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MUTAGENESIS WITH EMS IS AN ALTERNATIVE TO RECOMBINANT BREEDING FOR INDUCING ALLELIC DIVERSITY IN COTTON

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Abstract

Present study was conducted to develop and characterize 131 *G. hirsutum* mutant lines along with the wild type (FH-Lalazar) for plant height (PH), ginning out turn (GOT) percentage, upper half mean length (UHML), fiber strength (FS) and micronaire value (MIC). A total of 107 mutant lines depicted significant variation for plant height. Maximum plant height (133.33 cm) was observed for the mutant line LZM-51-2. High GOT percentage was measured for two mutant lines (1.53%). A total of 76 mutant lines (58.02%) depicted higher fiber strength while 51 mutant lines showed lower variation (38.93%) than that of the wild type. One of the mutant lines exhibited 3.26 MIC values which was significantly lower than the wild type. All the mutant lines showed reduced UHML value than that of the wild type owing to the selection of mutant lines made for resistance to cotton leaf curl disease as well as high boll number which may have some negative impact on UHML. Secondly, fixation of genes has been achieved by the breeders for developing cotton varieties with high UHML which may limit the scope of mutagenesis for improving such highly domesticated trait. This phenomenon can be explored in length by re-sequencing the mutant lines. Correlations among the traits have been observed in the same manner as reported for cultivated diploid as well as tetraploid cotton species, suggesting that the mutant traits were conferred by the new alleles generated at the same locus, thus eliminating the chances of creating new genes. The principal component analysis (PCA) was applied to group the mutant lines which may have significant importance in selecting the best mutant lines for developing resilient cotton cultivars. These mutant lines can also be used as a genetic resource for unraveling the genetic circuits of various traits using high-tech genomic assays.

Key words: Mutagenesis, LD50 value, TILLING, Cotton, Genetic diversity, Lint quality traits.

Introduction

Cotton is a source of natural lint fiber for textile industries (Ahmad *et al.*, 2021), feed for animals, and edible oil for human (Wang *et al.*, 2019, Pan *et al.*, 2021, Wang *et al.*, 2022). Cotton is a major cash crop for many developing countries including Pakistan (Naghera *et al.*, 2021). Total revenue generated by textile industry is around ~889 billion US$ worldwide (Rahman *et al.*, 2021). Out of four cultivated cotton species, *Gossypium hirsutum* L. (upland cotton, allotetraploid 2n=4x=52) alone provides around 95% of the total world lint production (Su *et al.*, 2019) while the remaining share is contributed by *G. barbadense*, also called Pima cotton, *G. herbaceum* (2n=2x=26) and *G. arboreum* (Rahman *et al.*, 2008, Iqbal *et al.*, 2015, Fang *et al.*, 2017).

Cotton production is largely depressed by several factors including the infection of cotton leaf curl disease (CLCuD), pest infestation particularly whitefly, jassid, lepidopterons, and high temperature (Rahman *et al.*, 2021; Ali *et al.*, 2022). For addressing these challenges, it is vital to adopt strategies which can sustain cotton production. Among these, development of resilient cotton varieties remains the most attractive approach which can sustain cotton yield. There are several approaches, among these inducing mutations in cotton germplasm may lead to create new alleles which can be used in mitigating the aforementioned challenges (Yadav *et al.*, 2016, Hussain *et al.*, 2018). The physical and chemical mutagens can be employed for inducing mutations in *Gossypium* species (Auld *et al.*, 2000). The most commonly used mutagens are fast-neutron, gamma-ray bombardments and ethyl methane sulfonate (EMS) (Sevanthi *et al.*, 2018). These mutagens can induce mutations in chromosomes or genes or changes in cytoplasmic genes (Kayalvizhi *et al.*, 2016). In polyploid species, induced mutations through exposing the genetic material with chemical mutagens can cause random changes across the genome at higher frequency than that of the naturally occurring mutations. This phenomenon can create multiple alleles of a gene in a small population even if the specie has a big genome (Till *et al.*, 2007, Sabetta *et al.*, 2011).

