Pak. J. Bot., 41(2): 797-814, 2009.

DIRECT REGENERATION OF SUGARCANE PLANTLETS: A TOOL TO UNRAVEL GENETIC HETEROGENEITY

IMTIAZ AHMED KHAN[*], MUHAMMAD UMAR DAHOT[**], NIGHAT SEEMA, SHAFQAT YASMEEN, SAJIDA BIBI, GHULAM RAZA, ABDULLAH KHATRI AND MAZHAR H. NAQVI

[*]*Plant breeding and Genetics Division, Nuclear Institute of Agriculture, Tando Jam 70060, Sindh, Pakistan*
[**] *Institute of Biotechnology and Genetic Engineering University of Sindh, Jamshoro, Sindh, Pakistan*.

Abstract

A simple and efficient protocol for *In vitro* direct regeneration of shoot from immature leaf explants of sugarcane is reported. Three sugarcane clonal lines, viz., NIA-98, BL4 and NIA-2004 were studied for direct regeneration potential on different concentrations of plant growth regulators. Ten different media were used for direct regeneration studies. The best regeneration was observed on medium containing 4 mg/l IAA+ 1.0mg/lKin + 0.2 mg/l 2,4-D followed by media containing 4 mg/l IAA+ 0.5 mg/l Kin + 0.5 mg/l 2,4-D. The maximum rate of plantlet regeneration was recorded in clone NIA-98 while the minimum was in NIA-2004. Four different shoot elongation medium were used and best elongation rate were observed on medium containing 1.5 mg/l Kin + 1 mg/l NAA. Best root induction was observed when shoots were transferred on to media containing 1mg/l BAP and 60gm /l commercial sugar. The regenerated plants were transferred to jiffy pots and after weaning into the field for evaluation. Development of chlorophyll mutants confirms that direct regeneration cannot maintain genetic fidelity but could be considered as a good source of exploring existing aneuploidy. Agronomic data and SSR study also confirms the variation in the population

Introduction

Sugarcane an important industrial crop of tropical and subtropical regions, is cultivated on 20 million hectares in more than 90 countries. It accounts for around 60% of the world's sugar (Khan *et al*., 2004). It is also an important cash crop in many developing/developed countries, because of its high trade value (Naz, 2003). The importance of sugarcane has increased in recent years because cane is an important industrial raw material for sugar and allied industries producing alcohol, acetic acid, butanol, paper, plywood, industrial enzymes and animal feed (Arencibia, 1998). The cane and sugar yield of Pakistan is very low as compared to other countries of the world (Anon., 2007) and genetic improvement through conventional hybridization in Pakistan is hampered due to the intricate flowering nature of sugarcane. Moreover, the perennial and highly heterozygous nature of the plant coupled with a prolonged juvenile period limits the speed of improvement using traditional methods (Khan *et al*., 2005). Besides, sugarcane is a highly polyploid and aneuploid crop (Jannoo, *et al.,* 1999). *In vitro* plant regeneration is often the most important step for successful implementation of various biotechnological strategies used for plant breeding. In sugarcane, there are only few reports dealing with direct regeneration from different explants (Gill *et al*., 2006) while there are many available on regeneration and multiplication through organogenesis *via* callus formation. In these studies, embryogenic calluses were induced in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D) or picloram and regeneration was obtained either by reducing the concentration of the auxin or deleting it from the medium (Karp, 1991; Liu, 1993; Guiderdoni *et al*., 1995; Mozdhorst *et al*., 1997; Snyman, 2000; Sahasrabudhe

et al., 2000; Eudes *et al*., 2003) or by media supplementation with thidiazuron (Gallo-Meagher *et al*., 2000).

Larkin & Scowroft (1983) defined two classes of somaclonal variation: based on genetic or epigenetic changes. Genetic changes include polyploidy, aneuploidy, mutation (point) and new insertions of (retro) transposons (Smulders, 2005). Darke *et al*., (1998) stated that in plants, nuclear mutations can be directly estimated with the frequency of chlorophyll mutants in the plant population obtained through mutagenic treatments. Plants obtained through *In vitro* cultures can show phenotypic variability which is due to true genetic changes (Orton, 1980). Anbalogan *et al*., (2000) 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. Aneuploids may have lower or higher number of chromosome and plantlets regenerated from these cells could express different genetic behaviour. Direct regeneration method enables the exploitation of existing genetic heterogeneity present within cells in the form of different cytotypes. For an efficient application of the direct regeneration system, according to Lakshmanan *et al*., 2006 regeneration of plants directly from explants presents an effective strategy to avoid or substantially reduce somaclonal variation but he did not deny the chance of somaclone in population regenerated directly from explant.

Our primary objective was to develop a simple and reproducible protocol for shoot regeneration directly from the explant tissue. Sugarcane being a high ploidy and aneuploidy crop can we use the existing genetic variability (aneuploidy) present within plant cells? The present study showing that direct regeneration can be a good tool for the exploitation of existing aneuploidy as a source of genetic variability. Field data has been computed to confirm the genetic variability and RAPD / SSR study were also carried out to investigate the genetic variation in sugarcane plantlets derived from direct regeneration method by explanting immature leaves.

Materials and Methods

Three sugarcane clonal lines viz., NIA-98, BL4 and NIA-2004 were used through out this study. Fresh plant material (immature leaf segments) was collected from 6 month-old field-grown plants (Experimental Farm of NIA, Tando Jam, Pakistan). The field-collected material was washed several times with tap water with a few drops of liquid soap. Explants were taken closely from the apical meristametic region containing leaf primordia, explants of different thickness i.e., 2, 3, 4, 5 and 6mm, sterilized by standard procedure (Siddiqui *et al*., 1994) and cultured on Modified MS medium (Murashige and Skoog, 1962) containing different concentration of auxins and cytokinines. Ten different media formulations were used (Table 1). All the explants were kept in dark for 15, 20, 25 and 30 days and then transferred to light. All media were solidified with 0.8% Difco bacto agar. The regenerated shoots were scored for chlorophyll mutations.

Multiplication of shoots: Shoots of 2–3 cm derived from leaves were cultured on modified MS medium supplemented with different plant growth regulators (Table 2). The production of shoots from the base of the explant and also from the axils of leaves was taken into consideration for calculating the multiplication frequencies. The average number of shoots induced per explant was recorded after 30 days of culture.

