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Full Length Research Paper

In vitro regeneration, detection of somaclonal variation and screening for mosaic virus in sugarcane (*Saccharum* spp.) somaclones

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Three sugarcane accessions susceptible to sugarcane mosaic virus; HSF-240, S-2000-US-359, and S-2003-US-704 were evaluated for callogenesis and regeneration ability. For callogenesis, five different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) was used. The best callogenesis was obtained when Murashige and Skoog (MS) was portified with 3 mg/L 2,4-D and the highest regeneration was obtained on media containing MS + kinetin 0.5 + 0.5 mg/L naphthalene acetic acid (NAA). After succesful regeneration and rooting on half strength MS medium, with 1.5 mg/L indole-3-butyric acid supplementation, plantlets were shifted to green house. Enzyme linked immunosorbent assay (ELISA) test was performed to detect the presence of sugarcane mosaic virus (SCMV) in the regenerated plantlets and simple sequence repeat (SSR) markers were used to evaluate the genetic variation at DNA level between the parent's plants and regenerated somaclones of the accession HSF-240. A total of 26 parent plants and 64 somaclones, among the regenerated plants were selected for the screening of virus through double antibody sandwich (DAS-ELISA) test. Four (4) parent plants out of the 26, showed negative reaction to the virus test. Ten (10) somaclones showed positive reaction to the disease, 9 somaclones showed mild reaction to virus and 45 somaclones showed negative reaction. For the detection of somaclonal variation, 38 primers pair were used and 15 simple sequence repeats (SSR) primer pairs were found to be polymorphic with 51.61% polymorphism. The study demonstrates that SSR genetic markers are the best tool for the investigation of genetic variation in sugarcane.

Key words: Callogenesis, somaclones, simple sequence repeats (SSR), genetic markers.

INTRODUCTION

Sugarcane is a tropical, tall growing, monocotyledonous perennial grass, belonging to the genus *Saccharum*. Sugarcane is grown on over a million hectares and provides the raw material for Pakistan's 84 sugar mills. Its shares in value added agriculture and GDP are 3.6 and 0.8%, respectively (Anonymous, 2010, 2011). Most of the existing sugarcane varieties in Pakistan are susceptible to different diseases like sugarcane mosaic virus, red rot, pokha boeng, etc. Therefore, sugarcane improvement for disease resistance, quality and yield is important. Sugarcane is a highly polyploid crop with chromosome numbers in somatic cells (2n), ranging from 80 to 124 in cultivated varieties and 48 to 150 in wild types (Garcia et al., 2006). It is a photo-thermal sensitive crop and flowering takes place at 5 to 23° latitude whereas Pakistan is situated at 24 to 37° latitude. Other conditions required for changing the vegetative to reproductive phase are; (1) Temperature range of 25 to 33°C for 70 days. (2) 70 to 80% humidity for 70 days. (3) Day length of 11.5 to 12.5 h for 70 days. These conditions are

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available only at three places in Pakistan that is, Murree, Sajawal and Dargai, but the fuzz produced in these areas is unviable or have very low viability percentage. For breeding purposes, fuzz is imported from abroad because Pakistan breeders do not have the ideal conditions for crossing and variety improvement (Khan et al., 2004). Tissue culture techniques are widely used in sugarcane improvement programs (Eldessoky et al., 2011).

Somaclonal variation can provide an alternative for improvement of existing genotypes (Shahid et al., 2011). Assessment of genetic variability in tissue culture derived is helpful for plant breeders to select appropriate material for their breeding program. Technological advances in molecular biology have contributed greatly to understanding the genetic diversity of plants. Simple sequence repeat (SSR) markers are the most commonly used molecular techniques to study polymorphism in sugarcane (Nair et al., 2002). We used new varieties for tissue culture, so the study aimed to optimize the protocol for these varieties by checking their responses to different hormonal levels, and the use of techniques like ELISA (enzyme linked immunosorbent assay) to check and screen the disease in plantlets produced. The study was also aimed for induction of somaclonal variation and further testing of these variations using SSR molecular markers.

MATERIALS AND METHODS

Tissue culture study

Study was conducted at Agriculture Biotechnology Research Institute (ABRI), Ayub Agriculture Research Institute (AARI), Faisalabad, Pakistan. The plant material was taken from the germplasm source of Sugarcane Research Institute, Ayub Agriculture Research Institute (AARI), Faisalabad. Three sugarcane accessions susceptible to sugarcane mosaic virus; HSF-240, S-2000-US-359 and S-2003-US-704 were used for the tissue culture study. Two kinds of explant sources were used: leaf explants taken from innermost 1 to 5 leaves with 2 to 3 mm in length and pith explants taken from 1 to 5 apical internodes, sterilized by standard procedure (Khan et al., 2009b). For callogenesis, MS (Murashige and Skoog, 1962) supplemented with 5 different doses (1, 2, 3, 4, 5 mg/l) of 2, 4-D were tried using 10 replications each. Cultured tubes were kept in the dark at 28 ± 2°C for the first two weeks and then were shifted under continuous florescent light of 2000 to 2500 lux intensity at the same temperature for the next two months. The callus produced was subcultured twice at an interval of fifteen days, to increase the amount of callus. It was done on the same medium at which callus was produced initially. The test tubes were transferred to incubation room with continuous florescent light of 2000 to 2500 lux intensity and a dark period of 8 h at 28 ± 2°C. Data was recorded on the basis of callus color, morphology and size. +++; ++; + and - were scored as best, better, good and no callus.

