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Establishment of plant regeneration protocol through callus induction of sugarcane (*Saccharum officinarum l.*) var. China using leaf-sheath

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Abstract

Establishment of a plant regeneration system through direct organogenesis or via callus is also a prerequisite to further *in vitro* genetic manipulation of the cultivar through somaclonal variation, *in vitro* mutagenesis and genetic transformation. Production of sufficient numbers of plants of a unique genotype is possible using *in vitro* culture system. In this study, the effect of various concentrations and combinations of plant growth regulators for *in vitro* regeneration of sugarcane via callus was described using leaf-sheath as explants. Thus, a reproducible protocol for induction of callus and regeneration into plantlets were developed in sugarcane var. china using leaf-sheath as explants. Potential callus induction was observed on MS medium supplemented with 4.5 mg/l 2,4-D, in which 80% explants induced callus after culture initiation of 90 days. Shoot formation from the callus was optimum on MS + 1.0 mg/l GA_3 + 0.5 mg/l Kin., in which 70% of the cultured calli formed shoots. The average number of shoot formed per culture callus was 27.0 ± 2.50 and the average shoot length of 6.5 ± 0.50 cm were achieved in this medium after culture of 60 days. Regenerated shoots rooted well when they were transferred into half strength of MS + 2.50 mg/l NAA, in which 90% shoots rooted within 30 days of culture. The average number of root produced per shoot was 18.0 ± 2.25 and the average root length of 7.50 ± 1.20 cm were observed in this medium. Ninety percent of the *in vitro* raised plantlets were survived in the natural environment.

Keywords: Callus induction; Plant regeneration; Leaf sheath explants; Sugarcane

1. Introduction

Sugarcane is one of the most important cash crop in Bangladesh and used to produce some essential foods in the form of sugar, gur and juice. It is one of the world largest crop that belongs to the family Gramineae [01]. The entire cultivated area of sugar cane worldwide was estimated to be 26.4 million hectares in 2020. Since sugarcane accounts for the majority of the world's sugar production, an estimated 1.8 billion tons of sugarcane are gathered each year. The breeding of sugarcane cultivars with high yields is desperately needed. It is an economically important crop in many tropical and subtropical region of the world [02] and also to export product of many developing countries [03]. It is a prime crop for fuel production of ethanol from its biomass and its efficient byproducts are molasses, stock feed, alcoholic drink, cane wax etc. [04] (Gallo *et al.*, 2000). It is a perennial herb and propagated vegetatively by three-budded setts. Many methods for sugarcane in vitro micropropagation have been proposed over the past few decades [05-08]. But this traditional method of propagation is limited due to its lack of rapid seed multiplication procedure, long breeding cycle for selection, low yield to attack by fungi, virus, bacteria and mycoplasma which cause up to 70% in yield reduction [09-13]. Therefore, seed cane production through micropropagation is a suitable and effective method for rapid propagation

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in comparison to conventional method and also to effective means for genetic transformation, *in vitro* mutagenesis, creation of genetic variants, conservation and international germplasm exchange. The variety under study having high sugar content but with short internode and vulnerable to insect-pest-diseases, which affect crop yield and sugar production. The technique of plant tissue culture has developed as a powerful tool for crop improvement [14]. In contrast initial attempts to regenerate plants through *in vitro* technique were conducted on sugarcane by Nickel [15] and Heinz and Mee [16]. Protocols for *in vitro* plant regeneration of sugarcane through callus culture, axillary bud, shoot tip and leaf sheath culture have been developed by many authors with different cultivars [17-28]. Attempt also made for genetic transformation and gene expression of various cultivars of sugarcane was reported [29-30]. The present investigation has been undertaken to establish a reproducible protocol for *in vitro* mass propagation and also to develop a potential method for future study of *in vitro* mutagenesis and genetic transformation of sugarcane var. china to meet the challenges of sugar production.