Physical mutagens including gamma radiations etc. are not preferred over the chemical mutagens as these induces mega changes in the genome i.e. deletions, inversions, translocations etc. (Muller, 1928, Waines & Ehdaie, 2007). These mega changes in the genome have little significance for designing gene cloning experiments. Chemical mutagens such as methyl methane sulphonate (MMS), EMS, hydroxylamine, sodium azide, N-methyl-N-nitrosourea (MNU) and hydrogen fluoride (HF) have been used to mutagenize multiple crop species, while in cotton, EMS was used extensively for inducing point mutations. The EMS induces alkylation of guanine and alkylates the 'G' to make pair with 'T' instead of 'C' that alters the base composition from G/C to A/T (Hussain *et al.*, 2018, Witt *et al.*, 2018). The mutants generated through chemical mutagenesis remained the preferred choice of molecular geneticist for identifying single nucleotide change in a gene. These mutations can be

detected using Targeting Induced Local Lesions IN Genome (TILLING). The TILLING experiments can be designed to identify unknown as well as known point mutations for identifying the allelic series in several genes simultaneously (Irshad *et al.*, 2020). It was first demonstrated in *Arabidopsis* (Lai *et al.*, 2012, Enders *et al.*, 2015, Sun *et al.*, 2017) and fruit fly (*Drosophila melanogaster*) (Winkler *et al.*, 2005, Cooper *et al.*, 2008). Later on, several crop species including wheat, maize, etc. were exposed to EMS for creating new alleles by converting GC to AT. These mutations can be identified by single strand conformation assays, sequencing the genes, TILLING, exome capture assay, whole genome sequencing, etc. (Grabowski *et al.*, 2017, Hussain *et al.*, 2018, Hussain *et al.*, 2021, Zulfiqar *et al.*, 2021).

The mutants generated can be evaluated through several means. Many classical statistical procedures like analysis of variance for determining significance among the mean values of phenotypic traits were applied. Unravelling the correlated traits provide opportunity in designing breeding strategies for improving the traits. The knowledge of existing association among the traits can be improved to interpret the data, resultantly the generated information will be helpful in planning comprehensive varietal improvement strategies. Several studies have been published in cotton which have explained the correlation among various agronomic and fiber traits (Iqbal & Rahman, 2017).

The estimation of the extent of genetic variability in mutant traits as compared to wild type of *G. hirsutum* is gold mines for future improvement in cotton production. New statistical methods coupled with parallel evolution in bioinformatics procedure helped in explaining the extent of genetic variability much more precisely in cotton germplasm. Multivariate assay has been extensively used to identify the most promising genotypes. In this analysis, data of multiple traits collected from several genotypes can be interpreted simultaneously. In multivariate assay, cluster and principal component analysis (PCA) are used to study the extent of genetic diversity among the breeding material including advanced lines, accessions and cultivars. The PCA explains the share of each trait understudy in shaping the total genetic variability. While, cluster analysis makes groups of the genotypes in order to maximize the homogeneity within a group and also exhibits the heterogeneity among the groups (Yehia & El-Hashash, 2021). It can further fractionate the clusters into sub clusters (Rizwan *et al.*, 2021). Thus, PCA and cluster analysis together show the genetic kinship among the genotypes. In cotton, the multivariate analysis was deployed extensively for the estimation of genetic variability among genotypes (Guan *et al.*, 2012).

Thus, for creating new allelic diversity, we exposed the seed of FH-Lalazar to EMS aiming to improve its several traits including plant height and fiber features. The newly developed mutants can be a potential reservoir of new alleles can help exploring several biochemical mechanisms conferring important traits. Ultimately, this information would assist in initiating breeding by design which may lead to produce resilient cotton variety.

Materials and Methods

A cotton variety FH-Lalazar was exposed to a chemical mutagen ethyl methanesulphonate (EMS). It was bred at the Cotton Research Institute (CRI), AARI, and Faisalabad Pakistan. It has few undesirable features including plant height, vulnerability to jassid, and producing average grade fiber features.

