Pak. J. Bot., 40(2): 547-564, 2008.

GENETIC VARIABILITY IN PLANTLETS DERIVED FROM CALLUS CULTURE IN SUGARCANE

IMTIAZ AHMED KHAN[*], MUHAMMAD UMAR DAHOT[**], NIGHAT SEEMA, SAJIDA BIBI AND ABDULLAH KHATRI

Agriculture Biotechnology Division
[*]Nuclear Institute of Agriculture, Tando jam, Sindh 70060, Pakistan
[**]Institute of Biotechnology and Genetic Engineering
University of Sindh, Jamshoro, Pakistan

Abstract

Three sugarcane clones, NIA-98, NIA-2004 and BL4 were studied for callus induction and regeneration potential under two concentrations (2mg/l and 4mg/l) of four auxins i.e., 2,4-dichlorophenoxyacetic acid (2,4-D), indol acetic acid (IAA), 3,6-dichloro 2-methoxybenzoic acid (Dicamba) and picloram. Best callus was observed using picloram followed by 2,4-D whereas best proliferation was achieved with picloram. A high percentage of non-regenerable callus was produced when callus were transferred for proliferation on dicamba. The maximum callus formation and plantlets regeneration were recorded in NIA-98 and minimum in NIA-2004. The maximum chlorophyll mutation frequency was noted in NIA-2004 and minimum in NIA-98. The auxin 2, 4-D induced more genetic variability as compared to other auxins. The analysis of variance (mean square) for all the characters under study revealed that all the phytohormone treatments were significantly different ($p \leq 0.5$). Genetic advance at 2% selection intensity was about two fold greater than that at 30% selection intensity and intermediate at 10% selection intensity. Low coefficient of variability was recorded in all traits. High heritability percentage in broad sense was recorded. Variability obtained through callus culture was also confirmed through random amplified polymorphic DNA (RAPD) techniques.

Introduction

In-vitro culture technique offers unique opportunity for the creation of genetic variability and rapid isolation of clones with desired characteristics in sugarcane (Heinz & Mee, 1969; Khan *et al.*, 1999). This technique provides a promising future in sugarcane breeding programme particularly in Pakistan, where the production of the natural viable seed is a major problem due to the non- or sporadic flowering and scarcity of artificial condition for hybridization (Khan *et al.*, 2004). Ahloowalia (1995) reported that the development of desired genotype could be obtained through somaclonal variation or through *In vitro* mutagenesis in case of vegetatively propagated sugarcane plants. Liu & Chen (1976, 1978a & b) have reported significant variation in somaclones for cane yield and its components, sugar contents and some morphological traits. Besides, development of improved sugarcane clones through *In vitro* culture technique endowed with high cane yield, resistance to disease and tolerance to stress have been reported by many researchers (Krishnamurthi & Tlaskal, 1974; Larkin & Scowcroft, 1981, 1983).

For a long time the regenerated plantlets, produced through callus culturing were screened for the high yield, high sucrose content and resistance against biotic and abiotic stresses under replicated field trials (Liu, 1984). Decisions on selection strategy to use and number of clones to be progressed to advanced stages in breeding program will

[*]Email Address: imtiaz19622000@yahoo.com

ultimately depend on the available resources (Kimbeng *et al.,* 2001). Cox *et al.,* (1996) stated that selection in the early stages of crop improvement programme, where many genotypes are involved, should be designed to make genetic gain rather than identify superior genotypes because genetic advance give clear picture of genetic diversity and helps in comparing the individual plants among the population. As sugarcane is a perennial crop, high inputs and long duration is required for its screening while agronomical practices trigger the inherent potential of variety (Khan *et al.,* 1998). Now-a-days, molecular markers are widely used to detect and characterize somaclonal variation at the DNA level (Zucchi *et al.,* 2002; Ford-Lloyed *et al.,* 1992; Cloutier & Landry, 1994; Barrett *et al.,* 1997). Of the available techniques, RAPD is among the most useful ones (Rani *et al.,* 1995; Taylor *et al.,* 1995; Shoyama *et al.,* 1997; Todoroviska *et al.,* 1997; Rout *et al.,* 1998), because it provides powerful tools for evaluating genetic diversity within and between plant populations, as well as for elucidating genetic relationships among accessions within species. Changes in the RAPD pattern may result from the loss/gain of a primer annealing, caused by point mutation or by the insertion or deletion of sequences or transposition elements (Peschke *et al.,* 1991). This study was conducted to identify somaclonal variation in regenerated plantlets produced through plant tissue culture technique.

Materials and Methods

i. *In vitro* culture

Three sugarcane clones, NIA-98, NIA-2004 and BL4 were used for tissue culture studies. Ten explants containing leaf primordia were taken from each genotype, sterilized by standard procedure (Siddiqui *et al*., 1994) and cultured on modified MS medium (Murashige & Skoog, 1962) containing 2 mg/l and 4mg/l, 2,4-D, dicamba, picloram and indole acetic acid for callusing and designated as M1 (2mg/l 2,4-D), M2 (4mg/l 2,4-D), M3 (2 mg/l picloram), M4 (4 mg/l picloram), M5 (2 mg/l dicamba), M6 (4 mg/l dicamba), M7 (2 mg/l IAA) and M8 (4 mg/l IAA). Medium was solidified with 0.8% Difco bacto agar. Commercial sugar was used instead of Analar grade sucrose as carbon source in the medium. After five weeks of explantation, the calli were weighed and cultured on shoot induction medium (MS +2 mg/I IBA + 2 mg/I IAA + 2 mg/I kinetin). The appearances of green shoots from callus were counted for calculating the shoot organogenesis. The regenerated shoots were scored for chlorophyll mutations. When the plantlets attained 7-8 cm height, these were subjected to rooting by culturing on different media viz., i) MS medium, ii) ½ MS medium, iii) MS medium + 1mg/l IBA + 3% sugar, iv) MS +1mg/l IBA+ 4% sugar, v) MS +1mg/l IBA + 5% sugar , vi) MS + 1 mg/I IBA + 6% sugar, vii) MS + 1 mg/I IBA + 7% sugar and viii) MS + 1 mg/I IBA + 8% sugar. All these operations were carried out under aseptic conditions and cultures were incubated at 28 ± 2^{o}C with 16 hours photoperiod. Rooted plantlets were acclimatized and transplanted to field. Each treatment was repeated in triplicate with 50 explants per treatment.

ii. Field study

Regenerated plantlets were transplanted in the field after hardening. The experiment was laid out in RCB design with 4 replications and the plot size was 8 x 10m, with row-to-row distance of one meter. The sowing was done in the month of September 2004 and normal agronomic practices were followed throughout the growth period.

