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Full Length Article

Creation of New Genetic Diversity in Cotton Germplasm through Chemically Induced Mutation

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Abstract

Being an important cash crop of Pakistan, cotton has a major share in the agriculture based economy. Due to continuous breeding of same germplasm to produce new high yielding cultivars, genetic base of the germplasm has become narrowed down and yield of cotton is stagnant since last two decades. Keeping in view these facts, major objective of the study was to enhance the genetic variation of the available germplasm of cotton by treating with chemical mutagens. To achieve this, an experiment was conducted using three treatments of mutagen (sodium azide) and a control. Data were collected for morphological traits, yield traits and fiber quality traits. Results showed highly significant differences among genotypes and treatments. Application of sodium azide at 5 m*M* concentration of sodium azide does not affect any traits and the performances of all traits were reduced with treatment of 15 and 25 m*M* concentration of sodium azide. Fiber traits, number of nodes per plant and number bolls per plant does not respond to the treatments of sodium azide. Genetic diversity was further analyzed with SSR markers. It is concluded from the results that sodium azide may be a useful mutagen (at 15 and 25 m*M* concentration) to create genetic variation in cotton germplasm to be used for a breeding program.

Keywords: Chemical mutagen; Yield and fiber traits; Genetic variation; Mutation

Introduction

Cotton is a major fiber crop of the world and has a significant role in fiber industry and economy of many countries including Pakistan. It is mainly grown in tropical and subtropical regions. It belongs to the genus *Gossypium* and has 52 reported species out of which only four are cultivated. Cultivated species include two tetraploid species Upland cotton (*Gossypium hirsutum* L.) and Egyptian cotton (*Gossypium barbadense* L.) and two diploid species *i.e*., Asian cotton (*Gossypium arboretum* L.) and African cotton (*Gossypium herbacium* L.). Upland cotton has a share of 90% in world cotton production, Egyptian cotton has a share of 8% and other two diploid species has a share of 2% in world's cotton production (Seyoum *et al.,* 2018; Shim *et al.*, 2018).

Genetic variability is always a primary concern for the plant breeder but continuous use of same germplasm for different varietal programs has abridged the genetic variation which resulted in the development of cotton genotypes with a narrow genetic base (Iqbal *et al.,* 2017). Cotton breeders use several ways to produce new genetic variability that consists of interspecific hybridization, conventional hybridization with exotic germplasm, production of transgenic plants and mutagenesis (Ganesan *et al.,* 2005; Iqbal *et al.,* 2017; Ul-Allah *et al.,* 2017). Out of this mutagenesis is a simple and non-conventional technique which creates new heritable variation by inducing small change in DNA with artificial mutation by chemical or radiations. Commonly used mutagens include nitric oxide, colchicine, sodium azide and ethyl methane sulphonate and X-rays, Beta rays and Gamma rays (Ahloowalia and Maluszynski, 2001).

Induced mutations can create heritable variation in several traits and its role in plant improvement programs has been well recognized (Aslam *et al.,* 2009; Haidar *et al.,* 2016). Therefore, these mutations have a potential to serve as a complimentary approach in creating useful heritable mutation. Induced mutations have been used to improve major crops such as wheat, rice, barley, cotton, peanut and vegetables, which are seed propagated (Naeem *et al.,* 2015; Hussain *et al.,* 2017; Mago *et al.,* 2017; Olawuyi and Okoli,

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2017; Warghat *et al.,* 2018). Chemicals mutagens can damage plant chromosomes *via* reactive oxygen-derived radicals. This alters the DNA by base pair repalcements, especially GC→AT and results in change of amino acid sequence. The change in sequence changes the proteins characteristics, but do not totally eradicate function of the protein as frame shift mutations or deletions mostly do (Khan *et al.,* 2009; El-Sayed *et al.,* 2012; Mago *et al.,* 2017) which leads to change in morphological and yield related traits. It is reported that different mutagens have specific effect on different crops and do not respond in the same way for all crops and species (Naeem *et al.,* 2015; Mago *et al.,* 2017; Warghat *et al.,* 2018).