The calli developed on different treatments of 2, 4-D was transferred to regeneration medium. Three media combinations were tried using 10 replications each. RM1 = MS basal media + 1 mg/L kinetin, RM2 = MS basal media + Kinetin 0.5 + 0.5 mg/L NAA, RM3 = MS basal media + 480 mg/L casein hydrolysate + 1 mg/L Kinetin. About 1 to 2 g of callus was inoculated to each tube containing 10 ml of regeneration medium. The developed calli proceeded for regeneration and response was determined by regeneration percentage. Shoots clumps of 3 to 4 inches in length were taken from the test tubes and aseptically separated into the single plantlets and transferred to the half strength MS medium with 1.5 mg/L indole-3-butyric acid supplementation for root formation. Incubation conditions were the same as used for shoot formation. When the plants attained a sufficient height in the test tubes and developed proper root and shoot system, they were transferred to the glass house. The roots were washed with the distilled water and then transferred to the pots having autoclaved mixture of sand, clay and well rotten farmyard manure. Initially, the glass house temperature was maintained as that of incubation room with a relative humidity of 80%, but after fifteen days when the plants established, they were exposed to the normal temperature and humidity in the glass house condition for the next one month. All the required inputs were provided including proper supply of water and fertilizers. The data recorded for callogenesis and regeneration were analyzed statistically using completely randomized design (Steel et al., 1997). The differences among genotypes, explant sources, 2,4-D levels and their interaction were compared by Duncan's multiple range test (DMRT) (Steel et al., 1997).

Sugarcane mosaic virus screening through ELISA

Sugarcane variety HSF-240 was used for ELISA test. The plants developed through tissue culture in green house were selected randomly for sugarcane mosaic virus study. Soft leaves of the glass house grown plants, with size of 2 to 3 cm were taken and ground in the 0.5 M phosphate buffer with mortar and pestle. Double antibody sandwich ELISA was performed according to the method given by Kemeny and Challacambe (1989) for the screening of sugarcane mosaic virus disease. Data was recorded for the number of virus positive and negative plants by using ELISA reader.

Molecular studies (SSR)

Sugarcane variety HSF-240 somaclones produced were used for comparison with parents at DNA level for detection of somaclonal variations. Fresh young leaves were collected from the field experiment for isolation of the DNA. Total genomic DNA of the plants was extracted by using modified (CTAB) method (Doyle and Doyle, 1990). DNA concentration was determined, using a NanoDrop spectrophotometer (ND1000). Primer selection was based on previous investigation on SSR analysis, carried out with sugarcane genotypes and somaclones in this laboratory. A total of 38 primer pairs obtained from Gene link company (USA) were used in PCR reaction for each genotype. For SSR analysis, concentration of genomic DNA, l0 × PCR buffer with $(NH_4)_2SO_4$, $MgCI_2$, dNTPs primers and taq DNA polymerase were optimized. A reaction mixture of 20 µl was used to amplify genomic DNA in a thermal cycler (Eppendorf DNA Thermal Cycler 9600). To confirm that the observed bands were amplified genomic DNA and not the primer artifacts, genomic DNA was omitted from control reaction. A negative control was also run to confirm if the master/reaction mixture is correctly prepared or not. The PCR products were electrophoresed at 90 V, in 2% agarose gel for approximately 2 h, using 0.5 × tris-boric acids EDTA (TBE) buffer, along with a DNA molecular size marker. The gel contained 0.5 µg/ml ethidium bromide to stain the DNA and photographed under UV light using gel documentation system. Reactions were duplicated to check the consistency of the amplified products. Only easily resolved bright DNA bands were scored as presence of band (1) and absence of bands (0). Coefficient of similarity among somaclones was calculated according to Nei and Li (1978). Similarity coefficient was utilized to generate a dendrogram, by means of Unweighted Pair

Table 1. Analysis of variance table for callus initiation frequency (CIF).

Source	D.F	S.S	MS	F-value
Genotypes (G)	2	4.087	2.043	3.5388*
2,4-D levels (D)	4	20.113	5.028	8.7085**
G × D	8	0.847	0.106	0.1833^{ns}
Explant (Exp.)	1	52.083	52.083	90.2021**
G × Exp.	2	5.087	2.543	4.4047*
D × Exp.	4	1.900	0.475	0.8226^{ns}
G × D × Exp.	8	0.580	0.073	0.1256^{ns}
Error	270	155.90	0.577	
Total	299	240.597		

Coefficient of variation: 27.17%; ** = highly significant at p< 0.01; * = significant at p < 0.05; ns = non significant.