iii. Molecular studies

a. DNA extraction: DNA was extracted from fresh leaves of sugarcane somaclones using DNA isolation Kit (Gentra system, Minnesota, USA.). Two hundred mg of fresh leaves were ground in liquid nitrogen. Three ml of the cell lysis solution (Tris [hydroxymethyl] aminomethane, Ethylenediaminetetra acetic acid and Sodiumdodecyl sulfate) was added with leaf sample in a 15 ml centrifuge tube and incubated at 65°C for 60 minutes. RNase solution (15 µl) was then added to the cell lysate and incubated at 37°C for 30 minutes. Protein precipitation solution (GENTRA Kit, Minnesota, USA) was added and vortexed for 20 seconds and the tubes were placed on ice for 30 minutes. The mixture was centrifuged at 2000 x g for 10 minutes. Supernatant containing DNA was poured in the separate 15ml centrifuge tube and DNA was precipitated by centrifuging at 2000 x g with 3 ml of isopropanol absolute. Ethanol (70%) was used to wash the pellet and the DNA samples were then hydrated with Tris EDTA buffer. DNA was quantified on spectrophotometer (BIOMATE 3).

b. DNA amplification: Twenty-four primers from Gene Link (NewYork, U.S.A), each ten bases in length, were used to amplify the DNA (Table 1). PCR reaction was carried out in 25µl reaction mixture containing 13ng of template (genomic DNA), 2.5mM $MgCl_2$ (Eppendorf, Hamburg, Germany), 0.33mM of each dNTPs (Eppendorf, Hamburg, Germany), 2.5 unit of Taq polymerase (Eppendorf, Hamburg, Germany) and 1µM of primer in a 1x PCR reaction buffer (Eppendorf, Hamburg, Germany). The amplification reaction was performed in the Eppendorf Master cycler with an initial denaturation for 5 min at 94°C, then 32 cycles:1 min denaturation at 94°C; 1 min annealing at 52°C; 2min extension at 72°C. Final extension was carried out at 72°C for 10 min. Amplified products were analyzed through electrophoresis on 1.5% agrose gel containing 0.5X TBE (Tris Borate EDTA) at 72 Volts for 2 hours, the gel contained 0.5µg/ml Ethidium bromide to stain the DNA and photograph was taken under UV light using gel documentation system(Vilber Lourmat, France).

iv. Data analysis

Plantlets developed through callus culture method were grown in the field and observations recorded for nine important agronomic characters viz., plant height (cm), plant girth (cm), number of stalks per stool, weight per stool (kg), sucrose %, commercial cane sugar (CCS)%, fiber %, cane yield (t/ha) and sugar yield (t/ha). Three stools were randomly taken from each plot to determine sugar contents according to sugarcane laboratory Manual for Queensland Sugar Mills (Anon., 1970) while three rows from each plot were harvested to record yield data. The variants were assessed for genetic parameters viz., coefficient of variability, heritability percentage in broad sense and genetic advance at 2%, 5%, 10%, 20% and 30% selection pressure (Kang *et al.,* 1983). The mean and variance were computed from each treatment. Data on one plant crop and two ratoon crop was computed on above mentioned parameters. Data were analysed following Steel & Torrie (1980).

Molecular data was scored as presence of band as (1) and absence of band as (0) from RAPD of amplification profile. Coefficient of similarity among cultivars was calculated according to Nei & Li (1979).

Table 1. **Sequence of the primers.**

Primer	Sequence	Mono-morphic DNA fragments	Poly-morphic DNA fragment
A-02	TGCCGAGCTG	2	4
A-03	AGTCAGCCAC	0	1
A-04	AATCGGGCTG	0	3
A-05	AGGGGTCTTG	….	….
A-06	GGTCCTGAC	….	….
A-07	GAAACGGTG	….	….
A-08	GTGACGTAGG	2	2
A-09	GGGTAACGCC	1	0
A-10	GTGATCGCAG	2	0
A-11	CAATCGCCGT	5	0
A-12	TCGGCGATAG	….	….
A-13	CAGCACCCAC	5	0
A-14	TCTGTGCTGG	….	….
A-15	TTCCGAACCC	3	0
A-16	AGCCAGCGAA	0	5
A-17	GACCGCTTGT	3	0
A-18	AGGTGACCGT	4	0
A-19	CAAACGTCGG	3	0
A-20	GTTGCGATCC	0	6
B-01	GTTTCGCTCC	….	….
B-02	TGATCCCTGG	….	….
B-03	CATCCCCCTG	….	….
B-04	GGACTGGAGT	….	….
B-05	TGCGCCCTTC	1	0

Results and Discussion

i. *In vitro* culture

a. Callus induction: Best callus induction and proliferation was observed on medium containing 4mg/I 2, 4-D and 4mg/I picloram. Based on morphological appearance, two types of calli were observed: (i) type A-yellowish white, compact, dry and nodular (Fig. 1) and (ii) type B- whitish globular, non-compact and wet (Fig. 2). Such type of calli has also been reported by Khan *et al.,* (1998) and Khatri *et al.,* 2002. Best callus induction and proliferation was observed on medium containing 4mg/I 2,4-D and 4mg/I picloram (Fig. 3). Picloram in 2mg/l also yielded good callus (Fig. 5). NIA-98 yielded the maximum callus followed by BL4 while NIA-2004 produced the minimum (Table 2). No callus induction was observed in indole acetic acid. Dicamba yielded very few calli in NIA-98 and BL4 but on second sub-culture the calli were turned into non-regenerable callus (Fig. 4). Similar trend was observed in callus proliferation on sub-culture. Callus weight reduced in BL4, because of high percentage of type B callus. According to Orton (1980), the type B callus of *Hordeum vulgare* has twice intrinsic growth rate as compared to type A, but in our study, it was observed that type B callus of sugarcane did not exhibit the same attributes, rather its growth substantially decreased. Explants of clones NIA-2004 yielded type A callus on dicamba, but on subculture it got converted into type B. Aging of the medium affected morphological status of callus in NIA-2004, whereas calli of NIA-98 and BL4 were converted into somatic embryos.

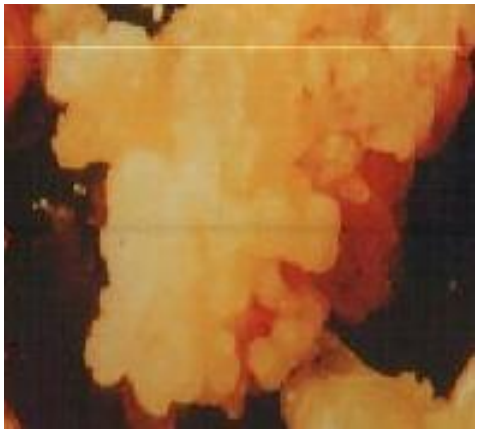

Fig. 1.Type A callus.