Sodium azide (NaN_3) is one of the most powerful chemical mutagens in crop plants. Ganesan *et al.* (2005) reported heritable variation in root traits of cotton (number of primary, secondary and tertiary roots improved) when treated with sodium azide. Ganesan *et al.* (2005) and Olawuyi and Okoli (2017) studied mutagenic variability in morphological and yield traits of maize genotypes treated with sodium azide and reported that all genotypes do not responds in same ways to the mutagenic effect. Hussain *et al.* (2017) investigated the effect of sodium azide on *Brassica napus* genotypes and reported improvement in proteins, moisture, linoleic acid and erucic acid percentage when treated with higher concentrations. Induced mutation in barley with combined application of sodium azide and N-methyl-N-nitrosourea was reported to be analyzed with TILLING techniques (Till *et al.,* 2007). The authors reported mutations affecting 32 genes related to plant performance, growth and development. They found a total of 382 mutations out of which, 61% were in coding region. These reported studies reveal important role of chemical mutagens especially sodium azide in creation of heritable mutations.

Genetic diversity and variation in traits among different genotypes is the basic requisite of plant breeders. But due to continuous inbreeding of existing germplasm, genetic diversity is reduced (Noormohammadi *et al.,* 2018; Seyoum *et al.,* 2018; Shim *et al.,* 2018). The present study was aimed to investigate the effect of sodium azide in the genetic variability of morphological ad yield and fiber traits of cotton genotypes.

Materials and Methods

Experimental Site and Plant Material

For conducting the experiment, cotton plants were grown in the research area of Department of Plant Breeding and Genetics, UCA&ES, The Islamia University Bahawalpur, Pakistan during cotton season of 2015. Soil of the experimental site was slightly alkaline (pH, 8.1) and sandy loam with a bulk density of 1.30 Mg/m^3 and soil fertility was low (N, available P and exchangeable K 456, 6.2 and 125 mg kg^{-1} respectively). Average day temperature ranged 32–41°C and average night temperature ranged 24–30°C during the experimental duration where maximum day and night temperature were observed during July. Rainfall was maximum during the months of July and August *i.e*., 80 and 55 mm respectively while in other months it was less than 20 mm.

Plant material included 10 true to type cotton genotypes obtained from the department of Plant Breeding and Genetics, UCA&ES, The Islamia University, Bahawalpur, Pakistan out of which nine genotypes were from *Gossypium hirsutum* (IUB-13, IUB-65, IUB-63, CIM-707, NIBGE-314, S-14, Sitara-11, NS-141, Bt-557) and one genotypes from *Gossypium arboreum* (Desi).

Mutagenic Treatments and Experimental Design

In order to mutate the cotton germplasm, Sodium Azide was used as a chemical mutagen. Treatment included were control where seed was treated with distilled water,5 m*M*, 15 m*M* and 25 m*M* sodium azide. First of all, 100 m*M* stock solution of sodium azide was prepared which was then diluted to each concentration for specific treatment. Fuzzy seed of cotton was soaked in the specific solution for six hours and was agitated after each hour. After six-hour seed was rinsed with distilled water various times to remove the solution of the chemical but again precautionary measures (use of gloves to touch the seed material) adopted to avoid any contact with seed material. Genotypes and mutagenic treatments were factorally combined and were sown in the field in four replications in a randomized complete block design.

Crop Husbandry

For sowing cotton crop, field was cleaned from surface flora and ploughed followed by planking. The cotton seeds were sown by dibbling method with a plant-to-plant distance of 30 cm and row-to-row 75 cm. After sowing, polythene bags to carry the mutated seed, and gloves used and rest of the material was buried in the soil as a precautionary measure to avoid contact of mutagenic chemical with any living organism.

The cotton crop was sown on raised beds in last week of June, 2015. The fertilizer *i.e*., (N, P, K and Zn) were applied @150, 60, 50 and 5 kg ha^{-1}, respectively where source of N was urea, sour of P was triple super phosphate, source of K was sulphate of potash and source of Zn was zinc sulphate. Whole amount of P, K and Zn was applied at sowing and N was applied in three equal splits *i.e*., at sowing, flower initiation and at late flowering stage. Weeds were controlled by applying herbicide (Stomp-330E @ 2.5 L ha^{-1}). Plant population was maintained @ 40,000 plants ha^{-1} after thinning. Recommended pesticides were used to protect the crop from

the attack of from insect pest. All agronomic and management practices were kept same for all treatments and genotypes to avoid any agronomic effect.

