Pak. J. Bot., 42(1): 605-614, 2010.

GENETIC VARIABILITY IN MUTATED POPULATION OF SUGARCANE CLONE NIA-98 THROUGH MOLECULAR MARKERS (RAPD AND TRAP)

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Abstract

Variability obtained from mutation breeding (gamma rays) was examined through molecular marker techniques (RAPD). A total of 85 loci were amplified, out of which 76.47% were polymorphic and 23.53% were monomorphic. Fragments size ranged from 220bp-2.1kb and fragments produced by various primers ranged from 3-13 with an average of 5 fragments per primer. The highest number of loci (13) was amplified with primer B-07, while the lowest number 3 with primer B-01. Results revealed that mutant P1 (20Gy) contained a specific segment of 2.03kb. Genetically most similar genotypes were P2 (10Gy) and P4 (20Gy) (95.55%) while most dissimilar genotypes were P4 (10Gy) and P3 (20Gy) (63.2%). On the basis of results achieved, the mutants could be divided into four clusters and three groups. Mutants P4 (40 Gy) and P4 (10 Gy) were genetically distinct from other mutants.

Introduction

Pakistan is the 5th largest country in the world in terms of area under sugar cane cultivation, 11th by production and 60th in yield (Anon., 2007). This state of affair needs concentrated efforts for the improvement of sugar cane. Sugar cane has a fairly lengthy reproductive cycle, high polyploidy, extreme heterozygosity, incompatibility, high sterility, maternal inheritance and an erratic flowering behaviour (Khan *et al.*, 2000, 2002). The imbroglio necessitates genetic improvement of sugarcane through non-conventional breeding methods. For instance mutation breeding can be used to augment the conventional procedure to produce varieties that can achieve maximum output potential (Khan *et al.*, 2004). In fact, our present day crop plants and related disciplines have made it possible to direct this evolution by utilizing hybridization, mutations and tissue culture techniques together through a synergetic approach (Ahmad *et al*., 1991; Khan *et al*., 2009).

Traditional identification methods that combine agronomic and morphological characteristics have been useful in describing the differences between members of the *Saccharum* complex (Artschwager & Brandes, 1958; Skinner, 1972; Skinner *et al*., 1987). The members of the *Saccharum* complex predominantly outcross and are maintained by vegetative propagation, hence, are highly heterozygous and display enormous plasticity in the phenotypic expression of traits. Although morphological traits are used to identify and classify clones, most of the traits are influenced by the environment under which the clones are grown or selected. With the advent of molecular markers, it is now possible to make direct inferences about genetic diversity and determine interrelationships among organisms at the DNA level. A vast number of molecular marker techniques such as isoenzymes (Glaszmann *et al*., 1989), RFLP (restriction fragment length polymorphism) (D'Hont *et al*., 1994; Jannoo *et al*., 1999; Coto *et al*., 2002), ribosomal DNA (Glaszmann *et al*., 1990; Pan *et al*.,2000),

microsatellites (Piperidis *et al*., 2001; Cordeiro *et al*., 2003), AFLP (amplified fragment length polymorphism) (Besse *et al*., 1998; Lima *et al*., 2002), and molecular cytogenetics (D'Hont *et al*., 1996) have been instrumental in explaining genetic diversity and interrelationships among accessions in sugarcane germplasm collections (Arcenaux, 1967). Yield is a polygenic character and dependent on many genetic and environmental factors. Plant breeders have their prime concern to enhance crop productivity by increasing yield through plant manipulation and exploring genetic diversity. The present research work was conducted to estimate genetic variability through molecular markers in a population obtained through the use of induced somatic mutation for the improvement of sugarcane.

Materials and Methods

The research material comprised of vegetative cuttings of sugar cane (*Saccharum* spp. hybrid) variety NIA-98. The cuttings were irradiated with different doses of gamma rays (0Gy, 10Gy, 20Gy, 30Gy and 40Gy) obtained from Cesium 137 source (Nigo 5, Bulgaria). The dose rate at the time of irradiation was 30.86Gy/minute. The irradiated cuttings were planted at NIA experimental farm in RCB design with 3 replications. The plot size was 8 x 10m, with row-to-row distance of one metre. The sowing was done in the month of September 2005 and normal agronomic practices were followed through out the growth period. Normal agronomic practices were followed through out the growth period. The young leaves were collected after three months of irradiation and the DNA was extracted.

