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RESEARCH ARTICLE

SUGARCANE IMPROVEMENT THROUGH MUTATION AND MOLECULAR TECHNIQUE

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ABSTRACT

Mutation breeding has been generally used for the improvement of plant characters in several crops. It is a powerful and effective tool in the hands of plant breeders. In mutation breeding program, selection of an effective and efficient mutagen is very essential to produce high frequency of desirable traits. The three sugarcane clones viz; NIA-0819, NIA-98 and BL4 were selected and subjected with Ethyl methanesulfonate (EMS) doses of 5, 10, and 15 mM and untreated of each variety were used as control. The results indicated for agronomic traits showed that maximum plant height was observed (331.67 cm) in BL4 under control and minimum plant height was observed (209 cm) at 15mM in BL4. The maximum number of tillers plant^{-1} (7.00) at 10 Mm. The higher number of internodes was noted (16.66) at 10 mM in BL4, maximum number of internodes length (17.31 cm) at 10 mM in NIA-98 and minimum (11.91 cm) at 5 mM in BL4. The results of quality traits indicated that maximum brix (20.33 %) was obtained at 15mM in BL4 and lowest (14.66 %) in 5mM in NIA-0819. The more purity was observed (72.43) at 10 mM in BL4, fibre (15.33) at 5 mM in BL4. The maximum sucrose (14.16 %) was recorded under at 15 mM in BL4, sugar yield (6.14 t ha^{-1}) under control in BL4 and cane yield BL4 was noted (331 t ha^{-1}) at 10 mM in BL4. The selected sugarcane mutants were analyzed by using simple sequence markers (SSR). Out of 13 SSR primers studied, 7 primers were found to be polymorphic, whereas 6 primers were counted to be monomorphic. A total of 495 conformers were analyzed by using 13 SSR primers as presented and 495 bands, 191 bands were polymorphic, showing 38.58 % polymorphism. The maximum number of locus (20) was produced by the primer SMC-703BS and minimum number of bands (8) was produced with primer EST-SSR29. The amplification products in 10 mM (EMS) produced multiple loci, in which the total numbers of 129 scroable bands, out of which 100 were monomorphic. They showed SMC 703BS bands15 was produced with lowest number of bands (8) was obtained with primer EST -SSR 29. The primer no amplification products found in EST-SSR30-5, EST-SSR38. The amplification products in 15 mM (EMS) produced multiple fragments in which the total number of 30 scroable bands was polymorphic. Some specific bands were also identified thus reflecting the SSR application for the identification of sugarcane mutants. The results showed that 15 mM (EMS) indicated 6 primers produced a single polymorphic band and 7 primers produced two polymorphic bands. The genetic similarity indices calculated by Jaccard's similarity coefficient varied from (0.98 to 0.85 % indicate a high level of genetic similarity among the mutants that was mainly attributed to intra specific diversity. Hence, this SSR technique helped to identify the genetic variation in mutant plants.

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INTRODUCTION

Sugarcane (Saccharum spp.) is polyploidy belonging to genus Saccharum of the family Poaceae**,** tribe *Andropogoneae*. It pays as economically important cash crop through its high cost products, sugar and ethanol. About 75 % of the global need of sugar has been achieved by the improved sugarcane varieties grown in tropical and subtropical regions of world (Singh *et al*. 2006. The polyploid/aneuploid nature with variation in chromosome number has been largely responsible for its genetic and taxonomic complexity (Parida *et al.* 2010).

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Mutation induction is a useful technique in plant breeding used to improve traits without disrupting the novel genetic make-up of the crop (Sleper *et al*., 2006). Exploitation of natural and induced genetic diversity is the basic condition of plant breeding to develop new varieties for maintainable crop production and plant breeders are handicapped due to lack of obtain ability or non-existence of favorite genotypes. But, they have positively recombined the desired genes from the existed available gene pool and associated with plant species by asexual and sexual hybridization, and successfully to grow new varieties with desirable traits such as high yield, abiotic and biotic stress resistance. The purpose of induced mutations is to enhance the mutation frequency amount in order to select suitable variants for plant breeders (Jain, 2010).

The mutation frequency rate of natural mutations is rather very low and very difficult to achieve by the plant breeders (Jain, 2010). Mutations are induced by chemical (e.g. ethylmethanesulfonate) physical (e.g. gamma radiation) and mutagen treatment of both seed and vegetatively propagated crops (Predieri, 2001, and Purwati, 2006). The mutagen treatment disruptions the nuclear DNA and during the process of DNA repair tool for new mutations which are induced randomly and heritable (*Wood, 2013).* The changes can occur in cytoplasmic organelles, and also results in chromosomal or genomic mutations and plant breeders enable to select useful mutants such as flower colour, flower shape, disease resistance, early flowering and early maturing (Crino, *et al.,* 1994 and Jain *et al.,* 2004). A specific advantage of mutation induction is the possibility to select genetic variation, improvement of vegetatively propagated plants when one or few characters of an outstanding cultivar are to be modified. The induced mutations played a major role in the development of superior crop varieties transforming into an incredible economic impact on agriculture and food production (Jain 2010). Induced mutation is a significant corresponding method for crop breeding (Khan *et al*. 2007).

Ethyl methanesulfonate (EMS) have been used for induced mutation on banana shoot tips, sugarcane sett and apical meristem, followed by regeneration of adventitious shoots. Hrishi, *et al.* (1968) was observed that the effective dose for methyl methanesulphonate (MMS) was 0.06 M. Baroda (1987) reported that effective dose for **e**thyl methanesulfonate (EMS) and sodium azide was 1x10-^{3} M and that the sodium azide was more effective than EMS. Srivastava *et al*., (1986) showed that 0.8 % of Nitroso Methyl Urethane (NMU), Di Ethyl Sulphate (DES) and EMS were effective mutagenic causes to induced mutations in sugarcane. The technology development in molecular biology contributed important to understanding the genetic variation of plant. Simple sequence repeats (SSR) markers are the most frequently used in molecular techniques to study polymorphism in sugarcane (Nair *et al*., 1999, 2002, Chen *et al.,* 2007, Abbas *et al.*, 2015, Singh *et al.* 2005, Smillah *et al.* 2012, Powell *et al.,* 1996, Cordeiro *et al*. 2000). Currently SSR markers have been widely used in genetic variability in several crops likely maize (Yao *et al*., 2008, Selvi *et al.,* 2003) rice (Kibria *et al.,* 2009), foxtail millet (Gupta *et al*., 2012) and as well as sugarcane (Pandey *et al*., 2011). The mutagenesis used to establish sugarcane mutants with positive impact with highly significant improvement in the general economy of the population. The aim of the present study was to identify the best yield characters with high sugar recovery in sugarcane mutants. This information will develop the ongoing breeding program by mutation breeding and genetic variability.

