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SCREENING OF MUTATED POPULATION OF SUGARCANE THROUGH RAPD

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Abstract

Variability obtained from mutation breeding (gamma rays) was examined through molecular marker techniques (RAPD). A total of 73 loci were amplified with 13 primers, out of which 75.34% were polymorphic and 24.66% were monomorphic. Fragments size ranged from 275bp-2.2kb and fragments produced by various primers ranged from 3-9 with an average of 3 fragments per primer. The highest number of loci 9 was amplified with primer B-09, while the lowest number 3 with primer B-02 and B-08. Results revealed that mutant P2(30Gy) contained three specific segment of 421bp, 2.0kb and 2.1kb while Mutant P1 (30Gy) contains another specific segment of 2.0kb amplified with primer B-06. Genetically most similar genotypes were P3 (10Gy) and P4 (10Gy) (96.5%) while most dissimilar genotypes were P2 (20Gy) and P1 (30Gy) (48%). On the basis of results achieved, the mutants could be divided into four cluster and three groups. Mutants P2 (30 Gy), P1 (20 Gy) and P2 (20 Gy) were genetically distinct from other mutants.

Introduction

The sugar industry in Pakistan is the second largest agro-based industry, comprising of 77 sugar mills, with daily crushing capacity of around 350 thousand tons. It provides employment to 47000 persons directly and about a million overall. Its byproducts have contributed significantly towards import substitution and in raising export earnings. It contributes 15 percent to value added of major crops and its share in agricultural value added and GDP is 6.3 percent and 1.5 percent respectively (Hashmi, 1995).

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., 2008). 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 such as mutation breeding need to be used to augment the conventional procedure to produce sugarcane varieties that can achieve maximum output potential (Khan *et al.*, 2004). Sugarcane improvement through plant breeding is an evolutionary process. 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 can be 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. Indeed, 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 obtained through the use induced somatic mutation for the improvement of sugarcane.

Materials and Methods

The research material comprised of vegetative cutting of sugar cane (*Saccharum officinarum*) variety, NIA-2004 was irradiated with different doses of gamma rays (0Gy, 10Gy, 20Gy, 30Gy and 40Gy) obtained from Cesium 137 source (Nigo 5, Belgaria). The dose rate at the time of irradiation was 30.86Gy/minute. The irradiated cuttings were planted at NIA experimental farm. 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-2004. 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 13 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 minute at 94^{o}C, then 33 cycles: 1 minute denaturation at 94^{o}C; 1 minute annealing at 52^{o}C; 2 minute extension at 72^{o}C. Final extension was carried out at 72^{o}C for 10 minute. 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.

Data analysis: 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

Sixteen sugarcane mutants of NIA-2004 were assessed through RAPD marker. Thirteen of the 45 random primers produced multiple fragments. The total number of scoreable loci were 73, out of which 75.34% were polymorphic and 24.66% were monomorphic. The number of fragments produced by various primers ranged from 03-09, with an average of 3 fragments. The size of fragments ranged from 275bp – 2.2kbp. Primer B-09 produced 09 fragments and primer B-02 and B-08 produced only 3 fragments (Table 1).

Some specific RAPD bands were also identified, reflecting the RAPDs application for the identification of sugarcane mutants. Mutant P2 (30 Gy) contained three specific DNA segments of 2.1kb, 2.0kb and 421bp and P1 (30 Gy) also contained a segment of 2.0kb with primer B-06 (Fig. 3 lane 9 and 10). Level of the individual genotypes of the sixteen mutants of NIA-2004 with parent produced polymorphism as shown in Figs. 1-3. Three major segments were amplified using primer B-02 (Fig. 1, bands a, b and c). Band 'a' was shared by all sixteen mutants; band b appeared in P1 (10 Gy), P4 (10 Gy), P4 (20 Gy), P2 and P3 (30 Gy), and band 'c' appeared in mutant P1-P4 (10 Gy), P1 and P4 (20 Gy), P1, P2 and P3 (30 Gy). Parent NIA-2004 contained all the three bands. On the other hand, there were 08 segments amplified by primer A-01 in sixteen mutant with parent (bands a, b, c, d, e, f, g, and h, in Fig. 2 and Table 1). Some segments (b, g and h) amplified only in one or a few genotypes, but other segments (e and f) amplified in all genotypes. Band 'a' appeared in P1, P3 of 30Gy and NIA-2004 and band 'd' was absent only in P2 (20 Gy), indicating the specificity to sub-species. Different combinations of the 08 segments formed DNA fingerprints in 16 mutants of NIA-2004.

