callus was sub cultured on medium containing cytokinins. It was noticed that lower concentrations of cytokinins induced higher number of multiple shoots from the callus with varying frequencies depending upon the type of growth regulator used. BAP (1.0mg/l) alone induced maximum multiple shoots (41.40±0.89) with 100% frequency, further increase in the concentrations of BAP resulted in decrease in the differentiation of shoots, however the frequency of shoot induction was not affected. Kn (1.0mg/l) and TDZ (0.2mg/l) were also found suitable for production of multiple shoots (29.12±0.33 and 18.91±2.03 respectively) but the frequency and number of multiple shoots inducted was lesser than shoots obtained on BAP supplemented medium (Plate: 2b, Table: 3). Similar reports were available on regeneration from callus using low concentrations of BAP (Naik et al., 1990; Gill et al., 2004; Kaur and Gosal, 2009; Alkantra et al., 2014). On the contrary Behara and Sahoo (2009) reported regeneration from callus cultures of sugarcane, using higher concentration of BAP. It appears from these reports that regeneration potential in sugarcane is highly dependent on the concentrations of plant growth regulators besides the genotypes (Maretzki, 1987). TDZ and Kn induced multiple shoots at 0.2mg/l and 2.0mg/l respectively with a frequency of 32 and 54.6%. Further increase in the concentration of both growth regulators gradually decreased the frequency and also number of shoots per callus. Among the three cytokinins, BAP was the most effective in inducing multiple shoots followed by Kn and TDZ. However many authors reported regeneration contrary to the present result using different cytokinins either alone or in combination like BAP + Kn (Rao and Jabeen, 2013), TDZ (Chengalrayan and Gallo-Meagher, 2001; Wamaitha et al., 2010) and BAP + NAA (Gopitha et al., 2010). From the reports and present result it is observed that for shoot induction from callus cultures of sugarcane, either BAP or Kn are essential. It is evident from the study that multiple shoot induction in sugarcane is highly dependent on plant growth regulator concentration. However Behra and Sahoo (2009), reported organogenesis on hormone free media but in the presence of high concentration of sucrose (60g/l) and casein hydrolysate (500 mg/l).

Rhizogenesis: *In vitro* developed plantlets 4-5cm height were transferred to MS medium supplemented with different concentrations of NAA or IBA for root induction. Highest frequency (100%) and maximum number (28.33±1.16) of thick and healthy roots per plantlet was obtained on medium containing 3mgl/l NAA (Table 4; Plate: 2c). The frequency of root induction remains same at lower concentration of NAA (1.0-2.0mg/l) with less number of roots than 3.0mg/l NAA supplemented medium; further increase in the concentration of NAA the frequency of root induction declined. Both NAA and IBA (Gopitha et al., 2010; Mittal et al., 2013; Behara and Sahoo, 2009) are reported to be useful in root induction in sugarcane; however, rooting on medium devoid of growth regulators but only at high concentrations of sucrose (6-8%) has been reported (Meretzaki and Hiraki, 1980). Well rooted plants were transferred to the poly cups (Fig. 2d) containing cocopeet, soil and sand mixture (1:1:1). Plantlets were initially covered with plastic bags to maintain high humidity in the culture rooms. MS medium was added to the plantlets on daily basis during this period. After 15-20 days, plastic bags were removed and thus they were acclimatized to the normal temperature later brought to the green house with 90% success rate.

5. CONCLUSION

From the present investigation and results of the earlier literature it can be concluded that sugarcane mainly depends on the concentrations and combination of growth regulators irrespective of the variety. Callus induction requires auxins especially 2, 4-D and it is played beneficial role when it is supplemented with BAP and for regeneration through callus requires low concentrations of cytokinins (BAP) for efficient regeneration and induction maximum multiple shoots.

Acknowledgement

The authors thank the Head, Department of Botany for the facilities provided during the course of investigation and Arjun Shetty is thankful to Department of Science and technology for financial support under Inspire scheme order No: DST/ INSPIRE Fellowship/2013, Reg No. IF130722 and plant material was kindly provided by Dr. N. R. Chikkagouda, Gulbarga.

