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Large-scale production, field performance and RAPD analysis of micropropagated sugarcane plants

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Abstract

An improved procedure has been developed for the micropropagation of true-to-type plants of two early maturing varieties of sugarcane, CoH92 and CoH99. The protocol involved (i) growth and proliferation of shoot tip explants in MS medium containing gibberellic acid, indole-3-acetic acid and kinetin, (ii) 3-6 rounds of shoot multiplication in MS medium enriched with 6-benzylaminopurine and kinetin, (iii) rooting in MS medium with (α-naphthalene acetic acid and sugar at higher concentrations, and (vi) hardening of plantlets and their transplantation into 1:1 mixture of unsterilized sand and soil under natural conditions. Shoot multiplication and rooting media contained food grade cane sugar and Isubgol™ as cheaper substitutes in place of sucrose (pure grade) and agar, respectively. This procedure does not require expensive equipment and facilities such as water purification units, greenhouse, polyhouse, etc. Plants propagated through micropropagation and conventional means using setts compared well for various agronomic (cane length, cane weight, number of internodes per cane, internode length) as well as sugar yield/quality traits (purity and CCS). Micropropagated plants had relatively higher number of millable canes, but they were thinner than the conventionally propagated cane. Plants propagated through setts of the micropropagated plants were genetically stable for all the traits. RAPD marker analysis using 20 primers clearly established the clonal fidelity in >90% of micropropagated plants.

Key words: Sugarcane, micropropagation, field performance, clonal fidelity, RAPD analysis.

Introduction

Sugarcane (*Saccharum* spp.) is an economically important, polysomatic, highly heterozygous, clonally propagated crop that accounts for more than 60 % of the world's sugar production [1]. Modern commercial sugarcane varieties are obtained through breeding and a multi-stage selection scheme over a period of 10-15 years. The cost of breeding of a new cultivar comes to about one million dollars from advance breeding programmes [2]. Tissue culture techniques have been widely used for large-scale micropropagation and can effectively reduce the time period between selection and commercial release of new sugarcane varieties [3-9]. The major problem in sugarcane tissue culture relates to the occurrence of somaclonal variation in micropropagated plants [10-14]. The degree of somaclonal variation depends upon a number of factors including the type and source of the explant and method of plant propagation [12]. Thus, it is important to determine the clonal fidelity of micropropagated plants to ascertain the value of a particular micropropagation procedure for commercial use. PCR-based DNA markers such as RAPD, AFLP, SSR and ISSR have been used to detect somaclonal variation in tissue-culture raised plants [15-16]. RAPD analysis is easy to perform, fast, reliable, relatively low cost and provides variation data at multiple loci [17]. In this paper, we report a procedure for cost-effective micropropagation of two commercially important sugarcane varieties, CoH92 and CoH99. Micropropagated plants were evaluated in the field for important phenotypic and sugar production/quality traits. RAPD analysis was conducted to assess the genetic uniformity/variability in micropropagated plants.

Materials and methods

Plant material and culture establishment: Shoot tip explants of about 1.5 cm were excised from six-month-old healthy, field grown plants of two early maturing varieties (CoH92 and CoH99) of sugarcane. Explants were treated with an aqueous solution of 0.2% (w/v) ascorbic acid and 0.4% (w/v) citric acid for 20 min, surface-sterilized with 0.1% (w/v) $HgCl_2$ solution with a few drops of Teepol™ (a surfactant) for 5 min, and washed once with sterile KCl solution (1.0% w/v) and thrice with sterile distilled water. The shoot tip explants were cut in four pieces vertically and transferred on to 0.8% (w/v) agar-solidified MS medium [18] supplemented with gibberellic acid (GA_3, 1.0 mg/l), indole-3-acetic acid (IAA, 1.0 mg/l), and kinetin (1.0 mg/l) (SS1, shoot tip proliferation medium) and sub-