Root induction from shoots: Healthy 6-7 cm long shoots were transferred to MS medium supplemented with different concentration of commercial sugar i.e., 2%, 4%, 6%, 8% and 1mg/l IBA for root induction. The duration for root induction, nature of roots induced and the frequency of root induction were recorded. For all the experiments on shoot induction, shoot multiplication and root induction from shoots, the cultures were kept at 25 ± 2^0C under a 16-h photoperiod with a photosynthetic photon flux density (PPFD) of 83.6 μE m^{-2} s^{-1} provided by white fluorescent tubes.

Acclimatization of regenerated plants: Regenerated plants bearing well developed roots were transferred to jiffy pots containing soil and organic manure (3:1) and kept in a screen house under shade for 15-20 days. In the first week of transfer, the plantlets were covered with polythene covers to maintain humidity. After 15- 20 days of acclimatization, the plantlets were transferred to earthen pots and planted out in field. The percentage survival was recorded after 4 weeks of transfer to field.

Field evaluation: The experimental layout was RCB design with 4 replications. The plot size was 8 x 10m, row-to-row distance one metre. The sowing was done in the month of September each year (2004, 2005 and 2006) and normal agronomic practices were followed through out the growth period. The data on plant height (cm), plant girth (cm), number of stalk per stool., weight per stool (kg), number of internodes, Internode length (cm), cane yield Kg/plot, TSS % and sugar yield kg/plot were recorded on one plant and two ratoon crop was computed. 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.

Molecular studies (SSR)

Plant material: Fresh young leaves of direct regenerants were collected from the field experiment at NIA Tandojam for isolation of the DNA using DNA isolation Kit (Gentra system, Minnesota, USA.).

PCR Reaction: Ten SSR Primers from CIRAD, France were used to amplify the DNA. PCR reaction was carried out in 10µl reaction mixture containing 2.5ng of template (Genomic DNA), 0.5mM MgCl, 0.2mM of each dNTPs, 0.1U of Taq polymerase, 0.08µM of Forward primer and 0.10µM of Reverse 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 minutes at 94ºC, then 35 cycles: 30 second denaturation at 94ºC; 30 second annealing at 55ºC; 1 minute extension at 72ºC. Final extension was carried out at 72ºC for 5 minutes. Amplified products were analyzed through electrophoresis on 3% agrose gel containing 0.5 X TBE (Tris Borate EDTA) at 100 volts for 3 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).

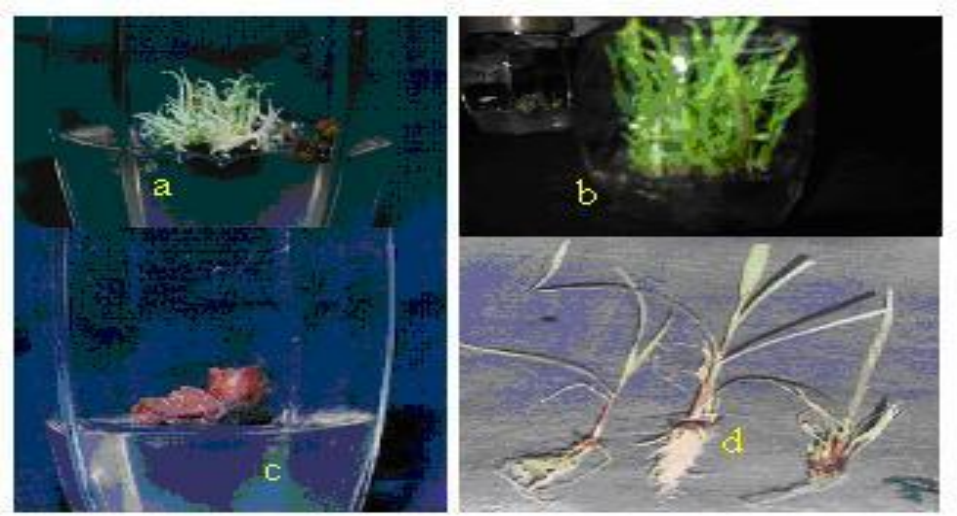

Fig. 1. a. Increased in the shoot regeneration with the increase concentration of Kinetin, b. Addition of 2, 4-D enhances the shoot regeneration, c. Swelling browning and subsequent death of the explant and d. Vigorous rooting with 1 mg/l IBA and 6% commercial sucrose.

Data analysis: Data was scored as presence of band as (1) and absence of bands as (0) from RAPD of amplification profile. Coefficient of similarity among cultivars was calculated according to Nei & Li (1979). Similarity coefficient was utilized to generate a dendrogram by means of Unweighted Pair Group Method of Arithmetic means (UPGMA).

Statistical analysis: Each treatment was repeated in triplicate with 50 explants per treatment. The appearances of green shoot from leaves were counted for calculating the shoot organogenesis from the leaf explants. The mean and standard deviations were computed from each treatment through DMR test (Steel & Torrie, 1980). 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.

Results

Plant regeneration via direct shoot organogenesis has been achieved from the immature leaf explants of all three cultivars. The size and thickness of explant remarkably influenced the response for direct organogenesis. The number of direct shoot organogenesis varied with concentration of phytohormones and it ranged from 16.50 to 78.78 (Table 1). It was observed that 25 days of dark incubation improved direct shoots induction. Immature leaf segments of 2-4 mm size showed swelling of the explant in the first week of culture (Fig. 1a). In the next two weeks, swellings of explant were visible at the cut end of explants. Shoot initiation was observed within four weeks of culture. The absence of any callus formation indicated that the process of shoot regeneration was direct. Initiation of direct regeneration was observed initially at the outer layer of explants. Regeneration started with the appearance of green dots on explants, which developed into green shoot with in 5 to 7 days. The number of shoots induced per explant (5.50-25.0) and the average length of shoots (0.82-3.52 cm) were higher in the presence of 4mg/l IAA + 0.2mg/l 2,4-D +0.5mg/l

kin in different cultivars. The frequency of shoot organogenesis, the number of shoots as well as the average length of shoots induced per explant was significantly less when IAA was used alone, it decreased with the increase in the concentration of 2,4-D and Kinetin in the medium, whereas, no shoot regeneration was observed when IAA was used along with BAP and TDZ. Significantly higher regeneration was observed on medium containing 4mg/l IAA+ 0.2mg/l 2,4-D + 0.5 mg/l Kin (Fig. 1a). The explants on media ii, iii, iv, v and vi initially showed slight swelling, but subsequently these structures turned brown resulting in the death of the explant (Fig. 1c). Addition of thidiazuron in medium along with IAA suppressed shoot regeneration (Table 1), whereas, direct regenerants that formed on medium containing 4mg/l IAA when supplemented with Kinetin (1.0 mg/l), 2,4-D (0.2 mg/l), showed vigorous shoot regeneration.

