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Field Performance and RAPD Analysis to Evaluate Genetic Fidelity of Tissue Culture Raised Plants *vis-à-vis* Conventional Setts Derived Plants of Sugarcane

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ABSTRACT

A comparative study was carried out to evaluate the field performance of meristem culture plantlets and conventional setts as seed source of two commercially popular sugarcane varieties, viz., Co86032 and CoC671 for various agro-morphological and quality traits. RAPD technique was used to assess the genetic fidelity of the meristem culture plants in relation to the mother plants. Plants derived from the meristem culture did not differ in the key agro-morphological traits from the plants raised by conventional setts. The analysis of plants of two varieties showed that there was no significant difference between conventionally produced plants and meristem culture plants with respect to brix %, sucrose%, purity% and CCS% and millable height of canes. Significant differences were observed in the case of girth of cane, internodes per cane and single cane weight of the setts derived plants over the plants derived from meristem culture plantlets due to spacing. The amplification products were monomorphic across all the micropropagated plants. RAPD marker analysis using 31 primers showed that clonal fidelity was >97% of meristem culture plants.

Key words : Sugarcane, micropropagation, field performance, RAPD analysis, genetic purity

INTRODUCTION

Sugarcane belongs to the Poaecae family and is a polyploid, highly heterozygous and clonally propagated crop cultivated in the tropical and sub-tropical regions of the world. The commercial sugarcane varieties are normally obtained through cross breeding and multi-stage selection schemes spanning a period of 15-18 years. Clonal multiplications in the field for one year and a small planting ratio (1:10) have been major constraints in the fast spread of the improved varieties.

Tissue culture techniques, used for large-scale micropropagation, can efficiently reduce the time period between commercial release and large-scale cultivation of new sugarcane varieties (Taylor and Dukic, 1993; Fieldmann *et al.*, 1994). Sugarcane micropropagation from apical meristem is very useful because of the time it saves in multiplying the promising varieties (Hendre *et al.*, 1983). One of the most crucial concerns in the *in vitro* propagation is to retain the genetic fidelity of the micropropagated plants with respect to the mother plant(s). Despite the advantage of *in vitro* propagation, phenotypic instability has been observed in micropropagated plants of many species, including sugarcane (Bailey and Bechet, 1989; Irvine *et al.*, 1991; Taylor *et al.*, 1995). The occurrence and degree of somaclonal variation in micropropagated plants (Nagai *et al.* 1991; Burner and Grisham 1995) depend upon a number of factors including the type and source of the explants and method of plant propagation (Larkin *et al.*, 1989). In this context, in the recent past, there has been an upsurge in assessing the genetic purity of tissue culture-derived plants of many important plant species using molecular markers.

During the last few years, various molecular DNA markers, which screen nuclear and organellar genomes have been utilized for the fast and unambiguous assessment of the genetic fidelity of micropropagated plants (Taylor *et al.*, 1995; Rani and Raina, 2000; Devarumath *et al.*, 2002; Saini *et al.*, 2004). RAPD analysis is simple, quick to perform and requires only

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small amount of DNA. It is relatively less expensive and provides variation data at multiple loci (Williams *et al.*, 1990).

In this paper we report observations on the micropropagated plants of two commercially popular sugarcane varieties, viz., Co86032 and CoC671 evaluated in the field for important morphological and quality traits. RAPD technique was used to assess the genetic fidelity of the micropropagated plants.

MATERIALS AND METHODS

Plant material and Production of micropropagated plants

Shoot tips of about 5-6 cm were excised from the disease free field grown plants of two commercially popular varieties of sugarcane (CoC 671 and Co 86032). Explants were washed in tap water then sterile distilled water and surface sterilized with 0.1% (w/v) $HgCl_2$ solution for 5 to 10 min, and rinsed thrice with sterile distilled water in aseptic conditions. The apical meristems of 4-5 month old plants were excised and cultured in liquid MS medium (Murashige and Skoog, 1962) supplemented with 0.1mg/l kinetin and 0.2 mg/l BAP for 30 to 45 days. The shoots were aseptically excised and transferred in the medium containing 0.1mg/l Kinetin, 0.1mg/l BAP and 0.1 mg/l IAA for shoot tip proliferation followed by sub culturing for up to 6 to 7 cycles for shoot multiplication. Shoots were individually transferred on to rooting medium (MS + IBA 5.0mg/ l). Well-rooted plantlets were transferred in polythene bags containing sterile mixture of loamy soil and sand (1:1) and hardened in the green house for 1 ½ month. After that the polybags were transferred outside the green house and plants hardened for 1 ½ month. The plants were then taken for planting in the field.