Plant material and DNA Extraction: RAPD studies were conducted to estimate the genetic variability among selected mutants of M_1V_3 generation of NIA-98. Sixteen promising mutants from different doses were selected for diversity studies. Genomic DNA was isolated through DNA isolation kit (Gentra system, Minnesota, USA) following the method described by Khan *et al.,* (2009). The DNA was quantified on spectrophotometer, at absorbance rate of 260/280nm. The quality was further checked on 0.8% agarose gel.

PCR with random primers: Of 45 primers from Gene Link 14 were able to amplify the DNA used in this study (Table 1). PCR reaction was carried out in 25µl reaction mixture containing 2.6ng/µl of template (Genomic DNA), 2.5mM $MgCl_2$, 0.33mM of dNTPs, 0.1U of Taq polymerase and 1µM of primer in 1x reaction buffer. The amplification reaction was performed in the Master cycler with an initial denaturation for 5 minutes at 94ºC, then 33 cycles: 1 minute denaturation at 94ºC; 1 minute annealing at 52ºC; 2 minute extension at 72ºC. Final extension was carried out at 72ºC for 10 minutes. Amplified products were electrophoresed on 1.5% agarose gels containing 0.5 x TBE (Tris Borate EDTA) and 0.5µg/ml Ethidium bromide to stain the DNA. The PCR product was electrophoresing at 72 volts for 2 hours. Photograph was taken under UV light using gel documentation system.

PCR with specific (SucSy) primer: National Center for Biotechnology Information (NCBI) website was used to obtain the EST nucleotide sequences of genes responsible for carbohydrate metabolism and sucrose accumulation and 18-mer primer was designed using a primer design software 'Primer3'. The first sequence is encoding the enzyme sucrose synthase (SuSy) NCBI accession No. AF263384 and also an 18-mer primer were used from the EST sequence of these enzymes paired with an arbitrary primer that targets the intronic and /or extronic region (AT- or GC-rich core).

Table 1. Sequence of the primers.

Primer	Sequence	Range of amplified loci	Polymorphic Loci	Monomorphic loci	Total no of loci
A-01	CAGGCCCTTC	300bp-1.9kb	04	02	06
B-01	GTTTCGCTCC	419bp-1.7kb	03	Nil	03
B-02	TGATCCCTGG	1.1kb-2.1kb	03	01	04
B-03	CATCCCCCTG	349bp-2.0kb	07	Nil	07
B-04	GGACTGGAGT	319bp-1.8kb	04	02	06
B-05	TGCGCCCTTC	301bp-2.0kb	04	02	06
B-06	TGCTCTGCCC	310bp-2.03kb	07	1	08
B-07	GGTGACGCAG	292bp-1.7kb	13	Nil	13
B-08	GTCCACACGG	316bp-1.3kb	05	02	07
B-09	TGGGGGACTC	451bp-1.3kb	02	03	05
B-10	CTGCTGGGAC	307bp-1.3kb	04	02	06
B-11	GTAGACCCGT	220bp-647bp	02	02	04
B-12	CCTTGACGCA	327bp-1.6bp	02	02	04
B-14	TCCGCTCTGG	396bp-1.989kb	05	01	06
			65	20	85
			(76.47%)	(23.53%)	

Table 2. Performance of selected mutants of NIA-98 developed through mutagenesis.

Clone	Cane yield (t/ha)	Commercial Cane Sugar (CCS) %	Sugar yield (t/ha)
NIA-98 P1-10Gy	55c	9.98bc	5.49c
NIA-98 P2-10Gy	55c	11.27ab	6.20b
NIA-98 P3-10Gy	75a	11.47ab	8.60a
NIA-98 P4-10Gy	45d	10.89b	4.90c
NIA-98 P1-20Gy	52cd	9.58c	5.03c
NIA-98 P2-20Gy	47de	11.37ab	5.40c
NIA-98 P3-20Gy	55c	9.00c	4.95c
NIA-98 P4-20Gy	35g	11.01b	3.85d
NIA-98 P1-30Gy	55c	10.17bc	5.59c
NIA-98 P2-30Gy	42ef	11.36ab	4.83c
NIA-98 P3-30Gy	45e	10.36b	4.66c
NIA-98 P4-30Gy	60b	10.93b	6.56b
NIA-98 P1-40Gy	50d	10.82b	5.41c
NIA-98 P2-40Gy	50d	10.11bc	5.06c
NIA-98 P3-40Gy	40f	12.05a	4.82c
NIA-98 P4-40Gy	35g	7.71e	2.70e
NIA-98 (Parent)	60b	8.81d	5.26c