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cultured at 10 d intervals. After 30 days, the shoots were aseptically excised and transferred in the medium containing BAP (1.0 mg/l) and kinetin (1.0 mg/l) (shoot multiplication medium, SS2CE medium). After 6-7 cycles (20 days each) of shoot multiplication, shoots were individually transferred onto SR1CE medium (MS+ α-naphthalene acetic acid, NAA, 5.0 mg/l) for rooting (20 d). All the media except SS1 contained Isubgol^{TM} (2.0 % w/v; Dabur Pvt. Ltd India) and food grade cane sugar (20 g/l w/v for shoot multiplication and 40 g/l for root induction) and tap water in place of agar, analytical grade sucrose and Milli-Q water, respectively. The cultures were sealed with Parafilm and incubated under light (50 µmol m^{-2} s^{-1}, 16 h photoperiod) at 28±1°C. Rooted plants (6-8 cm tall) were hardened by keeping in water for three days at room temperature in day light and transferred in polythene bags, containing a un-sterilized mixture of sand and soil (1:1) for three weeks.

Field trial for morphological and sugar yield characters: About 200 micropropagated plants and 100 conventionally propagated plants of each variety were planted in four replications using the randomized block design (RBD) and grown to maturity. There were 25 plants in each row with the plant-to-plant distance of 65 cm; the rows were spaced at 90 cm. At harvest, the number of millable canes per stool, cane length (cm), number of internodes per cane, inter node length (cm), cane diameter (cm), and cane weight (kg) were recorded for 20 random plants (5 from each replication) per treatment for each variety. These plants were also evaluated for quality traits like juice Brix, Pol, Purity and commercial cane sugar (CCS) following the standard analytical methods [19]. Purity is the ratio of Pol % to Brix % and it indicates the proportion of sucrose to non- sucrose components in the juice. Pol % and Brix % are the percentage of sucrose and total solids in cane juice, respectively. Commercial cane sugar (CCS) value represents the amount of sugar obtained per plant and is an important component in sugarcane breeding. In the next year, plants were raised from setts of the micropropagated as well as conventionally propagated plants and evaluated for various morphological and sugar productivity parameters. The statistical analysis (OPSTAT statistical software; http://hau.nic.in/spas.htm) involved establishing 95% confidence intervals for sample means of each trait and variety. Micropropagated population was then compared with the control population by F test for each trait.

RAPD analysis: Total genomic DNA was isolated from three- month-old leaf tissues of five control (field grown) and 20 randomly selected micropropagated plants of each variety by CTAB method [20]. A total of 20 arbitrary 10-mer oligonucleotide sequences (Operon Technologies, USA) were screened for genetic polymorphism/uniformity using PTC-100 TM 96V thermocycler (MJ Research Inc., Watertown, MA, USA) (Table 1). PCR was carried out in a reaction volume of 20 µl containing 50 ng genomic DNA, 1 unit Taq DNA polymerase, 2 µl 10 × PCR buffer, 0.25 µM each of dNTPs, 0.5 µM of primer and 2 mM $MgCl_2$. The PCR amplification was performed with a hot start of 94°C for 4 min and then 40 cycles of 1 min at 94°C, 1 min annealing at 38°C and 2 min extension at 72°C and 5 min at 72°C for final product extension. Amplified products were stored at –20°C till further use. The PCR products were resolved on 1.5% agarose gel and stained with ethidium bromide. The PCR reactions and electrophoretic separation of amplified products using each primer were repeated at least once to confirm the reproducibility of the results. The presence (1) and absence (0) of bands for each RAPD marker were recorded for all the micropropagated and control plants. Similarity (F) and dissimilarity (1-F) between two entries were computed as F = 2Mx/(My + Mz) where Mx is number of shared fragments between genotype y and z and My and Mz are the total number of bands for genotypes y and z, respectively.