Field trial

The material for the experiment comprised of well-hardened meristem cultured plantlets (MCP's) produced in the tissue culture laboratory of the Vasantdada Sugar Institute (VSI), Pune. Disease and pest free two-eye bud setts derived from the conventionally produced seed were used as the control. The variety Co86032 has high tillering habit, while CoC671 is a relatively shy tillering variety.

The experiment was laid out in a double nesting split-split plot design during 2005-06. The main treatment consisted of row spacing (M1: 90cm and M2: 120cm) and the sub treatment consisted of three fertilizer levels viz., S1: recommended seed nursery dose of fertilizer [450kg/ha N, 170 kg/ha of P_2O_5 and 170kg/ha of K_2O], S2: 125% recommended dose of fertilizer and S3: 150 % recommended dose of fertilizer. Four sub-sub treatments consisted of within row spacing, viz., SS1: two eye bud setts planting at 23 cm distance (Control), SS2: 60cm between MCP's, SS3: 75cm between MCP's and SS4: 90cm between MCP's. The gross plot size was 0.90m X 6.0m row length x 5 rows or 1.20m X 6.0m row length x 4 rows. The two separate experiments were conducted for the two varieties.

At the time of harvesting (i.e., ten month old crop), observations were recorded on millable height of cane, girth of cane, internodes per cane and single cane weight on individual canes cut from 10 randomly selected hills in each treatment. These canes were also evaluated for quality traits like juice brix, sucrose, purity and commercial cane sugar percent following the standard analytical methods (Meade and Chen, 1977). The data were statistically analyzed as per the methods described by Panse and Sukhatme (1967).

DNA isolation

Total genomic DNA was isolated from the fresh leaves of randomly selected 18 plants (field grown micropropagated plants) by modified Doyle and Doyle method (Aljanbi *et al.*, 1999). DNA samples were quantified and diluted in TE buffer. Similar determinations were made on the control plants.

RAPD fingerprinting

A total of 31 arbitrary 10-mer primers (Operon Technologies, USA) from Kit A, AB, C, G and K were used for the PCR amplification of DNA extracted from mother plant and micropropagated plants. Amplifications were done in 25µl of reaction mixture containing 50ng template DNA, 2.5µl of 10x PCR buffer, 2.5 mM $MgCl_2$, 2 mM dNTPs, 15ng of primer and 1 Unit of Taq. Polymerase (Bangalore Genei). DNA amplification was performed in BIO-RAD icycler programmed for 45 cycles as follows: 4.30 min at 92°C, 1 min at 35°C, 2 min at 72°C; followed by 44 cycles each of 1 min at 92°C, 1 min at 35°C, 2 min at 72°C followed by one by one final extension cycle of 15 min at 72°C. The amplification products were size-separated by gel electrophoresis in 1.4% agarose (Sigma) gel with 1x TBE and stained with ethidium bromide. All the reactions were repeated at least once, and only the consistently reproducible bands were considered.

RESULTS AND DISCUSSION

Field evaluation data of micropropagated plants: a comparison of the meristem culture plants (MCP's) and conventionally propagated plants of two sugarcane varieties Co86032 and CoC671 for important agronomic traits and sugar productivity parameters are presented in Table 1. It was observed that the meristem culture plants (MCP's) had no variation in both agro-morphological and quality traits over the crop raised by conventionally produced setts. There was no significant difference in height of millable cane of setts plants and the tissue culture plants due to row spacing and intra-row spacing, and fertilizer levels interaction (Sundara and Jalaja, 1994). Consequently, data on girth of cane, number of internodes per cane and single cane weight of the plants

Table1: Comparative performance of conventional setts (Control) and Meristem Culture Plantlets (MCP's) of sugarcane, varieties viz., Co86032 (A) and CoC671 (B) regarding morphological and quality traits