Shoot multiplication: Multiple shoots were induced from shoot tips differentiated from leaves at a significantly high number (101-227.75) on MS medium with 1mg/l NAA + 1.2mg/l Kinetin (Table 2) (Fig. 1b). The emergence of multiple shoots occurred in 9–10 days and the average length of shoots was 1.75–6.75 cm after 30 days of culture. Kinetin along with other growth regulators promoted shoot induction, whereas BAP showed less favourable effect (Table 2). The shoots produced on BAP containing medium exhibited stunted growth with development of dark green leaves. The shoot multiplication rate decreased with the subsequent subculture on the same medium but no such results were observed on medium containing kinetin. No significant difference was observed in shoot induction in all three clones. However, maximum shoots were observed in NIA-98 followed by BL4. Production of minimum shootlets was observed in NIA-2004 (Table 2).

Root induction from the shoots: When the plantlets reached 7-8 cm height, they were subjected to rooting. The frequency as well as the nature of roots induced from leaf regenerated shoots varied with the type and concentration of auxin used. Efficient root induction was observed on medium containing full strength MS salts +1mg/l IBA + 6% sucrose (Fig. 1d), in 12–14 days in all three genotypes, that enabled better survival upon transfer to soil. Higher and lower concentrations of sucrose decreased the frequency of root induction. Slender, thin roots were induced from shoots cultured on medium supplemented with low concentration of sucrose (4%) in all genotypes. Plantlets with well-developed root system were transferred in the field after 15 days of hardening. The regenerated plants of different cultivars survived with a higher frequency in autumn season than in summer season for all genotypes.

Chlorophyll mutants: Regeneration of albino and viridis plantlets suggested the appearance of chlorophyll mutation in direct regenerants. The frequencies of chlorophyll mutants were not significantly different in all the treatments for one given genotype. Clones NIA-98, BL4 and NIA-2004 gave rise to 6-17%, 16-19% and 24-27% chlorophyll mutants, respectively (Table 1).

Field evaluation: Mean square (analysis of variance) for all the nine characters under study are given in Table 3. The average performances of clones regenerated from different phyto-hormone treatments under study are presented in Table 4. The genetic parameters such as percent of coefficient of variability, heritability percentage in broad sense and genetic advance at 2%, 5%, 10, 20% and 30% selection pressure for different quantitative and qualitative characters are presented in Tables 5 and 6.

Table 4. Pooled performance of important characteristics of sugarcane somaclones of developed through direct regeneration method.

Traits/treatment	Control	M 1	M 2	M 3	M 4	M 5
			BL4			
Stalk/stool (no.)	3.60C	8.00A	8.66A	8.00A	5.50B	4.16C
Cane length (cm)	290.0B	297.7B	296.7B	313.3A	208.3C	175.0D
Cane thickness (cm)	3.20AB	3.06BCD	3.16ABC	3.23A	3.06BCD	2.93D
Cane weight (kg)	0.98B	0.88C	0.98B	1.05A	0.87C	0.74D
Cane yield (t/ ha)	170.8C	187.4B	194.8B	216.3A	158.2D	138.9E
Sucrose %	17.50C	17.50C	17.62C	19.05A	18.12B	12.62D
CCS %	13.52C	12.86D	13.47C	14.66A	14.11B	9.080E
Fiber %	12.58D	12.87C	12.16F	12.37E	12.85C	13.35A
Sugar yield (t/ha)	23.09CD	24.10C	26.23B	31.70A	22.32D	12.58E
			NIA98			
Stalk/stool (no.)	6.88A	7.50A	8.00A	8.88A	7.80A	4.50B
Cane length (cm)	272.2AB	283.3A	283.3A	276.7A	273.3AB	240.0B
Cane thickness (cm)	2.70A	2.50A	2.47A	2.60A	2.49A	2.33A
Cane weight (kg)	0.91A	0.88AB	0.90A	0.98A	0.91A	0.78B
Cane yield (t/ ha)	168.8BC	186.7AB	196.1AB	203.6A	183.7AB	146.7C
Sucrose %	17.44AB	16.46B	18.10A	17.15AB	17.03AB	16.50B
CCS %	14.30A	12.75BC	14.01AB	13.06ABC	12.85BC	12.48C
Fiber %	12.95A	12.48B	12.30B	12.78A	12.91A	12.91A
Sugar yield (t/ha)	24.14A	23.75A	27.47A	26.54A	23.68A	18.96B
			NIA2004			
Stalk/stool (no.)	4.00D	7.00B	9.00A	6.18C	4.00D	4.00D
Cane length (cm)	280.0B	280.7B	315.3A	257.3C	258.7C	256.0C
Cane thickness (cm)	2.41D	2.62A	2.58B	2.55C	2.32G	2.35E
Cane weight (kg)	0.82AB	0.77BC	0.83A	0.75C	0.68D	0.65D
Cane yield (t/ ha)	139.7D	161.2B	200.4A	149.9C	132.8D	129.9D
Sucrose %	18.55A	17.93B	18.18B	18.05B	18.02B	17.81B
CCS %	15.03A	14.14B	14.15B	14.07B	13.02C	12.79C
Fiber %	11.37D	12.08C	11.19D	11.36D	13.70A	12.68B
Sugar yield (t/ha)	20.99C	22.77B	28.33A	21.06C	17.26D	16.59D

DMR test (0.05): Means followed by the same letters are not significantly different from each other
M1 = MS + IAA 4mg; M2 = MS + IAA 4mg + 0.2 mg 2,4-D + 1.0 mg Kin; M3 = MS + IAA 4mg + 0.2 mg 2,4-D + 0.5mg Kin; M4 = MS + IAA 4mg + 0.5 mg 2,4-D + 0.5 mg Kin; M5 = MS + IAA 4mg + 0.5 mg 2,4-D + 1.0 mg Kin