MATERIALS AND METHODS

The field experiment was conducted at Experiment farm, Nuclear Institute of Agriculture (NIA), Tando Jam, Sindh, Pakistan. The experiment was layout in randomized complete block design (RCBD) replicated four times during 2013 and 2014. The plot size was (5 x 5 m); row to row distance was adjusted 1.5 meter. The sowing was done in the month of September during both years. Before sowing, sugarcane sets were treated with different concentration of **e**thyl methanesulfonate (5, 10 and 15 mM) and untreated as control was used on three sugarcane varieties i.e. NIA-98, NIA-0819 and BL4 respectively. Three stools were randomly taken from each plot to determine sugarcane contents according to sugarcane Laboratory Manual of Queensland Sugarcane Mills (Anon.1970). The observations of M_1 and M_2 generation were recorded for plant height (cm), cane thickness (cm), number of tiller plant^{-1}, internodes length (cm), brix (%), commercial cane sugar (CCS %), Fibre (%), cane yield (t ha^{-1}) and sugar yield (t ha^{-1}).

DNA extraction through MATAB Method

DNA was extracted from fresh leaves of sugarcane varieties (NIA-98, NIA-0819 and BL4) using MATAB method. Fresh leaves (2 g) were grounded in liquid nitrogen; MATAB buffer (20 ml) was placed in a 50 ml falcon tube, grounded material was transferred in MATAB solution (Tris-HCl pH 8.1, ethelenediaminetetra acetic acid (500 mM), sodium chloride (5 M), MATAB (10 g), PEG-6000 (5 g) and sodium sulphite). Incubate the mixture at 74^{o}C for 30 minutes and then cooled at room temperature. Equal amount of chloroform isoamyl alcohol (CIAA) (24:1) was added in the cool mixture and mixed by inversion 100 times. The mixture was centrifuged at 4000 rpm for 30 minutes. The supernatant was poured into a new clean falcon tube, which was already containing 20 ml CIAA, mixed the sample and then again centrifuge at 4000 rpm for 30 minutes. Supernatant containing DNA was collected in the separate with 50 ml falcon tube which contains 20 ml of absolute isopropanol. The collected the DNA with pasture pipette and transfer DNA in 2.0 ml Eppendrof tube. Ethanol 70% was used to wash the pellet and the DNA samples were then hydrated with TE buffer. The DNA was measured with spectrophotometer at absorbance 260/280 nm. The quality of DNA was checked on 0.8 % agarose gel.

PCR Amplification and gel Electrophoresis

For DNA amplification, 13 primers from Gene Link (NewYork, U.S.A), 20 bases pair length were used to amplify the DNA. PCR reaction was carried out in 25µl reaction mixture containing 13 ng of template (genomic DNA), 2.5 mM MgCl2 (Eppendorf, Hamburg, Germany), 0.33 mM of each dNTPs (Eppendorf, Hamburg, Germany), 2.5 U of Taq polymerase (Eppendorf, Hamburg, Germany) and 1 µM of primer in a 1 x PCR reaction buffer (Eppendorf, Hamburg, Germany). The amplification reaction was performed in the Eppendorf Master cycler with an initial denaturation for 5 minutes at 94°C, then 32 cycles: 1 minute denaturation at 94°C; 1 min annealing at 52°C; 2 minutes extension at 72°C. Final extension was carried out at 72°C for 30 minutes. Amplified products were analyzed through electrophoresis on 2 % agarose gel containing 0.5 x TBE (Tris Borate EDTA) at 72 Volts for 2 hours, the gel contained 0.5 µg/ml ethidium bromide to stain the DNA and photograph was taken under UV light using gel documentation system (Vilber Lourmat, France). Data was analyzed, mutants from each method were compared with each other using amplification profiles and band of DNA fragments were scored as presence of bands as (1) and absence of band as (0) from simple polymorphic DNA (SSR) of amplification profile. Coefficient of similarity among cultivars was calculated according to Nei and Li (1979). A dendrogram based on these similarity coefficients was constructed by using Euclidean and Ward's method (Kumar *et al*. 2009) a dendrogram or homology tree.

RESULTS AND DISCUSSION

The interaction of varieties x ethyl methanesulfonate (EMS) for agronomy traits indicated that plant height, number of tillers plant^{-1}, cane thickness, number of internodes plant^{-1},

Table 1. Sequence of the primers (SSR)

S. No	SSR Name*	Forward primer Sequence (5' to 3')	Reverse Primer Sequence (5' to 3')
1	EST-SSR30-2(GCC)-6	AGCTAGCAAGCGTGTCCCT	AGCTAGCAAGCGTGTCCCCT
2	EST-SSR31-1(CGA)5	AAGTGGAAGACCAACGCAGGA	GTGATCCGGGAACTTGAGGAA
3	EST-SSR42(TCG)5	CACGCATGCATCTGTGTTACT	CAGGATCTACGACGAGACGA
4	EST-SSR45(GGC)5	AGCCTCCCTCTCCTTCTCTG	GCTCACGTCGTAGAAGTCCA
5	EST-SSR47(GCC)5	AACGGCTCCATGGTCTACCT	TGGCTGATATGGACGACAAA
6	EST-SSR49(CGA)5	CAGTCCCACGTCGTACCAGTC	ATTCCCACACCTGCTACTCG
7	EST-SSR52(TA)5	TGCACACGGACGTGTCTATAA	TGGTACAACTACGCCCACCAA
8	SMC 703 BS(CA)12	GCCTTTCTCCAAACCAATTAGTG	GTTGTTTATGGAATGGTGAGGA
9	SMC 24 DUQ(TG)13	CGCAACGACATATACACTTCGG	GCCATGCCCATGCTGCTAAAGAT
10	EST-SSR29(CGGA)5	CGACTGCTGCTTCGACTACA	GACCGATCCCACCGAATCTC
11	EST-SSR38-1(CA)5	GCATTTTATTACACAAAACATCACAA	CGTTCCTCACCCTTGACG
12	EST-SSR39(AT)5	ACTGATTTGTGCCTGTGATCG	GCCGGGCCTGGACTACTAT

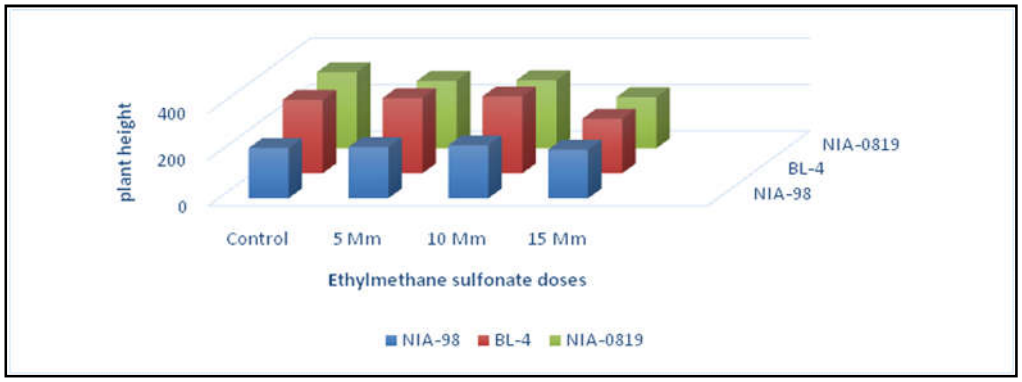

Figure 1. Effect of ethyl methanesulfonate (EMS) in plant height (cm)

