

SOMACLONAL VARIATIONS FOR RED ROT AND SUGARCANE MOSAIC VIRUS RESISTANCE AND CANDIDATE GENES INTEGRITY ASSESSMENT IN SOMACLONES OF SELECTED SUGARCANE VARIETIES (*Saccharum officinarum* L.)

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Sugarcane is a complex allopolyploid hybrid with erratic flowering due to various geo-climatic factors hence deliberate hybridization for varietal improvement is a dilemma in most of the countries where sugarcane is cultivated. Somaclonal variations is an alternate strategy used for development of disease resistance and improvement in yield attributes, but point mutation in candidate genes is questionable. The aim of this study was to develop somaclones resistant to red rot and *Sugarcane mosaic virus* (SCMV) in obsolete sugarcane varieties and their genetic integrity assessment in candidate gene's exon regions. A total of 581 somaclones were developed, of which 201 survived after hardening and 121 after transplantation and screened against red rot and SCMV. Only 10% somaclones were found resistant to red rot in susceptible and moderately susceptible varieties while SCMV concentration was recorded less than negative control in somaclones. Four candidate genes namely; catalase (*CAT1*), sucrose phosphate synthase (*SPS*), gibberellin 2-oxidase 4 (*GA 2-oxidase 4*) and tillering branched 1 (*TBI*) were evaluated for possible SNPs in their exon regions. Sequences of five representative somaclones were aligned with their parental clones and no possible SNP changes were observed. Somaclonal variations was a good source of genetic improvement of sugarcane for disease resistance with no SNP changes in candidate genes.

Keywords: Sugarcane, somaclonal variations, red rot, SCMV, candidate genes, genetic integrity.

INTRODUCTION

Sugarcane is a highly complex 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). About 173 million tons of sugar is consumed annually with an average of 24 kg per capita consumption on a world-wide basis (Statista, 2016). Its demand is continuously increasing with the increase of population but continuous decrease in yield has been observed for the last six years. During 2008 total world average cane yield was 717 million metric tons but it reduced to 699 million metric tons (FAOSTAT., 2016). Apart from biotic and abiotic stresses, development of new varieties is a dilemma due to absence of flowering for hybridization. Some common factors that cause yield decline in clonally propagated plant species include; long term use of vegetative sets as a seed for next crop that lead to clonal degeneration due to impact of microbial and viral infestations and their secondary metabolites that lead to fatal mutational changed in clones (Kumar, 2014 and Brown *et al.*, 2008).

Genetic improvement of sugarcane through conventional breeding is hindered by low fertility. Biotechnological tools

such as genetic transformation and induction of somaclonal variations are mostly employed in vegetative propagated crops. Genetic transformation can improve only qualitative or oligo-genic traits. *In vitro* culture techniques for somaclonal variation induction and induced mutation are being employed to create the new genetic variability for the selection of desired genotypes (Yasmin *et al.*, 2011). Somaclonal variation can provide an alternative for the improvement of existing genotypes (Shahid *et al.*, 2011). Clones are exact copies of the maternal genotype but clones obtained through tissue culture are not exact copies of the original plant used to initiate the culture. Such variation, which generated not from meiosis or normal sexual process but from the culture of somatic tissue, is known as somaclonal variation. Various factors responsible for somaclonal variation include; karyotype changes, cryptic changes associated with chromosome rearrangement, transposable elements, somatic gene rearrangements, gene amplification and depletion, somatic crossing over and sister-chromatid exchanges. Addition of auxin 2, 4-D in culture medium enhances the probabilities of somaclonal variation induction (Acquaah, 2012). Improvement of crops through somaclonal variation was first described by Heinz and Mee (1971). Phenotypic

variations among somaclones have been used as potential tools for crop improvement. Such variations associated with changes in chromosome number have led the breeders to exploit it in crop improvement programs (Rakesh *et al.*, 2011).

First *in vitro* raised somaclone of sugarcane, resistant to Fiji disease was reported by Heinz (1971). However, several studies have reported the improvement of commercially important crops via somaclonal variation. Gao *et al.* (2009) elaborated that somaclonal variation can be heritable in plant tissues raised *in vitro* and provides window of opportunity for plant breeders to produce novel variants in sugarcane. Various authors (Rastogi *et al.*, 2015; Seema *et al.*, 2014; Shahid *et al.*, 2011) reported the successful utilization of somaclonal variation in sugarcane for genetic improvement of agronomic traits. Rastogi *et al.* (2015) elaborated that somaclonal variation was successfully used for genetic improvement in sugarcane against diseases. Red rot (*Colletotrichum falcatum*) is one of the most devastating ascomycetous fungal groups, first reported from India during 1902 that infects sugarcane stem and causes huge yield losses in terms of cane yield (Viswanathan *et al.*, 2016). Sugarcane mosaic virus is also very devastating sugarcane disease worldwide and causes serious yield losses in susceptible varieties. Somaclonal variation is a rapid and robust genetic tool to improve the resistance mechanism or disease escape in sugarcane against red rot and sugarcane mosaic virus. Acquaah (2012), Kumar *et al.* (2012) and Singh *et al.* (2000) reported the development of somaclones in sugarcane with improved agronomic traits and resistance against red rot. Gaur *et al.* (2002), Oropeza and Garcia (1996) and Smiullah *et al.* (2012) reported development of sugarcane mosaic virus free somaclones.

Induction of somaclonal variation is a hit and trial method, along with beneficial mutations, there are equal chances of being conceiving deleterious nucleotide changes in important growth and development candidate genes. Genetic integrity of candidate genes is important to perform important metabolic pathways for normal growth and development processes. Various molecular techniques are routinely utilized for the detection of genetic integrity of somaclones like randomly amplified polymorphic DNA (RAPD), simple sequence repeats (SSR), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP) and methylation-sensitive amplified

polymorphism (MSAP) (Coste *et al.*, 2015). Several reports are available for the genetic fidelity of somaclones by using molecular markers. Jagesh *et al.* (2013) and Peyvandi *et al.* (2013) reported the utilization of ISSR and SSR marker to check the genetic stability of olive and potato somaclones respectively. Only few reports are available on nucleotide sequence integrity of candidate genes in somaclones. Coste *et al.* (2015) reported the integrity of lycopene gene's nucleotide sequence in tomato somaclones and detected a single nucleotide change. Recent trend is to use single nucleotide polymorphism (SNP) based markers that largely replace other marker types in several species, because SNPs markers are common in the genome, both within and between genes (Bundock *et al.*, 2009).

