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Assessment of Genetic Variability at Molecular Level in Sugarcane Somaclones Developed Through Tissue Culture

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Abstract: Three sugarcane accessions susceptible to sugarcane mosaic virus viz, HSF-240, S-2000-US-359 and S-2003-US-704 were evaluated for callogenesis and regeneration ability. For callogenesis five media combinations were used. The best callogenesis was observed in media containing M.S. + 3 mg/I 2, 4 D. The best regeneration was observed in media containing M.S basal media + Kinetin 0.5mg/l+0.5mg/l NAA. After shooting and rooting, plants were exposed to green house. ELISA test was performed and microsatellite or simple sequence repeats (SSR) markers were used to evaluate the genetic variation at DNA level between parents and somaclones of HSF-240 developed through tissue culture. Fifteen SSR primer pairs chosen randomly from the SSR primer collection were used to amplify DNA from plant material to assess the genetic variation between parents and somaclones developed. From present study it may be concluded that SSR genetic markers are best tool for investigation of genetic variation in sugarcane.

Key words: Callogenesis % Regeneration % Microsatellites % Simple sequence repeats

INTRODUCTION

Sugarcane (Saccharum officenarum) is a tropical, tall growing, monocotyledonous perennial grass, belonging to the genus Saccharum. Globally, sugarcane is an important source of commercial sugar accounting for almost two-thirds of world sugar production [1]. This crop is economically important because it is the main source of both sugar and alcohol and it accounts for two thirds of the world's sugar production [2]. Today, most of existing sugarcane varieties are susceptible to different diseases like sugarcane mosaic virus, red rot, pokha boeng etc that causes major reduction in yield. Therefore, efforts should be made to bring improvement in sugarcane varieties for disease resistance to overcome these losses. One of the strategies for protection sugarcane from diseases by using tissue culture techniques for screening of disease free genotypes. There are many problems in traditional breeding like a highly polyploid with chromosome numbers in somatic cells (2n) ranging from 80-124 in cultivated and 48- 150 in wild types [3].

Frequently aneuploid plant is impeded by complex genome and poor fertility but the most important is in-viable seed (fuzz) production due to the non availability of proper flowering conditions in many parts of the world especially in Asian countries because of its photo-thermal sensitivity. Other crucial conditions required to change vegetative to reproductive phase are temperature range of 25 - 33°C for 70 days, humidity 70 - 80% for 70 days, day Length 11.5 - 12.5 hrs for 70 days. The absence of any condition results in fuzz having very low viability percentage. For breeding purposes, fuzz is imported from abroad and only selection is done at sugarcane research institutes.

Biotechnology has been used as a tool to increase agricultural productivity in the context of sustainable agriculture [4]. These new techniques are not intended to replace conventional breeding methods, but rather to facilitate and supplement crop improvement. In sugarcane, variations can be created through tissue culture. These variation called somaclonal variation has emerged as an important parasexual tool for crop improvement. This technique has been developed as a breeding tool for improving the quality and production of vegetatively propagated crops such as sugarcane. Somaclones show variation for different parameters such as yield, sugar recovery, disease resistance, drought tolerance and maturity. Assessment of genetic variability in tissue culture-derived plants would be helpful for plant breeders to select appropriate material for their breeding program.

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Technological advances in molecular biology have contributed greatly to understanding the genetic diversity of plants. Molecular markers are used to measure the extent of variation at the genetic level, within and among populations. Random amplified

polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers are the most commonly used molecular techniques to study polymorphism in sugarcane. Use of molecular markers in the applied breeding programs can facilitate the appropriate choice of parents to make crosses. Molecular markers have been used for studying genetic diversity, cultivar identification and for marker-assisted selection (MAS) of major crops such as rice (*Oryza sativa*), maize (*Zea mays*), wheat (*Triticum aestivum*) and sugarcane (*Saccharum officinarum*). Moreover, molecular markers such as RFLP, RAPD and SSR have recently shown excellent potential in assisting selection of quantitative trait loci [5].

MATERIALS AND METHODS

Three sugarcane accessions which were susceptible to sugarcane mosaic virus viz, HSF-240, S-2000-US-359 and S-2003-US-704 used for tissue culture study. Two kinds of explant sources were used from the top portion of the plant.

- Leaf explants were taken from innermost 1-5 leaves and were 2-3 mm in length.
- Pith explant was taken from 1-5 apical internodes [6].

Media Used for Callogenesis: For callogenesis five media combinations were tried using 10 replications for each.