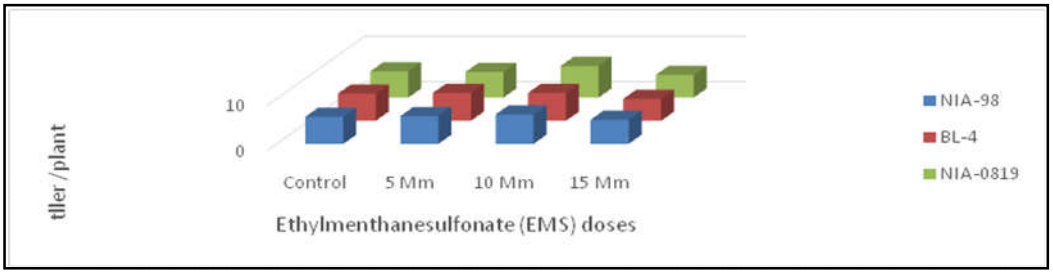

Figure 2. Effect of ethyl methanesulfonate (EMS) in number of tiller/plant

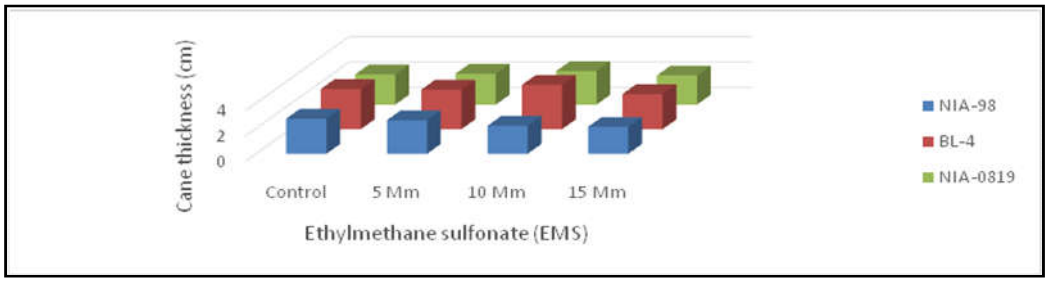

Figure 3. Effect of ethyl methanesulfonate (EMS) in cane thickness (cm)

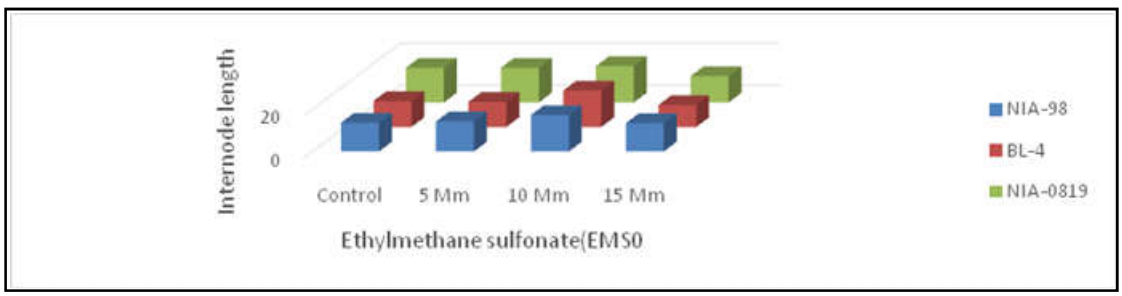

Figure 4. Effect of ethyl methanesulfonate (EMS) in internodes length (cm)

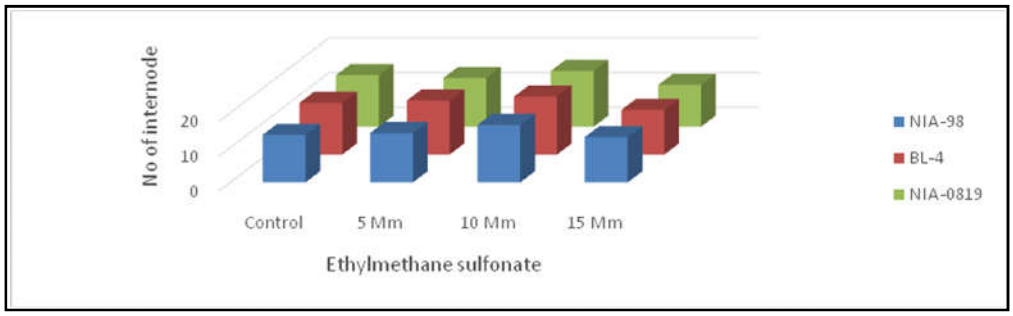

Figure 5. Effect of ethyl methanesulfonate (EMS) in number of internodes

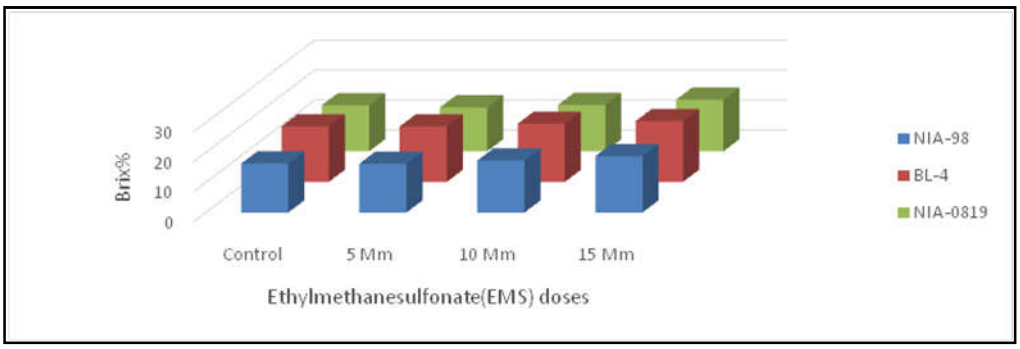

Figure 6. Effect of ethyl methanesulfonate (EMS) in brix (%)