Sugarcane (*Saccharum officinarum* L.) is a complex hybrid exhibiting chromosomes $2n=8X=80$ making its genome more sophisticated. Various efforts have been made to sequence sugarcane genome but multiple alleles of same gene and haplotype sequences due to repetition of same chromosome sets make genome sequencing complicated. Its high ploidy level and heterozygous nature is challenging for modern day high throughput short-read genome sequence technologies. SNPs discovery in genes has been done either by direct sequencing (Bundock *et al.*, 2006; Somers *et al.*, 2003) or on the basis of primer design for re-sequencing the genomic regions (Choi *et al.*, 2007; Rostoks *et al.*, 2005). Gene search is possible by sequence annotation of *Sorghum bicolor* genome, which is closest diploid relative of sugarcane (*S. spontaneum* L.) that served as a key source of sugarcane genomic studies (Zhang *et al.*, 2013). However, PCR amplified 1kb genomic DNA fragments can also be sequenced by using Sanger sequencing (Rizzo and Buck, 2012). Aims of this study were to develop somaclones resistant against red rot and free from sugarcane mosaic virus and their SNPs based stability assessment in candidate genes.

MATERIAL AND METHODS

Plant material: Plant material was collected from sugarcane field germplasm repository of Sugarcane Research Institute, Ayub Agricultural Research Institute (AARI), Faisalabad. Six

Table 1. Sugarcane genotypes used in the experiment their names, codes used in the paper, their nativity and disease status.

Genotypes/ varieties	Codes	Nativity	Disease susceptibility	Severity
S-03-SP-93	G1	Sao Paulo, Brazil	Red rot, SCMV	S
S-05-US-54	G2	Canal Point, USA	Red rot, SCMV	MS
S-03-US-694	G3	Canal Point, USA	Red rot, SCMV	MS
S-06-US-300	G4	Canal Point, USA	Red rot, SCMV	MS
HSF-240	G5	Sindh, Pakistan	Red rot, SCMV	MR to R
SPF-213	G6	Sao Paulo, Brazil	Red rot, SCMV	MR to R

obsolete sugarcane varieties were selected and used in this experiment (Table 1).

Callus induction: Experiment was conducted at Biotechnology Research Institute, AARI Faisalabad. Explant were prepared from meristem tissues of top young leaves and cultured in sterilized test tubes by using MS media (Murashige and Skoog, 1962). Composition of culture media contained MS media (Phytochemicals™) 4.43 g, Gel Grow 1.75 g (Phytochemicals™), 30 g sucrose, Iron 10 ml (100 g/L), 2,4-D (100 g/L) 1 ml/L, 3 ml/L, 5 ml/L and control without 2,4-D (2,4-Dichlorophenoxyacetic acid) separately, d₃H₂O (deionized double distilled) water up to 1 L and pH 5.75. Sets of twenty test tubes for each variety's explants containing different concentration levels of 2,4-D (0 ml/L, 1ml/L, 3ml/L 5ml/L and 7ml/L) were wrapped with paper and kept at dark for 19 days at incubation room contained 27°C temperature. All varieties responded to callus induction at 2,4-D level 3ml/L (Fig. 1), calli were sub-cultured twice with the interval of three weeks for the induction of somaclonal variations.

Regeneration, proliferation and field transplantation: Embryogenic calli were transferred to test tubes containing regeneration media. Regeneration had composition of; MS media (Phytochemicals™) 4.43 g, Gel Grow (Phytochemicals™) 1.75 g, 30 g sucrose, Iron 10 ml (100 g/L), 1 ml/L IBA (IBA 100 g/L), d₃H₂O up to 1 L and pH 5.75. Then test tubes were kept in incubation room at temperature 27°C and light intensity 300 Cd. After 4 weeks regenerated tissues were transferred to shooting media supplemented with Kinetin (100 g/L) 1ml/L media for shoot initiation and multiplication under similar conditions as for regeneration and proliferation. Regenerated plantlets were then transferred to rooting media composed of half strength

MS media 2.21g, Iron 5 ml/L (100 g/L), sucrose 30g, Gel Grow 1.75 g, NAA 1ml/L (NAA 100 g/L), d₃H₂O up to 1 L and pH 5.75. Roots were initiated within five to six weeks. After root initiation 581 plantlets raised from six different genotypes were transferred to polythene bags filled with canal silt and sand and kept at glass house for hardening. After six to eight weeks of hardening 201 survived somaclones were transplanted to the field at containment area by maintaining planting distance 50 cm and line spacing of 0.5 m. Parental clone of each variety from which somaclones were generated were also planted for comparison.

Screening of somaclones against SCMV: Serological examination of somaclones along with their parental clones was conducted against sugarcane mosaic virus (SCMV) by using double antibody sandwich, enzyme linked immunosorbent assay (DAS-ELISA) according to procedure described by Clark and Adam (1977). Reagents kit of DAS-ELISA, a product of Martin-Luther University, Germany with trade mark BIOREBA™ was used for the detection of sugarcane mosaic virus (SCMV). Microtiter plate containing 96 wells was used with three biological and two technical replicates for each sample and reading was taken at optical density (O.D) 405 nm on ELISA reader machine. Data were collected and exported to an Excel sheet. Mean of replicates was computed and plotted on graph along with -ve and +ve comparisons.

Screening of somaclones against red rot (*Colletotrichum falcatum*): A total of 134 somaclones from six varieties were evaluated against red rot in the field by inoculation of red rot suspension culture. Potato dextrose agar (PDA) medium containing 20 g agar, 20 g dextrose and d₃H₂O up to 1 liter was used to grow a culture of red rot for inoculation. Inoculum was collected from infected plants and multiplied over petri