Data Collection

Yield and yield related attributes: Data for yield and yield related traits was measured from five randomly selected plants in each replication and then averaged. Plant height (cm) was taken from soil level to the tip of the plant. Number of nodes was counted from the first fruiting branch. Seed cotton yield per plant was measured from five randomly selected plants and averaged. Average boll weight (g) was calculated as ratio of seed cotton yield per plant to total number of bolls per plant. Boll retention was measured as a ratio of total number of opened bolls per plant to total fruiting points and it was multiplied with 100 to get the boll retention percentage.

Fiber Traits

Data for fiber traits was calculated as an average of five random sample from each replication. GOT% was calculated by the following formula:

$$GOT\ (\%) = \frac{weight\ of\ lint\ (g)}{weight\ of\ seed\ cotton\ (g)} \times 100 \quad (1)$$

Fiber length (mm), strength (TPPSI) and fineness (micronair) were measured from Spin laboratory of central cotton research institute Multan. Fiber length was measured by Fibro graph 530, Fiber strength by pressly strength meter and fiber fineness by Micro mat Tester (F08 SDL England).

Genetic Mutation Screening

In order to detect induced mutations, fresh young leaves of 25 days old seedlings were taken from each genotype of all mutagenic treatments early in the morning and immediately dipped in liquid N prior to preservation at -20^{o}C for DNA extraction. 10 genotypes of cotton were analyzed with 50 SSR markers out of those 31 were found to be polymorphic. Equal proportion of genome wise thoroughly distributed BNL, JESPR and TM Microsatellite (SSRs) primer pairs obtained from BNL primers Research Genetics Cp. (Huntsville, A.L., U.S.A., https://www.resgen.com); JESPR from sequences of (Reddy *et al.*, 2001); TM from Dr. John Tu, USDA-ARS, Crop germplasm Research Unit, TE, USA;CIR from (Nguyen *et al.*, 2004) were utilized in present research. Polymerase chain reaction (PCR) amplification was done with 2 μL of DNA, 2 μL of 10X PCR Buffer, 2.4 μL of 10 m*M* dNTPs, 3 μL of 2.5 m*M* $MgCl_2$, 0.2 μL of Taq DNA polymerase, 2.0 μL of forward and reverse primers making the reaction mixture of 20 μL with dd.H_2O. The PCR reaction comprised of initial denaturation step of 5 min at 94°C followed by 35 cycles of 45 sec at 94°C for DNA denaturation, followed by annealing for 45 sec at 57°C and synthesis of DNA strand for 1 min at 72°C and final extension at 72°C for 10 min. Five microliters of PCR products were electrophoresised at 1.5% agarose gel with ethidium bromide stain for separating the amplified PCR segments. Five micro liters of 100 bp DNA ladder as well as PCR product were run in electrophoresis tank in TAE buffer for 50 min and 120 Volts. Agarose gel was examined in Biorad gel documentation system for determining the fragment length and counting of bands for subsequent gel scoring. Genetic diversity was determined by Power Marker V 3.25.10. Dendogram was constructed with UPGMA.

Statistical Analysis

All collected data were subjected to analyses of variance using Computer Software Statistics 8.1 considering randomized complete block design with two factor (*i.e.*, sodium azide treatments and genotypes) factorial. LSD test at 5% probability level was applied to separate the means of the treatment. Correlation and Biplot analyses were done with statistical software XLSTAT v. 2018.2 integrated with Microsoft Excel v. 365.

Results

Yield and Yield Related Attributes

Analyses of variance depicted that significant differences ($p < 0.05$) in genotypes for all yield related attributes. Similarly, all yield related traits were affected by application of mutagen sodium azide except plant height. Interaction of the two factors was non-significant for all the yield related traits (Table 1).

For morphological traits, genotypes IUB-13, IUB-65 and IUB-63 showed superior performance than other genotypes. Maximum plant height (88 cm) was observed in Desi cotton variety of *Gossypium arboreum*. Maximum number of nodes per plant (96 nodes) bolls per plant (34 bolls) was observed in the genotype IUB-13. The genotype IUB-63 showed maximum boll retention (67%). The Genotype IUB-13 also produced maximum boll weight (3.1 g) and seed cotton yield per plant (107 g) (Table 1).