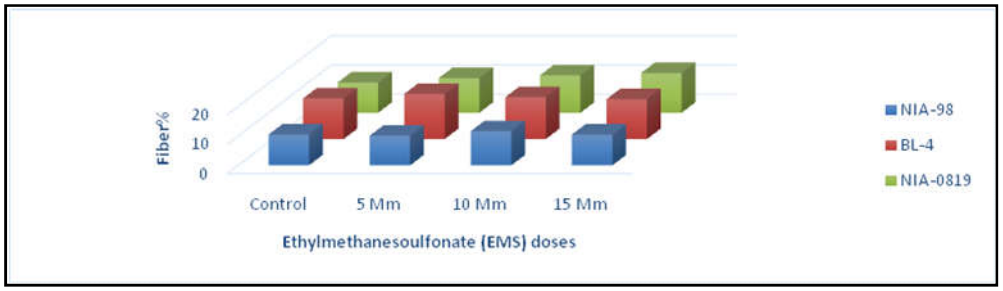

Figure 7. Effect of ethyl methanesulfonate (EMS) in fibre (%)

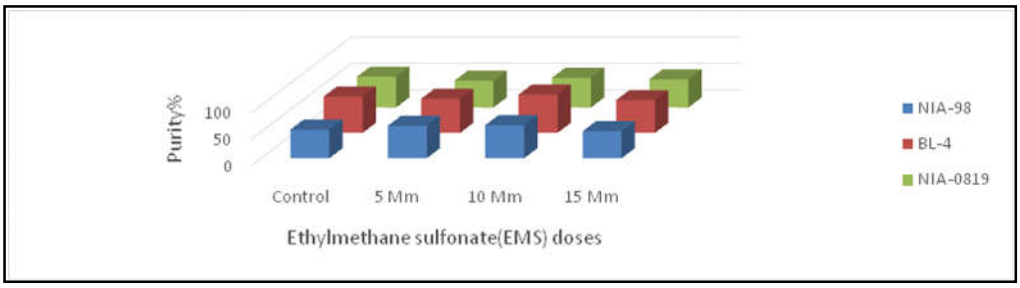

Figure 8. Effect of ethyl methanesulfonate (EMS) in purity (%)

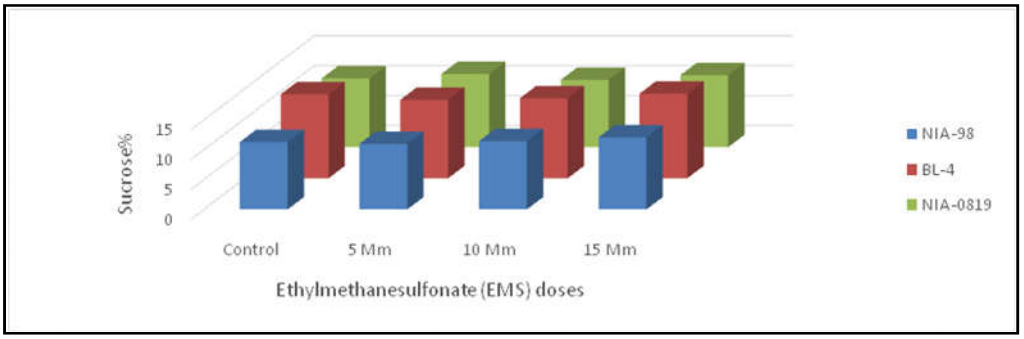

Figure 9. Effect of ethyl methanesulfonate (EMS) in sucrose (%)

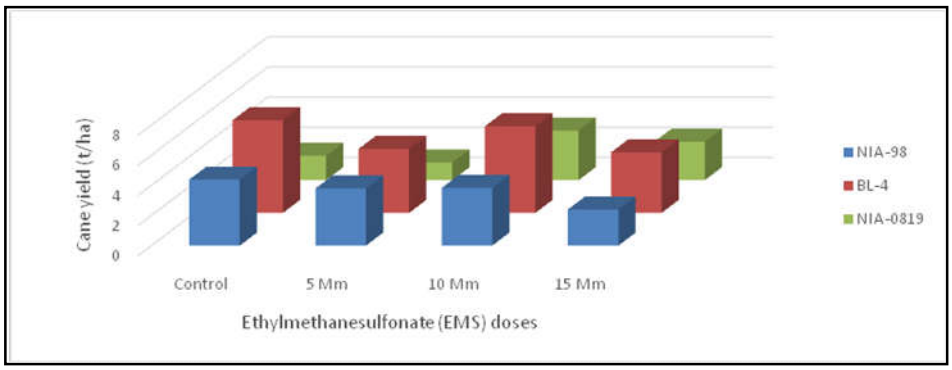

Figure10. Effect of ethyl methanesulfonate (EMS) in cane yield (t/ha)

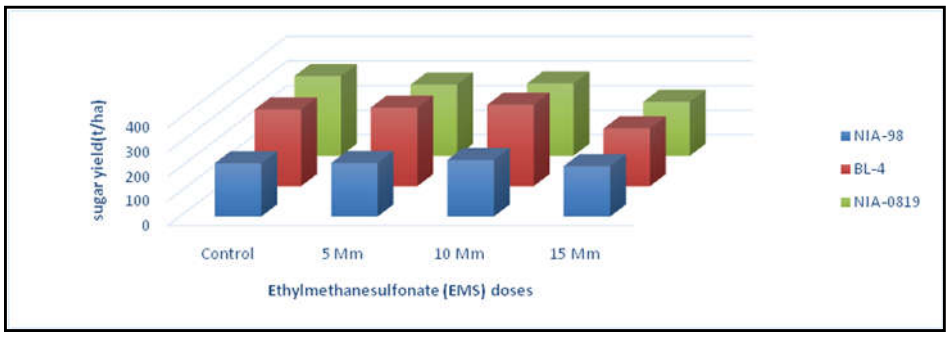

Figure 11. Effect of ethyl methanesulfonate (EMS) in sugar yield (t/ha)

fibre, sugar yield, commercial cane sugar, sugar recovery and purity were highly significant, while brix, sucrose and cane yield were non-significant at 5% probability level. Ethyl methanesulfonate showed stimulating effect on the plant height, the higher plant height (331.67 cm) were recorded in BL4 at dose of 10 mM EMS, while lower plant height (209.83 cm) were observed under the concentration of 15 mM in NIA-98 (Fig. 1). In case of maximum number of tillers plant^{-1} were produced (7.00) in NIA-0819 under at the dose of 10 mM EMS; however the lowest number of tillers plant^{-1} were recorded (4.83) at 15mM EMS concentration in each variety (Fig. 2). (Hrishi *et al.,* 1988) pyrocatechol and acenaphthene induced profuse tillering whereas quinol and coumarin suppressed tiller development. The maximum cane thickness (3.50) were recorded in BL4 at dose of 10 mM EMS, however the minimum cane thickness (2.13) was achieved in NIA-98 at the dose of 15 mM EMS (Fig. 3).