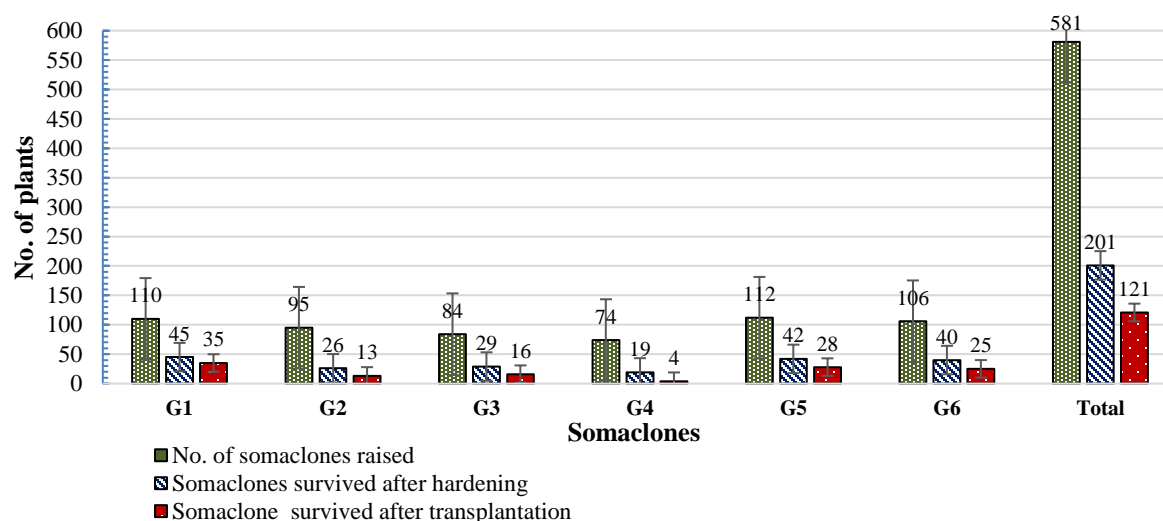


Figure 1. Number of somaclones raised, number of somaclones survived after hardening and number of somaclones survived after transplantation from six different genotypes.

plate containing PDA medium. After one week of infection spores of red rot pathogen were collected and spread over the PDA culture medium for regeneration of red rot spores in large quantity. Culture was suspended in 1 L distilled water and then purified in a flask. Inoculum containing 1ml of red rot cell suspension was injected via syringe into 6 month old plants in the central stem of each plant in the middle of the internode. Scoring of plant was done according to the 0-9 scale given by Srinivasan and Bhat (1961). Scale contained scoring (0-2.0) Resistant, (2.1-4.0) moderately resistant, (4.1-6.0) moderately susceptible, (6.1-8.0) susceptible and (above 8.0) highly susceptible.

Selection of candidate genes: Exon regions for four candidate genes i.e. *CAT1*, *SPS*, *GA 2-oxidase 4*, and *TBI* were selected.

Database search and annotation of candidate genes in sorghum and maize: Sequences of *CAT1*, *SPS* and *GA 2-oxidase 4* from sorghum (*Sorghum bicolor* L.) and *TBI* from Maize sub spp. *Teosinte* were used to identify the exon regions of genes in sugarcane. BLASTn search at NCBI (<http://www.ncbi.nlm.nih.gov>) and Phytozome 9.1 (<http://www.phytozome.net>) were performed to find out similar sequences in sorghum genome against full length CDS sequences and ESTs sequences from sugarcane at NCBI database.

Verification of candidate genes in sugarcane: From genomic sequences of sorghum and maize selected candidate genes intron and exon boundaries were identified and primers were designed from selected larger exon region by using Primer 3.0 software and NCBI primer BLAST. Genomic DNA of parental lines and their somaclones was isolated at Genomic Lab of Agricultural Biotechnology Research Institute Faisalabad, Pakistan according to the protocol described by Doyle (1991) and taken to CILR, SAFS University of Queensland Australia in pallet form contained in centrifuge tubes where DNA was resuspended in ultra-pure water then Qubit quantification was done for DNA concentration estimation. PCR reactions were conducted in a total volume of 25 µl containing 1.5 µl of 20 ng template DNA, 0.5 µl of 10 mM forward and reverse primer each, Mango *Taq*TM polymerase 0.5 µl (1U/µl), 5 µl reaction buffer provided with Mango *Taq*TM polymerase kit, 50 mM MgCl₂ 2 µl, 25 mM dNTPs 0.5 µl, ultra-pure water 14.5 µl. PCR conditions were; 5 min at 95°C followed by 35 cycles of 30 sec at 94°C, 30 sec annealing temperature (50-68°C), 45 sec at 72°C for extension and final extension after 35 cycle at 72°C for 6 min.

Verification of PCR amplified products: Amplified PCR products were run on 2% agarose (Sigma AldrichTM) gel. Two different molecular size DNA ladders i.e. 100bp and 1kb (Thermo ScientificTM) were also run alongside PCR products to compare the band size with desired known molecular weight for actual product identification. Gradient PCR was conducted with a range of temperatures to find out appropriate

annealing temperature of primer pairs for specific PCR products.

Authentication of amplified products with reference sequences: After identification of desirable PCR fragment by comparison with molecular weight marker desired bands were cut from agarose gel and eluted out by exposing gel at low index UV trans-illuminator in dark room. PCR products were then purified using Silica gel PCR product clean-up system as described by Boyle *et al.* (1995).

Quantification of purified PCR products and sequencing: Purified products were quantified by using 1 µl purified product at QubitTM fluorometry method. To reconfirm the actual band size, quality and single allele of purified PCR product 5 µl purified samples were again run on 2% agarose gel along with DNA ladders. Samples for sequencing were prepared from purified PCR products with bright single band on agarose gel. Sequencing was done according to the standard protocols of AGRF (Australian Genome Research Facility) Sanger Sequencing. Sequencing samples containing 1 µl of forward primer (10 mM) for forward direction sequencing, ultra-pure water and purified PCR sample after calculation with 12 µl final volume were put into 96 well plates and submitted to AGRF for sequencing.

Alignment of sequenced reads with reference sequences for conformation: Resulting sequenced reads were compared with reference sequences of sorghum and maize by doing pairwise sequence alignment using Geneious 6.1 software. Reads matched with reference sequences were confirmed and primers for these sequences were used for PCR amplification of our somaclone genetic integrity in comparisons with their parental clone's analysis. Sequence alignments of candidate genes exon region of parental and somaclonal lines were done by using Geneious® 6.0.6 software (Biomatters Ltd.).

RESULTS

Development of somaclones: Present study was conducted to develop somaclones from 6 sugarcane obsolete varieties/ genotypes (Table 1). Callus induction of these genotypes was initiated by subjecting young meristem as explants into our already standardized Lab protocol contained MS media supplemented with 3mg/L 2,4-D concentration that depicted 70 to 90 percent callus recovery (Fig. 4i). Callus regeneration was initiated on MS media supplemented with 1.0 mg/L BAP and all the varieties showed good callus regeneration (Fig. 4ii). Shoot initiation and multiplication was done with MS media supplemented with 1.0 mg/L kinetin that showed excellent shoot formation in all varieties in 4 to 6 weeks. Rooting was done with half strength MS media contained 1mg/L NAA that showed excellent root formation in all varieties after 3 weeks (Fig. 4iii).