Treatments (1-24)	Internodes Per cane		Single cane Weight (kg)		Millable height of cane (cm)		Cane girth (cm)		Brix %		Sucrose %		Purity %		CCS %	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
1. 90cm x 100 % RD x control	**18.96**	**21.20**	**1.24**	**1.33**	**218.66**	**234.66**	**8.60**	**8.73**	**20.51**	**22.59**	**18.87**	**20.43**	**91.98**	**90.40**	**13.47**	**14.47**
2. 60cm MCP's	17.10	19.80	1.06	1.00	201.20	222.10	6.86	7.40	20.89	22.47	19.36	20.37	93.58	90.64	14.06	14.45
3. 75cm MCP's	17.76	20.13	1.09	1.01	217.46	217.93	7.10	7.83	20.03	20.80	18.32	18.58	91.42	89.28	13.04	14.75
4. 90cm MCP's	16.16	20.10	1.10	1.04	214.56	214.80	7.43	8.06	19.10	22.05	17.92	19.89	93.52	90.19	12.90	13.81
5. 90cm x 125 % RD x control	**19.63**	**22.56**	**1.36**	**1.60**	**228.40**	**253.36**	**8.80**	**9.26**	**21.53**	**22.63**	**19.74**	**20.60**	**91.74**	**90.97**	**14.08**	**14.64**
6. 60cm MCP's	17.83	20.66	1.13	1.10	215.76	225.73	7.33	8.16	19.67	20.95	18.26	19.30	92.83	92.18	13.09	13.78
7. 75cm MCP's	18.26	20.70	1.22	1.11	226.10	227.90	7.40	8.36	20.83	21.18	18.90	19.55	90.76	92.18	13.41	13.98
8. 90cm MCP's	18.50	21.06	1.24	1.25	231.83	230.53	7.70	8.80	20.75	22.19	18.97	20.50	92.39	92.36	13.51	14.66
9. 90cm x 150 % RD x control	**19.06**	**21.43**	**1.37**	**1.63**	**227.16**	**249.63**	**8.66**	**9.33**	**20.52**	**22.07**	**18.68**	**19.57**	**91.04**	**88.67**	**13.27**	**13.74**
10. 60cm MCP's	17.73	20.50	1.07	1.10	225.70	225.20	7.46	8.36	20.60	22.28	19.00	20.45	92.21	92.79	13.58	14.59
11. 75cm MCP's	18.10	20.70	1.13	1.09	228.53	227.80	7.50	8.16	20.33	22.30	18.79	20.73	92.27	92.96	13.40	14.54
12. 90cm MCP's	17.10	21.10	1.11	1.20	226.73	233.43	7.60	8.53	20.00	22.58	18.18	20.58	90.87	91.02	12.91	14.62
13. 120cm x 100 % RD x control	**18.36**	**21.70**	**1.36**	**1.35**	**216.60**	**241.70**	**8.03**	**9.36**	**16.96**	**22.98**	**18.26**	**21.30**	**92.82**	**92.64**	**13.09**	**15.26**
14. 60cm MCP's	17.46	20.40	1.02	0.90	214.53	225.56	6.80	7.70	20.21	22.03	17.97	20.09	88.89	91.13	12.62	14.29
15. 75cm MCP's	17.16	20.46	1.08	1.01	219.16	223.60	7.00	8.00	20.05	22.03	18.18	20.22	90.73	91.81	12.90	14.44
16. 90cm MCP's	17.36	20.03	1.05	1.00	216.83	227.53	7.16	8.10	20.13	21.78	18.00	20.07	89.34	92.39	12.68	16.01
17. 120cm x 125 % RD x control	**19.60**	**22.20**	**1.47**	**1.53**	**219.86**	**239.86**	**9.40**	**9.50**	**20.04**	**22.59**	**18.35**	**20.65**	**91.57**	**91.41**	**13.07**	**14.70**
18. 60cm MCP's	17.66	20.50	1.07	1.11	218.06	229.10	7.70	8.20	19.88	22.70	17.87	20.49	89.89	90.23	12.63	14.50
19. 75cm MCP's	17.90	21.16	1.17	1.20	225.50	235.33	8.36	8.80	20.76	21.85	18.54	20.21	89.33	92.44	13.06	14.47
20. 90cm MCP's	17.53	20.83	1.12	1.30	222.73	229.83	7.93	8.33	20.29	22.22	18.27	20.16	91.11	90.79	13.14	14.30
21. 120cm x 150 % RD x control	**19.36**	**21.33**	**1.40**	**1.56**	**217.40**	**243.20**	**9.23**	**9.60**	**20.37**	**23.00**	**18.05**	**20.78**	**88.85**	**90.30**	**12.67**	**14.71**
22. 60cm MCP's	17.53	20.46	1.10	1.11	219.60	228.63	7.50	8.33	20.56	22.04	18.36	20.40	89.25	92.51	12.89	14.61
23. 75cm MCP's	17.76	20.63	1.18	1.14	220.60	235.43	7.86	8.50	20.51	21.68	18.18	20.09	88.69	92.50	12.76	14.98
24. 90cm MCP's	17.73	20.43	1.13	1.10	221.06	228.40	7.70	8.40	19.87	22.09	18.05	20.03	90.853	90.66	12.81	14.21
SE ±	**0.47**	**0.47**	**0.07**	**0.07**	**6.43**	**6.85**	**0.15**	**0.20**	**0.32**	**0.39**	**0.32**	**0.48**	**1.409**	**1.68**	**0.29**	**0.60**
CD at 5%	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**