The maximum number of internodes was observed (16.66) in NIA-98 at the dose of 10mM EMS and were recorded (12.83) at dose of 15 mM EMS in NIA-0819, respectively (Fig. 4 and 5). The more brix (20.33 %) were achieved in BL4 at the dose of 15 mM EMS and minimum brix were observed (14.66 %) at the dose of 5 mM in NIA-0819. The results for fibre indicated that maximum fibre were observed (15.33 %) in BL4 at the dose of 5 mM EMS, while low fibre were recorded (10.08 %) at the dose of 5 mM EMS in NIA-0819 (Fig. 6 and 7). The maximum sugar yield (331.00 t ha^{-1}) were obtained in BL4 at the dose of 10 mM EMS, and minimum sugar yield were noted (219.83 t ha^{-1}) in NIA-0819 at the dose of 15 mM EMS. The maximum cane yield was obtained (6.14 t ha^{-1}) in BL4 under control, while the lowest cane yield was recorded (1.17 t ha^{-1}) in NIA-0819 at the doses of 5 mM EMS (Fig. 10 and 11). The results regarding sucrose percentage showed that maximum sucrose were recorded (14.07 %) in BL4 at doses of 15 mM EMS and minimum sucrose was recorded (10.97 %) in NIA-98 at the dose 5 mM EMS.

The maximum purity (72.43 %) were recorded in BL4 at the dose of 10 mM and minimum purity were observed (51.05 %) at the dose of 15 mM EMS in NIA-98 (fig. 8 and 9). Higher concentration of mutagenic treatment was recorded with highest reduction in survival and lethality percentage. The present finding is agreed with Wang *et al*. (2006). The sugar yield was improved in two mutants with increases in stalk length, stalk number, sources% and stalk diameter (Oloriz *et al*., 2012). The selection of an effective and efficient mutagen is very important to produce high frequency of desirable mutant. The mutagenized populations exhibited significantly higher variability in the M_2 generation. The results in respect of ethyl methanesulfonate doses showed decrease in survival mutagen differences in genotypes to mutagens result conformity with Wang *et al*. (2006). The survival percentage and mean value of M_1 generation were decreased with increase the doses. The current results confirmed by in soybean (Pepol and Pepo, 1989, Cheng, and Chandlee, 1999 and Pavadai *et al*., 2009), mungbean (Khan and Wani 2005) and sesame (Prabhakar *et al.,* 1985) and sugar yield was improved in two mutants with increments in stalk length, stalk number, and stalk diameter and chemical mutation induction proved to be suitable for the generation in sugarcane (Oloriz *et al*., 2012).

Simple sequence markers (SSR) studies on plantlets developed through Chemical mutation breeding in sugarcane

Three sugarcane genotypes were analyzed by using simple sequence markers (Electrophoretic analysis of SSR markers showed the multiple band profiles for sugarcane genotypes which were determined on agarose gel (Fig. 13. 14 and 15). The number of total allele scored, number of polymorphic alleles obtained per SSR markers are shown in (Table. 2). Out of 13 SSR primers under studied, 7 primers were found to be polymorphic, whereas 6 primers were counted to be monomorphic.

A total of 495 conformers were analyzed in these mutants by using 13 SSR primers as presented in (Table 2). Of 495 bands, 191 bands were polymorphic, showing 38.58 % polymorphism. The average number of allele per loucs produced by each primer was 2.62 %. The Amplification products in 5 mM (EMS) of the mutant plants and their respective parent with 13 primers, and total number of scorable bands were 57 polymorphic. Fragments ranged in size from 1.0 bp-1 kb and the primer of the size ranging from 10.0 to 0.5 bp.

(Khan *et al.* 2009; Sharma *et al*., 2014) developed through tissue culture plants by using young leaf of sugarcane as explants. The polymorphic variation in amplification determined by SSR markers changes the genetic pattern between the selected genotypes. However, the monomorphic banding pattern shown the conserved coding nature of genome among the sugarcane. The binding efficiency of SSRs to the genomic DNA which, is readily achieved during amplification than the ESTs-SSRs (Cordeiro *et al.* 2001).

Table 2. PCR amplified details about the number of bands, polymorphism of the SSR markers in sugarcane mutant's plants

Sr. No	Primer	Product size (bp)	Annealing Temperature ^{0}C	Total bands	Polymorphic bands	Polymorphic status
1	EST-SSR30-2(GCC)6	19	59.5	0	-	F
2	EST-SSR31-1(CGA)5	20	55.4	57	30	P
3	EST-SSR42-(TCG)5	21	59.4	56	22	P
4	EST-SSR45-2(GGC)5	20	62.5	38	-	M
5	EST-SSR47-(GCC)5	20	60.5	28	-	M
6	EST-SSR49-(CGA)5	20	62.2	60	28	P
7	EST-SSR52-(TA)5	21	59.4	46	25	P
8	EST-SSR39-(GA)5	20	55.4	23	-	M
9	SMC703BS-(CA)12	23	61.1	56	40	P
10	SMC24DUQ-(TA)13	22	62.1	70	26	P
11	EST-SSR29-(CGGA)5	20	57.5	50	20	P
12	EST-SSR38-1(CA)5	26	56.6	0	-	M
13	EST-SSR30-(AT)5	20	55.4	11	-	F
Total				495	191	