A total of 581 somaclones were developed from six varieties (Fig. 1), of which 110 somaclones were raised from G1, 96 from G2, 84 from G3, 74 from G4, 112 from G5 and 106 from

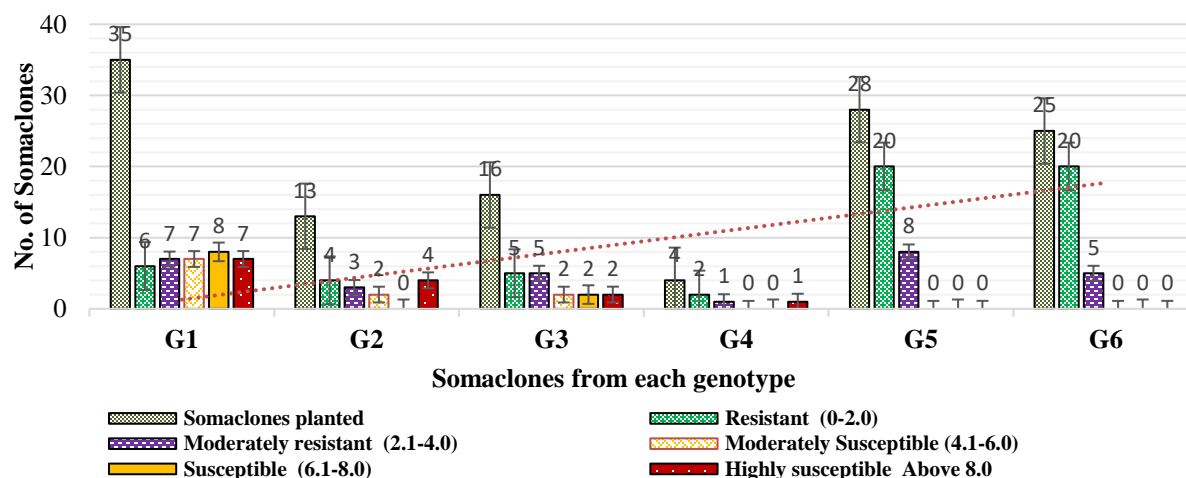


Figure 2. Screening of somaclones against red rot by using 0-9 scale as described by Srinivasan and Bhat (1961).

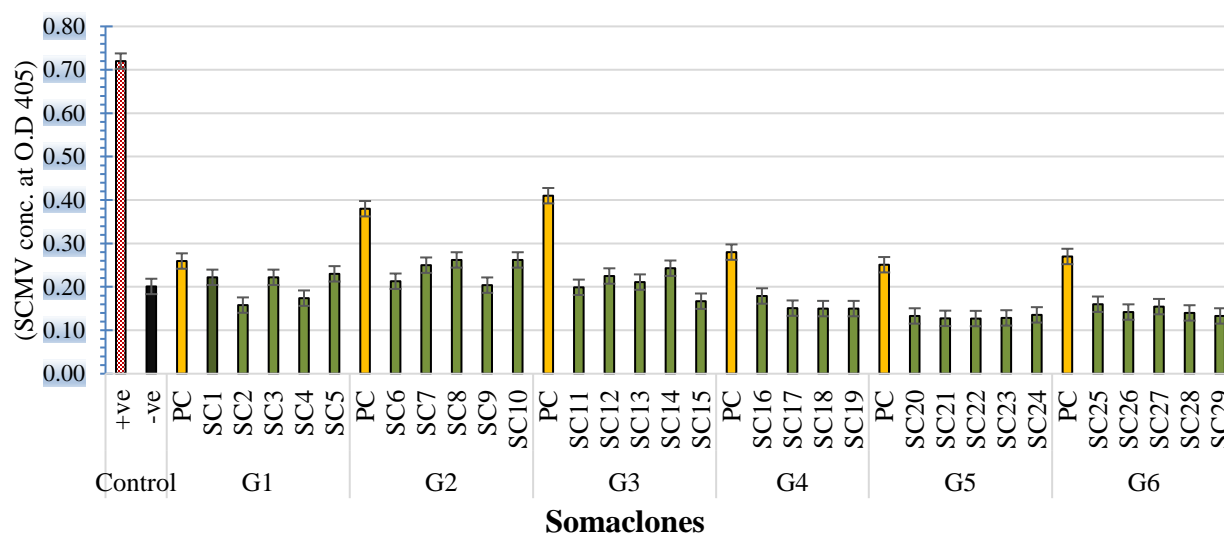


Figure 3. Screening of somaclones against sugarcane mosaic virus (SCMV). Where +ve and -ve represent positive and negative controls of SCMV respectively while PC represents parental clones and SC represents somaclones.

G6. After hardening 201 somaclones survived, of which 45 plants from G1, 26 from G2, 29 from G3, 19 from G4, 42 from G5 and 46 from G6. After transplantation 121 plants survived, of which 35 somaclones were from G1, 13 from G2, 16 from G3, 4 from G4, 28 from G5 and 25 from G6. The overall survival %age of somaclones after hardening was 33.3% with maximum survival percentage 40.9% in G1 while minimum 25.5% from G2. Overall survival %age after field transplantation was 60% with maximum 37% somaclones survived from G6 and minimum 5.4% survival rate was recorded from G6.

Screening of somaclones against red rot: A total of 121 somaclones from six varieties (Fig. 2) were inoculated with

red rot (*Colletotrichum falcatum* L.) suspension culture, of which 49 were resistant, 24 were moderately resistant, 15 were moderately susceptible, 10 susceptible and 19 were highly susceptible. Range of resistant to moderately resistant somaclones was 10 to 33 % while 8-15 % were susceptible to highly susceptible (Fig. 2). Ratio of resistant somaclones was recorded maximum in genotypes G5 and G6 while less in G4. In G1 a total of 35 somaclones were inoculated, of which 3 were found resistant, 5 moderately resistant, 8 moderately susceptible, 9 susceptible while 10 highly susceptible. In G2 total 13 somaclones were inoculated, of which 2 were found resistant while in G3 total 13 somaclones inoculated, of which 3 found resistant, in G4 total 4 plants were inoculated,

of them only 1 was resistant. In case of G5 total of 28 somaclones among them 20 found resistant in case of somaclones raised from G6 out of 25 somaclones 20 found resistant. A wide range of disease reactions were observed in somaclones, the ratio of susceptible and highly susceptible clones was less in all genotypes except somaclones from genotype G1, of which 9 somaclones found susceptible while 10 highly susceptible. This genotype was most susceptible to red rot than other genotypes under study.