2. Materials and Methods

Leaf sheath explants (Fig. 1) of 3 months old sugarcane var. china were collected from the experimental field of Plant Biotechnology and Genetic Engineering Division, Atomic Energy Research Establishment, Savar, Dhaka whilst the variety was collected from the sugarcane Research Institute, Ishurdi, Bangladesh. Explants were sterilized by rubbing with 70% alcohol (v/v) in the laminar air flow cabinet under aseptic conditions. The explants of about 1.5X0.5 cm were then excised and inoculated into MS media supplemented with different concentrations of 2,4-D and NAA alone or in combinations with BA + NAA for callus induction. Calli were maintained in the same media and also transferred to MS with different concentrations of BA and GA_3 + 0.5 mg/l Kin for shoot formation. Shoots were excised individually and transferred to half strength of MS supplemented with different concentrations of IBA, IAA and NAA for root initiation. The sucrose (Table sugar) concentration was used 30 g/l and the pH of the media adjusted to 5.8 prior to autoclaving. Cultures were incubated at 26 ± 2 °C with a 16 hour illumination of 21.8 μmol/cm^2/s provided by cool white fluorescent tubes. Data were recorded for callus induction at 90 days, for shoot proliferation at 60 days and for root induction at 30 days of inoculation period. Observations on cultures were carried out every alternate day. The experiments were arranged in a completely randomized design (CRD) with three replications for each treatment and five explants per replication. Each experiment was repeated twice. A descriptive analysis was carried out using the recorded data. Each value represents the Mean ± standard errors. *In vitro* raised plantlets were removed from culture vessels, washed thoroughly to remove traces of nutrient medium, transferred to poly bags and placed outdoor condition for acclimatization.

3. Results and Discussion

Swelling and browning of explants was observed within the culture of 15 days which might be due to the phenol exudation of the explants and also the explants experiencing with the media type. A yellowish and friable callus [Fig. 3. a &b] capable of regeneration into plantlets was found from the leaf sheath tissue within 90 days of culture period. Callus induction from explants tissue differed according to the concentrations and combinations of 2,4-D, NAA and BA + NAA used in the media (Table 1 and Figure 1). These might be due to the fact that explants tissue is responsible only with the competent media type and irresponsive with the imbalance media composition. Among the media type used, 2,4-D was found to be the best for callus induction and 4.5 mg/l was showed optimum, in which 80% explants induced callus. Similar observation was made by many authors [01,03,27, 31-32] in callus induction of different sugarcane cultivars. Callus became green and about to shooting (Fig. 3.c-d) was observed at the callus induction medium of MS + 10.0 mg/l NAA and also on transferring to concern regeneration media. The shooting response from the calli differed among the media type used in the study (Table 2 and Figure 2.B). Among the combinations, GA_3 + Kin was found to be the most responsive for shoot formation from the calli and 1.0 mg/l GA_3 + 0.5 mg/l Kin. performed optimum, in which 70% of the cultured calli produced shoots within 60 days of culture (Table 2, Fig. 3 e). The average number of shoot per cultured calli was 27.0 ± 2.50 and the average shoot length of 6.5 ± 0.50 cm was achieved in this medium. Reports on shoot formation from the sugarcane calli were mentioned by many authors using 2.0 mg/l Kin + 1.0 mg/l NAA [31] and using 2.0 mg/l Kin + 5.0 mg/l IAA [16] and also using 3.0 mg/l Kin. Alone [33]. These showed that different cultivars responses with different concentrations and combinations of cytokinin and auxin for morphogenic response of sugarcane callus. This is also in agreement with the study reported by Tripathi *et al.*[33], Kambaska and Santilata, [03], Imtiaz *et al*., [09], Sabaz *et al*., [34], Gopitha *et al*., [27], Kaiser and Makdoom, [35], Sandeep *et al*., [36], Arjumand *et al*., [01], Roy *et al*., [24] and also Roy and Kabir, [25] in callus induction and morphogenic response of various sugarcane cultivars.