C1= M.S. + 1 mg/I 2, 4 D

C2= M.S. + 2 mg/I 2, 4 D

C3= M.S. + 3 mg/I 2, 4 D

C4= M.S. + 4 mg/I 2, 4 D

C5= M.S. + 5 mg/I 2, 4 D

Each cultured tube was kept in the dark at 28±2°C for the first two weeks and then was shifted under continuous florescent light of 2000 to 2500 lux intensity at the same temperature for the next two months.

Media for regeneration/ organogenesis.

For regeneration three media combinations were tried using 10 replications for each given under.

RM1= M.S basal media + 1mg/l Kinetin

RM2=M.S basal media + Kn 0.5mg/l+0.5mg/l NAA

RM2 = M.S basal media + 480 mg/l Casein hydrolysate+ 1mg/l Kinetin

Culture for Regeneration: The callus developed on different treatments of 2, 4-D were subjected to regeneration in such a way that 1-2 g of callus was inoculated to each tube having 10ml medium. The test tubes were transferred to incubation room having continuous florescent light of 2000 to 2500 lux intensity with a dark period of eight hours at the temperature of 28±2°C.

Rooting of the Shoots: When the shoots attained the height of 3-4 inches in the form of bunch. They were taken from the test tube aseptically, separated into the single plantlets and transferred to the half strength M.S. medium for root formation. Tubes kept under same conditions as that for shoot formation.

Acclimatization in the Glass House and Field Transfer: 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.

Data Recording

Callogenesis: Data on the following characteristics of callus will be recorded.

Contamination Percentage: The contaminated test tubes were replaced to assure accuracy.

Callus Proliferation (Callus Scores): + + +, ++, +. - were scored as best, better, good and no callus.

Regeneration: The developed callus will be preceded for regeneration and response will be determined by regeneration %age.

Statistical Analysis: The data recorded for callogenesis and regeneration were analyzed statistically using completely randomized design [7]. The differences among genotypes, explant sources, 2, 4-D levels and their interaction were compared by Duncan's Multiple Range Test [7].

Sugarcane Mosaic Virus Screening Through ELISA: The ELISA test was performed for the detection and screening of sugarcane mosaic virus disease in somaclones that were produced through tissue culture.

Data Recording: The data recording for Sugarcane Mosaic Virus resistance.

- Number of virus positive plants.
- Number of virus negative plants

Molecular Studies (SSR): Plant material: Sugarcane variety HSF-240 somaclones produced through tissue culture were used for comparison with parents at DNA level for sugarcane mosaic virus. Fresh young leaves were collected from the field experiment for isolation of the DNA using DNA isolation Kit.

PCR Reaction: Fifteen SSR Primers were used to amplify the DNA. The PCR conditions for amplification were almost same for all the primers with little variation in the concentration of DNA. The following concentrations/amount of PCR reagents were used to make the final reaction mixture of 20 µL.

Reagents	1X (µL)
d_3H_2O	4.8
l0X PCR buffer$_+$ $(NH_4)_2S0_4$	2.0
$MgCl_2$ (25mM)	4.0
dNTP's (2.5mM)	3.0
Taq (5U/ µL)	0.2
Both forward and reverse Primers	3.0 (1.5+1.5)
DNA (30ng/ µL)	3.0
Total volume	20

Data Analysis: Data was scored from good quality photographs of each amplification reaction. The bands were counted by starting from top of the lanes to their bottom. All visible and unambiguously scorable fragments amplified by the primers were scored under the heading of total scoreabIe fragments. Amplification profiles of all the plants of sugarcane were compared with each other and bands of DNA fragments were scored as present (1) or absent (0). The data of the primers were used to estimate the dissimilarity on the basis of number of unshared amplified products. Dissimilarity matrix was generated using Nei's Measures of Genetic Identity and Genetic distance [8]. In addition population relationships were inferred using the unweighted pair group of arithmetic means (UPGMA) clustering method using popgen software (version 3.5).

RESULTS AND DISCUSSIONS

Tissue Culture Study: The present study was undertaken to check the response of three sugarcane accessions viz., HSF-240, S-2000-US-359 and S-2003-US-704 for callogenesis, organogenesis and to check the existence of somaclonal variations by SSR marker.

Callogenesis Studies

Callus Initiation Frequency: The interaction of genotypes x auxin levels, auxin levels x explant and interaction of all three factors i.e. genotypes x auxin levels x explant was non significant in the study.