Table 2 : RAPD primers between the mother and meristem derived plants of sugarcane varieties CoC 671 (A) and Co 86032 (B)

Primers	Number of loci produced		Size range (bp)	Number of Monomorphic loci produced		Number of polymorphic loci produced	
	A	B		A	B	A	B
OPA 01	10	12	1521 – 455	10	12	-	-
OPA 03	11	13	2281 – 355	11	13	-	-
OPA 08	08	08	1482 – 380	08	08	-	-
OPA 09	06	08	2013 – 285	06	06	-	02
OPA 11	03	03	1384 – 956	03	03	-	-
OPA 12	05	04	1868 – 590	04	04	01	-
OPA 19	06	05	2070 – 836	05	05	01	-
OPA 20	05	04	1228 – 490	05	04	-	-
OPAB 01	08	10	2139 – 514	08	09	-	01
OPAB 02	04	04	1840 – 334	04	04	-	-
OPAB 04	05	08	1583 – 276	05	08	-	-
OPAB 09	09	04	2000 – 653	09	04	-	-
OPC 01	09	07	2404 – 470	09	07	-	-
OPC 02	04	07	2460 – 460	04	07	-	-
OPC 03	06	06	1760 – 580	06	06	-	-
OPC 04	06	06	1784 – 885	06	06	-	-
OPC 05	10	10	2100 – 620	09	10	01	-
OPC 06	08	08	2210 – 568	08	08	-	-
OPC 07	05	04	2000 – 975	04	04	01	-
OPC 15	04	04	2200 – 675	04	04	-	-
OPC 16	05	05	2210 – 610	05	05	-	-
OPG 02	06	06	1245 – 500	06	06	-	-
OPG 05	06	04	1220 – 300	06	04	-	-
OPG 06	11	09	2780 – 650	09	09	02	-
OPG 08	01	01	2000 – 000	01	01	-	-
OPG 10	07	06	2000 – 500	07	06	-	-
OPK 01	03	04	1760 – 800	03	04	-	-
OPK 04	04	04	1210 – 450	04	04	-	-
OPK 17	05	05	2170 – 780	05	05	-	-
OPK 19	10	10	1500 – 350	10	10	-	-
OPK 20	07	07	1510 – 480	07	06	-	01
Total	**197**	**196**		**191**	**192**	**06**	**04**

from conventionally produced seeds was relatively higher than the plants grown from the MCP's and the difference was significant due to intra-row spacing. Hendre *et al.* (1983) indicated that meristem cultured plants showed very little deviation in the number of internodes, girth of cane and single cane weight compared to donor clone. Geetha and Padmanabhan (2002) observed that there was no significant difference for cane diameter between tissue culture plants and setts planted crop. Saini *et al.* (2004) reported that plants propagated through setts and micropropagation were genetically stable for all traits. Ranju Kumari and Shahi (2002) pointed out that the plant regeneration through tissue culture were identical to their parental clone, indicating that during tissue culture process genetic purity of the genotype is maintained and it is suitable for mass multiplication of clones through micropropagation.