Similarity matrix of mutant plants and their as revealed by SSR markers (Cont.)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
(NIA98-c)	1.00														
P2(NIA98-5mM)	0.53	1.00													
P3(NIA98-5mM)	0.64	0.15	1.00												
P6(NIA98-5mM)	0.77	0.31	0.82	1.00											
(NIA-0819) control	0.53	0.35	0.15	0.31	1.00										
P26(NIA-019)5mM	0.53	0.35	0.50	0.31	0.35	1.00									
P27NIA-0819)5mM	0.53	0.35	0.15	0.31	1.00	0.39	1.00								
P30NIA-0819)5mM	0.53	0.02	0.50	0.31	0.02	0.35	0.02	1.00							
BL4-C	0.64	0.50	0.63	0.82	0.15	0.15	0.15	0.15	1.00						
P50(BL4)5mM	0.46	0.53	0.05	0.25	0.22	0.22	0.22	0.22	0.38	1.00					
P52(BL4)5mM	0.77	0.69	0.42	0.56	0.69	0.31	0.69	0.31	0.42	0.22	1.00				
P53(BL4)5mM	0.53	0.35	0.84	0.69	0.02	0.35	0.25	0.35	0.84	0.22	0.31	1.00			
P7(NIA98-10mM)	0.53	0.25	0.15	0.31	0.35	0.02	0.35	0.67	0.15	0.53	0.31	0.02	1.00		
P9(NIA98-10mM)	0.46	0.22	0.72	0.59	0.22	0.22	0.22	0.22	0.38	0.23	0.59	0.53	0.09	1.00	
P10(NIA98-10mM)	0.77	0.31	0.42	0.56	0.31	0.31	0.31	0.31	0.42	0.22	0.56	0.31	0.31	0.59	1.00
P3(NIA-0819)10mM	0.77	0.31	0.42	0.67	0.31	0.31	0.31	0.31	0.42	0.22	0.56	0.31	0.13	0.59	0.56
P34NIA-0819)10mM	0.84	0.50	0.83	0.42	0.16	0.50	0.15	0.50	0.83	0.83	0.42	0.84	0.15	0.38	0.42
P35NIA-0819)10mM	0.46	0.22	0.38	0.59	0.22	0.09	0.22	0.22	0.38	0.23	0.59	0.22	0.22	0.38	0.25
P56(BL4)10mM	0.46	0.09	0.05	0.22	0.09	0.09	0.09	0.53	0.05	0.07	0.22	0.09	0.53	0.07	0.22
P58(BL4)10mM	0.53	0.35	0.50	0.69	0.25	0.25	0.25	0.25	0.84	0.22	0.31	0.67	0.02	0.22	0.31
P59(BL4)10mM	0.64	0.50	0.27	0.42	0.50	0.15	0.50	0.15	0.27	0.05	0.82	0.15	0.15	0.72	0.42
P13 (NIA98-15mM)	0.64	0.58	0.27	0.42	0.15	0.15	0.15	0.50	0.27	0.05	0.42	0.15	0.50	0.05	0.42
P15(NIA98-15mM)	0.64	0.58	0.27	0.42	0.15	0.15	0.15	0.50	0.27	0.05	0.42	0.15	0.50	0.05	0.42
P17(NIA98-15mM)	0.46	0.98	0.38	0.59	0.22	0.22	0.22	0.22	0.38	0.07	0.22	0.22	0.22	0.07	0.59
P36NIA-0819)15mM	0.33	0.62	0.15	0.05	0.62	0.30	0.62	0.35	0.18	0.41	0.43	0.02	0.02	0.22	0.43
P40(NIA-0819)5mM	0.33	0.30	0.18	0.43	0.30	0.02	0.30	0.35	0.18	0.09	0.43	0.02	0.22	0.41	0.05
P41NIA-0819)15mM	0.53	0.02	0.50	0.31	0.35	0.67	0.35	0.67	0.15	0.22	0.31	0.35	0.35	0.22	0.69
P62(BL4)15mM	0.53	0.02	0.50	0.31	0.35	0.67	0.35	0.35	0.15	0.09	0.31	0.35	0.02	0.53	0.69
P64(BL4)15mM	0.46	0.22	0.38	0.22	0.22	0.85	0.22	0.53	0.05	0.07	0.22	0.22	0.22	0.07	0.59
P65(BL4 1)5mM	0.46	0.53	0.05	0.22	0.53	0.22	0.53	0.22	0.05	0.69	0.59	0.9	0.53	0.07	0.22

The primer has no amplification products found in EST-SSR30-5, EST-SSR38. The size of fragments produced ranging from 0.08 bp- 1 kb. The number of fragment produced by various primers ranged from 1-1 with an average of 2.0 fragments per primers. They studied diversity in 96 genotypes with just two primer pairs. Cordeiro *et al*. (2001) applied 21 primer sets to five sugarcane genotypes and among them 17 pairs were polymorphic, but the level of polymorphism (PIC value) in the cultivars detected by these SSRs was low (0.23).

The optimum GC content is set to 50 %, with a minimum of 30 % and a maximum of 70 %. Screening of these SSR primers are under process for tagging of sugar trait gene among Co86011 (high sugar) and UP9530 (low sugar) parents reported by (Mishra *et al*., 2015). The amplification products in 15 mM EMS produced multiple fragments in which the total number of 30 scroable bands was polymorphic. The number of fragments products range from 0.02 bp- 1 kb. The number of fragments produced by various primers ranged from 1-1 with

Similarity matrix of mutant plants and their as revealed by SSR markers

	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
(NIA98-c)															
P2(NIA98-5mM)															
P3(NIA98-5mM)															
P6(NIA98-5mM)															
(NIA-0819) control															
P26(NIA-019)5mM															
P27NIA-0819)5mM															
P30NIA-0819)5mM															
BL4-C															
P50(BL4)5mM															
P52(BL4)5mM															
P53(BL4)5mM															
P7(NIA98-10mM)															
P9(NIA98-10mM)															
P10(NIA98-10mM)															
P3(NIA-0819)10mM	1.00														
P34NIA-0819)10mM	0.42	1.00													
P35NIA-0819)10mM	0.25	0.05	1.00												
P56(BL4)10mM	0.59	0.05	0.38	1.00											
P58(BL4)10mM	0.31	0.50	0.53	0.22	1.00										
P59(BL4)10mM	0.82	0.27	0.38	0.38	0.15	1.00									
P13 (NIA98-15mM)	0.42	0.27	0.72	0.50	0.27	0.15	1.00								
P15(NIA98-15mM)	0.42	0.27	0.72	0.72	0.50	0.27	0.15	1.00							
P17(NIA98-15mM)	0.22	0.05	0.38	0.38	0.53	0.05	0.53	0.38	1.00						
P36NIA-0819)15mM	0.58	0.18	0.09	.22	0.30	0-18	0.30	0.18	0.09	1.00					
P40(NIA-0819)5mM	0.43	0.15	o.41	0.09	30	0-32	0.30	0.18	0.09	0.35	1.00				
P41NIA-0819)15mM	0.31	0.50	0.09	0.22	0.02	0-15	0.35	0.15	0.53	0.02	1.35	1.00			
P62(BL4)15mM	0.69	0.50	0.09	0.22	0.02	0-05	0.35	15	0.22	0.02	1.02	0.67	1.00		
P64(BL4)15mM	0.22	0.38	0.07	0.07	0.09	0-05	0.22	0.38	0.07	0.98	0.22	0.53	0.53	1.00	
P65(BL4 1)5mM	0.22	0.05	0.07	0.07	0.09	0.38	0.22	0.05	0.07	0.41	0.41	0.22	0.09	0.07	1.00

1. (NIA98Control), 2.P2(NIA98)5mM, 3.P3(NIA98)5mM, 4.P6(NIA98)5mM, 5.(NIA-0819Control), 6.P26(NIA-019)5mM, 7.P27(NIA-0819)5mM, 8.P30(NIA-0819)5mM, 9. (BL4Control), 10. P50 (BL4)5mM, 11. P52 (BL4)5mM, 12. P53 (BL4)5mM, 13. P7 (NIA98)10mM, 14. P9 (NIA98)10mM, 15. P10 (NIA98)10mM, 16. P3 (NIA-0819)10mM, 17. P34 (NIA-0819)10mM, 18. P35 (NIA-0819)10mM, 19. P56 (BL4)10mM, 20. P58 (BL4)10mM, 21. P59 (BL4)10mM, 22. P13 (NIA98)15mM, 23. P15 (NIA98)15mM, 24. P17 (NIA98)15mM, 25. P36 (NIA-0819)15mM, 26. P40 (NIA-0819)5mM, 27. P41NIA-0819)15mM, 28. P62 (BL4)15mM, 29. P64 (BL4)15mM and30. P65 (BL4)15mM