DMR test (0.05):Means followed by the same letters are not significantly different from each other

PCR reaction was carried out in 25µl reaction mixture containing 50ng of template (Genomic DNA), 2.5mM MgCl, 0.33mM of each dNTPs, 0.05% gelatin, 0.5U of Taq polymerase and 0.15µM of each primer (one fixed & two arbitrary primers) in a 1X PCR reaction buffer. The amplification reaction was performed in the Eppendorf Master Cycler with an initial denaturation for 5 minutes at 94ºC, then 30 cycles: 1 minute denaturation at 94ºC; 2 minute annealing at 55ºC; 3 minutes extension at 72ºC. Final extension was carried out at 72ºC for 7 minutes.

Amplified products were analyzed through electrophoresis on 3% agarose gels containing 0.5X TBE (Tris Borate EDTA) and 0.5µg/ml ethidium bromide to stain the DNA. Gel electrophoresis at 72 volts for 2 hours and photograph was taken under UV light using gel documentation system.

Data analysis: Irradiated seed cuttings were grown in the field and data was recorded for three important agronomic characters viz., commercial cane sugar (CCS)%, cane yield (t/ha) and sugar yield (t/ha). Three stools were randomly taken from each plot to determine sugar contents according to sugarcane laboratory Manual for Queensland Sugar Mills (Anon., 1970) while three rows from each plot were harvested to record yield data. The mean and variance were computed from each treatment. Data were analysed by employing Duncan Multiple Range test (Steel & Torrie, 1980).

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

Results and Discussion

The results indicate that the genetic variability occurred in all the mutagenic treatments (Table 3). It was observed that mutant (P3 10Gy) produced highest cane yield (75 t/ha) and sugar yield (8.60 t/ha). The maximum CCS% was observed in mutant P3 of 40Gy (12.05). The mutant P3 of 10Gy showed peculiar behaviour and therefore in the dendrogram it positioned itself as an independent genotype. Moreover the mutagenic treatment also exhibited that genotype P3 (10Gy) has new genetic makeup in which quantitative and qualitative trait had shown some unique balance in it. Ahloowalia (1995) reported that the development of desired genotype could be obtained through mutagenesis in case of vegetatively propagated sugarcane plants. Present investigations suggested that the mutagenic doses ranging from 10 to 40Gy were effective in inducing desired genetic variability. For increasing per hectare sugar yield it is necessary to deem cane yield and sugar recovery together (Hashmi, 1995; Khan *et al*., 2009), and our studies have shown that there are tremendous possibilities of obtaining desirable mutants at intermediate dose.

Sixteen sugarcane mutant of NIA-98 were assessed through RAPD marker technique. Fourteen out of 45 random primers produced multiple fragments. There were 85 scorable bands of which 76.47% were polymorphic and 23.53% monomorphic. The number of fragments produced ranged from 03 to 13, with an average of 5 fragments, whereas, the size of fragments ranged from 220 bp to 2.1 kbp. Primer B-07 produced 13 fragments and primer B-01 produced only 3 fragments (Table 1).

Some specific RAPD bands were also identified, reflecting the RAPDs application for the identification of sugarcane mutant. We found P1 (20 Gy) containing a specific DNA segment of 2.03kbp amplified with primer B-06 (Figs. 1 lane 5). Level of the individual genotype of the 16 sugarcane mutants produced polymorphism in which few monomorphic bands were observed (Figs. 1, 2 & 3).