Results and discussion

Micropropagation is currently the only realistic means of achieving rapid, large-scale production of disease-free seed canes of newly developed varieties in order to speed up the breeding and commercialization process in sugarcane [3, 4, 6-8]. The potential of this technology has not been fully exploited due to: (i) the higher inputs it requires in terms of labour, infrastructure/equipment and expensive medium components, and (ii) occurrence of variability in *in vitro* propagated plants. In this study, an efficient and cost-effective procedure has been developed for large-scale production of genetically uniform, disease-free plants of two early maturing varieties of sugarcane being commercially cultivated in India. While the mechanism of shoot multiplication remains the same but composition of various media used in this study were quite different from those used earlier [4-6, 9]. The micropropagation frequencies obtained here are higher and/or comparable to that reported earlier in sugarcane [5-6].

Large-scale multiplication: Micropropagation of two Indian sugarcane varieties, CoH92 and CoH99, was accomplished by a three-step sequential culture of the shoot tip explants/ shoots onto different media. The first step involved the elongation and proliferation of shoot tip explants excised from the field grown plants in SS_1 medium. The pre-treatment of the shoot tip explants with an aqueous solution of ascorbic acid and citric acid, and sub-culture at 10 d intervals onto fresh medium, completely avoided the deleterious effects of

brown pigment secretion by the explants. On an average, three (range 0-6) shoots were obtained after 30 d of shoot tip culture. The second step involved 3-6 cycles of shoot multiplication of individual elongated shoots in the shoot multiplication medium (SS2CE). During the first two subcultures, the shoot multiplication rate was 20 shoots per culture, which declined gradually to 10 shoots per culture (Table 2). The third step involved rooting of the individual shoots in SR1CE medium. Shoot multiplication continued in this medium also. Four shoots could be cultured in 500 ml capacity bottles (jam bottles) without any adverse effect on the multiplication rate and as many as 10 shoots could be cultured in the rooting medium. Care was taken to carry out the subculture within 25 days. The optimum subculture period was 20 d, and anything above 25 d was detrimental (data not shown). Using this procedure, over six billion plants can be theoretically produced from a single shoot tip in the sugarcane variety CoH92 in about six months (Table 2). The response of variety CoH99 was similar to that of CoH92, with no significant differences in shoot multiplication and rooting frequencies.

The media, SS2CE and SR1CE, contained Isubgol^{TM}, ordinary sugar and tap water, as low-cost substitutes for agar, analytical grade sucrose and Milli Q water, respectively. This substitution of components reduced the medium cost by 80%. The use of cheaper medium substitutes did not affect shoot multiplication and rooting (data not shown). Agar and sucrose together constitute 90% of the medium cost. A number of cheaper gelling agents including Isubgol^{TM} (processed husk derived from the seeds of *Plantago ovata*) have been used for tissue culture in many plant species [22-24]. Removal of gelling substance from both shoot multiplication and rooting media resulted in the production of weak shoots, which subsequently had poor survival and transplantation frequencies.

Micropropagated plants were washed thoroughly to remove the medium components and hardened by keeping them in ordinary tap water for three days under direct sunlight. The plants were transplanted to un-sterilized 1:1 sand:soil mixture in a net house under natural climatic conditions with 89.3-98.0% survival during different times of the year. It, thus, indicates that the expensive equipment and facilities like water purification systems, growth chambers, greenhouses and polyhouse facilities may not be required for sugarcane micropropagation.

Field evaluation of micropropagated plants: Micropropagated plants initially showed considerable variation with respect to plant morphology and leaf size/ shape but most of these differences disappeared within a month of transplantation (data not shown). A comparison of the micropropagated and conventionally propagated plants of two sugarcane varieties for important agronomic traits and sugar productivity parameters is shown in Table 3. There were no significant differences between the two types of plants for cane length, number of internodes per cane and internode length. However, the micropropagated plants had a higher number of millable canes (range 3-8 canes) compared to plants propagated through setts (range 3-6 canes). Consequently, micropropagated plants had 28-40% higher number of internodes per stool compared to the conventionally propagated plants. There was a marginal decline in the diameter of canes of *in vitro* propagated plants; however, this difference was statistically significant ($P = 0.05$) only in CoH92. Plants obtained from shoot tip culture exhibited no significant differences in cane juice yield and quality (Table 3) in comparison to the conventionally propagated plants in both varieties. In variety CoH92, however, mean Pol (%) was marginally higher compared to the control donor plants. Micropropagated plants had both cane juice purity (%) and CCS (%) values comparable to conventionally propagated plants. In the second year, plants raised from setts of micropropagated plants resembled the conventionally propagated control plants for all the agronomic and sugar productivity traits (Table 3).