Regarding the effect of mutant sodium azide, it does not affect all the traits and affected only number of nodes, boll weight, boll retention, seed cotton yield and GOT. In all cases, sodium azide reduced all the traits, except 5 m*M* dose which was always at par control. The mutagen dose 15 m*M* and 25 m*M* were equally effective for boll retention, seed cotton yield whereas maximum reduction in number of nodes per plant and average boll weight were observed for 25 m*M* sodium azide (Table 1).

Fiber Traits

There were no significant differences ($p > 0.05$) among genotypes for fiber fineness while showed significant (p

Table 1: Morphological traits, seed cotton yield and fiber traits of cotton as affected by genotype and sodium azide application

Genotypes	Plant height (cm)	Number of nodes	Boll weight (g)	Number of bolls	Seed cotton yield (g)	Boll retention (%)	Ginning out turn (%)	Fiber fineness (μg/inch)	Fiber strength (tppsi)	Fiber length (mm)
IUB-13	73.66b	96.37a	3.10a	34.43a	106.73a	67.15a	37.98a	4.65	89.50a	28.12a
IUB-65	67.63c	71.50b	2.86ab	32.81ab	93.84b	64.75a	33.21ab	4.51	89.40a	27.84ab
IUB-63	67.12c	63.87bc	2.48bc	30.87ab	76.55c	58.12b	32.60abc	4.48	89.38a	27.57ab
CIM-707	60.68d	57.75bc	2.11cd	28.00bc	59.12d	52.09c	31.21bcd	4.41	88.76a	27.35ab
NIBGE-314	48.23e	53.87cd	2.13cd	23.75cd	50.58e	50.44c	28.20bcde	4.38	88.31a	26.65ab
S-14	38.00f	39.62de	2.05cd	22.87cd	47.12g	48.43cd	27.09bcde	4.34	87.87a	26.55abc
Sitara-11	34.76fg	38.62de	1.92cd	22.37d	44.73h	47.70cd	26.85bcde	4.30	87.45ab	26.03bc
NS-141	33.95g	30.81e	1.93cd	26.87d	52.13f	43.02d	26.44cde	4.30	85.22bc	25.03cd
Bt-557	76.09b	23.93e	1.73d	28.37d	49.07fg	50.10c	25.36de	4.29	83.18cd	24.15d
Desi	87.56a	23.06e	1.61d	22.25d	35.82i	42.42d	23.42e	3.68	81.59d	21.71e
Sodium azide level										
Control	59.04	54.60a	2.27a	26.72	60.78a	56.78a	30.76a	4.34	86.94	26.19
5 m*M*	59.15	57.87a	2.30a	27.30	62.02a	54.59a	30.87a	5.16	87.82	26.30
15 m*M*	58.66	48.07b	2.12b	25.60	54.13b	50.11b	28.13b	4.28	86.91	26.17
25 m*M*	58.22	40.22c	2.03c	25.22	54.01b	48.46b	27.18b	4.23	86.61	25.73
LSD (G) ($p \leq 0.05$)	3.54	17.08	0.59	1.60	4.98	6.17	6.38	ns	2.62	1.86
LSD (L) ($p \leq 0.05$)	ns	8.87	0.07	ns	3.50	4.09	1.27	ns	ns	ns
G × L ($p \leq 0.05$)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Values with same letters in each column do not have significant difference at $p \leq 0.05$

Table 2: Correlation matrix of yield and fiber traits of cotton

Variables	Number of nodes	Boll weight (g)	Number of bolls	Seed cotton yield (g)	Boll retention (%)	Ginning out turn (%)	Fiber fineness (μg/inch)	Fiber strength (TPPSI)	Fiber length (mm)
Plant height (cm)	0.18^{ns}	0.17^{ns}	0.41*	0.29^{ns}	0.32*	0.19^{ns}	-0.12^{ns}	-0.27^{ns}	-0.24^{ns}
		0.91**	0.76**	0.91**	0.91**	0.91**	0.57**	0.82**	0.82**
			0.83**	0.94**	0.93**	0.90**	0.54**	0.77**	0.80**
				0.92**	0.85**	0.84**	0.49**	0.49**	0.59**
					0.91**	0.93**	0.49**	0.67**	0.72**
						0.93**	0.53**	0.68**	0.73**
							0.63**	0.79**	0.82**
								0.59**	0.57**
									0.93**