The number of bands recorded per primer across the 16 mutants of NIA-98, with the primer A-01 six bands were amplified in which 4 bands were polymorphic and 2 monomorphic (segment of 1.0kb and 300bp), ranged between 300bp-1.9kp. All mutants with parent (NIA-98) containing a band of 1.9kbp and 685bp except P3 (30 Gy). P1-P4 (10 Gy), P1, P2 & P4 (20 Gy), P1, P2 (30 Gy), P1-P4 (40 Gy) with NIA-98 containing a band of 1.7kbp, while P1 (10 Gy), P3 (20 Gy), P1 (30 Gy), P4 (30 Gy) contains a band of 1.6kbp.

Table 3. Similarity coefficient among the NIA-98 mutants calculated according to Nei & Li's coefficient.

	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17
L1	1																
L2	0.91	1															
L3	0.791	0.851	1														
L4	0.68	0.774	0.699	1													
L5	0.845	0.884	0.856	0.708	1												
L6	0.846	0.892	0.764	0.709	0.829	1											
L7	0.856	0.847	0.729	**0.632**	0.787	0.836	1										
L8	0.824	**0.955**	0.861	0.778	0.89	0.879	0.849	1									
L9	0.925	0.914	0.781	0.692	0.849	0.884	0.845	0.876	1								
L10	0.797	0.824	0.756	0.692	0.784	0.839	0.76	0.819	0.845	1							
L11	0.814	0.845	0.808	0.676	0.802	0.829	0.806	0.868	0.864	0.85	1						
L12	0.872	0.846	0.806	0.703	0.803	0.776	0.833	0.845	0.838	0.801	0.858	1					
L13	0.884	0.89	0.797	0.689	0.855	0.818	0.859	0.892	0.878	0.794	0.849	0.89	1				
L14	0.853	0.861	0.805	0.700	0.821	0.813	0.829	0.853	0.866	0.808	0.861	0.856	0.87	1			
L15	0.781	0.791	0.803	0.656	0.779	0.773	0.776	0.829	0.801	0.836	0.869	0.815	0.824	0.859	1		
L16	0.797	0.854	0.796	0.682	0.781	0.763	0.756	0.867	0.79	0.739	0.766	0.761	0.856	0.778	0.754	1	
L17	0.841	0.919	0.846	0.787	0.849	0.854	0.849	0.949	0.857	0.819	0.87	0.819	0.86	0.849	0.81	0.842	1

10 Gy: P1-P4 (1-4); 20 Gy: P1-P4 (5-8); 30 Gy: P1-P4 (9-12); 40 Gy: P1-P4 (13-16) & NIA-98 (17)

With the primer-B-14 six bands were amplified, ranged between 396bp-2.0kb in which 5 were polymorphic and 1 was monomorphic bands (1.02kbp). All mutants with parents (NIA-98) having a bands of 545bp and 396bp except P4 (10 Gy). P2 (10 Gy) P2 (20 Gy), P1 and P2 of 30 Gy contains a segment of 1.989kbp. P2 (20 Gy) and P2 (30 Gy) containing a specific segment of 1.7kbp same as P2 (20 Gy), P1 & P2 (30 Gy) having a segment of 1.3kbp.

With the primer B-06 eight segments were amplified in which 7 were polymorphic only one segment was monomorphic (1.4kb), with the range of 310bp-2.03kbp. Only P1 (20 Gy) contain a specific segment of 2.036kb. 1.7 kbp segment disappeared in P1, P3, P4 (10 Gy) and P4 (30 Gy) and 1.66kbp segment was also disappeared in P4 (10 Gy) and P4 (30 Gy). All mutants with parent contains segment of 1.55kbp except P3, P4 (10 Gy). P1 (10 Gy), P2 (10 Gy), P1 (20 Gy), P2 (20 Gy), P3 (20 Gy), P1-P4 (30 Gy), P1 (40 Gy), P2 (40 Gy) and NIA-98 contains a differentiate segment of 796bp. P4 (20 Gy), P4 (40 Gy) and NIA-98 were not containing segment of 420 bp, while 310 bp segment only disappeared in P4 (40 Gy).