Table 2. Comparison of genotypes means for callus induction frequency.

Genotype	Callus induction frequency
S-2003-US-704	2.920^{a}
S-2000-US-359	2.830^{ab}
HSF-240	2.640^{b}

Table 3. Comparison of 2, 4-D level means for callus induction frequency.

2, 4-D level	Callus induction frequency
3	3.200^{a}
2	2.950^{ab}
4	2.783^{bc}
1	2.550^{c}
5	2.500^{c}

Group method of arithmetic means (UPGMA).

RESULTS AND DISCUSSION

Callus initiation frequency (CIF)

The present study was undertaken to check the response of three sugarcane accessions; HSF-240, S-2000-US-359 and S-2003-US-704 for callogenesis and organogenesis, and to check the existence of somaclonal variations by SSR marker. The interaction of genotypes × auxin levels, auxin levels × explant and interaction of all three factors that is genotypes × auxin levels × explant was non significant in the study (Table 1).

Effects of genotypes on CIF

Significant differences ($p < 0.05$) were observed among the genotypes using Duncan multiple range test (DMRT). The genotypes showed high value of callus score ranging from 2.92 to 2.64 (Table 2). Genotype S-2003-US-704 was the highest callus producer with an average of 2.92 callus score per test tube. Genotypes S-2000-US-359 and HSF-240 were statistically similar with an average of 2.83 and 2.64, respectively. These results reveal that callogenesis response is genotype dependent. Gandonou et al. (2005a) determined the response of three sugarcane varieties and found that callus induction ability was genotypes dependent. Similar results were also reported by Seema et al. (2011) and Raza et al. (2010).

Effects of auxin (2,4-D) levels on CIF

Comparison of auxin means, using DMRT showed that 3 mg/L of 2,4-D was the most potent concentration with an average of 3.2 callus score. There was no significant increase in response to callogenesic when MS was portified with 2 and 4 mg/L 2,4-D. Moreover, supplementing MS with 1 and 5 mg/L 2,4-D produced statistically similar response to callogenesis. However, their performance was less than 3 mg/L of 2,4-D. The average callus score of the five 2,4-D levels were 2.55, 2.95, 3.2, 2.78, and 2.5, respectively.

The lowest response was observed when MS was portified with 1 mg/L 2,4-D, with average callus score of 2.5. From this result, we can conclude that concentrations of 2,4-D from 1 to 5 mg/L proved to be optimum for callus induction in sugarcane, with best performance at 3 mg/L (Table 3). These results are consistent with the report of Mamun et al. (2004) who studied *in vitro*, micropropagation of two sugarcane varieties and found that 3 mg/l of 2,4-D was the best concentration for callus induction in sugarcane. Many scientists have used 2, 4-D for callus formation and found it effective. Like Ather et al. (2009) obtained 100% callus induction in 3.0 mg/L of 2,4-D. Badawy et al. (2008), Pandey et al. (2011) and Gandonou et al. (2005a) also obtained embryogenic callus from leaf bases at 3 mg/L 2,4-D. Jahangir et al. (2010) and Shahid et al. (2011)

Table 4. Comparison of explants source means for callus induction frequency.

Explants' source	Callus induction frequency
Leaf	3.213[a]
Pith	2.380[b]

Table 5. Comparison of genotypes × explants means for callus induction frequency.

Genotype × explants interaction	Callus induction frequency
V3 × leaf	3.520[a]
V2 × leaf	3.140[b]
V1 × leaf	2.80[b]
V2 × pith	2.520[c]
V3 × pith	2.320[c]
V1 × pith	2.300[c]

worked on callus inductions of sugarcane using different harmonal levels and found satisfactory results at 3.0 mg/L of 2,4-D. In contrast, Eldessoky et al. (2011) used sugarcane GT54-9 (C9) cultivar and obtained best results producing embryonic calli at 4 mg/L 2,4-D.

Effects of explant on CIF

Both explants showed good response to callus production but, leaf explant performed better with average callus score of 3.21, which is statistically different from pith explant (2.38) (Table 4). Thus, it can be inferred that leaf explant is a good source of callus induction than pith explant. This is primarily due to excretion of phenols which turned the whole pith brown, hindering proliferation. These results are in line with the work of Niaz and Quraishi (2002) who studied the response of leaf, lateral bud and pith explants, and found leaf to be the best source of explant for callogenesis in sugarcane. Shahid et al. (2011) demonstrated that leaf as explant with 3.0 mg/L 2,4-dichlorophenoxy acetic acid gave the best results, both for callus induction and proliferation. Mamun et al. (2004) also reported similar observations.