Effects of Genotypes on CIF (Callus Initiation Frequency): Significant differences ($p<0.05$) were observed among the genotypes using DMR test. The genotypes showed high value of callus score ranging 2.92 to 2.64. Genotype S-2003-US-704 was 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 2.83 and 2.64 respectively. The genotype HSF-240 appeared to be least callus producer with the average of 2.64. These results revealed that callusing response is under the influenced of genotype. Gandonou *et al*. (2005a) determined the response of three sugarcane varieties and found that callus induction ability is genotypes dependent. Burner[9] also studied the response of three sugarcane cultivars to callus induction using mature caryopses as explants and reached at the similar conclusions. Same results have also been reported in other members of the graminea family like on rice[10,11].

Effects of Auxin (2, 4-D) Levels on CIF (Callus Initiation Frequency): Mean of the doses was analyzed using DMR test which showed that the D3 medium having 3mg/l of 2, 4-D with an average of 3.2 callus score was the best callus producer. Callogenesis response seen at D2 and D4 media were very less statistically different from each other.

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

Source	D.F	S.S	M.S	F-VALUE
Genotypes (G)	2	4.087	2.043	3.5388*
2,4-Dlevels (D)	4	20.113	5.028	8.7085**
G x D	8	0.847	0.106	0.1833^{ns}
Explant (Exp.)	1	52.083	52.083	90.2021**
G x Exp.	2	5.087	2.543	4.4047*
D x Exp.	4	1.900	0.475	0.8226^{ns}
G x D x 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: 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 x RM	4	221.667	55.417	0.8544^{ns}
Explant (Exp.)	1	9606.806	9606.806	148.1135**
G x Exp.	2	236.944	118.472	1.8266^{ns}
RM x Exp.	2	36.944	18.472	0.2848^{ns}
G x RM x Exp.	4	75.556	18.889	0.2912^{ns}
Error	162	10507.500	64.861	
Total	179	25863.750		

Also media D1 and D5 were statistically similar with each other. However, there performance was different than D3 media. The callus score of five media levels are 2.55, 2.95, 3.2, 2.78 and 2.5 respectively. The poorest response was shown at D5 media (1mg/l) with average callus score of 2.5.

It can be deduced from the results that concentration of 2, 4-D from 1-5 mg/I was considered to be the good for callus induction with best performance at 3 mg/l.These results are consistent with Mamun [12] who studied invitro micro propagation of two sugarcane varieties and found that 3 mg/I of 2, 4-D produced maximum callus.

Effects of Explant on CIF (Callus Initiation Frequency): Both the explants showed good response to callus production but the leaf explant performed better with average callus score of 3.21 per test tube which is statistically different from pith explant (2.38). Thus it can be inferred that leaf explant is a good source of callus induction than pith explant. Thus is primarily due to excretion of phenols which turned the whole pith brown, hindering proliferation. These results coincide with work of Niaz and Quraishi [13] that used leaf, lateral bud and pith as an explant and found leaf as the best explant source. Barbe *et al.* [14] reported that leaf tissue showed better callus induction on modified M.S. medium supplemented with 2, 4-D. Taylor *et al.* [15] established callus on leaf explant tissue taken from a range of 18 genetically diverse sugarcane cultivars by culture on MS medium containing 2, 4-D. Mamun *et al.* [12] also used leaf explant for callus induction in two sugarcane varieties.

Interactive Effects of Genotype X Explant on CIF (Callus Initiation Frequency): Using leaf explant, maximum callus was produced by S-2003-US-704 with an average callus score of 3.52 per test tube. However, genotypes S-2000-US-359 and HSF-240 were statistically same with an average of callus score 3.14 and 2.98 per test tube with leaf explant. These results are opposed to the findings of Niaz and Quraishi [13] who reported that pith explant showed better performance than leaf explant. All other interactions between Genotype x 2,4-D level, 2,4-D level x Explant source and Genotype x 2,4-D level x Explant source were found non significant for callogenesis in this study.

Organogenesis Studies: Callus organogenesis or regeneration is also dependent on a number of factors. Regeneration response from callus was studied under the effects of three factors i.e., genotype, regeneration media and explants and their interactions also have been observed for regeneration response.