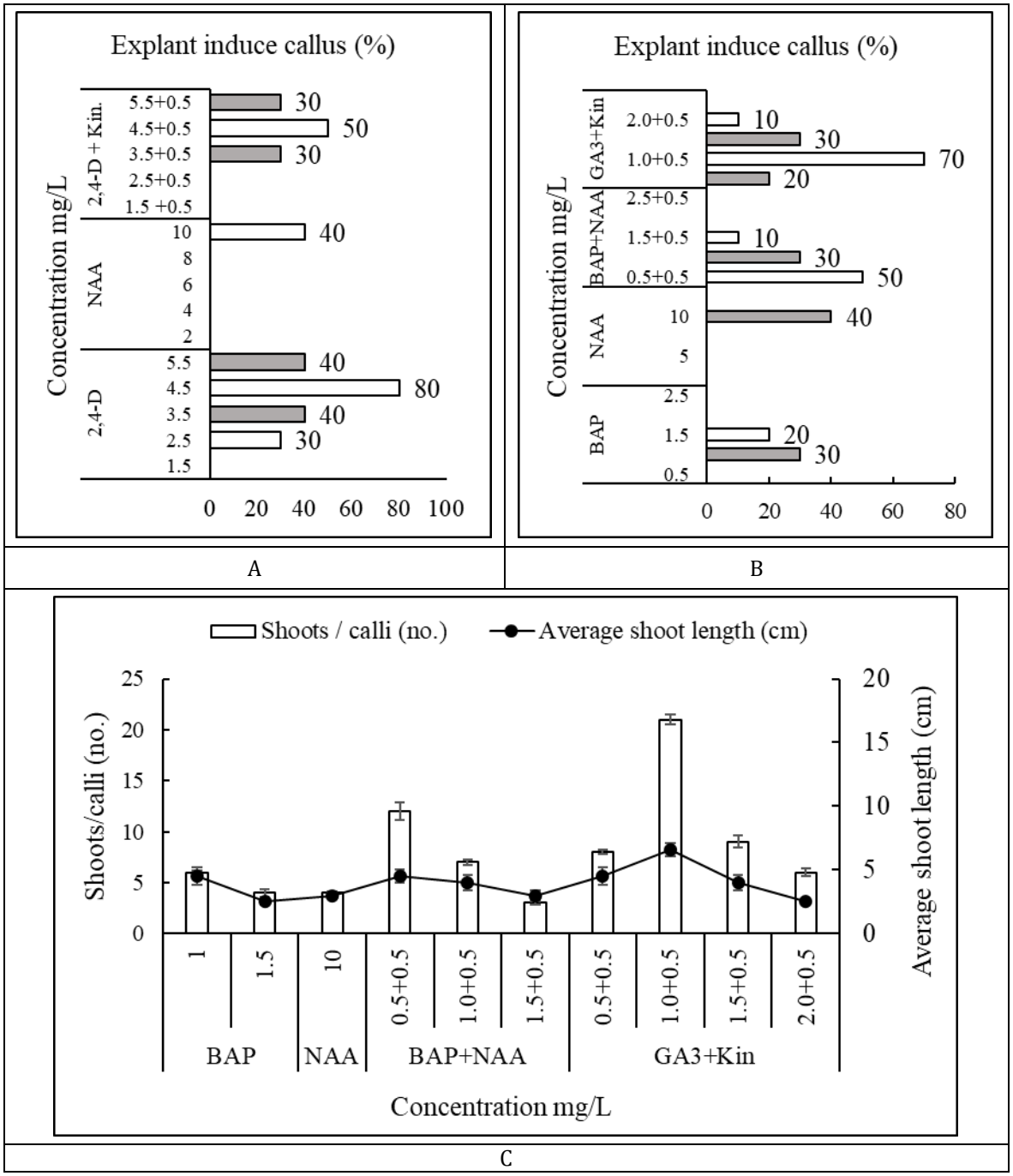

Figure 1 Effects of different concentrations and combinations of plant growth regulators (PGRs) on in vitro shoot induction from calli derived from leaf sheath. Response of PGRs on inoculated explants (A & B) and effects of responded PGRs on calli (C)