The data regarding the average performance of all the treatments and their parent for all the characters is presented in Table 4. Stalks /stool were significantly ($p \leq 0.05$) higher in M1 (8.00), M2 (8.66) and M3 (8.00) regenerants of BL4, non significant differences were observed in case of NIA-98 for this trait and M2 (9.00) produces maximum stalks in case of NIA-2004. Maximum plant height in case of BL4 was observed in M3 (313.3 cm), in case of NIA98 similar plant height was observed in the regenerants of M1 and M2 (283.3) and M2 treatment in case of NIA-2004 showed maximum length of plant (315.3 cm). Minimum plant height was observed in the regenerants of M5 in all three genotypes. Highest cane thickness was observed in M3 (3.23) and M1 (2.62) plantlets of BL4 and NIA-2004, respectively. In case of NIA-98 regenerated plantlets showed non-significant difference for cane thickness. Primary stalk weight (kg) were significantly higher in M3 (1.05) regenerants of BL4 and minimum in M5 (0.74), almost non significant differences were observed in case of NIA-98 for this trait and M2 (0.83) of NIA-2004 produces maximum primary stalks weight. Maximum sucrose % and CCS % in case of BL4 was observed in M3 19.05, 14.66 respectively, in case of NIA98 maximum value of sucrose % was

observed in M2 (18.10) and control showed highest CCS % (14.30) and in NIA-2004 no regenerants could surpass the control in sucrose % (18.55%) and CCS % (15.03%) Minimum sucrose % and CCS% were recorded in control (17.50%) and M5 (9.08%) regenerants in case of BL4, M1 (16.46%) and M5 (12.48%) in case of NIA-98 for sucrose % and CCS% respectively. In case of NIA-2004 minimum sucrose % and CCS % was observed in M5 regenerants. Regenerants of M5 in case of BL4, NIA-98 and M4 in case of NIA-2004 showed maximum value for fiber %. Statistically significant higher cane yield (t/ha) was recorded in regenerants of M3 in case of BL4, NIA-98 and M2 in NIA-2004 was observed in the regenerants of M5 in all three genotypes. Highest sugar yield (t/ha) was observed in regenerants of M3 (31.70) in case of BL4 and M2 (28.33) in NIA-2004. In case of NIA-98 regenerants of M1, M2, M3 and M4 were at par with control. Minimum sugar yield was recorded in M5 in all three genotype.

Table 5. Expected genetic advance under varying selection intensities for various traits.

Traits/treatment	Selection intensities (%)				
	2	5	10	20	30
			BL4		
Stalk/stool (no.)	15.85	13.50	11.53	9.17	7.60
Cane length (cm)	15.85	13.50	11.53	9.17	7.60
Cane thickness (cm)	0.84	0.72	0.61	0.48	0.40
Cane weight (kg)	0.79	0.67	0.58	0.46	0.38
Cane yield (t/ ha)	200.469	170.64	145.79	115.97	96.09
Sucrose %	16.40	13.96	11.93	9.49	7.86
CCS %	14.46	12.31	10.52	8.37	6.93
Fiber %	3.04	2.59	2.21	1.76	1.46
Sugar yield (t/ha)	45.14	38.66	33.03	26.27	21.76
			NIA-98		
Stalk/stool (no.)	1.11	0.94	0.811	0.64	0.53
Cane length (cm)	117.74	100.22	85.63	68.11	56.43
Cane thickness (cm)	1.01	0.86	0.73	0.58	0.48
Cane weight (kg)	0.81	0.69	0.59	0.47	0.38
Cane yield (t/ ha)	149.38	127.16	108.64	86.42	71.60
Sucrose %	4.52	3.85	3.29	2.61	2.17
CCS %	5.38	4.58	3.91	3.11	2.58
Fiber %	1.96	1.67	1.43	1.14	0.94
Sugar yield (t/ha)	23.12	19.76	16.88	13.42	11.12
			NIA 2004		
Stalk/stool (no.)	15.04	12.81	10.94	8.70	7.21
Cane length (cm)	15.04	12.81	10.94	8.70	7.21
Cane thickness (cm)	0.99	0.84	0.72	0.57	0.47
Cane weight (kg)	0.53	0.45	0.38	0.30	0.25
Cane yield (t/ ha)	190.59	162.23	138.61	110.25	91.35
Sucrose %	1.88	1.60	1.37	1.09	0.90
CCS %	6.00	5.10	4.36	3.47	2.87
Fiber %	7.10	6.04	5.16	4.11	3.40
Sugar yield (t/ha)	30.81	26.23	22.41	17.82	14.77

Table 6. Estimates of co-efficient of variability, genotype x year, genotype x replication and broad sense heritability of various traits.

Parameters	NIA-98	BL4	NIA2004
	Stalks/stool (Nos.)		
CV%	3.65	7.89	6.02
H^2 %	84.89	99.26	99.62
δ G x Y	0.071	0.125	0.166
δ G x R	0.044	0.842	0.242
	Cane length (cm)		
CV%	5.45	2.13	1.45
H^2 %	91.17	99.86	99.53
δ G x Y	14.35	12.5	12.5
δ G x R	739.35	110.5	54.1
	Cane thickness (cm)		
CV%	8.37	2.50	0.83
H^2 %	73.45	76.51	72.83
δ G x Y	0.147	0.125	0.146
δ G x R	0.104	0.022	0.116
	Cane weight (kg)		
CV%	3.57	3.08	4.05
H^2 %	97.47	99.89	99.77
δ G x Y	0.016	0.001	0.001
δ G x R	0.173	0.030	0.030
	Sucrose %		
CV%	5.22	0.77	1.14
H^2 %	83.39	99.89	85.07
δ G x Y	2.256	0..125	0.304
δ G x R	0.188	0.016	0.067
	CCS %		
CV%	6.10	1.00	1.81
H^2 %	88.94	99.71	97.60
δ G x Y	1.786	0.288	0.389
δ G x R	0.245	0.016	0.052
	Fiber %		
CV%	1.51	0.53	1.64
H^2 %	89.86	97.14	98.95
δ G x Y	0.125	0.006	0.025
δ G x R	0.124	0.136	0.150
	Cane yield (t/ha)		
CV%	9.57	4.13	4.07
H^2 %	92.47	99.08	99.25
δ G x Y	34.70	25.99	20.31
δ G x R	963.81	162.33	118.27
	Sugar yield (t/ha)		
CV%	11.86	4.53	4.64
H^2 %	92.11	99.99	99.36
δ G x Y	0.946	0.029	0.452
δ G x R	24.434	0.901	2.539