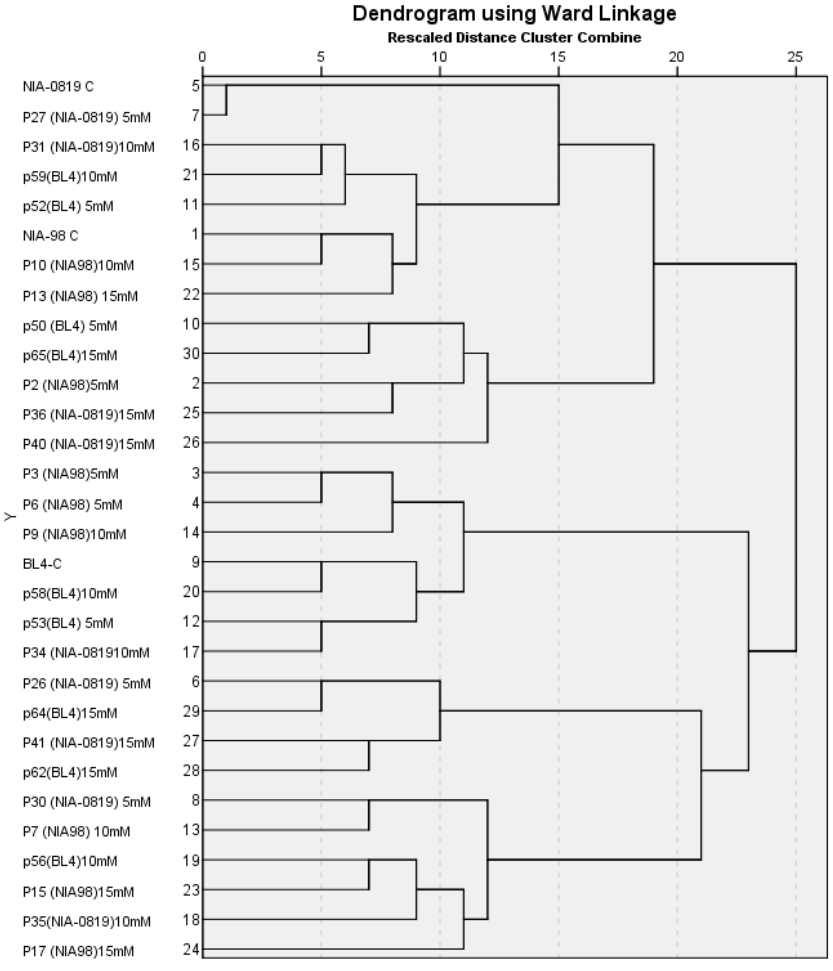

Fig.12 Dendrogram of sugarcane mutants, mM, 10mM, and 15mM plants from SSR data using unweight pair group method of arithmetic means (UPGMA)

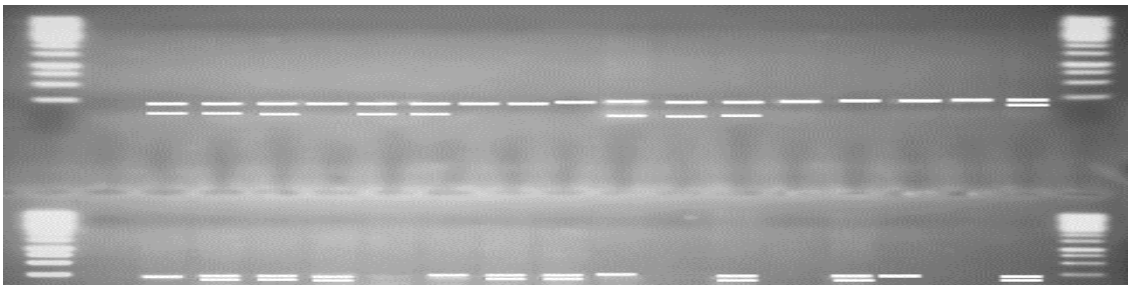

Fig. 13. SSR markers resulting from amplification with six different EST-SSR markers in the chemical mutagens DNA analyzer

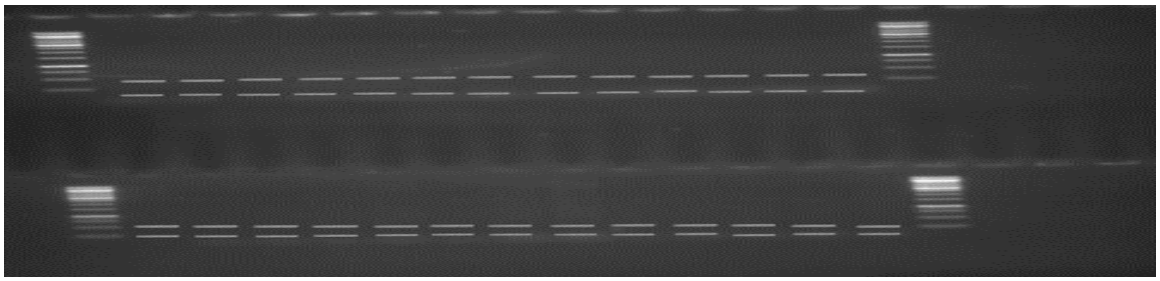

Fig. 14. SSR markers resulting from amplification with different EST-SSR markers in the chemical mutagens DNA analyzer

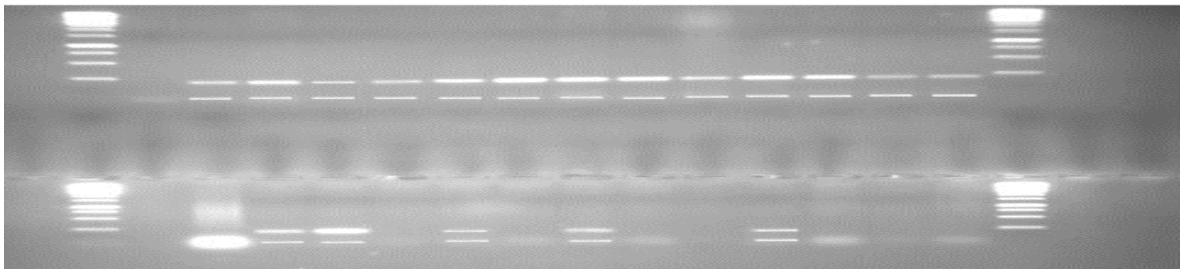

Fig.15. SSR markers resulting from amplification with different EST-SSR markers in the chemical mutagens DNA analyzer

an average of 2.0 fragments per primer. Some specific bands were also identified thus reflecting the SSR application for the identification of sugarcane mutants. The results shown that 15 mM EMS contains a specific band of 2.0 bp whereas 6 primers produced a single polymorphic band and 7 primers produced two polymorphic bands. Indian sugarcane genotypes with the 'Co' origin have presented the significant dissemination at 50-80 % GS through EST-SSR SSCP technique (Swapna *et al.* 2010).