Interactive effects of genotype × explant on CIF

Leaf explants produced maximum callus in CV S-2003-US-704 with an average callus score of 3.52. However, genotypes S-2000-US-359 and HSF-240 were statistically similar with an average callus score of 3.14 and 2.98, respectively (Table 5). In contrast to this, Niaz and Quraishi (2002) reported that pith explant demonstrated a better response to callogenesis when compared with leaf explants in the presence of 2,4-D. All the interactions between genotype × 2,4-D level, 2,4-D level × explant source ,and genotype × 2,4-D level × explant source were found not significant in their response to callogenesis.

Organogenesis studies

Callus organogenesis or regeneration is dependent on a number of factors. Regeneration response from callus was studied under the effects of three factors that is, genotype, regeneration media and explants and their interactions. The analysis of variance table for percentage regeneration (Table 6) showed that there were significant differences among genotypes, regeneration media and explant sources. While the interactions between regeneration media × explant sources (RM × Exp), genotype × regeneration media (G × RM), genotype × explant sources (G × Exp) and genotype × regeneration media × explant sources (G × RM × Exp) were non significant.

Genotypic response for regeneration

The analysis of variance table depicted that there was significant difference in the response of genotypes to the organogenesis in sugarcane. HSF-240 proved to be the most responsive to organogenesis with an average percentage regeneration of 41.67. The lowest regeneration response was observed in S-2000-US-359 with an average percentage regeneration of 34. Genotypes S-2003-US-704 and S-2000-US-359 were statistically different from HSF-240 in their response to regeneration, with an average percentage regeneration of 41.08 and 34, respectively (Table 7). On the basis of HSF-240 performance, it can be concluded that this genotype was observed to demonstrate a great regeneration ability and poor response to callogenesis, indicating that callogenesis and organogenesis are two independent phenomena. A genotype with poor response to callogenesis might demonstrate a better response in regeneration capability and vice versa. So, we can conclude that successful regeneration in sugarcane is greatly influence by genotypic behavior.

Similarly, Rahman et al. (2002) observed different genotypic response towards organogenesis. Gill et al. (2004) studied that factors affecting somatic embryogenesis and subsequent plant regeneration *in vitro* sugarcane cultures were highly genotype specific. Significant differences were observed among sugarcane genotypes in their regeneration ability, indicating that *in vitro* regeneration is a genotypic dependent trait (Gandonou et al., 2005a). Similar results were also

Table 6. Analysis of variance table for regeneration percentage.

Source	DF	SS	MS	F-value
Genotype (G)	2	2185.833	1092.917	16.8501**
RM level (RM)	2	2992.500	1496.250	23.0685**
G × RM	4	221.667	55.417	0.8544^{ns}
Explant (Exp)	1	9606.806	9606.806	148.1135**
G × Exp	2	236.944	118.472	1.8266^{ns}
RM × Exp	2	36.944	18.472	0.2848^{ns}
G × RM × Exp	4	75.556	18.889	0.2912^{ns}
Error	162	10507.500	64.861	
Total	179	25863.750		

Table 7. Comparison of genotypes means for regeneration percentage.

Genotypes	Regeneration percentage
HSF-240	41.67^{a}
S-2003-US-704	41.08^{a}
S-2000-US-359	34.00^{b}

Table 8. Comparison of regeneration media level means for regeneration percentage.

Regeneration media levels	Regeneration percentage
2	44.42^{a}
1	37.67^{b}
3	34.67^{c}

Table 9. Comparison of explants source means for regeneration percentage.

Explants' source	Regeneration percentage
Leaf	46.222^{a}
Pith	31.611^{b}

reported by Raza et al. (2010) in sugarcane. Khan et al. (2009b) observed non-significant difference in shoot induction from three different sugarcane cultivars.

Effect of regeneration media on organogenesis

The analysis of variance table showed that the regeneration media; RM1 (MS + 1 mg/L kinetin), RM2 (MS + 0.5 mg/L kinetin + 0.5 mg/L naphthalene acetic acid) and RM3 (MS supplemented with 480 mg/L casein hydrolysate + 0.5 mg/L kinetin), were statistically different in their ability to induce regeneration in sugarcane. Furthermore, means seperation using Duncan Multiple Range test showed significant difference among the three regeneration media. RM2 proved to be the best media for regeneration with average percentage regeneration of 44.42. RM2 proved to be superior when compared with RM1 and RM3, with average percentage regeneration of 37.67 and 34.67, respectively (Table 8). We can therefore conclude, based on our observation that high level of cytokinin and low level of auxin is required for successful *in vitro* regeneration of shoots in sugarcane. It was also observed that type and concentration of growth regulators used in culture medium had significant effect on shoot induction. Earlier reports showed that combination of NAA with kinetin promoted rapid plantlets regeneration from sugarcane callus culture. Gill et al. (2006) found out that highest frequency (83.12%) of shoot regeneration occurred on MS medium supplemented with NAA and kinetin in variety CoJ-83. Medium devoid of NAA and supplemented with only kinetin did not induce direct shoot regeneration in any of the varieties evaluated. Niaz and Quraishi (2002b) also reported that media containing kinetin and NAA significantly increased the number of shoot production. Eldessoky et al. (2011) used sugarcane GT54-9 (C9) cultivar, and observed vigorous shoot regeneration when NAA was used with benzyl amino purine (BAP). Similarly, Khan et al. (2009a) studied three sugarcane clonal lines and observed best results on the medium containing 1.5 mg/L Kin + 1 mg/L NAA.