The analysis of variance table for percentage regeneration response showed that there were significant differences among genotypes, regeneration media and explant sources. While the interactions between regeneration media x explant sources (RM x Exp), genotype x regeneration media (G x RM), genotype x explant sources (G x Exp) and genotype x regeneration media x explnt sources (G x RM x 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 percent of 41.67. The minimum regeneration response was observed in S-2000-US-359 with an average percent of 34. Genotypes, S-2003-US-704 and S-2000-US-359 were statistically different in regeneration behavior from HSF-240 with an average percent of 41.08 and 34 respectively. On the basis of HSF-240 performance; it can be concluded that this genotype was observed to be more responsive to regeneration response but less responsive to callus production showing that callogenesis and organogenesis are independent from each other. A genotype less responsive to callus production might be better in regeneration and vice versa. So we can say that successful regeneration is under the control of genotypic behavior. The similar results reported by Rahman *et al*. [6] who also observed different genotypic behavior towards organogenesis. Gill *et al*. [16] studied that factors affecting, somatic embryogenesis and subsequent plant regeneration *in vitro* sugarcane cultures were highly genotype specific, significant differences were observed between genotypes for plant regeneration ability in sugarcane indicating that it is a genotypic dependent trait. (Gandonou *et al*. [17].

The Effect of Regeneration Media on Organogenesis: As the analysis of variance showed that the regeneration media, RM1 (M. S. basal media + 1 mg/l Kinetin), RM2 (M. S. basal media + 0.5mg/l kinetin + 0.5mg/l Naphthalene acetic acid) and RM3 (M. S. basal media supplemented with 480 mg/l casein hydrolysate + 0.5 mg/l kinetin) were statistically different in response of regeneration. The following Duncan Multiple Range test table showed significant difference between three regeneration media and the RM2 showed better regeneration response with average percent of 44.42 as compared to RM1 and RM3 with average percent of 37.67 and 34.67 respectively, On the basis of above results, it can be concluded that high level of cytokinin and low level of Auxin is essential for regeneration of shoots in sugarcane leaf sheath callus. 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 regeneration from sugarcane callus. Niaz and Quraishi [13] also determined that media containing Kinetin, addition of NAA significantly increased the number of shoot production. However no shoots were observed on media containing Kinetin (2.0 mg/l) with and without NAA which confirmed that high concentration of growth hormones may, hinder the regeneration ability. (Siddiqui *et al*. [18].

The Influence of Explant on Regeneration: The leaf proved to be the best explant for regeneration of plantlets from callus with an average percent of 46.22 than pith with an average percent of 31.61. These results were according to the expectations. The leaf being soft in nature produced soft, granular and embryogenic callus in nature thus showed more regeneration response while hard callus of pith produced from mature part showed less number of plantlets. These results were consistent with the findings of Rahman *et al*. [6] and Shahid *et al.* [19]. It was also found that regeneration was dependent upon the concentration of growth hormones and type of explants used. All the interactions between genotype x RM level, genotype x explant source, RM level x explant source and genotype x RM level x explant source were found non significant. The regenerated plants when attained the height of 4 to 5 inches were shifted to half strength M.S. rooting media. When they established roots were transferred to pots for hardening and later shifted to field. At the age of three months in the field further tests (SSR marker) were performed.

Sugarcane Mosaic Virus Screening: Total 26 parent plants and 64 somaclones of HSF-240 were selected for screening of virus through Das- ELISA test. Four parent plants out of 26 showed negative reaction to the virus. Ten somaclones showed positive reaction to the disease and were declared as susceptible. Nine plants of

somaclones showed mild reaction to virus showing them tolerant. Fourty five plants showed negative reaction declaring them resistant against virus.

The experiment was conducted only in one ELISA plate due to the shortage of chemicals in the laboratory and for the ease of handling the experiment. In the plate a row of six wells was donated to the control treatments, two of them for positive control, two for negative control of the sugarcane mosaic virus and remaining for the extraction buffer. Positive control always showed positive reaction, negative control showed negative reaction and same did by the buffer. Proving that the experiment was 100% successful. The plants for the confirmation of virus elimination were selected randomly from the regenerated plants of Sugarcane accession HSF-240 at the age of three months after hardening. The ELISA plate was subjected to test the disease and each sample in the plate represented a separate plant. The result were taken by ELISA reader. The results were quite according to expectations because only ten somaclones out of 64 showed positive reaction and 22 out of 26 parent plants showed positive reaction. Parent plant samples were selected from the field for comparison of the test. While the control of positive, negative and buffer on all the six wells showed results as were supposed to prove that the experiment is successful. The results are in confirmation of Carnot [20]) who reported the production of virus free clones of sugarcane through callus culture.