Cotton seed of FH-Lalazar was exposed to various doses of EMS for determining the LD50 value. A series of experiments with different concentrations of EMS were conducted. Before exposing to EMS, seeds were immersed in concentrated (98%) H_2SO_4 (10% v/w) for 1-2 min. Then seeds were thoroughly washed with water to remove residues of acid. Thereafter, 5% sodium hypochlorite and 70% ethanol were used to sterilize the seeds, and washed three times to remove the residues. In total, 25 batches of 100 sterilized seed each were exposed to varying concentrations of EMS (ranging from 0.1 to 0.5%). Each concentration was also tested at two different temperature regimes at two-time intervals, i.e. 33°C and 35°C for 1 and 2 hours, respectively. After exposing the seeds with mutagen, seeds were washed under the running tap water for three hours to remove the residual EMS. These seeds were sown under controlled conditions in small trays filled with sand. Germination percentage was calculated (# of seeds germinated/total # of seeds sown X100). The batch (of seed) treated with 0.4% EMS for 2 hours at 35°C exhibited germination 45-55% and was selected as an optimized LD50 value for conducting the mutagenesis experiment for FH-Lalazar. The aforementioned procedure was performed for mutating 8000 seeds with the optimized EMS concentration. After an overnight drying, M_0 seed of the parent genotype (wild type) and the mutated seeds were sown manually in the experimental field of the National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad Pakistan in 2013. Recommended spacing between rows and plants were given, i.e. 75 cm and 30 cm row-to-row and plant-to-plant, respectively. We also applied the standard agronomic practices from sowing till maturity. In total, 3920 M_1 plants were germinated. The flowers were bagged to ensure self-pollination. At maturity, seed cotton from each plant was picked and kept separately. The cotton seed of each M_1 plant was ginned with small sawn gin machine. In total, 2000 M_2 progeny rows of each of the M_1 plant were sown in 2014. From each row, plants bearing high number of bolls were tagged. Seed cotton from each of the selected 450 plants was picked. Next year, 450 M_3 rows of each selected plant were sown in NIBGE cotton field followed by selecting one cotton plant (based on its boll bearing as well as high tolerance to cotton leaf curl disease) from each row. Here, we selected 151 M_3 plants from the selected rows. The seed cotton was ginned. The 151 M_4 progeny rows of each M_3 plant were sown in NIBGE field in 2016. A total of 20 segregating progeny rows were rejected. Seed cotton from each of the 131 M_4 progeny rows was harvested in bulk. During the next cotton growing season (2017), the same procedure was adopted for sowing M_5 lines. A total of 131 M_6 lines

along with the wild type were sown in alpha lattice design with three replications of 11 incomplete blocks consisting of 12 entries (genotypes) in each block in NIBGE field (Faisalabad, Pakistan) on 3rd June 2018, NIAB on June 05, 2018 and CCRI Multan on May 28, 2018. Alpha program was used to randomize the trial. The area allocated for each mutant line was 11.43 m^2 (3 rows 5 m long with spacing of 0.75 m). Like previous trials, all agronomic practices as well as integrated pest management (IPM) were undertaken for the whole trial from sowing till harvesting. Chemicals were sprayed to control chewing and sucking insect pests. It is worth to mention that stress was not given at any growth stage of the cotton crop. Data was collected from the central row from each entry in each replication.

Data pertaining to plant height (cm) and ginning out turn (GOT) percentage were recorded. Plant height of each mutant line was measured by measuring five plants from base to the top of a stem followed by taking average of these plants. The GOT percentage of all mutant lines was calculated by dividing the total weight of lint with the total weight of the seed cotton followed by multiplying with 100. Fiber traits including upper half mean length (UHML), fiber strength (FS) and micronaire value (MIC) were analyzed through High Volume Instrument (HVI-1000).