Screening of somaclones against SCMV: Out of 121 somaclones from six varieties almost five representative somaclones from each genotype along with its parental clones were evaluated against sugarcane mosaic virus (SCMV) and their results are presented in the Fig. 3. Positive control and negative control of sugarcane mosaic virus depicted that O.D

values 0.72 and 0.20 respectively at 405 nm wavelength on spectrometer. All the samples of somaclones and their mother clones were compared with controls. In case of G1, parental clone (PC) depicted O.D value 0.26 while its somaclones showed SMCV concentration ranged from 0.16 to 0.23 (O.D values). In case of G2, its mother clone showed O.D value 0.38 while its somaclones gave values ranged from 0.20 to 0.26 which were near to negative control. In case of G3, its mother clone showed O.D value 0.41 while its somaclones depicted O.D values ranged from 0.17 to 0.24.

Genetic integrity of candidate genes:

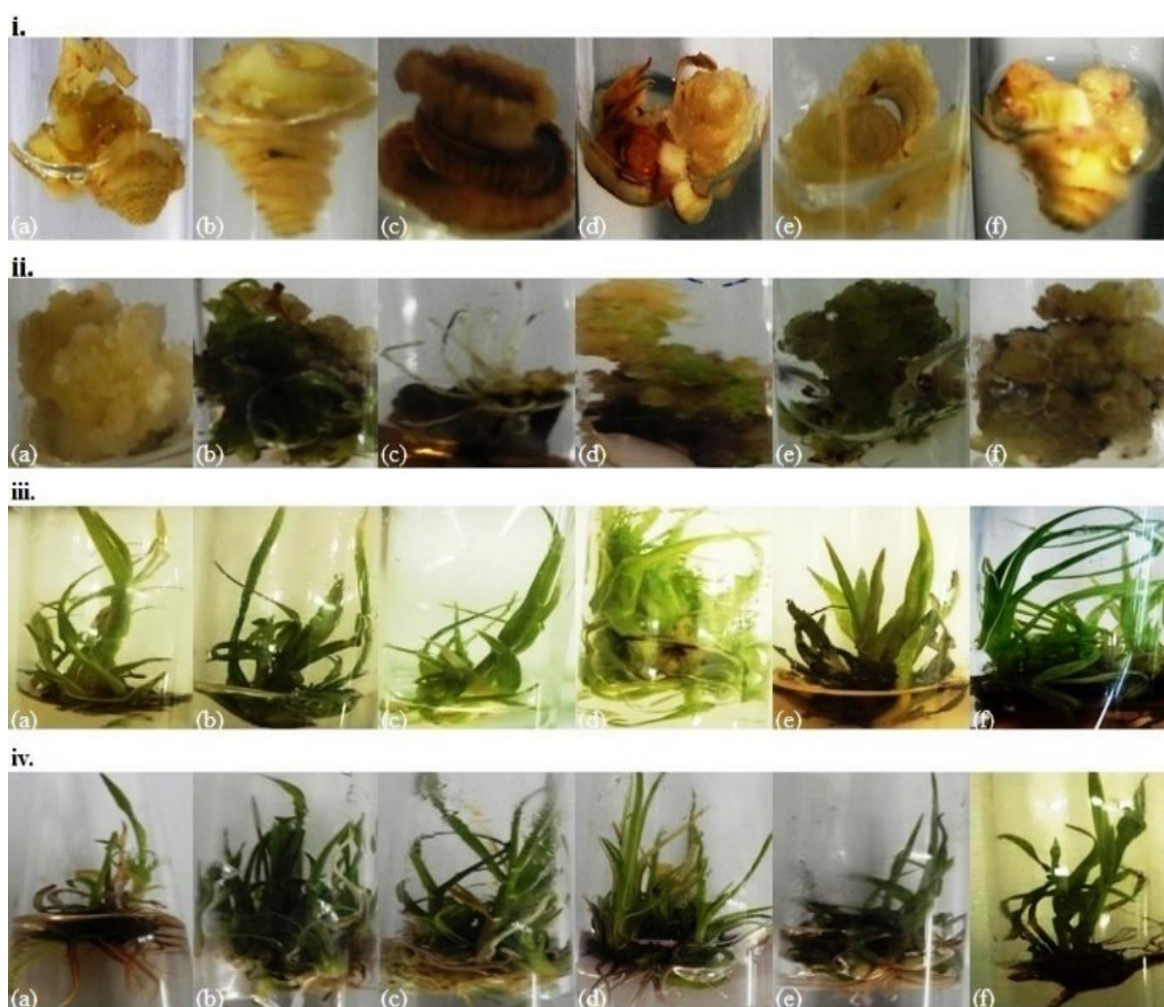
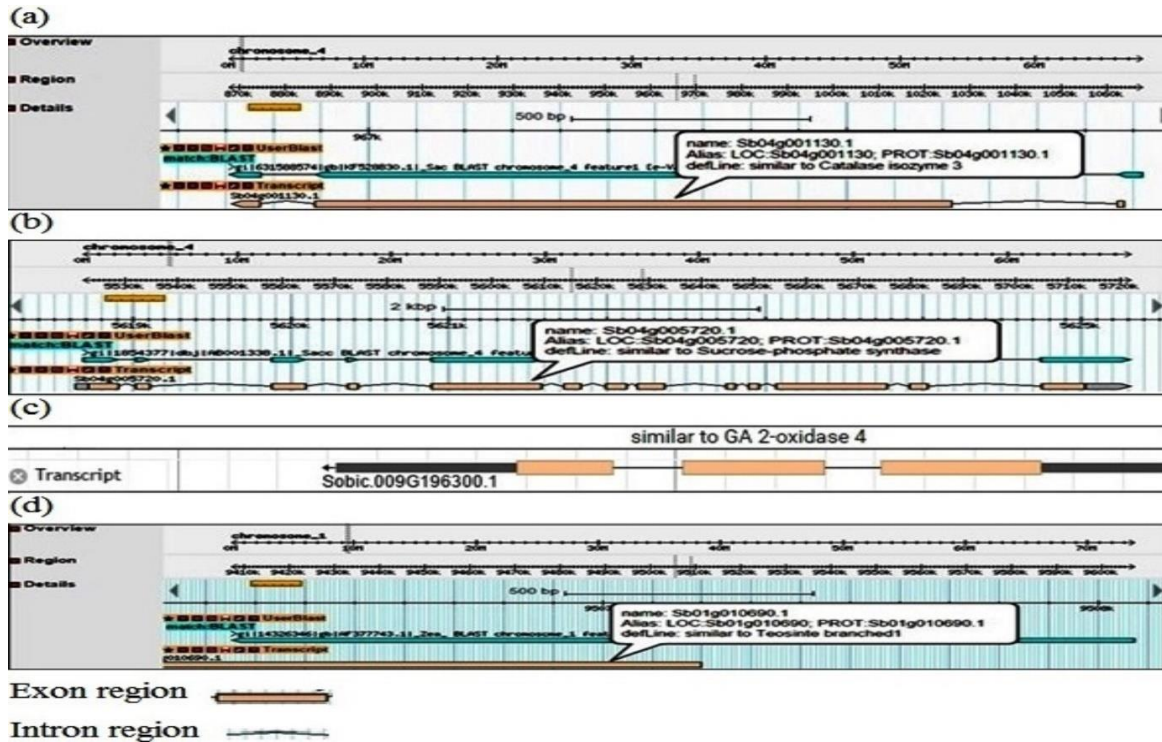