Table 1 Effects of different concentrations of BA, NAA, BA + NAA and GA_3 + Kin. with MS media on shoot formation from the calli derived from leaf sheath at 60 days

PGRs (mg/L)		Average shoots / cultured calli (no.) Mean ± SE	Average shoot length / cultured calli (cm) Mean ± SE
BAP	1.0	6.0 ± 0.20^{d}	4.5 ± 0.70^{ab}
	1.5	4.0 ± 0.40^{de}	2.5 ± 0.20^{c}
NAA	10.0	4.0 ± 0.10^{de}	3.0 ± 0.20^{b}
BAP + NAA	0.5 +0.5	12.0 ± 0.90^{b}	4.5 ± 0.50^{ab}
	1.0 + 0.5	7.0 ± 0.30^{cd}	4.0 ± 0.60^{ab}
	1.5 + 0.5	3.0 ± 0.20^{e}	3.0 ± 0.40^{b}
GA_3 + Kin	0.5 +0.5	8.0 ± 0.20^{c}	4.5 ± 0.70^{ab}
	1.0 + 0.5	27.0 ± 2.50^{a}	6.5 ± 0.50^{a}
	1.5 + 0.5	8.5 ± 0.60^{c}	4.0 ± 0.60^{ab}
	2.0 + 0.5	6.0 ± 0.40^{d}	2.5 ± 0.10^{c}

Here, PGR= Plant Growth regulator, SE= Standard error and Values in the same column with different lowercase letters are significantly different at $p < 0.01$

The rooting responses of shoots derived from the calli differed according to concentrations of auxins used (Table 2). Among the auxins, NAA was found to be the best and 2.5 mg/l NAA showed optimum, in which 90% shoots rooted (Fig. 2B & 3 f) within 30 days of culture. The average number of root produced per shoot was 18.0 ± 2.25 and the average root length of 7.50 ± 1.20 cm were observed in this medium. Roots obtained from callus derived shoots were reported by using half strength of MS devoid of plant growth regulators [31 & 37], NAA by MS supplemented media [03 &27], using MS + IBA + NAA (Roy *et al*., 2011) and using MS + GA_3 [01]. The number of shoots and roots were positively correlated with shoot and root lengths respectively whereas the shoot length and root lengths were negatively correlated with each other (Table 3). These indicates that rooting response also depends on competent media composition with the different genotypes and type of callus tissue from explants used. Healthy rooted shoots were taken out from the culture vessels and washed gently under running tap water to get rid of agar.