Primer B-06 produced four loci on amplification (band a, b, c, and d) (Fig. 3) in which one is monomorphic and three were polymorphic. The molecular weight of these loci ranged between 421bp to 2.5kp. Loci 'a' was shared by all sixteen mutants and parent. Loci 'b' appeared only in P1 and P2 (30 Gy) whereas, loci 'c' and 'd' appeared in P2 (30 Gy). Therefore, these loci can be designated as RAPD marker for P2 of 30Gy.

Primer B-03 on amplification yielded four loci on gel and all of them were polymorphic, ranged between 398bp-1.1kb. Primer B-09 on amplification exhibited 66.67% polymorphism and 33.33% monomorphic bands. Six loci were detected on the gel when the DNA was amplified with Primer B-04. These loci showed 83.33% polymorphism among the mutated population of NIA-2004. The amplified segments of DNA ranged between 275bp-936bp. Primer B-12 and primer B-14, amplified seven polymorphic loci, with the range of 386bp-1.2kb and 506bp-1.8kb respectively. Primer B-07 amplified five polymorphic alleles, ranged between 851 bp-1.7kb. Whereas, primer B-11 amplified seven loci, six were polymorphic and only one monomorphic, segment size ranged between 328bp-984kb. Primer B-08 amplified three loci in which two were monomorphic, ranged between 368bp-1.6kb. Primer B-10 (745bp-2.2kb) and C-03 (898bp-1.8kb) produces one and four polymorphic loci respectively.

Table 1. Sequence of the primers used.

Primer	Sequence	Range of amplified loci	Polymorphic loci	Monomorphic loci	Total no of loci
A-01	CAGGCCCTTC	309bp-1.3kb	04	04	08
B-02	TGATCCCTGG	425bp-951bp	02	01	03
B-03	CATCCCCCTG	398bp-1.1kb	04	Nil	04
B-04	GGACTGGAGT	275bp-936bp	05	01	06
B-06	TGCTCTGCCC	311bp-1.1kb	03	01	04
B-07	GGTGACGCAG	851bp-1.7kb	05	Nil	05
B-08	GTCCACACGG	368bp-1.6kb	01	02	03
B-09	TGGGGGACTC	451bp-1.3kb	06	03	09
B-10	CTGCTGGGAC	745bp-2.2kb	01	04	05
B-11	GTAGACCCGT	328bp-984bp	06	01	07
B-12	CCTTGACGCA	386bp-1.2bp	07	Nil	07
B-14	TCCGCTCTGG	506bp-1.8kb	07	Nil	07
C-03	GGGGGTCTTT	898bp-1.8kb	04	01	05
			55(75.34%)	**18(24.66%)**	73

Genetic variability: The similarity coefficients reflected the genetic relationship between the mutants and parent. The greatest similarity was observed between P3 (10 Gy) and P4 (10 Gy) (96.5%) and the least similarity occurred between P2 (20 Gy) and P1 (30 Gy) (48%) (Table 2).

On the basis of results achieved, the mutants could be divided into four clusters, designated a, b, c and d (Fig. 4). Cluster 'a' comprises of the P3 (10 Gy), P4 (10 Gy), P1 (10 Gy), P4 (20 Gy), NIA-2004 (parent), P3 (30 Gy) and P1 (30 Gy). Cluster 'b' contained predominantly P3 (20 Gy) and P4 (30 Gy) showing more genetic similarity among each other. Whereas, P2 (40 Gy) and P3 (40 Gy) were in cluster 'c' and P1 (40 Gy) and P4 (40 Gy) are grouped in Cluster d. Mutants P2 (30 Gy), P1 (20 Gy) and P2 (20 Gy) were genetically distinct from other mutants.

Mutagenesis disturbs the normal biological composition of an organism and the true genetic changes are desirable in mutation studies. 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 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 they cannot 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 to affect the phenotypic appearance in the population. The screening and selection of mutants have been reported to be preferable from second irradiated generation because in case of recessives, both *loci* are rarely mutated, and therefore segregation must be permitted so that 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).