Statistical analysis

The range, average, standard deviation, standard error, variance and coefficient of variation were computed for all the traits (plant height, GOT percentage, UHML, FS and MIC value) using Minitab17 software. Analysis of variance was calculated by deploying the alpha lattice design. For this, software was used to determine the significance level. Statistical significance of the data was tested at 1% level of probability. Correlations among the studied traits, were determined using statistix 8.1 software (https://statistix.informer.com/8.1/). Moreover, principal component analysis (PCA) was undertaken using Minitab17. Relative score was used to calculate principal components. Cluster analysis was performed using Minitab17 and determined the hierarchical similarity among the mutant lines. First and second principal component axes scores were plotted for the enhancement and visualization of differences among the mutant lines.

Results

In total, 107 mutant lines (out of 131 mutant lines) expressed significant variation (9.92% higher and 71.76% lower) for plant height than that of the wild type. High GOT percentage was depicted by two mutant lines (1.53%) while 125 mutant lines showed low GOT percentage (95.42%) than that of wild type. All the mutant lines showed reduced UHML value than that of the wild type. In total 76 mutant lines (58.02%) depicted higher fiber strength while 51 mutant lines showed lower variation (38.93%) than the wild type. A total of 79 mutant lines expressed higher substantial variation (60.31%) while 45 mutant lines showed lower variation (34.35%) for MIC value than that of wild type (Table 1).

Table 1. Phenotypic variations for five traits in 131 mutant lines and wild type.

Sr. No.	Traits	Value of wild type	Number and %age of significant mutants
1.	PH	107.67 cm	13 (9.92%) ⇑ 94 (71.76%) ⇓
2.	GOT	40.89 %	2 (1.53%) ⇑ 125 (95.42%) ⇓
3.	UHML	28.26 mm	0 (0%) ⇑ 131 (100%) ⇓
4.	FS	28.33 (g/tex)	76 (58.02%) ⇑ 51 (38.93%) ⇓
5.	MIC value	4.13 (ug/inch)	79 (60.31%) ⇑ 45 (34.35%) ⇓

PH= Plant height; GOT= Ginning out turn; UHML= Upper half mean length; FS= Fiber strength; MIC= Micronaire; ⇑ Higher; ⇓ Lower

The statistical values (range, average, standard deviation, standard error, variance and coefficient of variation) showed substantial genetic variations among all the studied traits (Table 2). Plant height was ranged from 72.0 to133.33 cm with a mean value of 96.76 cm. Out of 131 mutant lines, 18 surpassed the plant height of wild type (107.67 cm). Mutant line LZM-51-2 expressed 133.33 cm followed by LZM-14-2 and LZM-2-3-5. The GOT percentage ranged from 33.81 to 41.62% with a mean value of 38.91%. Two mutant lines exhibited high GOT percentage (41.62% for LZM-27-2 and 41.37% for LZM-50-1) than that of wild type (40.89%). Upper half mean length ranged from 24.75 to 27.87 mm while wild type expressed 28.26 mm. Fiber strength exhibited a high level of divergence i.e. 23.42 to 32.31 g/tex and wild type depicted 28.33 g/tex. A total of 79 mutant lines exhibited higher fiber strength than that of wild type. Out of these, three mutant lines revealed maximum fiber strength values (32.08 g/tex for LZM-10-1, 32.10 g/tex for LZM-39-1 and 32.31 g/tex for LZM-34-1). Micronaire value was in the range of 3.26 to 6.31 µg/inch. It was concluded that 82 mutant lines showed highest MIC value than that of wild type (4.13 µg/inch). Out of these, two mutant lines expressed the highest MIC values (6.03 µg/inch for LZM-30-2 and 6.31 µg/inch for LZM-33-1) while the lowest MIC value was observed for LZM-2-1-1 (3.26 µg/inch). Analysis of variance (ANOVA) was computed for all the studied traits (Table 3). It was found that highly significant differences were observed for all the phenotypic traits (plant height, GOT percentage, UHML, fiber strength and MIC value) among the mutant lines.