RAPD analysis: Analysis of genomic DNA of conventionally propagated plants of sugarcane varieties CoH92 and CoH99 using 20 primers resolved 122 and 116 scorable markers, respectively. RAPD primers produced an average of 6.0 bands per primer (range 4-12 bands) with size range of 350-3250 bp in both varieties. Banding profiles of RAPD markers were consistent between the replicates in both varieties, and no intra-cultivar polymorphism was detected in the samples analyzed for the five conventionally propagated plants.

Of the 40 randomly selected micropropagated plants of two varieties (20 of each variety) analyzed for 20 RAPD markers (Table 1), 37 plants (CoH92 - 19 plants, CoH99 - 18 plants) had banding profiles identical to their respective parental control plants. Two bands of 0.9 and 1.4 kb were not observed in a micropropagated plant of CoH92 for the primer OPI-06. While two CoH99 micropropagated plants showed polymorphism for two primers (OPC-01 and OPG-05) with a missing fragment in each plant compared to control (Fig. 1). The similarity coefficient between the micropropagated and conventionally propagated plants in both varieties as determined using the Nei and Li [21] equation was 99.8%, indicating good genetic stability and fidelity for the micropropagated plants.

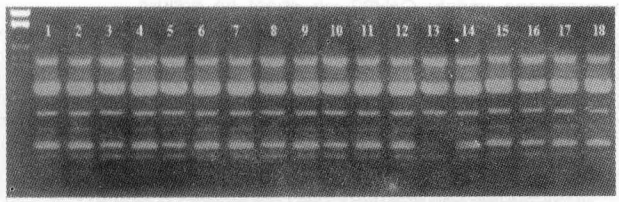

Fig 1. Banding profile of amplified DNA sequences from a RAPD reaction using primer OPG-05. Lane 1, 1 kb DNA ladder (EcoR1 and HindIII digested lambda DNA); Lane 2-4, Control CoH99 plants; Lane 5-18, micropropagated plants of CoH99.

Table 1. RAPD primers used and the number of scorable bands obtained for each in sugarcane varieties CoH92 and CoH99.

Primers	Sequence (5′-3′)	Number of scorable bands	
		CoH92	CoH99
OPC 01	TTCGAGCCAG	8	6
OPC 02	GTCAGGCGTC	6	3
OPC 03	GGGGGTCTTT	12	7
OPC 04	CCGCATCTAC	4	3
OPC 05	GATGACCGCC	7	11
OPG 05	CTGAGACGGA	5	6
OPG 08	TCACGTCCAC	7	6
OPG 09	CTGACGTCAC	3	2
OPG 10	AGGGCCGTCT	3	8
OPG 12	CAGCTCACGA	5	4
OPH 04	GGAAGTCGCC	8	5
OPH 05	AGTCGTCCCC	7	8
OPH 06	ACGCATCGCA	10	6
OPH 07	CTGCATCGTG	8	7
OPH 11	CTTCCGCAGT	1	1
OPI 06	AAGGCGGCAG	2	5
OPI 07	CAGCGACAAG	4	7
OPM 04	CGCGATTAGC	9	8
OPM 15	AACCGCGTCA	4	6
OPQ 01	GGCACGATGG	9	7
Total no. of bands		122	116

The occurrence of somaclonal variation is dependent upon the explant type, its source and the method of plant propagation and regeneration [12]. Among the *in vitro* methods commonly used to propagate sugarcane, shoot tip culture reportedly induces less variation than regeneration from callus cultures [6, 13]. The shoot tip consists of the shoot apex and the sub-apical meristematic region with subjacent leaf and bud primodia. Plants are propagated by proliferation of the apical and axillary meristems. Genetic stability is expected because the process merely involves proliferation, elongation and root differentiation of the already existing, differentiated apical and/or axillary meristems [25].