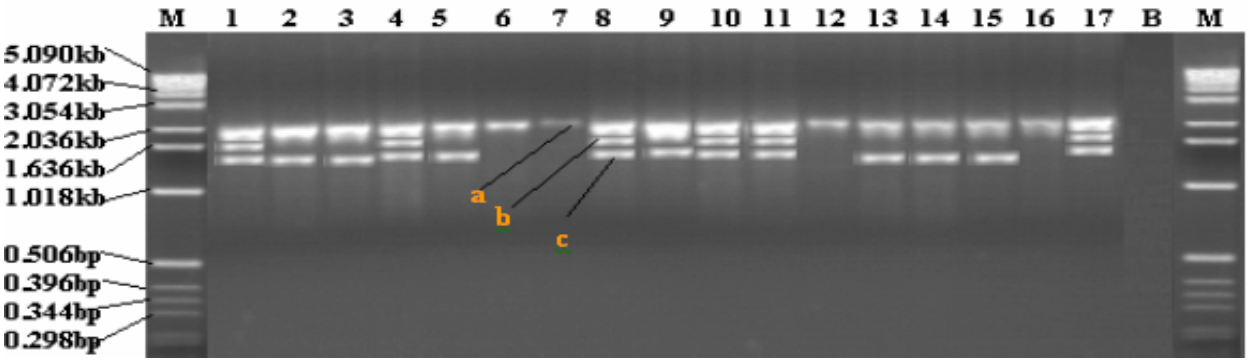

Fig. 1. Primer B-02, M=1Kb ladder, 1=P1, 2=P2, 3=P3, 4=P4 (10 Gy)., 5=P1, 6=P2, 7=P3, 8=P4 (20 Gy)., 9=P1, 10=P2, 11=P3, 12=P4 (30 Gy)., 13=P1, 14=P2, 15=P3, 16=P4 (40 Gy)., 17=NIA-2004, 18=Blank.

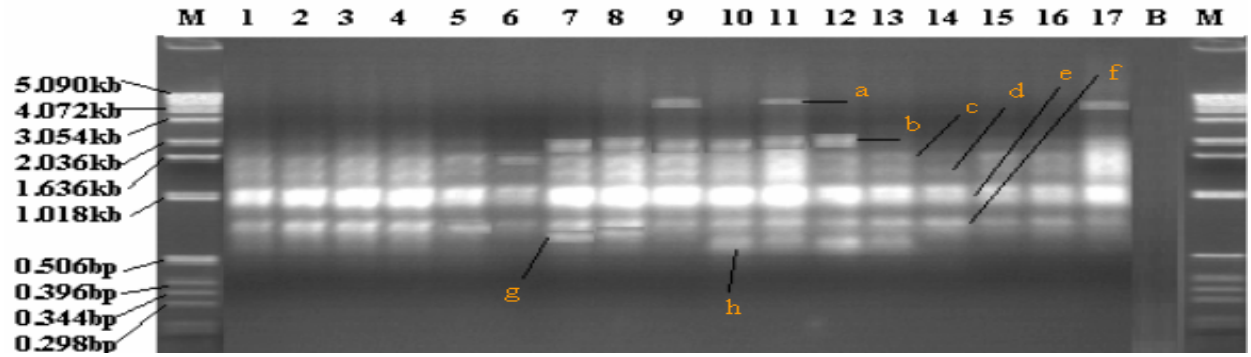

Fig. 2. Primer A-01, M=1Kb ladder, 1=P1, 2=P2, 3=P3, 4=P4 (10 Gy)., 5=P1, 6=P2, 7=P3, 8=P4 (20 Gy)., 9=P1, 10=P2, 11=P3, 12=P4 (30 Gy)., 13=P1, 14=P2, 15=P3, 16=P4 (40 Gy)., 17=NIA-2004, 18=Blank.