Polymorphism as Revealed by SSRs: SSR sequence is spreaded throughout the whole genome, highly polymorphic, co-dominant and requires a specific repeat DNA sequence as primer. PCR reactions for SSRs is run in the presence of forward and reverse primers that anneal at the 5` and 3` ends of the template DNA, respectively. All the conditions were optimized and found to be same in the case of concentrations of $MgCl_2$, Taq DNA polymerase and dNTPs. The concentration of template DNA, annealing temperature and concentration of primers were separately optimized for best possible results. Gelatin was not used in SSR reaction mixture.

The optimum template concentration was determined to be 30ng/µL and 3µL of template was used per 20µL tube. 3µL concentrations of primers both reverse and forward were used per 20µL reaction mixture. Annealing temperature was optimized separately for each primer.

Fifteen SSR primer pairs chosen randomly from the SSR primer collection were used to detect polymorphism in 10 sugarcane soaclones of HSF-240 as indicated before. The PCR product was observed by running on agrose gel to study polymorphism. All the primers were monomorphic except three primers those were polymorphic. Among them primer no.10 produced one band per primer and parent fragment size was different from all the somaclones. Primer no.6 produced five fragments per primer. One somaclone and parent showed polymorphic bands. Primer no. 3 produced two bands and

	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

Figure: Layout for samples loaded in ELISA plate for ELISA test

	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

Figure: Scores taken through ELISA reader for ELISA test

Scoring values:

Value less than 0.205 = Resistant

Value between 0.25 and 0.205= Tolerant

Value more than 0.25= Susceptible

Table 3: polymorphism table showing information about primers

S.No.	Primer No.	Annealing Temp. (°C)	Band size	Total Bands	Polymorphic Bands	Primer sequence
1	3	59	400	2		CATCTGCTCCCTCTTCCT
						CTCTGGCGGCTTGGTCCTG
2	6	55	600-1200	5	1	GACTCCTGTCACCGTCTTC
						ATACTTCAACCGTCTCCTCC
3	7	54.5	389-1250	6		CTAAGCAACACAGGAAAG
						AGCAACAGCAGAGAGCAG
4	8	53	383	2		CTGACTAAGGAGGAAGTGGAG
						GACGACGATAGATGAAACA
5	9	59.5	400	1		GAGCCGCAAGGAAGCGAC
						CATACAAGCAGCAAGGATAG
6	10	55	497	1	1	CTCTCTTCTCGTCTCCTCATT
						GTCCTTCTTCTTCTCGTGGT
7	11	57	400	1		ACACGCATCGCAAGAAGG
						AAGAACACTCAACAGAAGCAC
8	12	53.5	400-600	2		AAATGTCTTCGCACTAACC
						AAGGAGATGCTGATGGAGA
9	16	55	260-400	2		CCCAGAGGACAAGGAACT
						GTAATGGAAGGAAGCAACTGA
10	45	54	400	1	1	CTTCCCTCCCTCTCCTCT
						AGCCTTCTACTAAACTATCTGCT
11	46	53.5	400	2		GTGAGTGAGACCAGACCAG
						CCGTGCTGTAGTTGTTGTAG
12	47	54	400	1		ATACGCTACTCTGAATCCCAC
						CAATCACTATGTAAGGCAACA
13	48	55	400	1		ACTCCTCTTCCTCTTCCTCTT
						GTTCTTCGGGTCATCTGG
14	49	60.5	250-400	2		ACTCGGTCATCTCATCACTC
						GTTCTTCGGGTCATCTGG
15	50	55	400-500	2		ACGGTGAGCGAGGACTAC
						CTTGGGTGGCATCAGGAA
Total				31	3	

a somaclone produced polymorphic band. All the primers were found to give reproducible bands. 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 primer used were of the size ranging from 200-750 bp. Reactions were duplicated to check the consistency of the amplified products. Only easily resolved bright DNA bands were scored.

The tissue culture induced mutations are referred to as Somaclonal variations. The rate of mutation is quite high; in one study with rice more than 70% of the plants regenerated from tissue cultures differed in one or more phenotypic characteristics. It has been reported for both qualitative and quantitative plant characteristics. So it was concluded from the study that Somaclonal variation can be produced from tissue culture techniques. Actually variation are produced when mature cells are dedifferentiated to callus stage using different hormones like 2, 4-D. During these stages DNA replication takes place and mutation of single or more nucleotides may takes place or may be the break down of DNA take place and during repairing variation produced. During our experiment we used the explants from sugarcane mosaic virus diseased field and found some somaclones healthy by using ELISA test that were resistant or disease free. So two possibilities are there one is that mutation came on to the locus containing gene for disease and second is that explant used from diseased field were disease free that is why they produced healthy somaclones. Then healthy somaclones were taken and SSR molecular markers were used to asses' variation at DNA level between parents and healthy somaclones. There were some somaclones that were differed from parent fingerprints. It means that some variation is there and that the tissue cultural techniques can be used to exploit variations. Proposed research wok was also very helpful to rejuvenilize the extinct varieties like CO-1148 (Indian variety) that was banned in country due to more susceptible against a