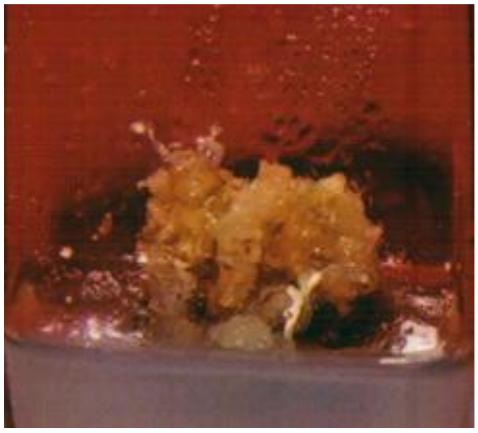

Fig. 2. Type B callus.

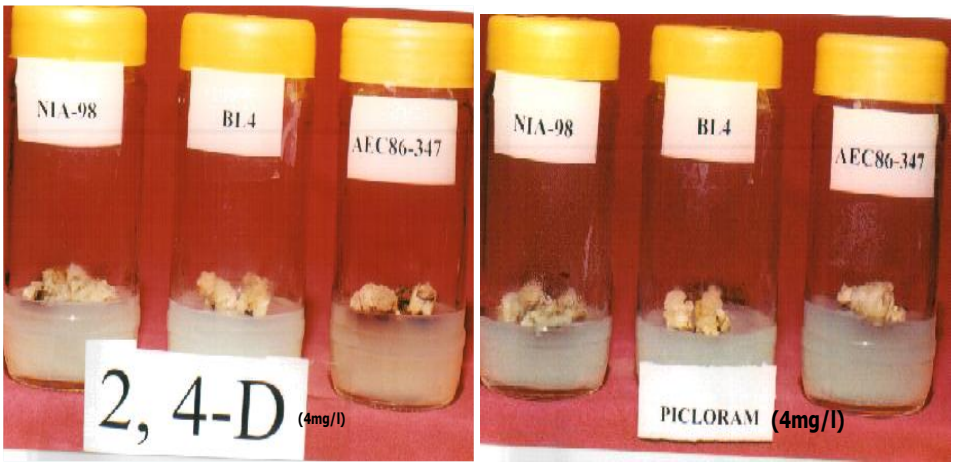

Fig. 3. Effect of 2,4-D and Picloram on callus induction on three different genotypes.

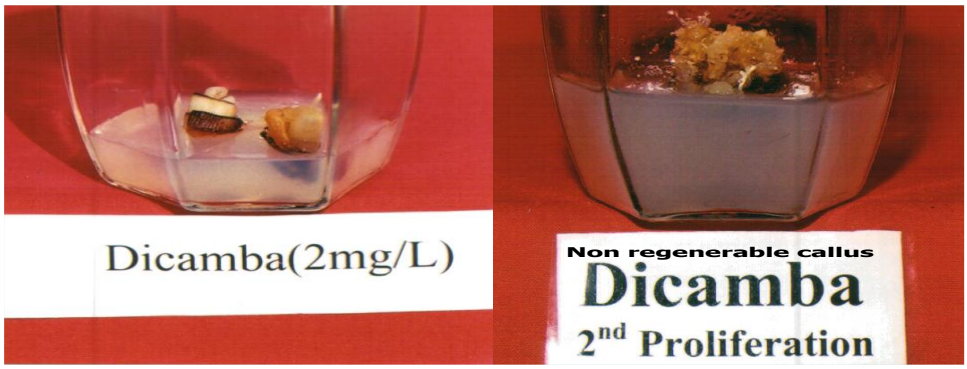

Fig. 4. Effect of Dicamba on callus induction.

Table 2. Effect of auxins concentration on callogenesis of different genotypes/clones.

Auxins Conc./Genotypes	Callus (g)	Proliferation of callus (g)
	NIA-98	
2mg/l 2,4-D (M1)	1.56c	1.83d
4mg/l 2,4-D (M2)	3.56b	2.83b
2mg/l Picloram (M3)	3.68b	2.90b
4mg/l Picloram (M4)	4.68a	3.61a
2mg/l Dicamba (M5)	0.78d	0.53e
4mg/l Dicamba (M6)	0.94d	2.2c
2mg/l IAA	--	--
4mg/l IAA	--	--
	BL4	
2mg/l 2,4-D (M1)	1.32c	1.20c
4mg/l 2,4-D (M2)	3.32b	2.24b
2mg/l Picloram (M3)	3.10b	2.25b
4mg/l Picloram (M4)	3.98a	2.98a
2mg/l Dicamba (M5)	0.70d	0.25d
4mg/l Dicamba (M6)	0.88d	0.37d
2mg/l IAA	--	--
4mg/l IAA	--	--
	NIA-2004	
2mg/l 2,4-D (M1)	0.93c	0.34c
4mg/l 2,4-D (M2)	1.93a	0.80a
2mg/l Picloram (M3)	1.55b	0.56b
4mg/l Picloram (M4)	1.85a	0.88a
2mg/l Dicamba (M5)	0.24e	0.35c
4mg/l Dicamba (M6)	0.58d	0.86a
2mg/l IAA	--	--
4mg/l IAA	--	--

DMR test (0.05):Means followed by the same letters are not significantly different from each other 2,4-D= 2,4 dichlorophenoxy acetic acid, IAA= Indol acetic acid

b. Regeneration: Regeneration started with the appearance of green dots on callus within a week on regeneration medium and generally produced normal stem and leaves (Fig. 6). NIA-98, yielded maximum plantlets on callus derived from picloram followed by 2, 4-D and minimum plantlet regeneration was recorded on callus derived from dicamba (Table 3). Similar trend of regeneration was observed in all the genotypes. Whereas, minimum plantlets was produced by NIA-2004 in all the calli as compared to other genotypes. Regeneration of albino and viridis plantlets exhibited the appearance of chlorophyll mutations in *In-vitro* plantlets (Fig. 7).

Regeneration potential was specific and genotype dependent phenomenon (Maretzki & Nickell, 1973; Maretzki, 1987). It was also observed that callus derived from different auxins showed different regeneration potential. Callus induction, proliferation and regeneration potential in sugarcane exhibited synchrony to each other (Khan *et al.*, 1999). However, regeneration was low as compared to its callus production in BL4. This might possibly be due to the conversion of regenerable callus type A to non-regenerable callus type B on sub-culturing of callus.

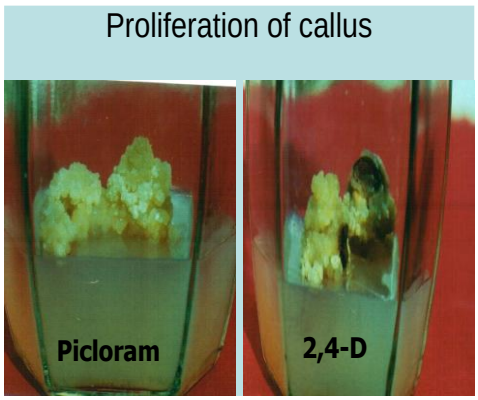

Fig. 5. Effect of picloram and 2,4-D.