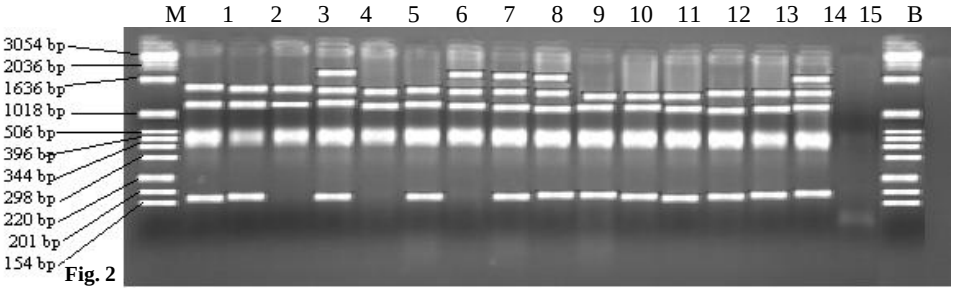

Fig. 2

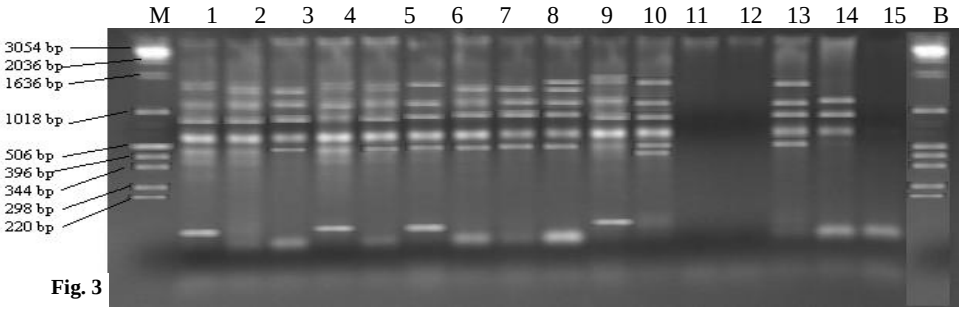

Fig. 3

L2= P-102, L3=P-105, L4=P-100, L5=P-110, L6=P-109, L7=P-108, L8=P-107, L9=(Parent), L10=P-104, L11= P-101, L12= P-97, L13=P-98, L14=P-99, L15=P-96, L16=P-95.

Fig. 2 & 3. Gel showing genetic dissimilarities between directly regenerated plantlets.

Expected genetic advance under selection with varying selection intensities (2, 5, 10, 20 and 30%) are shown in Table 5. 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. 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 6. 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 ^{2} σG x R component was larger than σ^2 G x Y in most of the traits, which indicated that this trait was less stable, σ^2G x R relates to performance with in the same year, which implied that more replication should be desirable to obtain more reliable results.

SSR study: Direct regenerants of sugarcane when tested for genetic fidelity with 10 SSR primers showed polymorphism, a total of 37 alleles were amplified, out of which 30 alleles are polymorphic, indicating an intrinsic polymorphism rate of 81% and only 07 (19%) alleles were monomorphic. The average number of alleles per locus was 3.7, with a range from two to eight. The amplified product resulted from SSR primers ranged from 117 to 2191 bp. Maximum of 08 bands were amplified with primers 55 (Fig. 3), primer-74 amplified with five polymorphic alleles (Fig. 2), ranged between 225-1442 bp and minimum two bands was amplified with primer 7, 12 and 27. Similarity coefficient matrix were calculated according to Nei's & Lei's (1979) method to estimate the genetic

divergence and relatedness among the 15 somaclones developed through direct regeneration, ranged from 0.366 (P-100 vs P-98) to 0.951 (parent vs P-104). Such a variation may derive from point mutations or alterations in chromosome number. The effects of pre-existing DNA polymorphism in clonally propagated plants were also taken into consideration. Plant meristems have an organized layer structure, particularly in dicots.

Dendrogram of the direct regenerants showed that plantlets under study can be divided into 5 clusters, designated A through E (Fig. 4). Cluster A consisted mainly of the P-102, P-105, P-108 (NIA-98) and P-107 (NIA-98). Cluster B contained predominantly P-110 (NIA-98) and P-109 (NIA-98) showing more genetic similarity among each other. Cluster A and cluster B are showing 75% similarity among each other which is forming a group and a distinct soma clone P-100 is showing 74% similarity with group of cluster A and cluster B. Control and P-104 (NIA-98) are in cluster C were genetically close to each other, same as P-101 (NIA-98) and P-97 (NIA-98) are in cluster D, both clusters are showing 77% similarity to each other. Four additional lines P-96 (NIA-98), P-95 (NIA-98), P-98 (NIA-98) and P-99 (NIA-98) were grouped separately in cluster E.

Discussion

In *Poaceae* species, plant regeneration has been facilitated by the identification of appropriate explant and *In vitro* culture conditions (Vasil, 1987). In this study, we identified explants and proper combinations of growth regulators for the induction of direct regeneration in three cultivar of sugarcane from Pakistan (NIA-98, BL4 and NIA-2004). The thickness of leaves was a determining factor affecting induction of direct shoot organogenesis from immature leaves of sugarcane. The best response for shoot organogenesis was observed on the leaves of 2-4 mm thickness. Yamanouchi *et al*., (1999) reported on the stable regeneration of plantlets from immature leaves isolated from winter buds of field-grown mulberry. It is inferred from these studies that the size of the leaf plays an important role in its endogenous potential to regenerate shoots. Kumar *et al*., (2003) reported that continuous exposure of explants to TDZ resulted in the complete loss of regeneration ability and callus production from explants. Debnath (2005) reported that moderate concentrations (1-5 μM) of TDZ supported bud and shoot regeneration from *In vitro* derived Lingonberry leaves, but strongly inhibited shoot elongation. Our results confirm that TDZ inhibits shoot elongation in sugarcane. The positive effect of auxins, especially 2,4-D, may be due to the fact that the stimulated auxin may have changed the balance of endogenous growth regulators and thus enhanced direct shoot regeneration. Our present study, shows that dark/light conditions are also effective for direct shoot regeneration. Darkness was generally observed to stimulate direct shoots production better than light condition. The effect of light can be interpreted as acting on metabolism and sugar uptake.