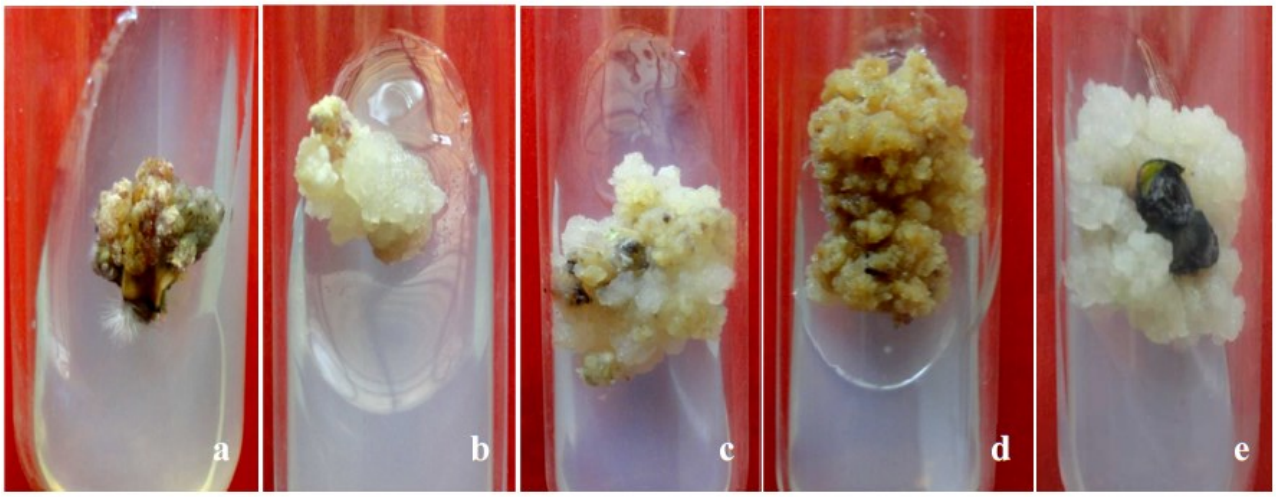

Fig. 1

Fig. 1 Callus induction from immature leaf whorl on **a.** MS + 1.0mg/l IAA **b.** MS + 1.0mg/l NAA **c.** MS + 3.5mg/l 2, 4-D **d.** MS + 3.5mg/l 2, 4-D + 5% CW **e.** MS + 3.5mg/l 2, 4-D + 0.5 mg/l BAP (Note: Maximum amount of callus).