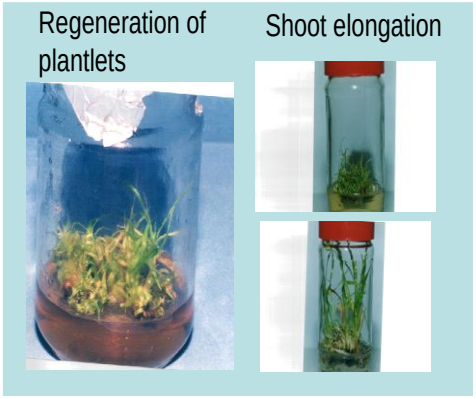

Fig. 6. Shoot regeneration and elongation.

Table 3. Response of genotype on regeneration and frequency of chlorophyll mutants.

Auxins concentration	NIA-98		BL4		NIA-2004	
	GP	CM	GP	CM	GP	CM
2mg/l 2,4-D	96d	5	90c	4	53c	9
4mg/l 2,4-D	112c	16	107bc	14	66b	21
2mg/l Picloram	138b	4	120b	3	84a	5
4mg/l Picloram	159a	8	150a	8	88a	10
2mg/l Dicamba	36f	2	34d	2	22d	3
4mg/l Dicamba	54e	4	41d	6	31d	5

DMR test (0.05): Means followed by the same letters are not significantly different from each other
GP= Green plants, CM= Chlorophyll mutants

Table 4. Effect of medium composition on root induction of sugarcane.

Medium	Root induction
MS medium	-
½ MS medium	-
MS + 1 mg/I IBA + 3% sugar	+
MS + 1 mg/I IBA + 4% sugar	+
MS + 1 mg/I IBA + 5% sugar	+
MS + 1 mg/I IBA + 6% sugar	+++
MS + 1 mg/I IBA + 7% sugar	++
MS + 1 mg/I IBA + 8% sugar	++

-, No root, +, weak root, ++, good rooting, +++, excellent rooting

Chlorophyll deficiency or albinism is a standard marker in plant cytoplasmic genetics. Its stability is consistent with mutations in the plastid genome because nuclear mutations induces plastid ribosome deficiency (Smulders, 2005). A chlorophyll deficient phenotype can also result of recessive mutations such as *iojap* in maize and *albostrians* in barley (Han & Ullrich, 1993). Maximum numbers of chlorophyll mutants were observed in callus derived from 2,4-D. and minimum was recorded in calli derived from dicamba. The highest percentage of chlorophyll mutants was recorded in NIA-2004 and the lowest in NIA-98 (Table 3). The presence of chlorophyll deficient plantlets confirmed the

induction of genetic variability (Shepard *et al.*, 1980). Present study revealed that the calli derived from 2,4-D produced more genetic variability as compared to the calli of dicamba. Plants showing phenotypic variability after *In vitro* culture indicate true genetic changes (Orton, 1980; Liu & Chen, 1978a & b). However, Chaleff & Keil, (1982) and Callebaut *et al*., (1987), reported that some phenotypic variability was the result of physiological changes during *In vitro* conditions; hence such plantlets normally revert to their parent type in field conditions. Among the four auxins, picloram and 2,4-D showed best results for callusing. Maximum chlorophyll mutant frequency was observed when 2,4-D was applied in 4mg/l concentration (31.8%) in NIA-2004 (Table 3).

c. Rooting: Roots grow from the nodal primordial when the plantlets are well developed (Khan *et al.,* 1998). Eight different media were used for root induction (Table 4). Root induction was observed in the regeneration medium when plant hormones were exhausted, but more vigorous root development was achieved, when the plantlets were separated, the leaves were trimmed and plantlets were cultured on the root induction medium containing MS + 1mg/I IBA + 6% sugar (Fig. 8). It was observed that sucrose concentration affects the root induction in sugarcane (Thrope, 1995; Thrope & Boindi, 1984). At low concentration, size and number of roots were very low as compared to high concentration of sucrose. The plantlets with well developed shoots and roots were transferred to jiffy pots having sterilized perlite. After acclimatization the plantlets were first transferred to the earthen pots for hardening and afterwards transplanted in the field. These plantlets are being evaluated for desired agronomic traits (Fig. 9).

Field evaluation: The mean squares for all the characters under study revealed that all the phytohormone concentration were significantly different at 1% and 5% levels of probability for all nine characters in all three genotypes (Table 5). The data regarding the average performance of all the treatments is presented in Table 6. Stalks/stool (No), were significantly ($p \leq 0.05$) higher in M2 (8.76), followed by M1 (8.44), in the regenerants of BL4; in case of NIA-98, M4 produced maximum stalks per stool (8.97) followed by M3 (8.83) and M1 (8.16) and minimum was observed in M5 and M6 (4.50). Regenerants of M2 produces maximum stalks (8.94) in case of NIA-2004. Maximum plant height (cm) in case of BL4 was observed in M3 (318.7) and minimum in M5 (169.0), in NIA-98 regenerants of M4 and M2 (290.0) exhibited maximum plant height and significantly different from parent (265.0). Treatment M2 in case of NIA-2004 showed maximum plant height (316.90). Maximum cane thickness (cm) was observed in M3 (3.33), M2 (2.73) and M1 (2.76) plantlets of BL4, NIA-98 and NIA-2004, respectively. Primary stalk weight (kg) was significantly higher in M3 (1.07, 0.98) regenerants of BL4 and NIA-98 respectively, whereas, M2 (0.84) regenerants of NIA-2004 showed maximum weight. Minimum primary cane weight (kg) was observed in M6 (0.67), control (0.74) and M4 (0.69) in BL4, NIA-98 and NIA-2004, respectively. Almost non-significant differences were observed in case of NIA-2004 for this trait.

Maximum sucrose % and CCS % (19.07, 14.65) was noted in case of BL4 in M3 respectively, in case of NIA-98 in M2 (18.36, 14.34) respectively. In NIA-2004 no regenerants could surpass the control in sucrose% (18.64%) and CCS% (15.11%). Regenerants of M6 (13.87%) and M4 and M5 (12.91%) showed maximum value for fiber % in BL4 and NIA-98, respectively. Non-significant differences were observed among the treatments in case of NIA-2004 for this trait.

Fig. 7. Chlorophyll mutant (Albino and viridis).

Fig. 8. Extensive root induction alongwith polyphenol production.

Fig. 9. *In vitro* regenerated sugarcane plantlets in the field for evaluation.

Table 6. Pooled performance of important characteristics of sugarcane somaclones of developed through callus culture.