The immature leaf segment of sugarcane consists of many parenchytmatous cells and is soft and light whitish yellow in colour. Explants from the different regions of sugarcane were cultured on various combinations of media for the induction of direct regeneration in sugarcane (Desai *et al*., 2003, 2004). The addition of cystein HCl in the culture media prevents the production of polyphenol during culture initiation as reported by Khatri *et al*., (2002). Direct regeneration without callus phase has been reported in several other monocots like orchard grass (Conger *et al.,* 1983), garlic (Sata *et al*., 2000) and minor millet (Vikrant & Rashid, 2001). Such a callus-free development and

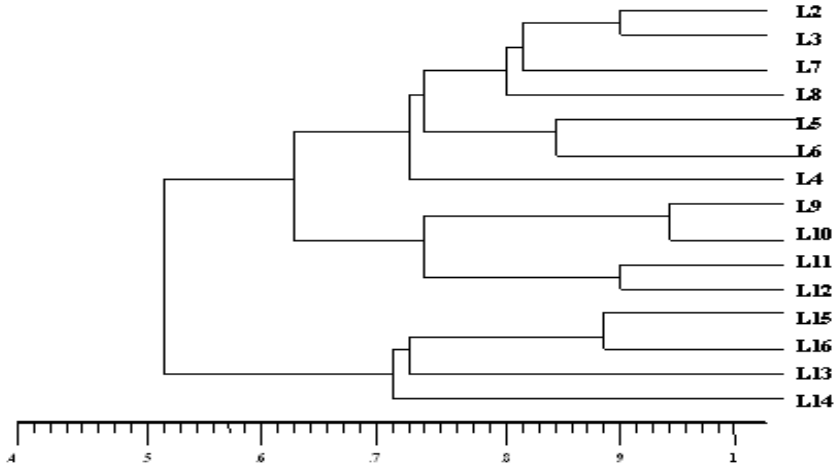

Fig. 4. Dendrogram of fifteen sugarcane soma clones developed from SSR data using unweight pair group method of arithmetic means (UPGMA).

regeneration pattern through the induction of direct regeneration can be advantageous since callus culture is associated with problems in embryo formation, maturity and plantlet regeneration (Thorpe, 1994). In barley, Cho *et al*., (1998) reported that the regeneration system through callus cultures produced plants with reduced fertility, suggesting that the callus culture could be at the origin of genome instability and regeneration problems. Direct regeneration from sugarcane leaf discs was earlier reported in selections through rapid regeneration for sugarcane mosaic virus resistance and in a transformation protocol (Snyman *et al*., 2000). In the present study, induction of direct regeneration from immature leaf segment yielded a large number of plants in a short time. Healthy and well-grown rooted plants were obtained within eight weeks. The type of cytokinin used in the medium had a marked effect on shoot proliferation with better results obtained with Kinetin in relation to BAP. Furthermore, in presence of the BAP shoots were fused and appeared fasciated with the development of thick dark green leaves. Variation in the activity of different cytokinins can be explained by their differential uptake (Blakesey, 1991), varied translocation rates to meristematic regions and metabolic processes, in which the cytokinin may be degraded or conjugated with sugars or amino acids to form biologically inert compounds, as reported by Tran Thanh Van & Trinh (1990) and Kaminek (1992).

According to Svetleva (2004), chlorophyll mutants are used as marker in genetics, physiological and biochemical investigations. Van Harten (1998) demonstrated that chlorophyll synthesis is under the control of nuclear and cytoplasmic genes and chlorophyll mutants are used as test for the evaluation of genetic fidelity. The production of chlorophyll mutant confirms that this process will allow us to utilize natural background heterozygosity reachable through an access to aneuploid cells.

In *Morus alba* L., the stimulatory effect of auxins was found to depend partly on the type of auxin employed (Anuradha & Pullaiah, 1992). Kim *et al*., (1985) observed substantial amount of callus formation at the base of the leaf in regenerated shoots on higher concentration of IBA > 3mg/l whereas in the presence of 1mg/l IBA, healthy and more vigorously growing roots were formed directly at the base of shoots. The presence

of callus between the root and shoot resulted in poor vascular connection, which made field survival of the plantlets difficult. Bhau & Wakhlu (2001) reported that IBA was the most effective auxin for root induction from shoots regenerated from the callus whereas higher levels of auxins encouraged callus formation from the cut ends of the explants. Our results confirm these observations since IBA at 1mg/l was found to be the best treatment for root induction from regenerated shoots of all three studied cultivars. In the present study, we have developed a simple and efficient protocol involving dark incubation of explant for 25 days and subsequent transfer into light on medium containing low concentration of 2,4-D along with high concentration of kinetin for promoting shoot development from leaves of three elite cultivars of sugarcane. Shoot proliferation rates were higher on medium containing kinetin as compared to BAP. Efficient rooting from the shoots of all three cultivars was observed on medium containing 1mg/l IBA + 6% sucrose. In this context the protocol developed in this study could be useful for creating new genetic variability.

The results indicate that the genetic variability did occur in all the phytohormone treatments and both quantitative and qualitative traits showed wide range of genetic variability. Plant height and plant girth are the main contributing trait for cane yield. Significant differences in plant height and plant girth were observed among the clones. The plant height and cane girth are the major contributing factors for high cane yield (Rehman *et al*., 1992; Khan *et al*., 1998, 1999). Khan *et al*., 2004 also reported that plant height and plant girth are the important cane yield contributor alongwith stalk per stool. All the mutagenic treatment showed significant difference in the stalks per stool. Singh *et al*., (1985) have reported that number of canes were the most important character contributing directly to higher yield. According to Raman *et al*., (1985) and Javed *et al*., (2000), number of stalks was the major contributing factor for cane yield. Quebedeadux & Martin (1986) proposed that both the stalk number and weight should be assessed to have an accurate yield potential of the variety. Khan *et al*., (1997, 2002) have reported that excessive stalks in stool showed adverse effect on cane yield due high intra plant competition. Sugar yield per unit area can be increased only if there is a break through in the production of sugarcane and the recovery of sugar.

Meristem of vegetatively propagated plants can represent a complex chimerical structure. A possible chimeric nature of the cultivar used may also be the reason for its high phenotypic instability, rather than an intrinsic genetic factor. Tissue culture was thus responsible for the generation of new variability therefore high rate of molecular polymorphism was observed. A large number of variant loci were observed from the beginning of the process, indicating that culturing meristems *In vitro* is stressful to the plant genome. An explanation for this finding is that the pattern observed is in accordance with the segregation of genotypes from a chimeric meristem, resulting in high polymorphisms in the initial generation, due to the breakdown of the meristematic organization, forming a heterogeneous population of homogeneous plants, but over consecutive generations, lower polymorphisms, due to a stochastic process.