In this study, initial variation observed among the micropropagated sugarcane plants may have been the effect of many environmental factors and culture conditions. For example, micropropagation as opposed to natural cultivation is carried out in a sugar-rich environment, under poor illumination, inadequate gas exchange, and high humidity conditions and in the presence of growth regulators, which may result in epigenetic changes [7]. The shoot tip-derived sugarcane plants compared well with the control plants propagated through setts for most of the cane yield and juice yield and quality traits, except for the significantly higher number of millable canes per stool and number of internodes (potential setts for next generation) per stool and a marginal decline in cane diameter (significant in CoH92 at $P = 0.05$). Plants raised from setts of micropropagated plants were similar to those raised conventionally for all agronomic and sugar productivity parameters. In sugarcane, several authors have reported an increased number of canes (stalks) per stool and/or decrease in stem diameter and mass and other morphological changes attributable to the tissue culture process [3, 4, 13]. The differences in the number of stalks per stool in the micropropagated plants may be due to residual plant growth regulator activity and should not be interpreted as heritable somaclonal variation. Such an increase in the number of stalks generally leads to a decrease in the stem diameter and/or mass. From an economic point of view, commercial cane sugar percentage is most important in terms of the amount of sugar obtained per unit area of land. The mean CCS value of shoot tip culture raised plants did not differ from the respective control plants.

RAPD analysis has been employed by many research groups to assess the clonal fidelity of micropropagated plants and somaclonal variation in a number of plant species including sugarcane [16, 17]. The present results of RAPD analysis using a large number of primers demonstrate a high degree of genetic fidelity of *in vitro* propagated plants using shoot tip explants in the two sugarcane varieties. More than 90% of the micropropagated plants had RAPD banding profiles identical to that of the parental stocks. The rest of the micropropagated plants showed polymorphism at one or two of the 122 or 116 loci. These plants, however, did not show any gross morphological changes or changes for cane juice yield or quality. These changes may have occurred due to a mutation within priming sites, primer amplicons spanning a highly mutable genomic site, or loss of all or part of specific chromosome(s) [14, 16]. This study indicates good clonal fidelity and little somaclonal variation in the plant saplings produced, confirming the utility of the micropropagation procedure for exploitation at the commercial level.

As shown in Table 2, theoretically > 0.5 million plants (sufficient for planting ~25 hectares) can be

Table 2. An estimate of plant production by micropropagation in sugarcane variety CoH92 via shoot tip culture.

Culture stage/step	Medium	Culture period (days)	Number of bottles in multiplication process	Shoot multiplication rate	No. of shoots/or plants produced	Period from beginning (days)
Shoot tip elongation and proliferations*	SS_1	30	1	3	3	30 (25)
Shoot multiplication						
Culture cycle I	SS_2CE	20	3 (3)	20 (22.1)	60 (67)	50 (49)
Culture cycle II	SS_2CE	20	15* (25)	20 (22.0)	1200 (2200)	70 (71)
Culture cycle III	SS_2CE	20	300* (25)	15 (15.4)	18000 (1540)	90 (94)
Culture cycle IV	SS_2CE	20	4500* (25)	12 (12.6)	216000 (1260)	110 (115)
Culture cycle V	SS_2CE	20	54000* (25)	10 (10.3)	2160000 (1030)	130 (138)
Culture cycle VI	SS_2CE	20	540000* (25)	10 (10.6)	21600000 (1060)	150 (161)
Rooting	SR_1CE	20	2160000** (75)	3 (3.7)	64800000 (2775)	170 (180)

*The shoot tip explant was cut in four pieces vertically and transferred onto the SS1 medium.
A total of four* and ten** shoots were cultured maintaining an equal distance in a 500 ml bottle.
Values in the brackets refer to the results actually obtained. In practice, 25 bottles were used for each sub-culture in the shoot-multiplication medium and 75 bottles for rooting.

Table 3. A comparison of micropropagated and conventionally propagated sugarcane plants for important morphological, agronomic, sugar productivity and quality characteristics.