Traits/treatment	Control	M 1	M 2	M 3	M 4	M 5	M6
				BL4			
Stalk/stool (no.)	3.86c	8.44a	8.76a	8.40a	5.72b	4.26c	4.25c
Plant height (cm)	294.3b	299.7b	301.2b	318.7a	200.3c	169.0e	185.0d
Plant girth (cm)	3.167ab	3.10bc	3.17ab	3.33a	3.10bc	2.95c	3.10bc
Cane weight (kg)	0.89b	0.90bc	1.00ab	1.07a	0.90bc	0.78cd	0.64d
Cane yield (t/ ha)	169.8c	193.3b	197.9b	217.4a	157.2d	132.9e	130.3e
Sucrose %	17.19d	17.53c	17.55c	19.07a	18.05b	12.70f	14.19e
CCS %	13.12c	12.86c	13.21c	14.65a	14.06b	9.201d	9.636d
Fiber %	12.58e	12.87c	12.16g	12.43f	12.85d	13.37b	13.87a
Sugar yield (t/ha)	22.27c	24.86b	26.15b	31.84a	22.10c	12.22d	12.55d
				NIA-98			
Stalk/stool (no.)	6.16c	8.16ab	7.83b	8.83ab	8.97a	4.50d	4.50d
Plant height (cm)	265.0d	280.0b	290.0a	270.6c	290.0a	240.0e	240.0e
Plant girth (cm)	2.52c	2.52c	2.73a	2.62b	2.63ab	2.32d	2.33d
Cane weight (kg)	0.74c	0.88ab	0.87abc	0.98a	0.97a	0.80bc	0.77bc
Cane yield (t/ ha)	164.6b	186.8a	195.4a	196.3a	207.2a	143.3c	139.3c
Sucrose %	17.14b	15.35d	18.36a	17.09b	17.16b	16.67c	15.35d
CCS %	13.57b	11.84e	14.34a	12.98c	13.07c	12.75d	12.55d
Fiber %	12.83a	12.66b	12.13c	12.71b	12.91a	12.91a	12.90a
Sugar yield (t/ha)	22.31b	22.11 b	27.99a	25.48a	27.08a	17.94c	17.76c
				NIA2004			
Stalk/stool (no.)	3.67d	7.00b	8.94a	5.91c	4.00d	4.00d	4.00d
Plant height (cm)	283.8b	280.4bc	316.9a	258.2d	271.2c	257.3d	254.3d
Plant girth (cm)	2.57bc	2.76a	2.67ab	2.68ab	2.43d	2.45cd	2.46cd
Cane weight (kg)	0.84a	0.78ab	0.84a	0.76ab	0.69b	0.70ab	0.76ab
Cane yield (t/ ha)	155.1b	159.9b	202.1a	153.6b	137.8c	134.9c	135.6c
Sucrose %	18.64a	17.92d	18.32b	18.19bc	18.09cd	18.01cd	18.00cd
CCS %	15.11a	14.18b	14.29b	14.33b	13.13c	13.13c	12.93c
Fiber %	11.48a	12.27a	11.29a	11.47a	13.80a	12.76a	12.81a
Sugar yield (t/ha)	23.44b	22.65b	28.86a	21.99b	18.08c	17.71c	17.52c

DMR test (0.05): Means followed by the same letters are not significantly different from each other M1 = MS + 2mg/l 2,4-D, M2= MS + 4mg/l 2,4-D, M3= MS + 2mg/l Picloram, M4= MS + 4mg/l Picloram, M5= MS + 2mg/l Dicamba, M6= MS + 4mg/l Dicamba

Statistically significant higher cane yield (t/ha) was recorded in regenerants of M3 (217.4), M4 (207.2) and M2 (202.1) in BL4, NIA-98 and NIA-2004 respectively. Lowest cane yield was recorded in treatment M6 in BL4 and NIA-98, M5 in NIA-2004. Highest sugar yield (t/ha) was observed in regenerants of M3 (31.84), M2 (27.99) and M2 (28.86) in BL4, NIA-98 and NIA-2004 respectively. In case of NIA-98 regenerants of M2, M3 and M4 were at par with each other.

The average performance of all the treatments and their control (parents) for all the characters indicate that stalks/stool were significantly ($p \leq 0.05$) higher in regenerants obtained from high concentration of 2,4-D in BL4 and NIA-2004. In case of NIA-98 high concentration of picloram yielded similar results. Maximum plant height in case of BL4 was observed in the regenerants obtained from low concentration of picloram, in case of NIA-98 and NIA-2004, high concentration of 2,4-D gave similar results. Highest cane thickness, primary stalk weight (kg), sucrose %, CCS %, cane yield and sugar yield was observed in the regenerants obtained from low concentration of picloram in case of BL4. In case of NIA-98 primary stalk weight, cane yield and sugar yield were higher in the regenerants obtained from low concentration of 2,4-D while sucrose % and CCS % were

higher in the regenerants obtained from high concentration of 2,4-D. In case of NIA-2004, primary stalk weight, cane yield and sugar yield were higher in the regenerants obtained from high concentration of 2,4-D while sucrose % and CCS % were higher in the control.

Expected genetic advance under selection with varying selection intensities (2, 5, 10, 20 and 30%) are shown in (Table 7). For any given trait, genetic advance at 2% selection intensity was about 2 times greater than that at 30% selection intensity and intermediate at 10% selection intensity. The magnitude of genetic advance from selection for a character in population determines; the total variation in the population, heritability of the character concerned and selection pressure. In vegetatively propagated crop like sugarcane where heritability is near 100%, little progress can be made by selection unless considerable genetic variability is present. In other words, the magnitudes of genetic advance might be equal by selecting in a population that exhibited low heritability and high genetic variability, and in one with high heritability and low genetic variability. Therefore, estimates of heritability bear a direct relation to the response that can be expected from artificial selection.

Estimates of variance components (σ^2 G x Y/y and σ^2 G x R/r) and broad sense heritability (H value in percentage) for the traits studied are given in (Table 8). Heritability estimates based on three replications in each of plant cane, and first and second ratoon crops were relatively high for all the traits. This is not surprising since the soil type and other environmental conditions were uniform in the three replications for each crop year. Therefore, heritability estimates based on any crop year would be expected to be high also. A comparison of σ^2 G x Y and σ^2 G x R components indicated that σ^2 G x R component was larger than σ^2 G x Y in most of the traits, which indicated that this trait was less stable and σ^2G x R relates to performance within the same year. The σ^2 G x Y component was larger than σ^2 G x R for sucrose % and CCS %, this is understandable since it is commonly observed that a ratoon cane crop has higher sucrose and CCS values as compared to plant crop.

RAPD studies: Genomic DNAs of the fifteen arbitrary primers produced multiple fragments (Fig. 10). The total number of scorable bands was 52, out of which 21 (40.38%) were polymorphic and only 31 (59.62%) were monomorphic. The number of fragments produced by various primers ranged from 1-5 with an average of 2.16 fragments per primer. The level of polymorphism varied with different primers. Maximum 6 bands were amplified with primer A-20 while minimum one band was amplified with primer A-03, A-09 and B-05. The size of fragments ranged from 200 bp – 3.05 kbp. The amplification of monomorphic loci is depicting sharing of common blood among the genotypes (Asif *et al*., 2005).