***** significant at 5% probability level; ****** significant to 1% probability level; **ns** not significant

≤ 0.05) genotypic differences for GOT, fiber length and fiber strength. Maximum fiber strength (89 TPSSI) and GOT (38%) was observed in the genotype IUB-65 and maximum fiber length (28 mm) in the genotype IUB-13 (Table 1).

Effect of mutagen treatment was non-significant ($p > 0.05$) for fiber fineness, fiber length, and fiber strength whereas it significantly ($p \leq 0.05$) affected GOT. Mutagenic treatment made change in GOT only when applied at 15 and 25 m*M* concentration where the two concentration had non-significant ($p > 0.05$) difference for this traits (Table 1).

Correlation and Biplot Analysis

Correlation analyses depicted significant ($p \leq 0.05$) and highly significant ($p \leq 0.01$) association among yield and fiber traits except plant height where association was weak or non-significant ($p > 0.05$) (Table 2). This was also depicted in biplot (Fig. 2) where plant height lies on one side separated from all other traits. Biplot also depicts diversity among the genotypes as affected by chemical mutagen. Creation of genetic diversity in the genotypes is confirmed, as same genotypes with different mutagenic treatments have been grouped differently (Fig. 2).

Mutation Analysis

On morphological basis, mutation caused variation in the performance of all genotypes for different fiber and yield related traits which ultimately increased genetic variation for the effected traits. This genetic variation was also confirmed on molecular level by the use of 50 SSR markers most of which showed polymorphism. Number of bands at in each genotypes type at each level were counted and a dandogram was constructed which showed that use of mutagen sodium azide created genetic variation within the genotypes (Fig. 1).

Discussion

Sodium azide is widely used chemical mutagen to create mutation in crop plants (Kalwar and Dahot, 2017; Warghat *et al.*, 2018). It causes mutation in DNA which effects plant morphology, physiology and yield (Hussain *et al.*, 2017; Kalwar and Dahot, 2017). In current study, interaction of chemical mutagen and genotype was found non-significant for all the traits studied which depicts that all genotypes showed same response and same type of mutation occur in all genotypes. From morphological yield related traits, all were influenced by the

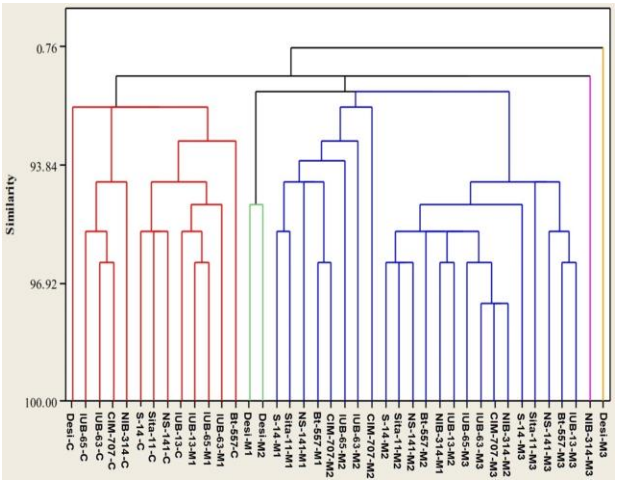

Fig. 1: Genetic diversity of cotton genotypes after treatment with mutagen sodium azide (C- control, M1-5 m*M*, M2- 15 m*M* and M3- 25 m*M*)