With the primer B-01 three polymorphic bands were amplified, ranged between 419bp-1.7kbp. With the primer B-11 and primer B-12 half of the bands were polymorphic and half monomorphic, with the range of 220-647bp and 327bp-1.6kbp respectively. With the primer B-04, primer B-05 and primer B-10 six bands were amplified, in which 66.67% of segments were polymorphic and 33.33% were monomorphic, ranged between 319bp-1.8kbp, 301bp-2.0kbp and 307bp-1.3kbp respectively. With the primer B-03 seven polymorphic bands were amplified, with the range of 349bp-2.0kbp and primer B-07 amplified with 13 polymorphic alleles, ranged between 292 bp-1.7kbp.

With the primer B-02 three bands were amplified in which one was monomorphic (2.1kb), ranged between 1.1kbp - 2.1kbp. P1 (10 Gy), P2 (10 Gy), P4 (10 Gy), P1-P4 (20 Gy), P1 (30 Gy), P2 (30 Gy), P2 (40 Gy), P4 (40 Gy) and parent NIA-98 contained a segment of 1.636kbp, while P1, P2, & P4 (10 Gy), P1, P2, & P4 (20 Gy) P1 & P2 ((30 Gy), P1 & P2 (40 Gy) and NIA-98 possessed a segment of 1.229kbp.

Primer B-08 amplified 7 bands in which 5 were polymorphic, with the range of 316bp-1.3kbp. P2 ((30 Gy) and P3 (40 Gy) had a specific RAPD bands of 996bp. Primer B-09 yielded 5 bands, 2 polymorphic and 3 monomorphic, ranging between 451bp-1.3kbp.

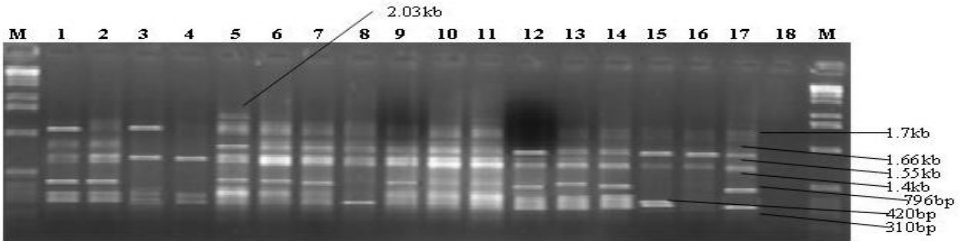

Fig. 1. Primer B-06, M=IKb ladder, 1=P1, 2=P2, 3=P3, 4=P4 (10 Gy)., 5=P1, 6=P2, 7=P3, 8=P4 (20 Gy)., 9=P1, 10=P2, 11=P3, 12=P4, (30 Gy)., 13=P1, 14=P2, 15=P3, 16=P4 (40 Gy)., 17= NIA-98, 18=Blank.

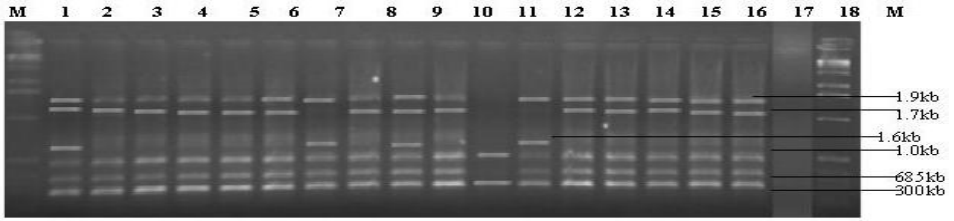

Fig. 2. Primer A-01, M=IKb ladder, 1=P1, 2=P2, 3=P3, 4=P4 (10 Gy)., 5=P1, 6=P2, 7=P3, 8=P4 (20 Gy)., 9=P1, 10=P2, 11=P3, 12=P4, (30 Gy)., 13=P1, 14=P2, 15=P3, 16=P4 (40 Gy)., 17= NIA-98, 18=Blank.