Correlation coefficients were estimated for all the phenotypic traits. Significant associations were found for few traits (Table 4). Plant height exhibited non-significant negative association with GOT percentage while non-significant positive association of plant height was found with UHML, fiber strength and MIC value. Ginning out turn percentage showed non-significant negative association with UHML and fiber strength. The GOT showed non-significant positive correlation with MIC value. The UHML exhibited highly significant positive association with fiber strength while non-significant negative correlation with micronaire value. Fiber strength depicted highly significant negative association with MIC value.

Table 2. Descriptive statistics of five phenotypic traits of 131 mutant lines.

Sr. No.	Traits	Range	Mean	Standard deviation	Standard error	Variance	Coefficient of variation
1.	PH (cm)	72-133.33	96.76	12.27	1.76	150.65	12.69 %
2.	GOT (%)	33.81-41.62	38.91	1.23	0.047	1.52	3.17 %
3.	UHML (mm)	24.75-27.87	26.41	0.72	0.03	0.51	2.71 %
4.	FS (g/tex)	23.42-32.31	28.61	1.84	0.29	3.38	6.43%
5.	MIC value (µg/inch)	3.26-6.31	4.29	0.49	0.01	0.24	11.51%

PH= Plant height; GOT= Ginning out turn; UHML= Upper half mean length; FS= Fiber strength; MIC= Micronaire

Table 3. Mean square values for various traits of cotton mutant lines.

S.O.V	df	PH	GOT	UHML	FS	MIC value
Replications	2	3613.3410	0.0018	0.2525	0.3621	0.0054
Blocks	30	10.3719	0.0038	0.0015	0.2489	0.0005
Genotypes	131	451.9789	4.5586	1.5365	10.1458	0.7292
Genotypes (Adjusted)	131	409.2411**	4.2454**	1.4678**	9.2212**	0.6846**
Error	232	9.0781	0.0067	0.0023	0.2508	0.0004

S.O.V= Source of variance; df= Degree of freedom; PH= Plant height; GOT= Ginning out turn; UHML= Upper half mean length; FS= Fiber strength; MIC= Micronaire; **= Highly significant at 1% level; *= Significant at 1% level; N.S= Non-significant at 1% level

Table 4. Correlation coefficients among five traits of 131 mutant lines and wild type.

Traits	PH	GOT	UHML	FS
PH	1			
GOT	-0.0128 N.S	1		
UHML	0.1492 N.S	-0.0767 N.S	1	
FS	0.0042 N.S	-0.1382 N.S	0.4477**	1
MIC	0.0080 N.S	0.0605 N.S	-0.0350 N.S	-0.4258**

PH= Plant height; GOT= Ginning out turn; UHML= Upper half mean length; FS= Fiber strength; MIC= Micronaire; **= Highly significant at 0.05 % level; *= Significant at 0.05 % level; N.S= Non-significant at 0.05 % level

Table 5. Principal component analysis (PCA) of different traits of 131 mutant lines.

Variables	PC1	PC2
Eigenvalues	1.69	1.09
Percent of variance	34	22
Cumulative percent of variance	34	56
Factor loadings by various traits		
Variables	**PC1**	**PC2**
PH	0.115	0.752
GOT	-0.236	0.031
UHML	0.514	0.434
FS	0.672	-0.119
MIC value	-0.464	0.480

PC1= Principal component 1; PC2= Principal component 2; PH= Plant height; GOT= Ginning out turn; UHML= Upper half mean length; FS= Fiber strength; MIC= Micronaire

The PCA is a multivariate statistical technique that is used to study the pattern of variation as well as to establish relationship among the phenotypic traits under study. It can be performed on all variables simultaneously. The eigenvalues, variability and cumulative percentages are presented in (Table 5). Out of the five PCs, two exhibited eigenvalues >1 and accounted for 56% of the total genetic variation among the mutant lines. Out of these, PC1, contributed 34% towards the total variability with an eigenvalue of 1.69. The mutant lines in PC1 exhibited positive effects for fiber strength (0.672) and UHML (0.514) while negative effects were shown for MIC value (-0.464) and GOT percentage (-0.236). Contribution of PC2 towards the variability was 22% with the eigenvalue of 1.09. The mutant lines in PC2 exhibited positive values for plant height (0.752), UHML (0.434) and MIC value (0.480) while negative value was shown for fiber strength (-0.119).