The influence of explant on regeneration

Leaf proved to be the best source of explant for plantlets regeneration from callus culture in sugarcane, with an average percentage regeneration of 46.22. Leaf explants produced a significantly higher regeneration when compared with pith explants with an average percentage regeneration of 31.61 (Table 9). This result is in line with our expectations, the leaf explants being soft in nature, produced soft, granular and embryogenic callus. Thus,

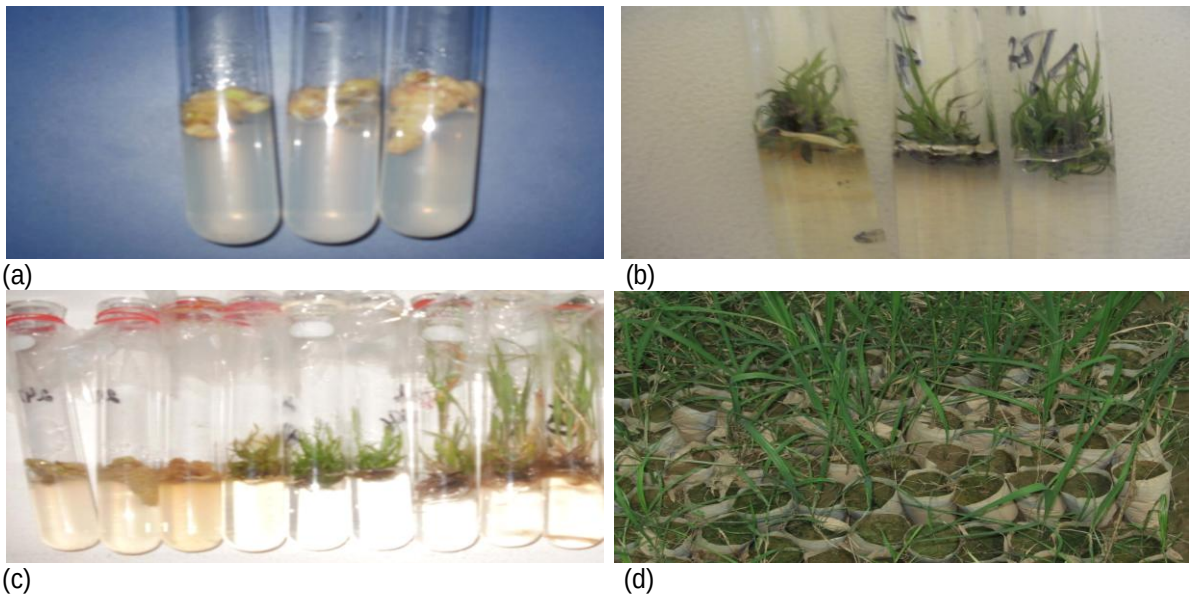

Figure 1. (a) Callus response of different genotypes at 2,4-D level of 3 mg/L; (b) Regeneration responses of different genotypes at RM2 media; (c) General procedure of plantlet development; (d) Growth of plantlets in pots for hardening.

Table 10. Layout for samples loaded in ELISA plate for ELISA test.

Parameter	1	2	3	4	5	6	7	8	9	10	11	12
A	PC	PC	p11	p19	SC1	SC9	SC17	SC25	SC33	SC41	SC49	SC57
B	NC	NC	p12	p20	SC2	SC10	SC18	SC26	SC34	SC42	SC50	SC58
C	BLK	BLK	p13	p21	SC3	SC11	SC19	SC27	SC35	SC43	SC51	SC59
D	P1	P6	p14	p22	SC4	SC12	SC20	SC28	SC36	SC44	SC52	SC60
E	P2	P7	p15	p23	SC5	SC13	SC21	SC29	SC37	SC45	SC53	SC61
F	P3	P8	p16	p24	SC6	SC14	SC22	SC30	SC38	SC46	SC54	SC62
G	P4	P9	p17	p25	SC7	SC15	SC23	SC31	SC39	SC47	SC55	SC63
H	P5	P10	p18	p26	SC8	SC16	SC24	SC32	SC40	SC48	SC56	SC64

showed more regeneration response, while hard callus from the pith explants produced from presumably mature cells produced less number of plantlets. This result is consistent with the findings of Rahman et al. (2002) and Shahid et al. (2011). It was also discovered that regeneration was dependent upon the concentration of growth regulators and type of explants. All the interactions between genotype × RM level, genotype × explant source, RM level × explant source and genotype × RM level × explant source were found to be non significant. The regenerated plantlets with an average height of 4 to 5 inches were subcultured in half strength MS medium with 1.5 mg/l indole-3-butyric acid supplementation. When rooting was succesfully established, plantlets were transferred to pots for hardening, and later shifted to field. After a period of three months in the field, further evaluation was performed (Figure 1).