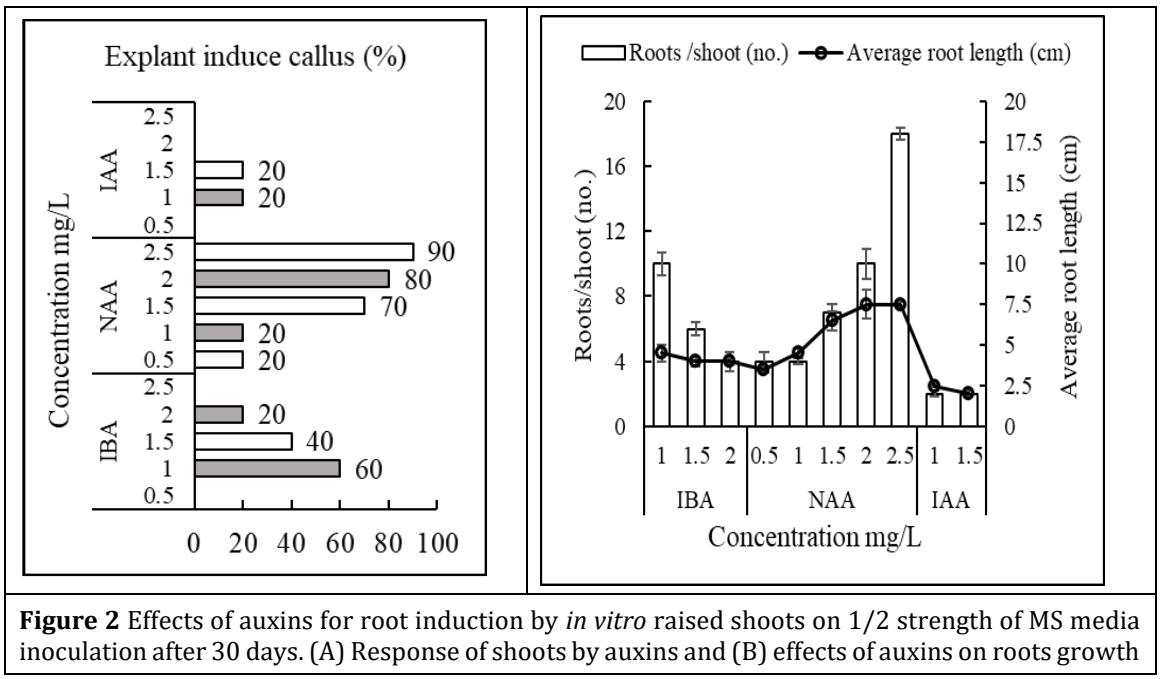

Figure 2 Effects of auxins for root induction by *in vitro* raised shoots on 1/2 strength of MS media inoculation after 30 days. (A) Response of shoots by auxins and (B) effects of auxins on roots growth