Figure 4. (i) A view of crystalline compact and embryogenic calli formed from young meristematic enfold leaves explant after 24 days of inoculation in first subculture in Murashige Skoog (MS) medium supplemented with 3mg/L 2,4-D. Where (a), (b), (c), (d), (e) and (f) represents calli from genotypes; G1, G2, G3, G4, G5, and G6 respectively as mentioned in Table 1. (ii) Regeneration from calli after 70 days of inoculation in third subculture in MS medium supplemented with 1mg/L BAP. (iii) Shooting of four weeks old regeneration tissues in MS medium supplemented with 1mg/L Kinetin. (iv) Rooting of shootlets in half strength MS medium supplemented with 1mg/L NAA.

Table 2. Candidate gene's ID, putative functions, source of sequences, location on sorghum chromosome, transcripts name, exon(s), primer sequences and product size.

Gene ID	Putative function	Source of sequences	Location on sorghum Chromosome	Transcript Name	Gene Bank /Phytozome ID and Accession number	Exon(s)	Primer sequences (5'-3') (3'-5')	Product size (bp)
(CAT1) Catalase 1	Catalytic activity in cell specially conversion reactive oxygen species i.e. H ₂ O ₂ to H ₂ O and O ₂ response to oxidative stress	Sugarcane/ Sorghum	Chr04	Sobic.004G0xx11500.1	KF528830.1/ Sb04g001130		F: GGCTTCTTCGAGTGCACCCAC R: CGCCATCACTCACATGTTTGG	1275
(SPS) Sucrose phosphate synthase	Sugar metabolism pathway, sucrose biosynthesis	Sugarcane/ Sorghum	Chr04	Sobic.004G068400.1	AB001338.1/ Sb04g005720	Exon-I	F: TCCTGGAGTTTACCGGGTT R: TACATCTTGCACTAATTGCCT	740
			Chr04	Sobic.004G068400.1		Exon-II	F: TCGGATGCACTATATAAACTT R: AAGGAATGCACAATGCACG	722
(GA2 oxidase 4) gibberellin 2-oxidase 4	Oxidoreductase activity, control the stem elongation in plants	Sorghum	Chr09	Sobic.009G196300.1	Sb09g025470		F: TCGTCTCGCGAAGCCACC R: TGATTGGTTACCGCACCCGACG	422
(TB1) Tilling gene	Control tillering in most of the species of family Gramineae	Zea mays sub.spp teosinte/ Sorghum	Chr01	Sobic.001G121600.1	AF377743.1/ Sb01g010690		F: TCCTTTCTGTGATTCTCAAGCC R: TCAGTAGAAGCGTGAGTTCTGC	1143

**Figure 5. Sequence annotations of sorghum candidate genes searched from gene database Phytozome 9.1. where (a) represents catalase isoform 3 transcript sequence, (b) sucrose phosphate synthase, (c) gibberellin 2 oxidase 4, (d) Teosinte branched1.**

In-silico candidate gene identification: Four well annotated candidate genes in sorghum responsible for growth and development were used to find out their corresponding homologous genes exon regions in sugarcane. The candidate genes responsible for enzymes included catalase, sucrose phosphate synthase, gibberellin 2 oxide 4 and teosinte branched1. Nucleotide sequences of these genes were searched from sorghum gene database (Phytozome database version 9.0. [www.http://phytozome.jgi.doe.gov](http://phytozome.jgi.doe.gov)). Annotation

sequences of these genes exons and introns are listed in Fig. 5, while their function, location on chromosome, transcript name and GenBank accession number/Phytozome ID are listed in Table 2. Intron and exon boundaries of these sequences were identified and the only exon regions were used for primer synthesis. Polymerase chain reactions were performed on sugarcane gDNA for expected bands amplification and later these PCR products were used for sequencing. Sequences of PCR products were pairwise

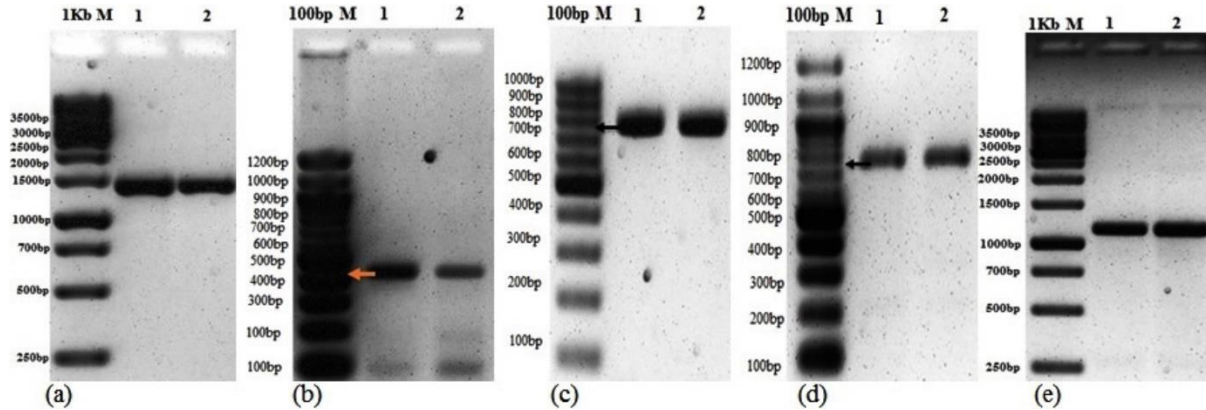


Figure 6. Combine picture of candidate genes with exon regions gel purified PCR products amplified from sugarcane gDNA samples, where (a) is *CAT1*, (b) *SPS* exon-I, (c) *SPS* exon-II, (d) *GA 2-oxidase 4*, and (e) *TBI*.