Sugarcane mosaic virus screening

Plants that were used to run the ELISA test were selected randomly from both the parent and the regenerated plants of sugarcane accession HSF-240, at the age of three months after field establishment. A total of 26 parent plants and 64 somaclones among the regenerated plants were selected for screening for virus through DAS-ELISA test. Four (4) parent plants out of 26 showed negative reaction to the virus test. Ten (10) somaclones showed positive reaction to the disease and were declared as susceptible, 9 somaclones showed mild reaction to virus and were called tolerant, 45 somaclones showed negative reaction, declaring them resistant against virus (Table 11). In the plate, a row of 6 wells was donated to the control treatments, 2 for positive control, and another 2 for negative control of the sugarcane mosaic virus and the remaining for the extraction buffer

Table 11. Scores taken through ELISA reader for ELISA test.

Parameter	1	2	3	4	5	6	7	8	9	10	11
A	0.339	0.394	0.4	0.336	0.184	0.16	0.177	0.303	0.178	0.333	0.143
B	0.159	0.138	0.317	0.33	0.145	0.216	0.182	0.284	0.146	0.222	0.134
C	0.160	0.131	0.133	0.322	0.166	0.222	0.148	0.12	0.188	0.213	0.215
D	0.33	0.305	0.29	0.32	0.167	0.154	0.153	0.13	0.137	0.334	0.166
E	0.265	0.333	0.28	0.311	0.167	0.137	0.215	0.133	0.204	0.131	0.156
F	0.333	0.304	0.223	0.19	0.189	0.184	0.155	0.14	0.169	0.333	0.131
G	0.311	0.332	0.291	0.334	0.144	0.154	0.185	0.17	0.161	0.366	0.141
H	0.322	0.306	0.287	0.222	0.161	0.156	0.469	0.141	0.333	0.132	0.142

Scoring values; values less than 0.205 = resistant**,** values between 0.25 and 0.205 = tolerant, values more than 0.25 = susceptible.

Table 12. Polymorphism revealed by SSR markers between parents and somaclones.

S/N	Primer	Band size	Total band	Polymorphic bands	Polymorphic (%)	Primer sequence FR	PIC
1	SMs3	400	2	1	50.00	CATCTGCTCCCTCTTCCT CTCTGGCGGCTTGGTCCTG	0.1103
2	SMs6	600 - 1200	5	2	40.00	GACTCCTGTCACCGTCTTC ATACTTCAACCGTCTCCTCC	0.4041
3	SMs7	389 - 1250	6	1	16.67	CTAAGCAAGAACACAGGAAAG AGCAACAGCAGAGAGCAG	0.1103
4	SMs8	383	2	1	100.00	CTGACTAAGGAGGAAGTGGAG GACGACGATAGATGAAACA	0.1948
5	SMs9	400	1	1	100.00	GAGCCGCAAGGAAGCGAC CATACAAGCAGCAAGGATAG	0.1103
6	SMs10	497	1	1	100.00	CTCTCTTCTCGTCTCCTCATT GTCCTTCTTCTTCTCGTGGT	0.1103
7	SMs11	400	1	1	100.00	ACACGCATCGCAAGAAGG AAGAACACTCAACAGAAGCAC	0.1103
8	SMs12	400 - 600	2	1	50.00	AAATGTCTTCGCACTAACC AAGGAGATGCTGATGGAGA	0.1103
9	SMs16	260 - 400	2	1	50.00	CCCAGAGGACAAGGAACT GTAATGGAAGGAAGCAACTGA	0.3706
10	SMs45	400	1	1	100.00	CTTCCCTCCCTCTCCTCT AGCCTTCTACTAAACTATCTGCT	0.2146
11	SMs46	400	1	1	100.00	GTGAGTGAGACCAGACCAG CCGTGCTGTAGTTGTTGTAG	0.2146
12	SMs47	400	2	1	50.00	ATACGCTACTCTGAATCCCAC CAATCACTATGTAAGGCAACA	0.1103
13	SMs48	400	1	1	100.00	ACTCCTCTTCCTCTTCCTCTT GTTGTTCCCGTTCCCGCC	0.2146

Table 12. Contd.

14	SMs49	250 - 400	2	1	50.00	ACTCGGTCATCTCATCACTC GTTCTTCGGGTCATCTGG	0.1948
15	SMs50	400 - 500	2	1	50.00	ACGGTGAGCGAGGACTAC CTTGGGTGGCATCAGGAA	0.1103
Total			31	16			

(Table 10). Positive control always showed positive reaction, while negative control and the buffer showed negative reaction. This clearly demonstrated that the test was succesful. The results are in confirmation with the study of Carnot (2009) who reported the production of virus free clones of sugarcane through callus culture.