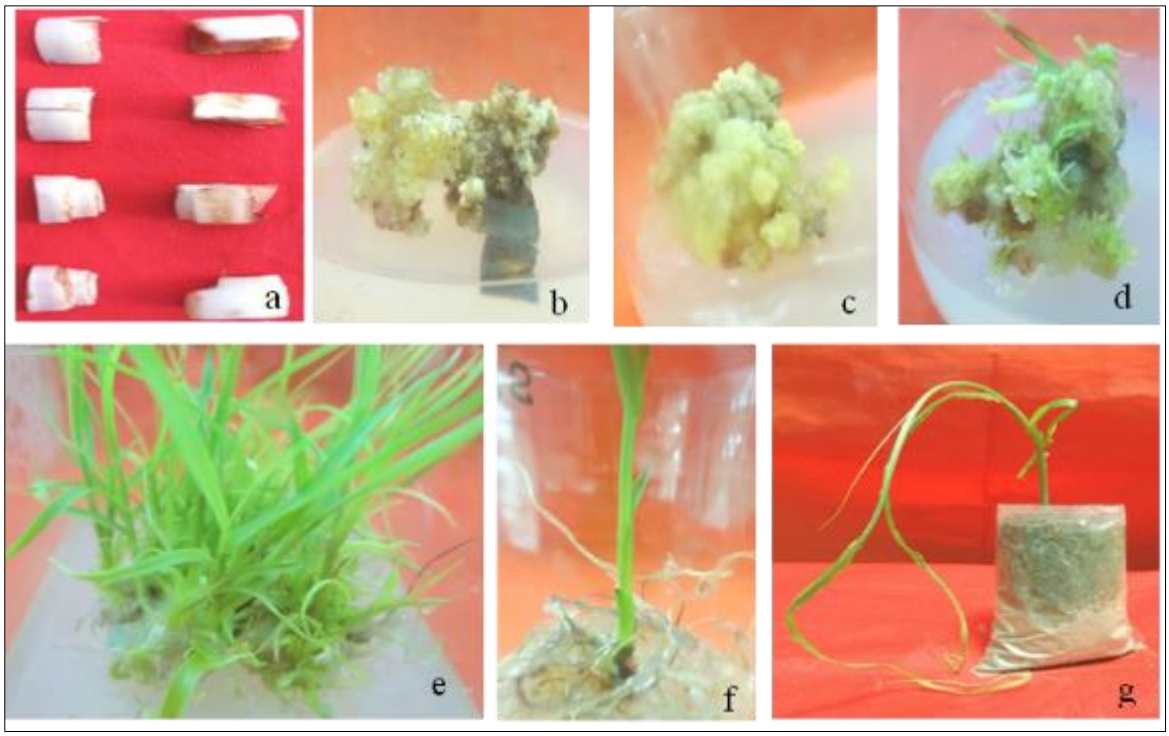

Figure 3 *In vitro* plant regeneration via callus of sugarcane (*Saccharum officinarum* L.) var. china). Collected leaf-sheath explants and callus induction as well as proliferation on MS + 4.5 mg/L 2,4-D (a, b & c). 3). Regeneration of callus into plantlets followed by proliferation on MS + 1.0 mg/l GA_3 + 0.5 mg/l Kin (d &e). Root induction on 1/2 strength of MS + 2.5 mg/l IBA and acclimatized (f & g).

The *in vitro* rooted plantlets were then transferred to poly bags (Fig. 7) containing a mixture of soil and compost (2 : 1) and covered with transparent polyethylene lid to maintained high humidity. The polyethylene lids were removed after 7 days. The plantlets were kept in a shade and misted twice a day. About 90% of the plantlets were resumed new growth within 30 days and were transferred to the field.

Table 2 Effects of IBA, IAA and NAA on half strength of MS media in root induction from *in vitro* raised shoots derived calli of sugarcane var. China at 30 days

PGRs (mg/L)		Average roots induced / shoot (no.) Mean ± SE	Average root length (cm) Mean ± SE
IBA	1.0	10.0 ± 0.70^{b}	4.5 ± 0.50^{b}
	1.5	6.0 ± 0.40^{cd}	4.0 ± 0.30^{bc}
	2.0	4.0 ± 0.20^{de}	4.0 ± 0.60^{bc}
IAA	1.0	2.0 ± 0.20^{e}	2.5 ± 0.10^{cd}
	1.5	2.0 ± 0.20^{e}	2.0 ± 0.20^{d}
NAA	0.5	4.0 ± 0.60^{de}	4.5 ± 0.20^{bcd}
	1.0	4.0 ± 0.20^{de}	4.5 ± 0.30^{b}
	1.5	7.0 ± 0.50^{c}	6.5 ± 0.60^{a}
	2.0	10.0 ± 0.90^{b}	7.5 ± 0.90^{a}
	2.5	18.0 ± 2.25^{a}	7.5 ± 1.20^{a}

Here, PGR= Plant Growth regulator, SE= Standard error and Values in the same column with different lowercase letters are significantly different at $p < 0.01$

Table 3 Pearson's correlation coefficients for *in vitro* plantlet growth characteristics

	Shoots / explant (no.)	**Average shoot length (cm)**	**Roots /shoot (no.)**	**Average root length (cm)**
Shoots / explant (no.)	1			
Average shoot length (cm)	0.891196932	1		
Roots /shoot (no.)	0.638496638	0.76243094	1	
Average root length (cm)	0.34814913	0.552303838	0.821818554	1

4. Conclusion

The protocol described in this study is reproducible and providing disease free propagules of an elite clone for commercial cultivation, conservation and international germplasm exchange whilst adequate planting material of an elite clone is concern in the country. From the above investigation it may be concluded that among the growth regulators used in the study 4.5 mg/l 2,4-D was found most effective for callus induction, 1.0 mg/l GA_3 + 0.5 mg/l Kin showed excellent performance for shoot induction from the callus and 2.5 mg/l NAA appeared better results for root induction from shoots derived from the callus. Therefore, this protocol might be facilitate *in vitro* mutagenesis, genetic transformation programme and also the selection of useful somaclonal variants of a wide range of sugarcane cultivars.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

References

[1] Arjumand, A., K. Saifullah, A. Rahman and M. Nazir. Optimization of the protocols for callus induction, regeneration and acclimatization of sugarcane cv. Thatta-10. Pak. J. Bot. 2009; 41(2): 815 – 820.

[2] Chatenet, M., C. Delage and M. Ripolles.. Detection of sugarcane yellow curl virus in quarantine and production of virus free sugarcane by apical meristem culture. Plant Disease. 2001; 85(11): 1177 – 1180.