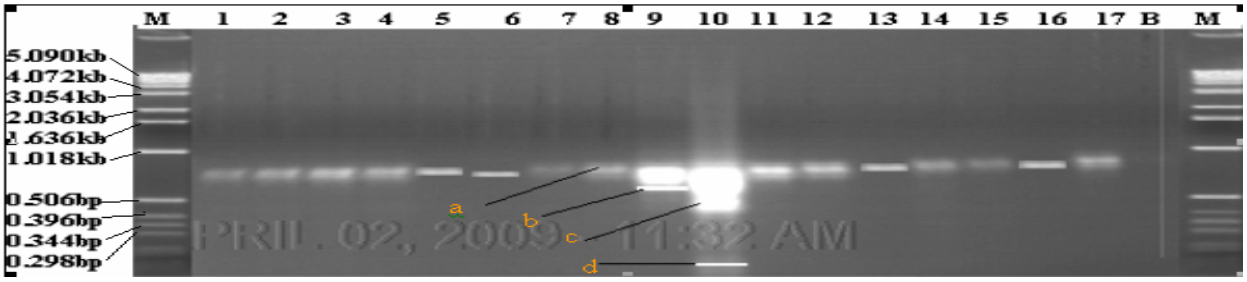

Fig. 3. Primer B-06, M=1Kb ladder, 1=P1, 2=P2, 3=P3, 4=P4 (10 Gy)., 5=P1, 6=P2, 7=P3, 8=P4 (20 Gy)., 9=P1, 10=P2, 11=P3, 12=P4 (30 Gy)., 13=P1, 14=P2, 15=P3, 16=P4 (40 Gy)., 17=NIA-2004, 18=Blank.

The results revealed that all selected mutants of 10 Gy showed more than 80-96% similarity. Population of 20Gy (P1-P4) exhibited 31-50% dissimilarity, which is the highest polymorphism in the mutated population of NIA-2004. Irradiation dose of 30 and 40Gy gave 59% genetic variability. Two mutants of 20Gy and one of 30Gy occupied a distinct place in dendrogram, and might be due to enhancing effect of low doses (Khan *et al.*, 2009). In the present study it was observed that low dose 20Gy treatment generated more variation than 30 and 40Gy. Mutants of 30Gy population showed 13-52% dissimilarity with 10, 20 and 40Gy, whereas, mutants of 40Gy showed more than 60% similarity with parent.

Table 2. Similarity coefficient among the NIA-2004 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	0.802	1															
L3	0.924	0.878	1														
L4	0.957	0.843	**0.965**	1													
L5	0.652	0.616	0.662	0.664	1												
L6	0.503	0.507	0.52	0.513	0.564	1											
L7	0.816	0.837	0.874	0.851	0.693	0.6	1										
L8	0.894	0.817	0.94	0.927	0.64	0.512	0.859	1									
L9	0.83	0.729	0.818	0.821	0.588	**0.48**	0.739	0.847	1								
L10	0.777	0.643	0.751	0.771	0.592	0.506	0.728	0.783	0.747	1							
L11	0.847	0.813	0.884	0.875	0.578	0.496	0.803	0.869	0.858	0.711	1						
L12	0.79	0.782	0.776	0.794	0.665	0.617	0.863	0.792	0.775	0.672	0.834	1					
L13	0.713	0.703	0.702	0.72	0.668	0.642	0.805	0.697	0.657	0.699	0.687	0.826	1				
L14	0.786	0.809	0.808	0.773	0.602	0.615	0.8	0.796	0.726	0.699	0.798	0.831	0.82	1			
L15	0.825	0.843	0.855	0.851	0.652	0.597	0.861	0.845	0.777	0.7	0.87	0.894	0.795	0.899	1		
L16	0.735	0.731	0.777	0.742	0.682	0.609	0.783	0.767	0.699	0.684	0.692	0.752	0.812	0.824	0.786	1	
L17	0.86	0.745	0.868	0.857	0.574	0.504	0.791	0.899	0.852	0.722	0.861	0.743	0.672	0.798	0.819	0.732	1