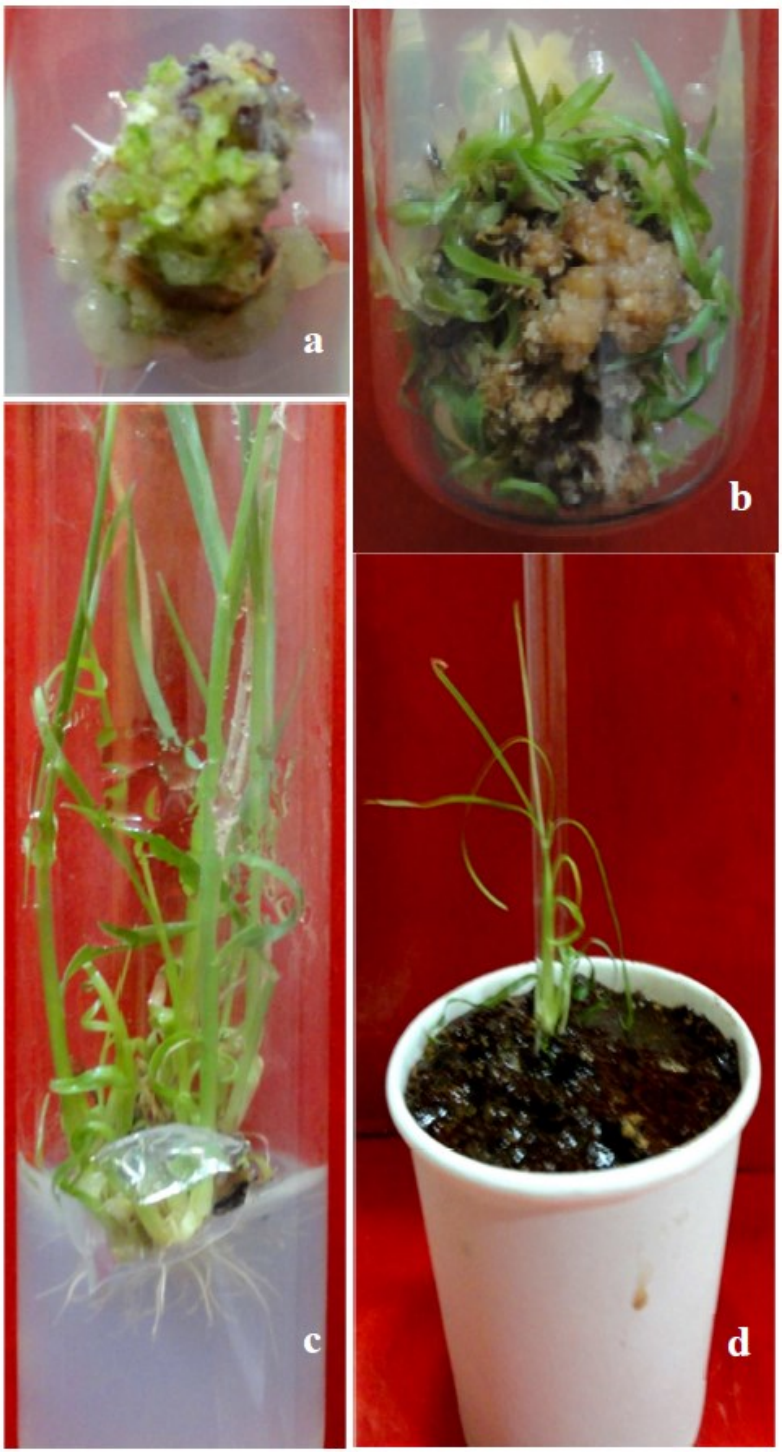

Fig. 2

Fig. 2 Differentiation of callus into shoots **a. & b.** Initiation and growth of multiple shoots on MS + BAP (1.0mg/l), **c.** Rooting of regenerated shoots on MS + 3.0mg/l NAA **d.** Potted plant.

Table 1. Effect of different auxins at different concentrations on frequency of induction and growth of young leaf whorl derived callus in sugarcane 93v298

Growth Regulators (mg/l)	Frequency of callus induction	Growth of callus after 30 days	
		Fresh weight (mg)	Dry weight (mg)
2, 4-D			
1.0	81	1371.67±0.33	141.67±0.67
2.0	87	1562.67±0.33	148.33±0.33
2.5	89	1892.57±0.57	201.89±0.89
3.0	100	2351.33±0.67	210.33±0.57
3.5	**100**	**2740.33±0.94**	**280.89±0.33**
4.0	100	2632.89±0.66	271.57±0.94
NAA			
1.0	70	712.33±0.54	72.89±0.94
2.0	67	654.89±0.89	61.33±0.89
3.0	57	421.33±0.33	39.67±0.33
IAA			
1.0	50	320.67±0.33	29.33±0.89
2.0	35	274.67±0.88	25.67±0.89
3.0	29	192.33±0.88	21.33±0.94

Data represent average of three replicates, and each replicate consists of 30 cultures. Mean ± Standard error. Mean followed by the superscript in a column are significantly different from each other according to ANOVA..

Table 2. Effect of BAP and coconut water (CW) on growth derived young leaf whorl callus in sugarcane

	Fresh weight(mg)	Dry weight(mg)
2, 4-D + BAP (mg/l)		
3.5 + 0.5	3602.33±0.88	357.33±0.66
3.5 + 1.0	3287.89±0.33	314.33±0.89
3.5 + 1.5	3123.89±0.89	309.89±0.88
2, 4-D + CW (%)		
3.5 + 5	3208.67±0.33	331.67±0.88
3.5 + 10	3109.33±0.67	318.89±0.67

Data represent average of three replicates, and each replicate consists of 30 cultures. Mean ± Standard error. Mean followed by the superscript in a column are significantly different from each other according to ANOVA.