[3] Kambaska, K. B. and S. Santilata. Rapid in vitro micropropagation of sugarcane (Saccharum officianarum L. cv-Nayana) through callus culture. Nature and Science. 2009;7(4): 1 - 10.

[4] Gallo, M. M., R. G. English and A. Abouzid. Thidiazuron stimulates shoot regeneration of sugarcane embryogenic callus. In vitro cell Dev. Biol. Plant. 2000; 36: 37 –40.

[5] Lakshmanan P, Geijskes RJ, Wang L, Elliott A, Grof CPL, Berding N, Smith GR. Developmental and hormonal regulation of direct shoot organogenesis and somatic embryogenesis in sugarcane (Saccharum spp. interspecific hybrids) leaf culture. Plant Cell Rep. 2006; 25:1007–1015. doi: 10.1007/s00299-006-0154-1.

[6] Lal M, Tiwari AK, Gupta GN, Kavita Commercial scale micropropagation of sugarcane: constraints and remedies. Sugar Tech. 2015;17(4):339–347. doi: 10.1007/s12355-014-0345-y.

[7] Kavita, Saxena S, Anand A, Lal M. Use of antibiotics to control bacterial contamination during in vitro micropropagation of sugarcane. Agrica. 2015; 4:41–44. doi: 10.5958/2394-448X.2015.00011.5.

[8] Pathak S, Lal M, Tiwari AK, Sharma ML. Effect of growth regulators on in vitro multiplication and rooting of Sugarcane. Sugar Tech. 2009;11(1):80–82. doi: 10.1007/s12355-009-0017-5.

[9] Imtiaz, A. K., M. U. Dahot, S. Yasmin, A. Khatri, N. Seema and M. Naqvi. Effect of sucrose and growth regulators on the micropropagation of sugarcane clones. Pak. J. Bot. 2006; 38(4): 961 – 967.

[10] Siddiqui, S. H., I. A. Khan, A. Khatri and G. S. Nizamani. Rapid multiplication of sugarcane through micropropagation. Pak. J. Agri. Res. 1994; 15: 134 – 136.

[11] Xue, L. P. and R. K. Chen. Elimination of sugarcane mosaic virus by callus tissue culture and apical culture. J. Fujian Agric. Univ. 1994; 23: 253 – 256.

[12] Bhavan, K. and G. Gautam. Micropropagation technology through tissue culture. Indian council for Agric. Res. 2002; pp. 24 – 25.

[13] Oropez, M. P., R. Guevara and J. I. Ramiez. Identification of somaclonal variants of sugarcane resistant to sugarcane mosaic virus via RAPD markers. Plant mol. Biol. Rep. 1995; 13: 182 – 191.

[14] Carison, P. S. Crop improvement through techniques of plant cells and tissue culture. Bioscience. 1975; 25: 747 – 749.

[15] Nickell, L. G. Tissue culture of sugarcane, another research tool. Hawaiian PlantersRes. 1964; 57: 223 – 229.

[16] Heinz, D. J. and G. W. Mee. Plant differentiation from callus tissue of Saccharum species. Crop Sci. 1969;9: 346 – 348.

[17] Lee, T. S. G. Multiplication of sugarcane by apex culture. Tumalba. 1986;36: 231 – 235.

[18] Lee, T. S. G. Micropropagation of sugarcane (Saccharum spp.). Plant cell, tissue and organ culture. 1987; 10: 47 – 55.

[19] Baksha, R., R. Alam, M. Z. Karim, S. K. Paul, M. A. Hossain, M. A. SW. Miah and A. B. M. M. Rahman. In vitro shoot tip culture of sugarcane (Saccharum officianarum) variety LSD 28. Biotechnology. 2002;1(2– 4): 67 – 72.

[20] Hendre, R. R., R. S. Lyer, M. Kotwal, S. S. Khuspe and A. F. Mascavenhas. Rapid multiplication of sugarcane by tissue culture. Sugarcane. 1983;1: 5 – 8.