Polymorphism as revealed by SSR

All the primers were monomorphic except 15 primers which were polymorphic (Table 12). They produced 31 bands, out of which 17 bands were found to be polymorphic, showing 51.61% polymorphism. A total of 31 DNA fragments were generated by the 15 primers with an average of about 2.1 bands per primer. Bands that a primer yielded in the study ranged from 1 to 6. Generally, the size and the number of bands produced were dependent upon the nucleotide sequence of the primer pair, size of the primer used and the source of the template DNA. In this study, the primers used were of the size ranging from 200 to 750 bp. The size of the amplification products ranged from 250 to 1250 bp. The maximum number of bands (6) was produced by the primer SMs7. Primer SMs7 was also the least polymorphic, with 16.67% polymorphism. Shahid et al. (2011) studied the somaclonal variation in sugarcane genotype S97US297, by using 50 primers and found 67% polymorphism, using SSRs. Jannoo et al. (2001) studied diversity in 96 sugarcane genotypes with just two primer pairs and reported a high level of heterozygosity. Cordeiro et al. (2001) applied 21 primer sets to five sugarcane genotypes, and among them, 17 pairs were polymorphic, but the level of polymorphism (PIC value) in the cultivars detected by these SSRs was low (0.23). Khan et al. (2009a) developed tissue culture plants using young leaf of sugarcane as explant. Genetic fidelity, with 10 SSR primers was studied. A total of 37 loci were amplified, of which 30 were polymorphic, indicating 81% polymorphism. The average number of loci per locus was 3.7, with a range of 2 to 8. The amplified product resulting from SSR primers ranged from 117 to 2191 bp.

Using SSR primers, the present study showed polymorphic information content (PIC) values ranging from 0.11 to 0.40. A maximum PIC value of 0.40 was obtained with SSR primers SMs06. The Parent had the greatest genetic distance 40%, with somaclone HSF-240-S-04. Among the somaclones, greatest genetic distance was found 73% between the HSF-240-S-01 and HSF-240-S-04, followed by 67% between HSF-240-S-01 and HSF-240-S-06. Khan et al. (2009a) determined a similarity coefficient matrix based on genetic distance according to Nei and Li (1978) to estimate the genetic divergence and relatedness among the somaclones developed, which ranged from 0.366 (P-100 versus P-98) to 0.951 (parent versus P-104).

A dendrogram was constructed using fifteen somaclones and a parent HSF-240, on the basis of genetic similarity matrix generated from the fifteen polymorphic primers (Figure 2). The clustering pattern showed that the somaclones formed four clusters. Cluster 1 consisted of two somaclone, HSF-240-S-10 and HSF-240-S-11. Similarly, cluster 2 consisted of three somaclones, HSF-240-S-03, HSF-240-S-13 and HSF-240-S-14, respectively. Somaclones, HSF-240-S-02, and HSF-240-S-05 were found to be halfway between cluster 2 and 3, respectively. Similarly cluster 3 consisted of four somaclones, HSF-240 (Parent), HSF-240-S-01, HSF-240-S-12 and HSF-240-S-15. Cluster 4 comprised five somaclones HSF-240-S-04, HSF-240-S-06, HSF-240-S-07, HSF-240-S-08 and HSF-240-S-09. Clustering and subclustering as depicted in the dendrogram certified the presence of variability at the DNA level.

Results of SSR analysis confirmed the generation of genetic variability through somaclonal variation in sugarcane genotype HSF-240. The data obtained in this study also confirmed the ability to use SSR markers for the determination and estimation of genetic similarity and dissimilarity among different sugarcane somaclones developed from the same parent. The information about genetic similarity will be helpful to create a population of genetically uniform somaclones or to select a variable somaclone with improved traits and increased productivity, with respect to the parent. SSR analysis may also be very useful in breeding for early identification of the most diverse clones in a large population. This study identified the usefulness of somaclonal variation in generating variability in sugarcane genotype HSF-240 and also the ability of SSR markers to determine the diversity among somaclones along with the parent. Very limited reports on the use of DNA markers for the estimation of somaclonal variation are available, and