Heinz & Mee (1971), working with callus-derived cultures from sugarcane variety H50-7209, detected clones with chromosomal numbers ranging from 2n = 94 to 120. In contrast, chromosome stability was described for varieties NA56-79 (2n = 114) and Co419 (2n = 213) by Silvarolla & Aguiar-Perecin (1994), who developed a technique to obtain intact somatic metaphase sugarcane cells. Together, these observations suggest either that some genotypes are more susceptible to somaclonal variation, or that the *In vitro* instability is actually a consequence of a genotype *versus* culture medium

interaction. It is suggested that SSR bands represent mainly repetitive DNA (Grattapaglia & Sederoff, 1994). Polymorphism in repetitive DNA sequences has frequently been observed during plant propagation by tissue culture (Smulders *et al*., 1995) and undergoes more alterations than the coding sequences. *In vitro* stress may provoke changes at preferential sites, such as repetitive DNA, thereby activating transposable elements.

Direct regeneration cannot be used for commercial propagation in sugarcane because of the genetic heterogeneity presents within the cells naturally. In genetically stable crops direct regeneration can be used for large-scale plant production. It would suggest that, the induction of direct regeneration using immature leaf segments as described in the present study could be useful in exploring genetic variability or it may allow improving the existing cultivar by altering one or few traits keeping the whole genome intact.

Acknowledgement

The authors are thankful to Dr. Mazhar H. Naqvi, Director, Nuclear Institute of Agriculture, Tando Jam, Sindh, Pakistan for the support during the research work. Thanks also due to Dr. J. L. Noyer, CIRAD, France, for critical review of the paper.

References

Anbalogan, S., A. Kalamani and M. Sakila. 2000. *In vitro* propagation of sugarcane: nature of callus and morphological variation. *Res. Crops*, 1: 138-140.

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

Anonymous. 2007. *Agricultural Statistics of Pakistan*, 2006-07. Govt. Pakistan, pp. 27-28 and 106.

Anuradha, M. and T. Pullaiah. 1992. Micropropagation of mulberry (*Morus alba* L.). *Annali Di Botanica*, 15: 35-41.

Arencibia, A. 1998. Gene transfer in sugarcane. In: *Biotechnology of Food Crops in Developing Countries.* (Eds.): T. Hohn and K.M. Leisinger. Springer-Verlag, New York, pp.79-104.

Bhau, B.S. and A.K. Wakhlu. 2001. Effect of genotype, explant type and growth regulators on organogenesis in *Morus alba*. *Plant Cell Tiss. Org. Cult*., 66: 25-29.

Blakesey, D. 1991. Uptake and metabolism of 6-benzyladenine in shoot proliferation of *Musa* and *Rhododendron*. *Plant Cell Tiss. Org. Cult*., 25: 69-74.

Cho, M.J., W. Jiang and P. Lemaux. 1998. Transformation of recalcitrant barley cultivars through improvement of regenerability and decreased albinism. *Plant Sci*., 138: 229-244.

Conger, B.V., G.E. Hanning, D.J. Gray and J.K. McDaniel. 1983. Direct embryogenesis from mesophyll cells of orchard grass. *Science*, 221: 850-851.

Debnath, S.C. 2005. A two-step procedure for adventitious shoot regeneration from *In vitro* derived Lingonberry leaves: shoot induction with TDZ and shoot elongation using zeatin. *Hort Science*, 40: 189-192.

Desai, N.S., P. Suprasanna and V.A. Bapat. 2003. Improvement in somatic embryogenesis, analysis of somaclonal variation and genetic transformation of Indian sugarcane varieties. *Proceedings of the International Seminar on Sugarcane Genomics and Genetic Transformation*, VSI, Pune, 2003, pp. 28-30.

Desai, N.S., P. Suprasanna and V.A. Bapat. 2004. Molecular characterization of culture-induced genetic variability among regenerated plants obtained through direct and indirect somatic embryogenesis in sugarcane. In: *26th Annual Meeting PTCA* (India) and *National Symposium on Biotechnology for a Better Future*. St. Aloysius College, Bangalore, 15-17 January 2004, pp. 34-35.

Drake, J.W., B. Charlsworth, D. Charlsworth and J.F. Crow. 1998. Rate of spontaneous mutation. *Genetics*, 148: 1667-1686.

Eudes, F., S. Acharya, L.B. Selinger and K.J. Cheng. 2003. A novel method to induce direct somatic embryogenesis, secondary embryogenesis and regeneration of fertile green cereal plants. *Plant Cell Tiss. Org. Cult*., 73: 147-157.

Gallo-Meagher, M., R.G. English and A. Abouzid. 2000. Thidiazuron stimulates shoot regeneration of sugarcane embryogenic callus. *In Vitro Cell Dev. Biol. Plant*., 36: 37-40.

Gill, R., P.K. Malhotra and S.S. Gosal. 2006. Direct plant regeneration from cultured young leaf segments of sugarcane. *Plant Cell Tissue Org. Culture*, 84: 227-231.

Grattapaglia, D. and R. Sederoff. 1994. Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross: mapping strategy and RAPD markers. *Genetics*, 137: 1121-1137.

Guiderdoni, E., B. Merot, P.F. Klsomtramage, P. Feldman and J.C. Glaszman. 1995 Somatic embryogenesis in sugarcane. In: *Biotechnology in Agriculture and Forestry*. (Ed.): Y.P.S. Bajaj. Springer-Verlag, Berlin, pp. 92-113.

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

Janno, N., L. Grivet, M. Segiun, F. Paulet, R. Domaingue, P.S. Rao, A. Dookun, A. D'Hont and J.C. Glaszmann. 1999. Molecular investigation of the genetic base of sugarcane cultivars. *Theor. Appl. Genet*., 99: 171-184.

Javed, M.A., A. Khatri, I.A. Khan, M. Ashraf, M.A. Siddiqui, N.A. Dahar, M.H. Khanzada and R. Khan. 2000. Prospects of enhancement / improvement of sugarcane productivity in Pakistan. *Pak. Sugar J*., 15(6): 14-19.

Kaminek, M. 1992. Progress in cytokinin research. *TIBTECH*, 10: 159-162.