Genetic similarity: The similarity coefficients reflected the genetic relationship between the somaclclones (Sc) and maximum similarity was observed between Sc7 and Sc8 (55%) and the minimum similarity was recorded between Sc2 and Sc4 as well as between Sc2 and Sc9 (17%) (Table 9). The dendrogram constructed on the basis of the similarity matrix showed that the somaclones could be divided into two groups. Four somaclones viz., Sc1, Sc2, Sc3 and Sc4 were genetically close to each other and hence forming a group. Another distinct group is formed among parent, Sc5, Sc6, Sc7 and Sc8. However Sc9 was found to be distinct from the rest two groups (Fig. 11).

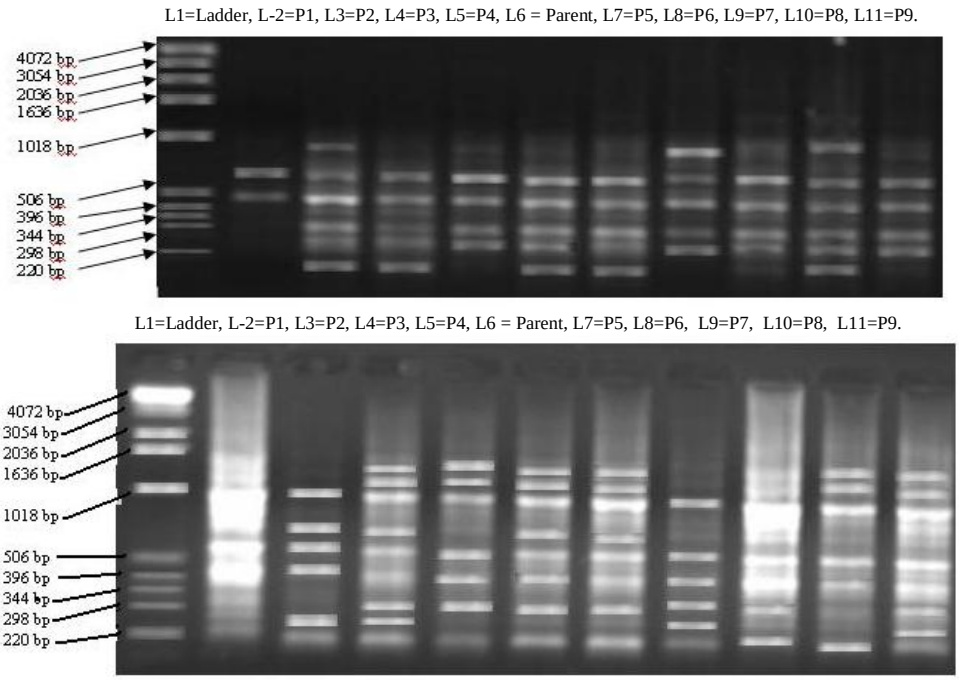

Fig. 10. Genetic polymorphism in somaclones regenerated through callus culture at primer A-16 and A-18.

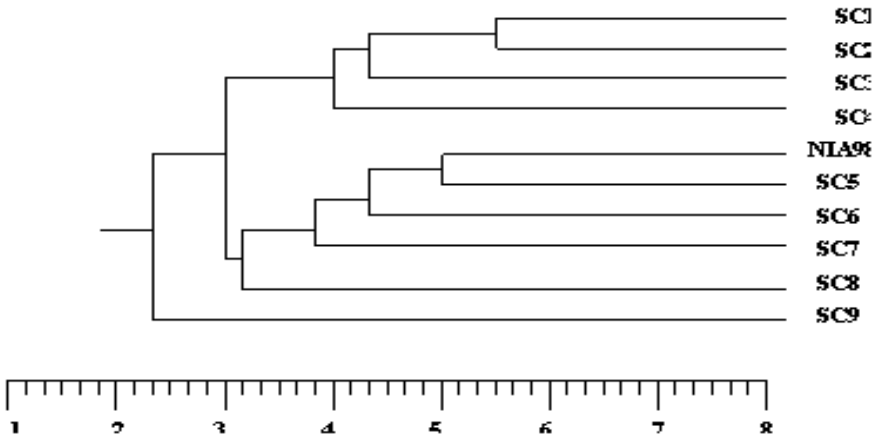

Fig. 11. Dendrogram showing genetic similarity between 9 somaclones of NIA-98 developed through callus culture

The data obtained in this experiment confirmed the efficiency of the RAPD technique for determination and estimation of genetic distances and relatedness among different sugarcane somaclones. The RAPD analysis has been found a valuable DNA marker system to evaluate genetic diversity. The information about genetic similarity will be helpful to avoid any possibility of elite germplasm becoming genetically uniform. RAPD analysis may be very useful in breeding for rapid and early identification of most

diverse clones in large population of perennial crop like sugarcane. Keeping in view the useful information about the close genetic relationship, it is suggested that goal oriented breeding programs with the help of RAPD technology will be helpful to produce distinct genotypes with diverse genetic background and improve crop productivity (Rout *et al.*, 1998; Zucchi *et al.*, 2002).

Table 7. Expected genetic advance under varying selection intensities.

Traits	Expected genetic advance				
	Selection intensity %				
	2%	5%	10%	20%	30%
			BL4		
Stalk/stool (no.)	15.27	13.00	11.10	8.83	7.32
Plant height (cm)	439.39	374.03	319.56	254.19	210.62
Plant girth (cm)	0.75	0.64	0.55	0.43	0.36
Cane weight (kg)	0.96	0.81	0.69	0.55	0.46
Cane yield (t/ ha)	242.84	206.72	176.61	140.48	116.40
Sucrose %	16.60	14.13	12.07	9.60	7.96
CCS %	15.42	13.13	11.21	8.92	7.39
Fiber %	4.22	3.59	3.07	2.44	2.02
Sugar yield (t/ha)	51.98	44.24	37.80	30.07	24.91
			NIA-98		
Stalk/stool (no.)	14.14	12.03	10.28	8.18	6.77
Plant height (cm)	153.88	130.99	111.91	89.02	73.76
Plant girth (cm)	1.11	0.94	0.80	0.64	0.53
Cane weight (kg)	0.71	0.60	0.51	0.41	0.34
Cane yield (t/ ha)	197.57	168.18	143.68	114.29	94.70
Sucrose %	6.54	5.57	4.76	3.78	3.13
CCS %	5.80	4.94	4.22	3.35	2.78
Fiber %	2.03	1.73	1.48	1.17	0.97
Sugar yield (t/ha)	30.02	25.55	21.83	17.36	14.39
			NIA-2004		
Stalk/stool (no.)	14.60	12.42	10.61	8.44	6.99
Plant height (cm)	162.08	137.97	117.88	93.76	77.69
Plant girth (cm)	0.93	0.79	0.68	0.54	0.44
Cane weight (kg)	0.47	0.40	0.34	0.27	0.22
Cane yield (t/ ha)	171.11	145.65	124.44	98.99	82.02
Sucrose %	2.06	1.75	1.49	1.19	0.98
CCS %	6.03	5.13	4.39	3.49	2.89
Fiber %	6.68	5.69	4.86	3.86	3.20
Sugar yield (t/ha)	29.90	25.45	21.74	17.30	14.33