The genetic evaluation studies conducted on sugarcane initiate the average range of PIC 0.69 generated by 365 EST-SSRs (Marconi *et al*. 2011), 0.73 by 342 EST-SSRs (Oliveira *et al*. 2009) and 0.66 by 30 EST-SSRs (Pinto *et al*. 2004) on similar 15 varieties and 3 species of sugarcane but there was lack of 'VSI' or 'Co' varieties involvement in their parentage background. The SSR amplification data were used to obtain a similarity matrix and for the generation mutants plants dendrograrm. Similarity matrix reflects the genetic relationship between the sugarcane genotypes mutants plants (Table.3) showed the highest similarity was obtained between at 5 Mm EMS in (0.98%), P2 NIA-98 and followed by in (0.85 %), P26 NIA-0819. The similarity coefficient matrix was observed in (0.84 %) in BL4 under control, P26 NIA-0819, P3 NIA-98 at 5 Mm EMS respectively. The sugarcane mutants' minimum similarity was obtained in (0.02%) in P2 NIA-98, NIA-0819 under control, P27 NIA-0819, BL4 P52 at 5 Mm EMS and P5 BL4, P36 NIA-0819 10 Mm EMS. Khan *et al*. (2009) determined a similarity coefficient matrix based on genetic distance according to Nei and Li (1979) to estimate the genetic divergence and relatedness among the somaclones developed, which ranged from 0.366 (P-100 vs P-98) to 0.951 (parent vs P-104). The dendrogarm of genetic similarity was observed among the mutants. The cluster analysis based on similarity values has classified the sugarcane genotypes mutant accession in eight clusters (Fig. 12). Cluster 1 comprised of one parent and one mutants, NIA-0819 under control, P27 NIA-0819 5Mm EMS. Cluster 2 consisted of three P31 NIA-08195, P59 BL4 10Mm (EMS) and P52 BL4 5Mm (EMS) Cluster 3 consisted of three mutants NIA-98 control, P10 NIA-98 10Mm (EMS) and P13 NIA-98 15Mm (EMS). Cluster 4 consisted of five mutants P50 BL4, P65 BL4, NIA-98 5Mm (EMS) and P36 NIA-0819, P40 NIA-081915Mm (EMS) similar relation. Cluster 5 consisted of three mutants P3 NIA-98, P6 NIA-98 5Mm (EMS) and P9 NIA-98 10Mm (EMS). Cluster 6 contained of four mutants BL4 control, P58 BL4 10Mm (EMS), P5 BL4 5Mm (EMS) and P34 NIA-0819 10Mm (EMS) control genotypes is dependent cluster different similar occupied. Cluster 7 Cluster four consisted of four mutant P26 NIA-0819, P62 BL4 5Mm (EMS), and P41NIA-0819, P64 BL4 15Mm (EMS) similar pair. Cluster 8 consisted of six mutants P30 NIA-0819 5Mm (EMS), P7 NIA-98, P56 BL4, P35 NIA-0819 10Mm (EMS), and P15 NIA-98, P17 NIA-98 15Mm EMS genetically closely.

Clustering and sub clustering as depicted in the dendrogram certified the presence of variability at the DNA level (Nair *et al.* 2002). Cluster I genotypes may be used for the introgression of resistance traits in spite of the low sugar content. The sucrose accumulation associated enzymes activities in these low sucrose sugarcane genotypes and other high sucrose genotypes showed better findings about the selection of high and low sugarcane representative which used in gene expression analysis (Kalwade *et al*. 2014). However, sugarcane genotype POJ2878 use as progenitor parents in crop improvement program in several commercial breeding programs in most of the international sugarcane research station (Lu *et al.* 1994), however, most of the similar genotypes used in the ISSR marker (Kalwade *et al*. 2012).

Results of SSR analysis confirmed the generation of genetic variability through mutation variation in sugarcane genotype. The collected data also confirmed that the ability to use SSR markers for the determination and estimation of genetic similarity and dissimilarity among different sugarcane mutants which developed from the same parent. The information about genetic similarity will be helpful to produce a population of genetically identical mutants to select a variable mutant with improved traits and increased productivity with respect to their parent. SSR analysis may also be very useful in breeding programme for initial and identification of the most different mutant plants in a large population. It is confirmed that SSR markers proved markers with respect to the ability of 291 polymorphism detected through SSR. However, it is also suggested that a larger number of primers should be surveyed to get more clear results.

Conclusion

It is concluded that among the different ethyl methanesulfonate (EMS) treatments (5, 10, and 15mM) showed that the best to achieve for plant height, number of tillers plant^{-1}, cane thickness, number of internodes plant^{-1}, fibre, sugar yield and purity, brix, sucrose and cane yield at 10 and15 mM EMS. The concentration of 10 mM was superior for high frequency of desirable mutant in sugarcane. The results of SSR analysis confirmed that the generation of genetic variability through mutagenesis variation in sugarcane. The results confirmed that the ability for to use SSR markers to determine and estimation of genetic similarity and dissimilarity among different sugarcane mutants.

SSR markers resulting from amplification with six different EST-SSR markers in the chemical mutagens DNA analyzer; according with sample number and mutants name respectivelyNIA98-c) P2(NIA98) 5 mM P3 (NIA98) 5 mMP6 (NIA98) 5 mM (NIA-0819) control P26 (NIA-0819) 5 m MP 27 (NIA-0819) 5 mM P30 (NIA-0819) 5 mM BL4 CP50 (BL4) 5 mM P52 (BL4) 5 mM P53 (BL4) 5 mM P7 (NIA98) 10 mM P9 (NIA98)10 mM P10 (NIA98) 10 mM P3 (NIA-0819) 10 mM P34 (NIA-0819) 10 mM P35 (NIA-0819)10 mM P56 (BL4) 10 mM P58 (BL4) 10 mM P59 (BL4) 10 mM P13 (NIA98) 15 mM P15 (NIA98) 15 mM P17 (NIA98) 15 mM P36 (NIA-0819) 15 mM P40 (NIA-0819) 5mM P41 (NIA-0819) 15 mM P62 (BL4) 15 mM P64 (BL4)15 mM P65 (BL4 1) 5mM.

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