Characters	CoH92				CoH99			
	Year I		Year II		Year I		Year II	
	Control plants	Micropropagated plants	Control plants	Plants raised from setts of micropropagated plants	Control plants	Micropropagated plants	Control plants	Plants raised from setts of micropropagated plants
No. of millable canes/ stool	3.23±0.09	3.93±0.08*	3.60±0.51	3.81±0.58	3.23±0.09	4.97±0.23*	3.20±0.51	3.42±0.58
Cane length (cm)	167.70±1.94	169.57±0.69	168.82±6.11	165.64±5.48	177.13±1.69	177.00±2.20	176.20±5.66	174.40±4.47
No. of internodes/cane	19.40±0.53	20.43±0.61	19.21±1.56	20.62±1.12	20.60±0.95	19.47±0.64	20.00±1.22	21.20±1.77
Internode length (cm)	11.36±0.03	12.03±0.15*	11.76±0.24	11.74±0.75	11.90±0.06	12.90±0.3	12.04±0.63	11.92±0.48
Cane diameter (cm)	2.56±0.02	2.43±0.03*	2.38±0.06	2.34±0.05	2.29±0.07	2.24±0.07	2.32±0.06	2.28±0.05
Cane weight (kg)	1.28±0.02	1.21±0.04	1.00±0.02	1.06±0.06	1.02±0.01*	1.00±0.07	1.01±0.02	1.03±0.02
Cane juice (%)	48.81±0.71	44.20±1.89	44.21±1.36	44.40±1.69	49.04±0.78	48.68±0.60	42.20±0.73	43.05±1.34
Brix (%)	21.00±0.35	20.17±0.88	20.07±0.37	19.91±0.46	18.77±0.32	18.80±0.35	19.36±0.31	19.33±0.40
Pol (%)	18.56±0.05	17.79±0.66	17.92±0.36	18.04±0.47	16.86±0.07	16.97±0.15	16.83±0.24	16.32±0.33
Purity (%)	88.12±1.26	89.10±0.57	87.83±0.92	87.83±0.92	87.31±0.15	85.57±0.62	87.83±0.92	86.94±1.02
CCS (%)	14.62±0.14	13.97±0.09	13.68±0.22	13.51±0.23	13.14±0.12	12.71±0.14	13.0±0.38	12.90±0.44

*Significant at 5% level.

produced after three subcultures on shoot multiplication medium in three months with an additional two months for rooting, hardening and transplantation. Any laboratory with a 15' × 15' culture room with a storing capacity of 10,000 bottles could produce this amount. Since each multiplication cycle takes only 20 days, the culture storage facility can be optimally utilized to further increase the production capacity. The protocol reported here should allow cost-effective and rapid multiplication of newly released varieties or promising breeding lines.

References

1. **Guimarces C. T. and Sobral W. S.** 1998. The *Saccharum* complex: relation to other andropogoneae. Plant Breeding Rev., **16**: 269-288.
2. **Birch R. G.** 1996. New gene technologies and their potential value for sugarcane. Outlook on Agriculture, **25**: 219-226.
3. **Feldmann P., Sapotille J., Gredoire P. and Rott P.** 1994. Micropropagation of sugarcane. *In*: Teisson, C., ed. In vitro culture of tropical plants. France:CIRAD: 15-17.
4. **Lal N. and Krishna R.** 1994. Sugarcane and its problems: Tissue culture for pure and disease free seed production in sugarcane. Indian Sugar, **44**: 847-848.
5. **Lee T. S. G.** 1986. Multiplication of sugarcane by apex culture. Turrialba, **36**: 231-235.
6. **Lee T. S. G.** 1987. Micropopagation of sugarcane (*Saccharum* spp.). Plant Cell Tiss. Org. Cult., **10**: 47-55.

7. **Lorenzo J. C., Ojeda E., Espinosa A. and Borroto C.** 2001. Field performance of temporary immersion bioreactor-derived sugarcane plants. *In vitro* Cell. Dev. Biol.,Plant, **37**: 803-806.