Plants obtained through *In vitro* cultures gave phenotypic variability, which was mainly due to true genetic changes. Some physiological changes also accomplished during *In vitro* conditions; hence some plantlets were reverted to their parent type in field conditions. Maximum chlorophyll mutant frequency was observed when 2,4-D was applied in 4mg/l concentration expressing that 2,4-D induced more genetic variability as compared to other auxins under study. Present investigations suggested that the phytohormone concentrations that were applied to regenerate sugarcane somaclones dicamba exhibited negative impact on the agronomic traits under study. Whereas, 4mg/l

of 2,4-D and 2mg/l picloram showed stimulating and enhancing effect on plant height and cane yield (kg/plot). The phytohormone treatments also gave high heritability percentage and genetic advance values reflecting the scope of phytohormone treatment can yield better clones with good agronomic traits. The genetic variability created in sugarcane through callus culture (somaclonal variation) was efficiently screened with molecular marker technique (RAPD). As RAPD is a dominant marker therefore, minor changes which may be caused by the recessive gene could not be identified during the screening processes.

Present investigations suggested that the phytohormone treatments were effective in inducing genetic variability; however the picloram and 2,4-D showed stimulating and enhancing effect on plant height and cane yield (kg/plot). This was also confirmed by the high heritability percentage and genetic advance. For increasing per hectare sugar yield it is necessary to consider cane yield and sugar recovery together (Hashmi 1995), and the genotypes being selected accordingly. Our studies showed that there were possibilities of obtaining desirable variants at 2mg/l concentration of 2, 4-D.

Table 8. Estimates of genotypic x year, genotypic x replication variances and broad-sense heritability for various traits.

Traits	σG x Y/y	σG x R/r	H%
		BL4	
Stalk/stool (no.)	0.021	0.200	99.98
Plant height (cm)	37.46	0.0030	99.99
Plant girth (cm)	0.0003	0.007	99.88
Cane weight (kg)	0.001	0.004	99.78
Cane yield (t/ ha)	6.678	51.027	99.98
Sucrose %	0.008	0.004	99.99
CCS %	0.180	0.032	99.98
Fiber %	0.0003	0.0003	99.99
Sugar yield (t/ha)	0.16	1.30	99.99
		NIA-98	
Stalk/stool (no.)	0.0003	0.4320	99.99
Plant height (cm)	0.13	0.13	99.99
Plant girth (cm)	0.001	0.001	99.84
Cane weight (kg)	0.001	0.005	99.61
Cane yield (t/ ha)	14.686	32.146	99.71
Sucrose %	0.090	0.019	99.91
CCS %	0.008	0.004	99.99
Fiber %	0.0003	0.0070	99.98
Sugar yield (t/ha)	0.54	0.63	99.79
		NIA-2004	
Stalk/stool (no.)	0.004	0.070	99.96
Plant height (cm)	79.09	79.92	99.64
Plant girth (cm)	0.0003	0.0006	93.18
Cane weight (kg)	0.002	0.003	96.74
Cane yield (t/ ha)	10.357	19.96	99.80
Sucrose %	0.170	0.014	98.45
CCS %	0.210	0.027	99.61
Fiber %	0.005	0.023	99.82
Sugar yield (t/ha)	0.520	0.290	99.85

Table 9. **Similarity coefficient among the sugarcane somaclones calculated according to Nei & Li.**

	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11
L2*	1									
L3	0.36	1								
L4	0.42	0.24	1							
L5	0.31	0.17	0.38	1						
L6	0.37	0.24	0.47	0.38	1					
L7	0.38	0.24	0.39	0.26	0.49	1				
L8	0.35	0.31	0.31	0.26	0.33	0.26	1			
L9	0.37	0.19	0.41	0.30	0.38	0.36	0.41	1		
L10	0.35	0.30	0.36	0.28	0.40	0.36	0.45	0.55	1	
L11	0.28	0.17	0.25	0.23	0.31	0.29	0.36	0.39	0.45	1

*L-2=Sc1, L3=Sc2, L4= Sc3, L5=Sc4, L6=Parent, L7=Sc5, L8= SC6, L9=Sc7, L10=Sc8, L11= Sc9.

References

Ahloowalia, B.S. 1995. *In vitro* mutagenesis for the improvement of vegetatively propagated plants. In: *Extended Synopsis FAO/IAEA Int. Symp. on Induced Mutation and Molecular Techn. for Crop Improvement, IAEA-SM*, 340: 203.

Anonymous, 1970. *Sugarcane Laboratory Manual for Queensland Sugar Mills, Bureau of Sugar Experiemntal Station, Queensland 2, 9th Edition*.

Asif, M., M. Rahman and Y. Zafar. 2005. DNA fingerprinting studies of some wheat (*Triticum aestivum* L.) genotypes using random amplified polymorphic DNA (RAPD) analysis. *Pak. J. Bot*., 37(2): 271-277.

Barrett, C., F. Lefort and G.C. Douglas. 1997. Genetic characterization of oak seedlings, epicormic, crown and micropropagated shoots from mature tree by RAPD and microsatellite PCR. *Sci. Hortic*., 70: 319-330.

Callebaut, A., J.C. Motte and W. De Cat. 1987. Substrate utilization by embryogenic and non-embryogenic cell suspension cultures of *Cucumis sativus* L. *J Plant Physiol*., 127: 271-280.

Chaleff, R.S. and Keil, R.L. 1982. Origins of variability among cultured cells and regenerated plants of *N. tabacum*. In: *Variability in Plants Regenerated from Tissue Culture*, (Eds.): E.D. Earle and Y. Demarly. Praeger Publication, pp. 175-187.

Cloutier, S. and B.S. Landry. 1994. Molecular markers applied to plant tissue culture. *In Vitro Cell. Develop. Biol*., 30: 32-39.

Cox, M.C., T.A. McRae, J.K. Bull and D.M. Hogarth. 1996. Family selection improves the efficiency and effectiveness of a sugarcane improvement program. In: *Sugarcane: research towards efficient and sustainable production*, (Eds.): D.M. Hogarth, J.A. Campbell and A.L. Garside. pp. 287-290.

Ford-Llod, B.V., A. Sabir, H.J. Newbury, C. Todd and J. Catty. 1992. Determination of genetic stability using isozymes and RFLPs in beet plants regenerated *In vitro*. *Theor. Appl. Genet*., 84: 113-117.

Han, F. and S.E. Ullrich. 1993. Mapping of quantitative trait loci associated with malting quality in barley. *Barley Genetics Newsletter*, 23: 84-97.

Hashmi, S.A. 1995. It is time to take stock: Sugar Technologist Convention. The DAWN, Karachi, Wednesday, August 30, pp: 8.