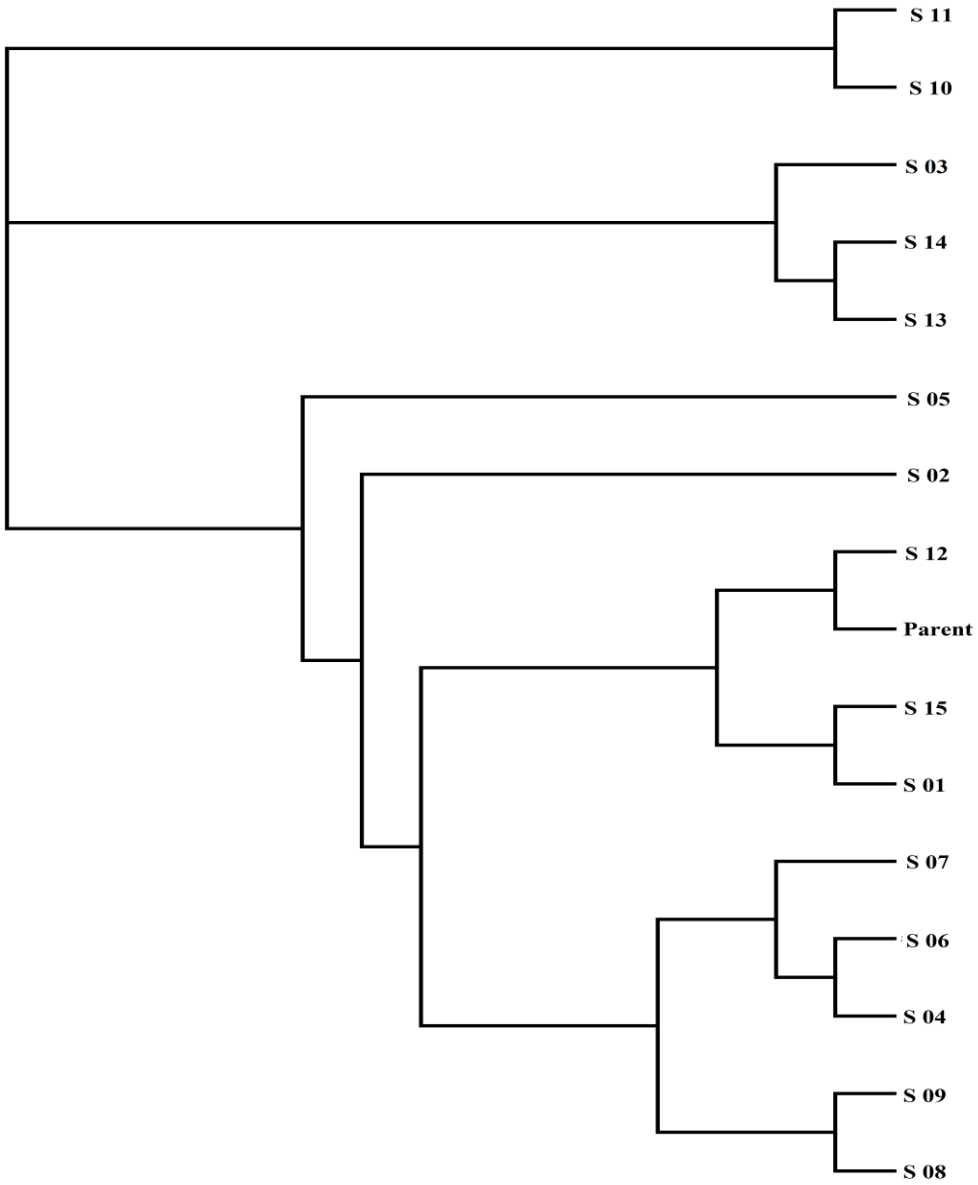

Figure 2. Dendrogram of somaclones and their parent HSF-240 as revealed by SSR markers. S-01 = HSF-240-S-01; S-02 = HSF-240-S-02; S-03 = HSF-240-S-03; S-04 = HSF-240-S-04; S-05 = HSF-240-S-05; S-06 = HSF-240-S-06; S-07 = HSF-240-S-07; S-08 = S97US297-S-08; S-09 = HSF-240-S-09; S-10 = HSF-240-S-10; S-11 = HSF-240-S-11; S-12 = HSF-240-S-12; S-13 = HSF-240-S-13; S-14 = HSF-240-S-14; S-15 = HSF-240-S-15; HSF-240 = parent.

hence, this study should provide a benchmark for further studies.

Conclusion

Somaclonal variation does occur in the process of regeneration under *in vitro* condition. Actually, genetic variation may have occured as a result of dedifferentiation of mature cells into callus stage, in the presence of plant growth regulators such as 2,4-D. During this process, DNA replication takes place and mutation of a single or multiple nucleotides must have occurred. On the other hand, the break down of DNA must have taken place and during repairing, variation occured. For the purpose of this study, explants were obtained from sugarcane infested with mosaic virus and some of the regenerated somaclones were proved to be healthy following ELISA test, which indicated that they are either resistant or disease free. There are two

possibilities to this; one, mutation must have occured on the locus containing gene for disease and two, the explants were disease free as result of which healthy somaclones were obtained. Healthy somaclones were taken for comparison with the parent plant in order to detect variation at DNA level using SSR molecular markers. There were differences in the genetic fingerprint of the parent plant and some of the somaclones, which means that some genetic variations might have occured during the tissue culture process. Plant tissue culture could be used in rejuvinating aging varieties like CO-1148 (Indian variety) that was banned in the country due to susceptibility to red rot. In the face of current global climate challenges, somaclonal variation could provide an oppurtunity for *in vitro* selection of genotypes that are resistant to conditions such as flood and extream weather, which are frequently cause by global climate changes.

Abbreviations: MS, Murashige and Skoog; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **NAA,** naphthalene acetic acid; **ELISA,** enzyme linked immunosorbent assay; **SCMV,** sugarcane mosaic virus; **SSR,** simple sequence repeat; **DAS-ELISA,** double antibody sandwich-ELISA.

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