10 Gy: P1-P4 (L1-L4); 20 Gy: P1-P4 (L5-L8); 30 Gy: P1-P4 (L9-L12); 40 Gy: P1-P4 (L13-L16) & NIA-2004 (17)

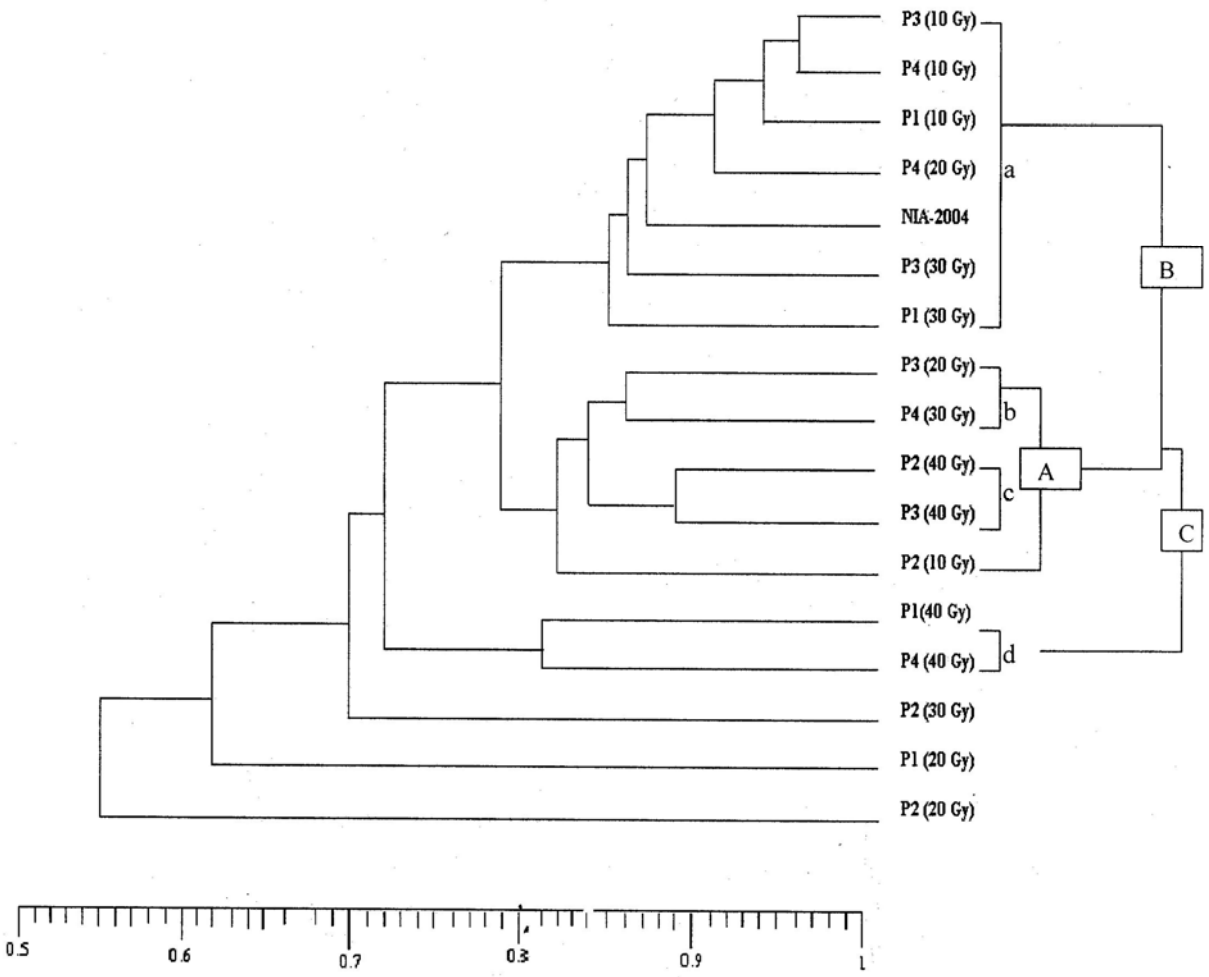

Fig. 4. Dendrogram of sixteen NIA-2004 mutants with parent developed from RAPD data using un-weight pair group method of arithmetic means (UPGMA).

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). Since 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.

In conclusion, genetic diversity among the sugarcane mutants was mainly based on the morphological and anatomic characters, which are governed by the gene and influenced by the environment due to which these features are changeable and sometimes difficult to distinguish. Therefore, it is necessary to be supported by molecular techniques 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), SSR (Simple Sequence Repeat), TRAP (Target Region Amplified Polymorphism) will improve the accuracy of resolution of accurate genetic variation among the mutant/variants of sugarcane.

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