Heinz, D.J. and G.W.P. Mee. 1969. Plant differentiation from callus tissue of *Saccharum* species. *Crop Sci*., 9: 346-348.

Kang, M.S., J.D. Miller and P.Y.P. Tai. 1983. Genetic and phenotypic path analyses and heritability in sugarcane. *Crop Sci*., 23: 643-647.

Khan, I.A., A. Khatri, M. Ahemad, S.H. Siddiqui, G.S. Nizamani and S. Raza. 2004. Performance of promising clone for yield and quality traits in different ecological zones of Sindh. *Pak. J. Bot*., 36(1): 83-92.

Khan, I.A., M.D. Gaj and M. Maluszynski. 1999. *In vitro* mutagenesis in sugarcane callus culture. *Mutation Breeding Newsletter*, 44 pp 19-20.

Khan, I.A., A. Khatri, M. Ahmad, S.H. Siddiqui, G.S. Nizamani, M.H. Khanzada, N.A. Dahar and R. Khan. 1998. *In vitro* mutagenesis in sugarcane. *Pak. J. Bot*., 30(2): 253-261.

Khatri, A, I.A. Khan, M.A. Javed, M.A. Siddiqui, M.K.R. Khan, M.H. Khanzada, N.A. Dahar and R. Khan. 2002. Studies on callusing and regeneration potential of indigenous and exotic sugarcane clones. *Asian J. Plant Sci*., 1(1): 41-43.

Kimbeng, C.A., T.A. McRae and M. Cox. 2001. Optimizing early generation selection in sugarcane breeding. *Proc. Int. Soc. Sugarcane Technol*., 24: 488-494.

Krisnamurthi, M. and J. Tlaskal. 1974. Fiji disease resistant. *Saccharum officinarum* L. var. Pindar subclone from tissue culture. *Proc. ISSCT*, 15:130-137.

Larkin, P.J. and W.R. Scowcroft. 1981. Somaclonal variation: A novel source of variability from cell cultures for plant improvement. *Theor. Appl. Genet*., 60: 197-214.

Larkin, P.J. and W.R. Scowroft. 1983. Somaclonal variation and eyespot toxin tolerance in sugarcane. *Plant Cell Tiss. Org. Cult*., 2: 111-121.

Liu, M.C. 1984. Sugarcane. In: *Hand book of Plant Cell Culture*. (Eds.): W.R. Sharp, D.A. Evans, P.V. Ammirato and Y. Yamada. Macmillan Publishing Company, New York. pp. 572-605.

Liu, M.C. and W.H. Chen. 1976. Tissue and cell culture as aids to sugarcane breeding I. Creation of genetic variability through cell culture. *Euphytica*, 25: 393-403.

Liu, M.C. and W.H. Chen. 1978a. Tissue and cell culture as aids to sugarcane breeding II. Performance and yield potential of callus derived clones. *Euphytica*, 27: 272-282.

Liu, M.C. and W.H. Chen. 1978b. Significant improvement in sugarcane by using tissue culture methods. In: *Fourth Inter. Cong. Pl. Tiss. Cell Cult*., (Abstr.) p. 163. Univ. Calgary, Alberta, Canada.

Maretzki, A. 1987. Tissue culture: Its prospects and problems In: *Sugarcane Improvement through breeding*. (Ed.) D.J. Heinz. Elsevier Science Publisher B.V. pp. 343-384.

Maretzki, A. and L.G. Nickell. 1973. Formation of protoplasts from sugarcane cell suspensions and the regeneration of cell cultures from protoplasts. In: *Protoplastes et Fusion de Cellules Somatiques Vegetales. Colloq*. Int. C. N. R. S. 212: 51-63.

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

Nei, N. and W. Li. 1979. Mathematical model for studying genetical variation in term of restriction endonucleases. *Proc. Natl. Acad. Sci. USA*, 76: 5267-5273.

Orton, T.J. 1980. Chromosomal variability in tissue cultures and regenerated plants of *Hordeum. Theor. Appl. Genet*., 53: 101-112.

Peschke, V.M., R.L. Phillips and B.G. Gengenbach. 1991. Genetic and molecular analysis of tissue- culture-derived Ac elements. *Theor. Appl. Genet*., 82: 5222-5226.

Rani, V., A. Parida and S.N. Raina. 1995. Random amplified polymorphic DNA (RAPD) markers for genetic analysis in micropropagated plants of *Populus deltoids* Marsh. *Pl. Cell Rep*., 14: 459-462.

Rout, G.R., P. Das, S. Goel and S.N. Raina. 1998. Determination of genetic stability of micropropagated plants of ginger using random amplified polymorphic DNA (RAPD) markers. *Botan. Bull. Acad. Sin*., 39:23-27.

Shepard, J.F., D. Bidiney and E. Shahin. 1980. Potato protoplast in crop improvement. *Science*, 208: 17-24.

Shoyama, Y., X.X. Zhu, R. Nakai, S. Shiraishi and H. Kohda. 1997. Micropropagation of Panax notoginseng by somatic embryogenesis and RAPD analysis of regenerated plantlets. *Pl. Cell Rep*., 16: 450-453.

Siddiqui, S.H., A. Khatri, M.A. Javed, I.A. Khan and G.S. Nizamani. 1994. *In-vitro* culture: A source of genetic variability and an aid to sugarcane improvement. *Pak. J. Agric. Res*., 15(1): 127-133.

Smulders, M.J.M. 2005. Are there adequate methods for assessing somaclonal variation in tissue culture–propagated plants?. In: *COST 843 Final Conference / COST 843 and COST 851 Joint Meeting*, (Eds.): G. Libiakova, A. Gajdosova. Stara Lesna, Slovakia, June 28- July 3, pp 201-203.

Steel, R.G.D. and J.H. Torrie. 1980. *Principles and Procedures of Statistics*. McGraw-Hill Book, New York.

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

Thorpe, T.A. 1995. In vitro Embryogenesis in Plants. Current Plant Science and Biotechnology in Agriculture, Kluwer, Boston MA, Vol. 20.

Thrope, T.A. and S. Biondi. 1984. *Fiber and Wood (Conifers). Hand Book of Plant Cell Culture*. Vol. 2, Crop species. (Eds.): W.R. Shrap, D.A. Evans, P.V. Ammirato and Y. Yammada. Macmillan Publishing Company. New York, pp. 435-470.

Todoroviska, E., A. Trifonova, M. Petrova, Z. Vitonova and E. Marinova. 1997. Agronomic performance and molecular assessment of tissue culture derived barley lines. *Plant Breed*., 166: 511-517.

Zucchi, M. I., H. Arizono, V.A. Morais, M.H.P. Fungaro and M.L.C. Vieira. 2002. Genetic instability of sugarcane plants derived from Meristem cultures. *Genetics and Molecular Biology*, 25: 1:91-96.

(Received for publication 27 October 2007)