[21] Hu, C. Y. and P. J. Wang. Meristem, shoot tip, bud culture. In: Plant cell culture. Ed. Evans et al., New York. Macmillan. 1983; 1: 177 – 227.

[22] Milton, J. and D. Alien. Breeding of field crops. 4th edition. 1995; pp. 134 – 135.

[23] Nagai, C. Micropropagation of sugarcane. Laboratory methodology. Annual Report. Experimental station. Hawaiian sugar planters association. 1988; pp. 34 – 37.

[24] Roy, P.K., A. N. K. Mamun, M.H. Kabir, M. R. Islam, M. T. Jahan and M. Z. Rahman. In vitro indirect regeneration of sugarcane (Saccharum officinarum L.) var. Isd16 through apical leaf culture. Bangladesh J. Life Sci. 2011; 23(1): 123 – 128.

[25] Roy, P.K. and M.H. Kabir. In vitro mass propagation of sugarcane (Saccharum officinarum L.) var. Isd 32 through shoot tips and folded leaves culture. Biotechnology. 2007;6(4): 588-592.

[26] Sabaz, A., R. Hamid, M. F. Chaudhary, Z. Chaudhry, Z. Fatima, S. U. Siddiqui and M. Zia. Effect of cytokinins on shoot multiplication in three elite sugarcane varieties. Pak J. Bot. 2009; 41(4): 1651 – 1658.

[27] Gopitha, K., A. L. Bhavani and J. S. Manickam. Effect of different auxins and cytokinins in callus induction, shoot, root regeneration in sugarcane. International journal of pharma and bio sciences. 2010;1(3): 1 – 7.

[28] Karim, M. Z., R. Alam, R. Baksha, S. K. Paul, M. A. Hossain and A. B. M. M. Rahman. In vitro clonal propagation of sugarcane (Saccharum officinarum) variety Isd 31. Pak. J. Biol. Sci. 2002; 5: 659 – 661.

[29] Franks, T. and R. G. Brich. Gene transfer into intact sugarcane cells using microprojectile bombardment. Aust. J. Plant Physiol. 1991; 5: 471 – 480.

[30] Chowdhury, M. K. U. and I. K. Vasil. Stably transformed herbicide resistant callus of sugarcane via microprojectile bombardment of cell suspension cultures. Plant cell Rep. 1992;11: 494 – 498.

[31] Aftab, F., Y. Zafar, A. Kouser, M. Malik and J. Iqbal. Plant regeneration from embryogenic cellsuspensions and protoplasts in sugarcane (Saccharum spp. hybrid cv. Col-54). Plant cell Tiss. Org. Cult. 1996;44: 71 – 78.

[32] Gandonou, C. H., J. Abrini, M. Idaomar and N. S. Senhaji. Response of Sugarcane (Saccharum sp.) varieties to embryogenic callus induction and in vitro salt stress.African J. Biotech. 2005;4(4): 350 – 354.

[33] Tripathi, J. P., S. K. Singh and S. B. Singh. High frequency plantlet regeneration through callus for improvement in sugarcane variety cose 95436. Plant tissue cult. 2000; 10(1): 89 -92.

[34] Sabaz, A., H. Rashid, M. F. Chaudhury, Z. Chaudhury and A. Afroz. 2008. Rapid micropropagation of three elite sugarcane varieties by shoot tip culture. African Journal of Biotechnology. 7: 2174 – 2180.

[35] Kaiser, L. C. and M. Hussain. Micropropagation of sugarcane through apical bud and axillary bud. Int. J. Agri. Biol. 2004;6(2): 257 – 259.

[36] Sandeep, B., D. P. Biradar, V. C. Patil, S. S. Patil and N. S. Kambar. In vitro plant regeneration using shoot tip culture in commercial cultivar of sugarcane. Karnataka J. Agric. Sci. 2009;22(1): 21 – 24.

[37] Chen, W. H., M. R. Davey, J. B. Power and E. C. Cocking. 1988a. Control and maintenance of plant regeneration in sugarcane callus cultures. J. Exp. Bot. 39: 251 – 256.