8. **Taylor P. W. J.** 1997. Micropropagation of sugarcane (*Saccharum* spp. Hybrid). *In*: Biotechnology in Agriculture and Forestry, Volume 39, High-Tech and Micropropagation V. (ed Bajaj Y.P.S.), Springer-Verlag Berlin; 256-271.

9. **Taylor P. W. J. and Dukic S.** 1993. Development of an *in vitro* culture technique for conservation of *Saccharum* spp. hybrid germplasm. Plant Cell Tiss. Org. Cult., **34**: 217-222.

10. **Burner D. M. and Grisham M. P.** 1995.Induction and stability of phenotypic variation in sugarcane as affected by propagation procedure. Crop Sci., **35**: 875-880.

11. **Heinz D. J. and Mee G. W. P.** 1971. Morphologic, cytogenetic and enzymatic variation in Saccharum species hybrid clones derived from callus tissue. Amer. J. Bot., **58**: 257-262.

12. **Larkin P. J., Banks P. M., Bhati R., Bretell R. S., Davies P. A., Rayan S. A., Scowcroft W. R., Spindler L. H. and Tanner G. H.** 1989. From somatic variation to variant plants: mechanisms and applications. Genome, **31**: 705-711.

13. **Lourens A. G. and Martin F. A.** 1987.Evaluation of in vitro propagated sugarcane hybrids for somaclonal variation. Crop Sci., **27**: 793-796.

14. **Nagai C., Ahloowalia B. S. and Tew T. L.** 1991. Somaclonal variants from an intergeneric hybrid : *Saccharum* spp. × *Erianthus arundinaceum*. Euphytica, **53**: 193-199.

15. **Chowdhury M. K. U. and Vasil I. K.** 1993. Molecular analysis of plants regenerated from embryogenic cultures of hybrid sugarcane cultivars (*Saccharum* spp.). Theor. Appl. Genet., **86**: 181-188.

16. **Taylor P. W. J. and Geijskes J. R., Ko H. -L., Fraser T. A., Henry, R. J. and Birch R. G.** 1995. Sensitivity of random amplified polymorphic DNA analysis to detect genetic change in sugarcane during tissue culture. Theor. Appl. Genet., **90**: 1169-1173.

17. **Williams J. G. K., Kubelik A. R., Livak, K. J., Rafalski J. A. and Tingey S. V.** 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., **18**: 6531-6535.

18. **Murashige T. and Skoog F.** 1962. A revised medium for rapid growth and bioassays with tobacco tissues cultures. Physiol. Plant., **15**: 473-479.

19. **Spencer G. L., Mead G. P.** 1963. Cane Sugar Hand Book, John Willey and Sons, Inc. New York.

20. **Saghai-Maroof M. A., Soliman K. M., Jorgenson R. A. and Allerd R. W.** 1984. Ribosomal spacer length polymorphism in barley: Mendelian inheritance, chromosomal location and population dynamics Pro. Natl. Acad. Sci. (USA), **81**: 8014-8019.

21. **Nei M. and Li W. H.** 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Pro. Natl. Acad. Sci. (USA), **76**: 5269-5273.

22. **Bhattacharya P., Dey S. and Bhattacharya B. C.** 1994. Use of low cost gelling agents and support matrices for industrial scale plant tissue culture. Plant Cell Tiss. Org. Cult., **37**: 15-23.

23. **Babbar S. B. and Jain N.** 1998. 'Isabgol' as an alternative gelling agent in plant tissue culture media. Plant Cell Rep., **17**: 318-322.

24. **Lal N. and Singh H. N.** 1993. Evaluation of gelling and support materials for *in vitro* shoot multiplication in sugarcane. Sugarcane, **2**: 2-3.

25. **Hu C.Y. and Wang P. J.** 1983. Meristem, shoot tip and bud cultures. *In*: Handbook of Plant Cell Culture, Vol 1. Techniques for propagation and breeding (eds. D.A. Evans, W.R. Sharp, P.V. Ammirato, Y. Yamada), Macmillan, New York, USA: 117-227.