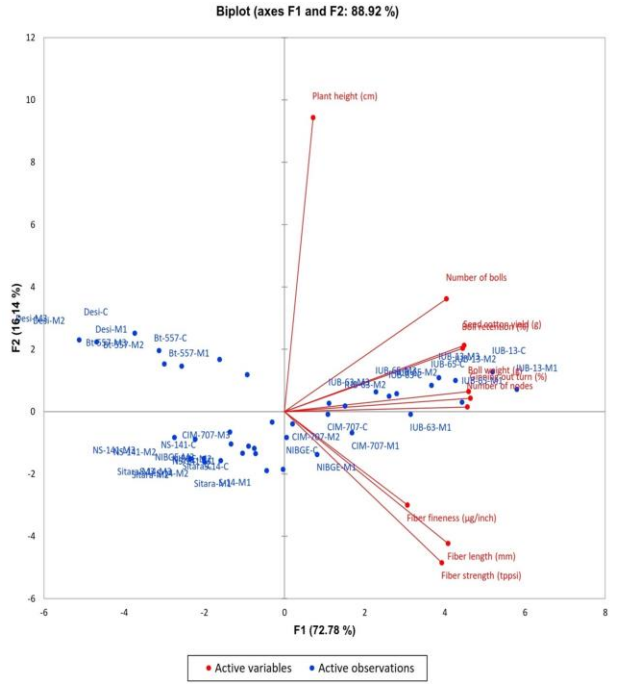

Fig. 2: A biplot showing the diversity of genotypes as effected by chemical mutagen (C- control, M1-5 m*M*, M2- 15 m*M* and M3- 25 m*M*) and relationship among different studied variables. Two components (F1 and F2) represents 88.92% of the total variability

genotypes and sodium azide also affected these traits except plant height and number of bolls. As sodium azide affects only part of DNA (Olsen *et al.*, 1993; Gruszka *et al.*, 2012) and it is quite possible that regions controlling the plant height and number of bolls per plant may not be affected by the mutation. Another reason for non-significant difference in these two traits may be the recessive mutations for these traits (Castillo *et al.*, 2001; Sugihara *et al.*, 2013) which are not visible in M1 generation and may be visible in proceeding generation. Therefore, further generations may be grown to assess the complete effect of mutations and to find out beneficial mutation.

Regarding the dose of mutant, it is evident that all the genotypes tolerated 5 m*M* concentration of sodium azide and showed no mutation effect. Similarly, mutation effect of 15 m*M* and 25 m*M* was also found statistically similar for boll weight, seed cotton yield and GOT (Muthusamy and Jayabalan, 2011). This depicts that some genotypes resist mutation due to small changes in the concentration of mutant (Muthusamy and Jayabalan, 2011; Kalapchieva and Tomlekova, 2016).

Effect of mutation found non-significant on all fiber traits that contrary to the many researchers (Herring *et al.*, 2004; An *et al.*, 2010; Patel *et al.*, 2016), where chemical mutations were used to improve the fiber traits. There might be three possible reasons for these contrary results. First is the genotypic resistance against the mutation to fiber traits, as where change in fiber traits has been reported might use different genotypes than in present study (Muthusamy and Jayabalan, 2011; Kalapchieva and Tomlekova, 2016). Second possibility may the concentration and duration of treatment may be less than required to cause mutation in the DNA regions that control fiber traits. It is reported in literature that some regions of DNA resist small concentrations of mutagen but become mutated when treated with higher concentration or for more duration (Oladosu *et al.*, 2016). Third reason may be the creation of recessive mutations (Castillo *et al.*, 2001; Sugihara *et al.*, 2013) not visible in early mutated generation and may become visible in advance generation where plants gain homozygosity. Studies presenting the effect of mutation of fiber traits referenced above also presented data of advanced mutation generations.

Only ten genotypes were used in present study which mutated with four different concentrations of chemical mutagen, but when genetic diversity was analyzed after mutation, all genotypes showed genetic differences among different treatments and this diversity is also evident in biplot analyses (Fig. 2). This depicts that new genetic variation has been created in the germplasm (Naeem *et al.*, 2015; Onda and Mochida, 2016; Olawuyi and Okoli, 2017) and only some of this variation become visible in M1 generation.

Conclusion

It is confirmed from the morphological (seed cotton yield and fiber quality) and molecular attributes that chemical mutation is a successful way to create new genetic variation in cotton germplasm. Sodium azide concentration of 15 m*M* and 25 m*M* was found useful to create successful mutation for maximum yield and fiber traits. It is suggested to extend study of mutation to beyond the M1 generation to discover recessive mutations.

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