Meristem culture plants (MCP's) and conventionally produced plants were also evaluated for quality traits like juice brix, sucrose, purity and commercial cane sugar percent following the standard analytical methods at 10 month age. A comparison of the meristem plantlets and conventionally propagated plants for important quality traits is shown in Table 1. The analysis of variance showed that there was no significant difference between conventionally produced plants

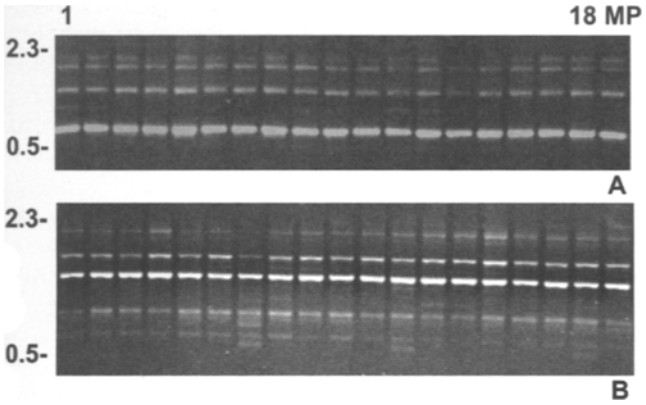

Fig. 1. A-B, RAPD profiles obtained by primer OPC- 07 with sugarcane var. CoC 671 (A) and OPA- 09 with sugarcane var. Co 86032 (B). Lanes 1-18 show RAPD products from micropropagated plants. Lane 19 RAPD bands from the field grown control mother plants (MP).

and meristem culture plants (MCP's) with respect to brix %, sucrose%, purity% and CCS% due to row, intra-row spacing and fertilizer levels (Baily and Bechet, 1989; Sreenivasan, 1995).

Several molecular markers, cytological, and isozymes have been used to detect somaclonal variation among the micropropagated plants of *Populus deltoides* clone L34 (Rani *et al.*, 1995, Rani and Raina, 2000), ginger (Rasut *et al.*, 1998), and sugarcane clones (Heinz and Mee, 1971; Irvine *et al.*, 1991; Taylor *et* al., 1995; Saini *et al.*, 2004; Jain *et al.*, 2005).

In the present study RAPD technique was used to detect variation, if any in the mother plants and TC raised plants of two sugarcane varieties viz., CoC 671 and Co 86032. Of the 55 primers initially tested with single plant, 31 primers produced clear amplification profiles that were easily scorable. A total of 197 and 196 bands were produced in CoC 671 and Co 86032 respectively. RAPD primers produced on an average 6.0 bands per primer (range 1-13 bands) with size range of 455-2521 bp in both the varieties (Table 2). In CoC 671, a total of 26 primers produced amplification products that were monomorphic across all the micropropagated plants and their respective mother plants. Further, out of the 37 bands 6 were polymorphic to five primers (OPA-12, OPA-19, OPC-05, OPC-07 and OPG-06). In Co 86032, out of the 21 bands 4 were polymorphic to three primers (POA-09, OPAB-01, OPK 20) (Table 2) and (Fig. 1). These 8 RAPD markers were monomorphic among 16 -17 plants and polymorphic in 1 or 2 micropropagated plants. Saini *et al.* (2004) have reported that RAPD analysis of sugarcane clones CoH92 and CoH99 showed 90% of genetic purity in micropropagated plants. Studies of Jain *et al.* (2005) indicated that isozyme and RAPD analysis showed no variation in meristem derived sugarcane clones. Chowdhury and Vasil (1993) also indicated that plants derived from embryogenic cultures were genetically identical and were less prone to genetic changes compared to organogenic cultures. In the present studies, meristem derived micropropagated sugarcane clones were found genetically identical and >97% fidelity was maintained. The rest of the micropropagated plants showed polymorphism at one to two of the loci 197 or 196 loci. These plants, however, did not show any gross morphological changes or other quality traits. These changes may have occurred due to mutation within priming sites or changes, which alter the size or prevent the successful amplification of a target DNA Variation, could have been induced by the *in vitro* process or by added biochemicals and stresses (Swartz, 1990; Taylor *et al.*, 1995).

CONCLUSION

Based on the above observations recorded in the present investigation, the field evaluation studies and RAPD analysis showed that the regeneration through micropropagation were identical and true-to-type to the conventionally produced plants, indicating that the meristem culture technique is feasible for maintaining the genetic purity of the genotype and is effective for production of quality seed material of sugarcane varieties.

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