Figure 7. Pairwise sequence alignments of candidate genes exon(s) regions, here (a) *CAT1* sugarcane mRNA gene bank accession (KF528830.1) and sugarcane gDNA obtained sequence, (b) *SPS* sorghum sequence (Sb04g005720) and sugarcane gDNA obtained sequence, (c) Gibberellin 2 oxidase 4 sorghum exon sequence and sugarcane GA2 oxidase obtained sequences, (d) Teosinte branched1 sorghum sequence and sugarcane obtained sequence.

aligned with sorghum candidate genes sequences for confirmation. Gel purified bands of putative homologues of sorghum from sugarcane gDNA are presented in Fig. 6, whereas sequence alignments of gel purified bands of sugarcane and their homologous sorghum sequences are presented in Fig. 7.

Authentication of candidate genes exon region(s) in sugarcane: For catalase (*CAT1*) gene exon region authentication, sugarcane mRNA sequence was used as a reference sequence with 1275 bp sequence length and GenBank (<http://www.ncbi.nlm.nih.gov>) accession number KF52883601 (Table 2). Almost similar sized band was obtained by PCR amplification in sugarcane (Fig. 6a). A sequence of 1180 bp was recovered from gel purified PCR product after sequencing and its pairwise sequence alignment with *CAT1* sugarcane mRNA GenBank accession

(KF52883601) showed almost 100% similarity. Sucrose phosphate synthase (*SPS*), sorghum sequence with Phytozome (<http://www.phytozome.net/>) ID Sb04g005720 was used as a reference sequence with two larger exons (Fig. 7b) with exon-I 740 bp and exon-II 722 bp (Table 2). Almost similar sized PCR amplification products were isolated from sugarcane genomic DNA (Fig. 6b & 6c). In case of *GA 2-oxidase 4*, sorghum sequence was used, having Phytozome (<http://www.phytozome.net/>) ID Sb04g005720 as a reference sequence with three small exon regions (Fig. 5c), among them two exons were obtained by PCR amplification in sugarcane and only one was truly sequenced with size of 422 bp (Fig. 7c) and other smaller exons had multiple haplotypes, they had strong GC rich regions hence could not be sequenced completely. Sequencing of gel purified band, almost 400 bp product, showed 100% similarity with its



Figure 8. (a) Multiple alignment of *CAT1* sequence reads obtained from parental clone of genotype G1 and its 5 somaclones; SC1, SC2, SC3, SC4 and SC5, (b) Multiple alignment of *SPS* Exon-I sequence reads obtained from parental clone of G2 and its five somaclones; SC6, SC7, SC8, SC9 and SC10, showed no SNPs, (c) Multiple alignment of *GA2* oxidase 4 sequence reads obtained from parental clone of G3 and its somaclones; SC11, SC12, SC13, SC14 and SC15, (d) Multiple alignment of *TB1* sequence reads obtained from parental clone of SPF-213 and its 5 somaclones; SC16, SC17, SC18 and SC19, showed no SNPs.

homologue of sorghum by doing pairwise alignment. Tillering branched1 gene was searched in sorghum Phytozome gene database with gene ID *Sb01g010690* and CDS sequence 1143 bp (Table 2). Polymerase chain reaction amplification of this sequence generated similar sized band in sugarcane.

Candidate gene integrity assessment of somaclones: After confirmation of target sequences of candidate genes in sugarcane with their reference sequences, PCR amplification of exon regions of candidate were done by using gDNA of somaclones and their parental (control) clones. By doing

sequencing of each candidate gene exon(s) from gel purified products of each candidate gene parental clone and its five somaclones from six varieties, the sequence reads were aligned and SNPs changes were examined. In case of *CAT1* no possible SNPs were observed in all the somaclones. Alignment of sequence reads of *SPS* exon-I and exon-II obtained from somaclones and their parental clones in all varieties showed no SNPs changes. Multiple sequence alignment of *GA 2-oxidase 4* sequence reads obtained from somaclones raised from 2,4-D sub-culture callus and their

parental clones from all the varieties depicted no true SNPs changes in sequences (Fig. 8).

DISCUSSION

Development of somaclones: Somaclones developed from 6 sugarcane obsolete varieties by callus induction from young meristem as explants by using MS media supplemented with 3mg/L 2,4-D concentration depicted 70 to 90 percent callus recovery. Several authors (Badawy *et al.*, 2008; Gopitha *et al.*, 2010; Lawan *et al.*, 2012; Tarique *et al.*, 2010; Wang *et al.*, 2003; Yadav and Ahmad, 2013) reported the best callus induction at 3 gm/L 2,4-D supplemented in MS media for sugarcane. All varieties depicted good callus regeneration at MS media supplemented with 1.0 mg/L BAP while rapid shoot initiation and multiplication was observed in MS media supplemented with 1.0 mg/L kinetin in 4 weeks. Rooting was initiated on half strength MS media contained 1mg/L NAA in 3 weeks. Some studies have been reported to find out the most appropriate regeneration, shoot and root initiation in sugarcane. Behera and Sahoo (2009) initiated multiple shoot induction on MS medium with BAP 2.0 mg/L + NAA 0.5 mg/L while rooting on the half-strength MS basal media supplemented with 3.0 mg/L NAA. Naz *et al.* (2008), Tarique *et al.* (2010), Shahid *et al.* (2012), Srinath and Jabeen (2013) and, Yadav and Ahmad (2013) reported 1.0 mg/L BAP for calli regeneration and 0.5 mg/L kinetin for shoot initiation and multiplication with 100% regeneration recovery. In our study 1 mg/L BAP for callus regeneration, 1 mg/L Kinetin for shooting while 1 mg/L NAA for rooting were found best supplements.

The overall survival percentage of somaclones after hardening was 33.3% with maximum survival percentage of 40.9% in G1 while minimum 25.5% in G2. Overall survival percentage after field transplantation was 60% with maximum 37% somaclones survived in G6 and minimum 5.4% survival rate was recorded in G6. Some authors like Behera and Sahoo (2009) observed 90 percent survival rate of somaclones after greenhouse and field transplantation while Srinath and Jabeen (2013) recorded 90 percent survival percentage after transplantation of somaclones in greenhouse. These results do not match with our findings, where the survival rate was 60 percent after field transplantation. These differences may be due to seasonal changes, environmental differences, temperature variations, field conditions, soil type and irrigation water. Nikam *et al.* (2015) reported survival of 18 somaclones out of 138 somaclones grown to maturity for their agronomic attributes with improved sugar yield and brix percentage.