Table 3 Effect of cytokinins on the frequency and average number of shoots from young leaf whorls derived callus in sugarcane

Growth regulator (mg/l)	Frequency (%)	Average number of shoots
MS media only	35	
BAP		
0.5	100	27.31±1.58
1.0	100	41.40±0.89
1.5	100	37.54±2.03
2.0	100	34.71±1.58
Kn		
1.0	51.9	29.12±0.33
2.0	54.6	26.24±0.89
3.0	52.3	25.24±0.67
TDZ		
0.1	28	16.27±0.33
0.2	32	18.91±2.03
0.3	30	17.66±1.59
0.4	24	16.74±0.87

Data represent average of three replicates, and each replicate consists of 30 cultures. Mean ± Standard error. Mean followed by the superscript in a column are significantly different from each other according to ANOVA.

Table 4. Effect of auxins on frequency, number of roots and root length per plantlet in sugarcane

Auxins (mg/l)	**% frequency of root induction**	**No of roots per explant**	**Root length (cm)**
NAA			
1.0	89	16.89±2.08	2.98±0.67
2.0	93	24.66±2.00	3.19±0.33
3.0	100	28.33±1.16	3.40±0.67
4.0	87	18.27±1.2	3.31±1.05
IBA			
1.0	81	19.91±1.46	2.98±2.08
2.0	89	21.45±1.46	3.14±0.57
3.0	89	18.32±1.73	3.00±1.2
4.0	86	15.65±1.20	2.57±0.33

Data represent average of three replicates, and each replicate consists of 30 cultures. Mean ± Standard error. Mean followed by the superscript in a column are significantly different from each other according to ANOVA.

References

[1] Ahloowalia, B.S. and Meretzki, A., Plant regeneration via somatic embryogenesis in sugarcane. Plant Cell Rep 2 (1983) 21-25.

[2] Alam, R., MafizurRahaman, A.B.M. and Gupta, S., *In vitro* plant regeneration from leaf sheath callus of sugarcane via organogenesis.Plant Cell Biotech. Mol.Biol.3 (2003) 131-136.

[3] Alcantara G. B., Dibax R., Oliveira R. A. Filho J. C. B. and Daros E., Plant regeneration and histological study of the somatic embryogenesis of sugarcane (*Saccharum* spp.). Acta Scientiarum Vol. 36 (1) (2014) 63-72.

[4] Barba, R. and Nickel, I. G., Nutrition and organ differentiation in tissue cultures of sugarcane-a monocotyledon. Planta 89 (1969) 299-302.

[5] Behara, K.K. and Sahoo, S., Rapid *in vitro* micropropagation of sugarcane (*Saccharum officinarum* L. cv-Nayana) through callus culture. Nature and Science7 (2009) 1-10.

[6] Chatenet, M., Delage, C. and Ripolles, K. Detection of sugarcane yellow leaf curl virus in quarantine and production of virus-free sugarcane by apical meristem culture. Plant Disease 85 (2001) 1177-1180.

[7] Chengalarayan K. and Gallo-Meagher M. Effect of various growth regulators on shoot regeneration studies in sugarcane variety CO-86032. Ad Plant Sci 6 (2001) 649-651.

[8] Dibax R., Alcantra G. B., Filho J. C. B., Machado M. P., Oliveira Y. and Silva A. L. L., Plant regeneration of sugarcane cv. RB931003 and RB98710 from somatic embryos and acclimatization. Journal of Biotechnology and Biodiversity Vol. 2 (3) (2011) 32-37.

[9] FAOSTAT, (2014). Food and Agriculture Organization Stitistics, accessed on 2015.

[10] Gallo-Meagher M., English R. G. And Abouzid A., Thidiazuran stiumaltes shoot regeneration of sugarcane embryogenic callus. In Vitro Cell Dev Biol- Plant 36 (2000) 37-40.

[11] Gandonou C., Errabii T., Abrini J., Idaomar M., Chibi F. and Skalisenhaji N., Effect of genotype on callus induction and plant regeneration from leaf explants of sugarcane (*Saccharum* Sp.). African J Biotech.4 (2005) 1250-1255.

[12] Gill N. K., Gill R. and Gosal S. S., Factor enhancing somatic embryogenesis in sugarcane (*Saccharum officinarum* L.). Indian Journal of Biotechnology. Vol. 3 (2004) 119-123.

[13] Gopitha K., Bhavani L. and Senthilmanickm J., Effect of different auxins and cytokinins in callus induction, shoot, root regeneration in sugarcane. Int J Pharma Bio Sci1 (2010) 1-7.