Distance of each variable with respect to PC1 and PC2 exhibited the contribution of the variables towards the genetic diversity of each mutant line. Fiber strength, UHML, PH and MIC value were well represented. Ginning out turn percentage exhibited least variability in the scatter diagram (Fig. 1). Variability among the phenotypic traits explored in this study showed the extent of diversity among the mutant lines which can be utilized in future cotton breeding program.

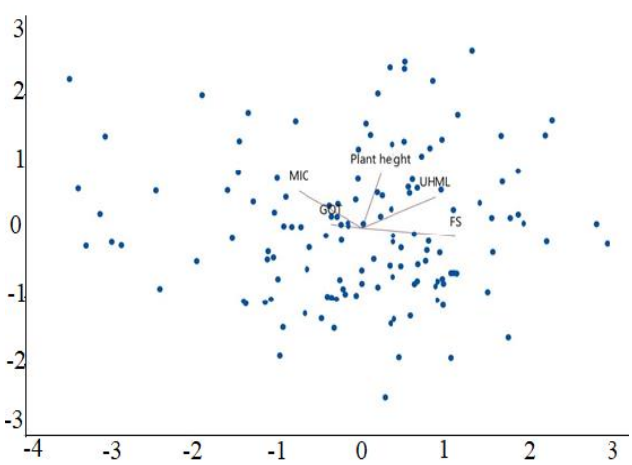

Fig.1 Principal component's Biplot of 131 mutant lines along with wild type showing contribution of various traits towards variability.

Owing to the difficulty in clustering genotypes with PCA and factors corresponding to PCs, cluster analysis was performed using Euclidean distance matrix to group these mutant lines. The newly developed mutant lines were grouped into eight clusters using the data of five traits (Table 6). These clusters contained six to 29 mutant lines, depending upon the extent of genetic similarity among the traits. The cluster-I contained 28 mutant lines while cluster-II comprised of 13 mutant lines. Cluster-III consisted of 11 mutant lines while cluster-IV comprised of 29 mutant lines. Similarly, the cluster-V contained 24 mutant lines. In cluster-VI, 14 mutant lines were grouped. Clusters-VII comprised of six mutant lines, while cluster-VIII contained seven mutant lines. Mean performance of five phenotypic traits for each cluster exhibited a vast range of variation. Cluster mean for each phenotypic trait has been shown in (Table 7). Maximum contribution in cluster-I originated from UHML. Similarly, cluster-II comprised of mutant lines having highest values for GOT percentage and MIC. The GOT percentage and fiber strength contributed large share towards the variability in cluster-III. The mutant lines in cluster-IV showed maximum values for GOT percentage as compared to the other traits. Plant height and MIC values contributed maximum towards cluster-V. In cluster-VI, all the phenotypic traits showed maximum contribution except micronaire value. In cluster-VII, maximum contributions were derived from plant height, UHML and MIC value. Cluster-VIII was considered best among all the studied clusters because all the phenotypic traits contributed large share towards the genetic variability.

Table 6. Cluster analysis of 131 cotton mutant lines.