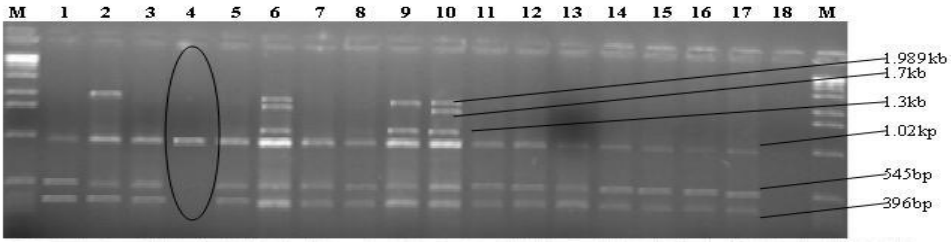

Fig. 3. Primer B-14, M=IKb ladder, 1=P1, 2=P2, 3=P3, 4=P4 (10 Gy)., 5=P1, 6=P2, 7=P3, 8=P4 (20 Gy)., 9=P1, 10=P2, 11=P3, 12=P4, (30 Gy)., 13=P1, 14=P2, 15=P3, 16=P4 (40 Gy)., 17= NIA-98, 18=Blank.

Genetic similarity: The similarity coefficients reflected the genetic relationship between the mutants and parent. The highest similarity (95.55%) was observed between P2 (10 Gy) and P4 (20 Gy) and the least similarity (63.2%) occurred between P4 (10 Gy) and P3 (20 Gy) (Table 3).

Mutants P1(10 Gy) and P1 (30 Gy) were in one group on the dendrogram, showing more genetic similarity among each other. Mutants P2 (10 Gy), P4 (20 Gy) and parent NIA-98 grouped in the second subclass, while P4 (30 Gy) and P1 (40Gy) grouped in third subclass and P3 (30 Gy) lattlerly join the main group. There were three individuals in the 4th sub class i-e P3 (30 Gy), P3 (40 Gy) and P2 (40 Gy), same as P2 (20 Gy) and P2 (30 Gy) in 5th subclass and P3 (10 Gy) and P1 (20 Gy) were found in 6th subclass. The other two mutants P4 (40 Gy) and P4 (10 Gy) not forming any group with other individuals.

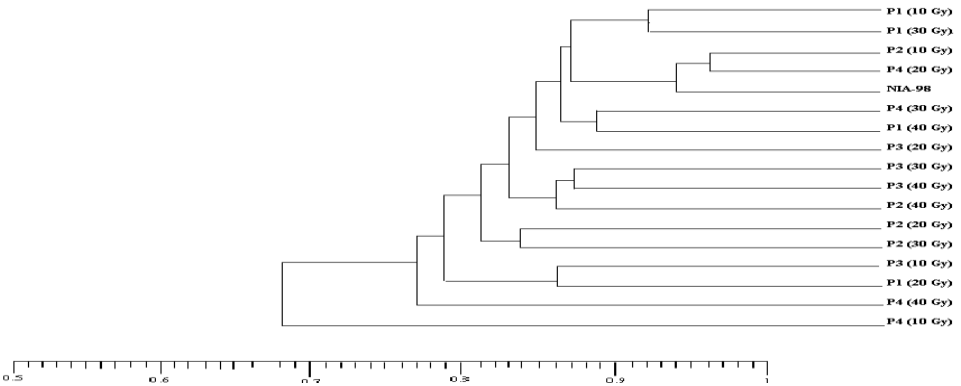

Fig. 4. Dendogram of sixteen NIA-98 mutants with parent developed from RAPD data using un-weight pair group method of arithmetic means (UPGMA).

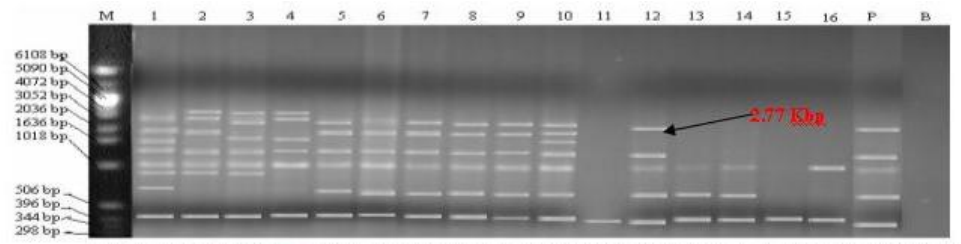

Fig. 5. Primer sucrose pyrovate, M=IKb ladder, 1=P1, 2=P2, 3=P3, 4=P4 (10 Gy)., 5=P1, 6=P2, 7=P3, 8=P4 (20 Gy)., 9=P1, 10=P2, 11=P3, 12=P4, (30 Gy)., 13=P1, 14=P2, 15=P3, 16=P4 (40 Gy)., 17= NIA-98, 18=Blank.