[14] Heinz D. L., Krishnamurthy G. and Nickell G., Cell tissue and organ culture in sugarcane improvement. In: Applied and fundamental aspects of Plant Cell, Tissue and Organ Cult. (Ed.) Reinert J. and Balaji Y.P.S., Narosa publishing House, New Delhi, India. Pp 13-17. 1990.

[15] Heinz D. J. and Mee G. W., Plant differentiation from callus tissues of *Saccharum* species. Crop Sci 9 (1969) 324-348.

[16] Ho W. And Vasil I. K., Somatic embryogenesis in sugarcane (*Saccaharum officinarum* L.): Growth and plant regeneration from embryogenic cell suspension cultures. Ann Botany 51 (1983) 719-726.

[17] Kaur A. and Gosal S. S., Desiccation of callus enhances somatic embryogenesis and subsequent shoot regeneration in sugarcane. Indian Journal of Biotechnology Vol. 8 (2009) 332-334.

[18] Lee T.S.G., Micropropagation of sugarcane (*Saccharum* spp.) Plant Cell Tiss Org Cult 10 (1987) 47-55.

[19] Liu M. C., Factors affecting induction, somatic embryogenesis and plant regeneration of callus from cultured inflorescences of sugarcane. J Plant Physiol 141 (1993) 714-720.

[20] Maretzki A. Tissue culture: Its prospects and problems In: sugarcane improvement through breeding. (Ed.) DJ Heinz. Elsevier Science Publication BV pp. 343-384 1987.

[21] Matsuoka M., Ideta O., Tanio M., Hayakawa A. and Miwa H.. *Agrobacterium tumefaciens*-mediated transformation of sugarcane using cell suspension culture with a novel method. Inter Soci Sugarcane Tech Proc of the XXIV Congress, Brisbane, Australia, 2 (2001) 660-662.

[22] Meretzaki A. and Hiraki P. Sucrose promotion of root formation on plantlets regenerated from callus of *Saccharum* spp. Phyton 38 (1980) 85-88.
[23] Mittal Pallavi, Ruma Devi and S.S. Gosal, Direct Plant Regeneration from Spindle Leaf Roll Explants in Sugarcane. *Indian J. Ecol.* 40 (1) (2013) 67-70.
[24] Murashige, T. and Skoog, F., A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15 (1962) 473-497.
[25] Naik G. R., Harinath Babu K. and Lingappa G., Studies on *in vitro* selection of Fe efficient lines in sugarcane. Plant and Soil 129 (1990) 183-186.
[26] Nandlal P., High frequency plant regeneration from sugarcane callus. Sugar Tech 5 (2003) 89-91.
[27] Rahulbaksha A. K. M., Alam-Ziaulkarim R., Mannan S. K., Mafizur-Rahaman A. B. M. and Gupta S., Anther culture and plant regeneration in sugarcane (*Saccharum officinarum* L.). Plant Cell Biotech. Mol. Biol. 4 (2003) 179-184.
[28] Ramgareeb S., Snyman S. J., Van Antwerpen T. and Rutherford R. S., Elimination of virus and rapid propagation of disease free sugarcane (*Saccharum* spp. Cultivar NCo376) using apical meristem culture. Plant Cell Tiss Organ Cult. 100 (2010) 175–181.
[29] Rao S. and Jabeen F. T. Z. Optimization of Protocols for Callus Induction, Regeneration and Acclimatization of Sugarcane (*Saccharum officinarum* L.) Cultivar CO-86032. Current trends in biotechnology and pharmacy 7 (4) (2013) 861-869.
[30] Schenck S. and Lehrer A. T. Factors affecting the transmission and spread of sugarcane yellow leaf virus. Plant Disease 84 (2000) 1085-1088.
[31] Soares R. R., Ferreira M. E., Gamarano M. C., Rebeiro R. C. Sabino V. M., and Pereira B. M. H., The use of histological analysis for the detection of somatic embryos in sugarcane. African Journal of Biotechnology Vol. 13 (6) (2014) 762-767.
[32] Wamaitha M. J., Suwa K., Fukuda K., Mir M. Daimon H. and Mishiba K., Thidiazuran-induced rapid shoot regeneration via embryo like structure formation from shoot tip-derived callus cultures of Sugarcane. Plant Biotechnology 27 (2010) 365-368.