Karp, A. 1991. On the current understanding of somaclonal variation. In: *Oxford Surveys of Plant Molecular and Cell Biology* (Ed.): H.F. Miflin. Oxford University Press, New York, pp. 1-58.

Khan, I.A, A. Khatri, M. Ahmad, K.A. Siddiqui, N.A. Dahar, M.H. Khanzada and G.S. Nizamani. 1997. Genetic superiority of exotic clones over indigenous clones for quantitative and qualitative traits. *The Nucleus*, 34: 153-156.

Khan, I.A., A. Khatri, G.S. Nizamani, M.A. Siddiqui, M.H. Khanzada, N.A. Dahar, N. Seema and M.H. Naqvi. 2004. *In vitro* studies in sugarcane. *Pak. J. Biotech*., 1: 6-10.

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.

Khan, I.A., A. Khatri, M.A. Javed, S.H. Siddiqui, M. Ahmad, N.A. Dahar, M.H. Khanzada and R. Khan. 2002. Performance of promising sugarcane clone AEC81-8415 for yield and quality characters II. Stability studies. *Pak. J. Bot*., 34(3): 247-251.

Khan, I.A., A. Khatri, M.A. Siddiqui, G.S. Nizamani, S. Raza, M.H. Khanzada, N.A. Dahar and R. Khan. 2005. Effect of NPK fertilizers on the growth of sugarcane clone AEC86-347 developed at NIA, Tando Jam. *Pak. J. Bot*., 37: 355-360.

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

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, 1: 41-43.

Kim, H.R., K.R. Patel and T.A. Thorpe. 1985. Regeneration of mulberry plantlets through tissue culture. *Bot. Gaz*., 146: 335-346.

Kumar, P., G.C. Srivastava, J.D.S. Panwar, R. Chandra and P. Raghuveer. 2003. Efficiency of thidiazuron on in vitro shoot regeneration from cotyledonary node explant in mungbean. *Indian J. Plant Physiol*., 8: 398-401.

Lakshmanan, P., R.J. Geijskes, L. Wang, A. Elliott, C.P.L. Grof, N. Berding and G.R. Smith. 2006. Developmental and hormone regulation of direct shoot organogenesis and somatic embryogenesis in sugarcane (*Saccharum* spp. interspecific hybrids) leaf culture. *Plant Cell Reports*, 25: 1007-1015.

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. 1993. Factors affecting induction, somatic embryogenesis and plant regeneration of callus from cultured immature inflorescence of sugarcane. *J. Plant Physiol*., 141: 714-720.

Mozdhorst, A.P., A. Marcel, J. Toonen and C. Sacco de Vries. 1997. Plant embryogenesis. *Crit. Rev. Plant. Sci*., 16: 535-576.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*., 15: 473-497.

Naz, S. 2003. Micropropagation of promising varieties of sugarcane and their acclimatization response. Activities on Sugar Crops in Pakistan. In: *Proc. Fourth Workshop Res*. Dev., 1-9.

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 plant of *Hordeum*. *Theor. Appl. Genet*., 56: 101-112.

Quebedeadux, J.P. and F.A. Martin. 1986. *A comparision of two methods of estimating yield in sugarcane*. Repot of Projects. Deptt. Of Agronomy Louisiana Agric. Expt. Stn. Louisiana State Univ. Baton Rouge, Louisiana, pp. 228.

Raman, K., S.R. Bhat and B.K. Tripathi. 1985. Ratooning ability of sugarcane genotypes under late harvest conditions. *Indian Sugar*, 35: 445-448.

Rehman, S., G.S. Khan and I. Khan. 1992. Coordinated uniform national varietal trial on sugarcane. *Pak. J. Agric. Res*., 13: 136-140.

Sahasrabudhe, N.A., M. Nandi, R.A. Bahulikar, P.S. Rao and P. Suprasanna. 2000. A two-step approach to scale up green plant regeneration through somatic embryogenesis from *In vitro* cultured immature inflorescence of a male sterile line and a maintainer line of rice. *J. New Seed*., 2: 1-10.

Sata, S.J., S.B. Bagatharia and V.S. Thaker. 2000. Induction of direct embryogenesis in garlic (*Allium sativum*). *Methods Cell Sci*., 22: 299-304.

Siddiqui, S.H., I.A. Khan, A. Khatri and G.S. Nizamani. 1994. Rapid multiplication of sugarcane through micropropagation. *Pak. J. Agric., Res*., 15: 134-136.

Silvarolla, M.B. and M.L.R. Aguiar-Perecin. 1994. Evaluation of chromosome number stability in two sugarcane varieties. *Rev. Bras. Genet*., 17: 237-242.

Singh, R.K., R.S. Tehlan and A.D. Taneja. 1985. Investigating some morphological and quality traits in relation to cane and sugar yield. *Indian Sugar*, 35: 267-271.

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 28-3 July, pp. 201-203.

Snyman, S.J., M.P. Watt, F.C. Huckett and B.I. Botha. 2000. Direct somatic embryogenesis for rapid, cost-effective production of transgenic sugarcane (*Saccharum* spp. hybrids). *Proceedings of the South African Sugar Technologists Association*, 74: 186-187.

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

Svetleva, D.L. 2004. Induction of chlorophyll mutants in common bean under the action of chemical mutagens ENU and EMS. *J. Central European Agric*., 5: 85-90.

Thorpe, T.A. 1994. *In vitro* Embryogenesis in Plants. *Current Plant Science and Biotechnology in Agriculture*, Kluwer, Boston MA, 1995; vol. 20.

Tran Thanh Van, K. and T.H. Trinh. 1990. Organogenic differentiation. In: *Plant Tissue Culture*. *Application and Limitations*. (Ed.): S.S. Bhojwani. Elsevier, Amsterdam, pp 34-53.

Van Harten, A.M. 1998. *Breeding, Theory and Practical Applications*. Cambridge University Press; 1-40.

Vasil, I.K. 1987. Developing cell and tissue culture systems for the improvement of cereals and grass crops. *J. Plant Physiol*., 128: 192-218.

Vikrant, A. and A. Rashid. 2001. Direct as well as indirect somatic embryogenesis from immature (unemerged) inflorescence of a minor millet *Paspalum scrobiculatum* L. *Euphytica*, 120: 167-173.

Yamanouchi, H., A. Koyama and H. Machii. 1999. Effect of medium conditions on adventitious bud formation in immature mulberry leaves. *JARQ*, 33: 267-274.

(Received for publication 23 August 2008)