RAPD results revealed that mutants of 10Gy were 68-91% similar with each other. Population of 20Gy (P1-P4) exhibited 11-37% dissimilarity, which is the highest polymorphism in the mutated population of NIA-98 followed by 40Gy (7.5-32%). Irradiation dose of 20Gy gave 27.3%, 58% and 41% different population as compared to 10, 30 and 40Gy respectively. Irradiation dose of 10Gy produced more variation in the sugarcane population as compared to 30Gy. Mutants 30Gy exhibit more than 62% similarity with parent.

The RAPD profile has the advantage of representing full genome of an individual unlike morphological differences which are more dependent on environmental conditions (Singh, 1994). RAPD-PCR method provides a direct analysis of the genome which is not possible with morphological methods and, therefore, serves as a powerful tool for biosystematics studies (Taylor-Grant, 1999). The RAPD technique does not require any previous knowledge of the target genome (Nair *et al.*, 2002), it is relatively simple and rapid to carry out, RAPD markers have been extensively used in population genetics, analyses of biodiversity and studies of relationships among species at different levels (Thorman & Osborn, 1992; Williams *et al.*, 1993; Ma *et al.*, 2004). However, the mismatching resulting in non-specific amplification products, which occur due to RAPD assay, this employs short nucleotide sequences of unknown locations and/or functions and the technique sometimes may be limited to obtain a result because of representing

only one segment of DNA (Taylor-Grant, 1999). Therefore, application of more primers will help in developing concrete results.

TRAP studies in mutated population of NIA-98 were assessed with sucrose synthetase (SuSy) primers alongwith two arbitrary primers (Fig. 5). It produced 9 loci in which 8 were polymorphic. Occurrence of a highly polymorphic band profile in sugarcane can be attributed to the fact that it is a highly self-incompatible, cross pollinating, complex polyploid grass species with homologous and homeologous chromosomes (D'hont *et al*., 1995; Ming *et al*., 2001). Sucrose Synthase (SuSy) gene present in P1-P4 (10 Gy), P1-P4 (20 Gy), P1, P2, and P3 (30 Gy) and also present in NIA-98, specific loci (3.0kbp) has been observed in the mutant of 10Gy (P1, P2 and P3), and this variation could be due to the combined effect of tissue culture and irradiation on the sugarcane. TRAP markers were able to amplify small to large fragment of DNA (356 bp- 2.0kb) simultaneously (Hu & Vick, 2003; Alwala *et al*., 2003).

Mutagenesis alters the normal biological composition of an organism and the true genetic changes are desirable. Ionizing radiations and chemical mutagens have been extensively used in mutation breeding in different crops. The use of gamma rays to create additional variability has also led to some progress in sugarcane mutation breeding (Srivastava *et al*., 1986). Mutations may be recessive or dominant but the former is more common although these do not express phenotypically unless two recessive genes come together as homozygotes (Hrishi *et al*., 1968; Smith, 1997; Edme *et al*., 2005). This expression requires one or more generations of recombination of two or more similar recessives for the phenotypic expression in the population. The selection of mutants have been more preferable from second irradiated generation because in case of recessives, both loci are rarely mutated and mutants can be isolated in homozygous condition (Donini *et al*., 1984; Chengalrayan *et al*., 2005). In sugarcane, most recessive mutants have been selected for the improvement of sucrose percentage, cane yield and resistance to pests and diseases (Jeswiet, 1929; Srivastiva *et al*., 1986; Cox, *et al*., 1996; Allen *et al*., 1997; Novak, 1991).

Genetic diversity among the sugarcane mutants was mainly based on the morphological and anatomical characters, which are governed by the gene and influenced by the environment due to which these features are changeable and sometimes difficult to distinguish. Molecular techniques serve as a device for the estimation of genetic dissimilarity among the mutants. In the future studies, the analysis of additional primers of RAPD-PCR and the use of different types of molecular markers such as AFLP (Amplified Fragment Length Polymorphism) and SSR (Simple Sequence Repeat) will improve the accuracy of resolution of accurate genetic variation among the mutant/variants of sugarcane.

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(Received for publication 27 August 2009)