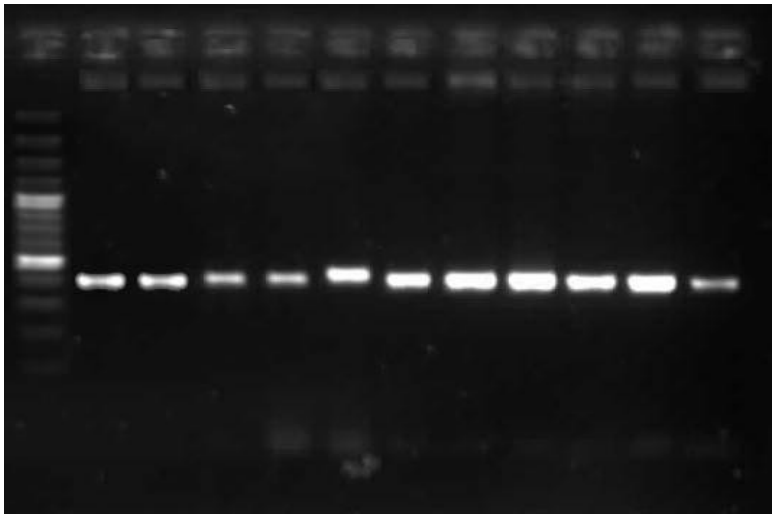

Fig. 1: Result of electrophoresis of SSR product of 10 somaclones and a parent using Sugarcane Microsatellite primer no.03

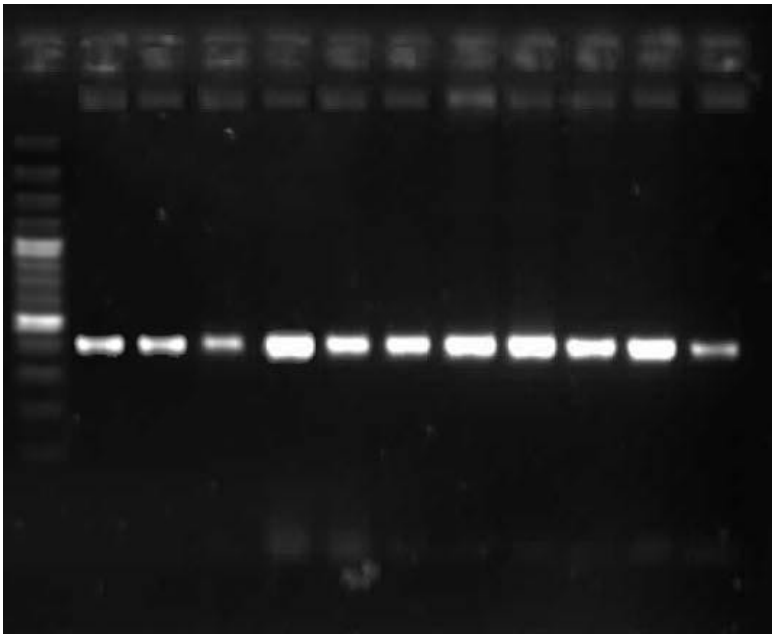

Fig. 2: Result of electrophoresis of SSR product of 10 somaclones and a parent using Sugarcane Microsatellite primer no.12.

disease called red rot. Also variation can be produced in crops that are propagated asexually using proposed method. This work also will be helpful for the screening of crop species that are more adaptive to climatic conditions and through Somaclonal variation technique the extinct crop species will be made more resistant and more adaptive to adverse conditions. This effort will also be helpful to improve some value able traits in different crop species in order to face the new challenges of global climatic changes because the use of somaclonal variation is likely to be of most interest where insufficient natural variation is available to provide the level sought for the trait; or existing variation is not easily used in breeding. So it is quite evident from study that tissue culture induced mutations are source of genetic variability for use in plant breeding programs. As with radiations and chemical induced mutations, a large proportion of the mutations are accompanied by deleterious effects.

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