Screening of somaclones against red rot: In 121 somaclones from six varieties, the range of resistant to moderately resistant somaclones was recorded 10 percent in susceptible to moderately susceptible genotypes. Ratio of resistant somaclones was observed maximum in genotypes G5 and G6

because these geneotypes were already resistant to moderately resistant and were used as a check. A wide range of disease reactions were observed in somaclones, the ratio of susceptible and highly susceptible clones was less in all genotypes except somaclones from genotype G1, this genotype was observed more susceptible to red rot as compared to other genotypes under study. Several studies have been reported about the development of somaclones resistant to red rot by *in-vitro* culture and irradiation mutagenesis. Singh *et al.* (2000) reported that somaclones developed from callus culture of leaf depicted wide variability for resistance against red rot and documented that out of 42 somaclones, three were moderately resistance against red rot by inoculation method. Ali *et al.* (2007) and Sengar *et al.* (2009) inoculated red rot pathogen *ex situ* for a season and reported 70% of selected somaclones revealing enhanced resistance as compared to their parental clones. Singh *et al.* (2008) inoculated 228 somaclones with red rot pathotype Cf 08 and identified three resistant, four moderately resistant somaclones whereas, inoculation with pathotype Cf 03, they found fourteen resistant and nineteen moderately resistant somaclones.

Screening of somaclones against SCMV: All somaclones from G4, G5 and G6 showed O.D values ranging from 0.12-0.18 while their mother clones gave values 0.26 and 0.28. Somaclones from these genotypes showed far less O.D than negative control indicating complete absence of virus concentration. All the somaclones from six varieties showed far less virus concentration than their parental clones. Oropeza and Garcia (1996), Gaur *et al.* (2002) and Smiullah *et al.* (2012) also reported somaclones with complete absence of virus. Young meristematic tissues of sugarcane plants are almost free from virus particles and somaclones developed from these tissues remain almost free from virus. Newly developed somaclones showed various responses against sugarcane mosaic virus. Resistant mode of plant also contains presence of infection but disease pathogen fails to proliferate due to hypersensitive reaction (Acquaah, 2012). Mosaic virus streaks were found absent on the leaves of newly developed somaclones.

In-silico candidate gene identification: Four well annotated candidate genes in sorghum responsible for growth and development were used to find out their corresponding homologous genes exon regions in sugarcane. These candidate genes have pivotal role in the plant defence mechanism against biotic and abiotic stresses, sugar production, growth and development and increase number of tillering in sugarcane. Catalase (CAT1) avoid oxidative damage by scavenging reactive oxygen species (ROS) to avoid oxidative damage (Su *et al.*, 2014). Catalases also has an important role in the defence mechanisms of plants like stress response, delay in aging and cellular redox balance (Lui *et al.*, 2015). Sucrose-phosphate synthase (SPS) is a plant enzyme that play a vital role in sucrose biosynthesis. It is

controlled by metabolites and by reversible protein phosphorylation in photosynthetic and non-photosynthetic tissues (Huber and Huber, 1996). Gibberellin 2-oxidase controls shoot apex and height of the plants (Sakamoto *et al.*, 2001). Teosinte branched1 gene controls the tillering in most of the species of family poaceae.

Authentication of candidate genes exon region(s) in sugarcane: In case of sucrose phosphate synthase, sequencing of the gel purified PCR products were almost same size as sorghum *SPS* exons, while pairwise sequence alignment with sorghum homologue showed almost 100% similarity in both the exons. Sucrose phosphate synthase gene was cloned for the first time in maize by Worrall *et al.* (1991). McIntyre *et al.* (2006) cloned gene family of sucrose phosphate synthase in sugarcane with 400 bp sequence. Verma *et al.* (2010) also utilized similar sequence with (GenBank accession No. GI161176315) for sucrose phosphate synthase expression analysis. Komatsu *et al.* (2002) reported the similar sequences for sucrose phosphate synthase in sugar beet, arabidopsis, carrot, barley, wheat and citrus. In case of *GA 2-oxidase 4*, sequencing of gel purified band, almost 400 bp product showed 100% similarity with its homologue of sorghum when pairwise alignment was performed. In Tillering branched1 gene gel purification of PCR product in sugarcane was sequenced in forward as well as reverse direction due to strong GC region in the middle of the product that block the reaction by self-pairing and making hairpin loops. Reverse direction sequencing gave true read until the GC rich region. Almost 900 bp true sequence was aligned with sorghum sequence that showed similar sequence pattern.

Candidate gene integrity assessment of somaclones: Sequence reads of four candidate gene exon(s) from gel purified products of each parental clone and its somaclones were aligned and SNPs changes were examined in somaclones. In all the somaclones, candidate genes showed no possible SNPs variation in the coded regions of exon nucleotide sequences. The findings clearly depicted that somaclones raised from callus sub-culture with 2,4-D neither affected single nucleotide sequence nor caused SNPs changes in nucleotide sequences. However, rapid cell proliferation induced by 2,4-D might have caused additions, deletions, inversions or transversions of large chromosomal segments or large DNA fragments. A change in coded region nucleotide sequence definitely changes the amino acid sequences in a polypeptide upon translation of coded region. A change in amino acid sequence leads to alter the phenotype of organism. A nucleotide change in coded region of important candidate genes affects the phenotype. Genetic integrity of candidate genes is important in mutated population for normal growth and development, metabolic functions and defence mechanism. Caste *et al.* (2015), Jagesh *et al.* (2013) and Peyvandi *et al.* (2013) reported the genetic fidelity of somaclones in sugarcane by using ISSR and SSR markers but

no report is available for genetic integrity of candidate genes in sugarcane somaclones except Coste *et al.* (2015) that reported SNPs variations in lycopene gene of tomato somaclones. In case of plant species with complex genome where whole genome sequence is not available due to high ploidy level and haplotypes, SNPs based integrity in genes is usually done either by direct discovery or on the basis of primer design for sequencing (Bundock *et al.*, 2006; Choi *et al.*, 2007; Rostoks *et al.*, 2005; Somers *et al.*, 2003). Our findings are first ever report on the genetic fidelity of candidate genes exon region of sugarcane somaclones.

It is concluded from our study that, all the varieties used in the experiment showed good response to callus induction at 2,4-D level 3mg/L supplemented in MS media. Genetic integrity assessment in candidate genes exons regions revealed intact nucleotide sequences with no SNPs variation as their parental clones in case of somaclones raised from sub-culturing of callus with 2,4-D. Almost all the somaclones showed SCMV concentration less than negative control with variable disease reaction against red rot, except somaclones raised from G1, rest of them depicted maximum resistance. It can be suggested that somaclonal variation is a good source of disease resistance as an alternative methodology for genetic improvement in the sugarcane.

Acknowledgements: The authors gratefully acknowledge the assistance and Lab. facilities extended by the University of Queensland, Australia and to the International Research Support Initiative Program (IRSIP) under Higher Education Commission (HEC), Pakistan for the grant of six months Fellowship Award (NO: I-8/HEC/HRD/2014/2959) for the conduct of research.

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