Clusters	Numbers	Name of mutant lines
Cluster I	28	LZM-1-1, LZM-2-1, LZM-6-2, LZM-12-1, LZM-15-1, LZM-15-2, LZM-17-1, LZM-21-2, LZM-24-1, LZM-24-2, LZM-27-1, LZM-29-2, LZM-30-1, LZM-31-1, LZM-31-2, LZM-32-1, LZM-32-2, LZM-33-2, LZM-35-2, LZM-37-2, LZM-41-1, LZM-42-2, LZM-46-2, LZM-47-2, LZM-48-2, LZM-1-2-2, LZM-2-4-3, LZM-2-6-3
Cluster II	13	LZM-1-2, LZM-7-1, LZM-16-2, LZM-22-1, LZM-22-2, LZM-27-2, LZM-30-2, LZM-36-2, LZM-37-1, LZM-40-1, LZM-44-2, LZM-1-1 (g), LZM-2-6-1
Cluster III	11	LZM-2-2, LZM-3-2, LZM-7-2, LZM-17-2, LZM-19-1, LZM-21-1, LZM-40-2, LZM-41-2, LZM-45-2, LZM-49-2, LZM-1-1-3
Cluster IV	29	LZM-3-1, LZM-4-2, LZM-5-1, LZM-9-2, LZM-11-2, LZM-12-2, LZM-16-1, LZM-18-1, LZM-18-2, LZM-19-2, LZM-20-2, LZM-23-2, LZM-25-1, LZM-29-1, LZM-33-1, LZM-34-1, LZM-36-1, LZM-43-2, LZM-44-1, LZM-45-1, LZM-46-1, LZM-47-1, LZM-50-1, LZM-50-2, LZM-1-2-3, LZM-2-1-1, LZM-2-5-1, LZM-2-5-2, LZM-2-5-3
Cluster V	24	LZM-4-1, LZM-5-2, LZM-6-1, LZM-8-2, LZM-9-1, LZM-10-1, LZM-10-2, LZM-13-2, LZM-20-1, LZM-23-1, LZM-25-2, LZM-26-1, LZM-26-2, LZM-28-1, LZM-42-1, LZM-48-1, LZM-49-1, LZM-1-1-2, LZM-1-2-1, LZM-1-3-2, LZM-2-2-2, LZM-2-2-3, LZM-2-3-4, FH-Lalazar
Cluster VI	14	LZM-8-1, LZM-11-1, LZM-28-2, LZM-34-2, LZM-35-1, LZM-39-1, LZM-39-2, LZM-43-1, LZM-1-3-3, LZM-2-1-2, LZM-2-2-1, LZM-2-4-1, LZM-2-4-2, LZM-2-6-2
Cluster VII	6	LZM-13-1, LZM-52-1, LZM-1-3-1, LZM-2-1-3, LZM-2-3-1, LZM-2-3-2
Cluster VIII	7	LZM-14-1, LZM-14-2, LZM-51-1, LZM-51-2, LZM-52-2, LZM-2-3-3, LZM-2-3-5

Table 7. Cluster means and general mean for various traits of 131 mutant lines.

Traits	Cluster I	Cluster II	Cluster III	Cluster IV	Cluster V	Cluster VI	Cluster VII	Cluster VIII	Over all mean
PH	89.88	81.85	77.79	95.75	106.32	101.50	117.72	125.67	96.75
GOT	38.68	39.12	39.12	39.06	38.83	38.99	38.52	39.05	38.91
UHML	26.46	26.04	26.34	26.41	26.31	26.67	26.56	26.75	26.41
FS	28.00	27.08	29.75	28.55	28.29	29.09	27.96	28.96	28.61
MIC value	4.27	4.48	4.17	4.22	4.38	4.16	4.33	4.37	4.29

PH= Plant height; GOT= Ginning out turn; UHML= Upper half mean length; FS= Fiber strength; MIC= Micronaire

Discussion

Development of new diverse crop varieties has been remained a major breeding objective (Waines & Ehdaie, 2007, Tester & Langridge, 2010). Like many other crop species, the cultivated cotton varieties has a very narrow genetic base (Rahman *et al.*, 2002, Iqbal & Rahman, 2017) resultantly it offers limited scope to improve its genetic potential for combating biotic and abiotic stresses, high yield and lint quality features (Hu *et al.*, 2019). Cultivation of genetically diverse cotton was suggested as a tool for sustaining cotton production (Rahman *et al.*, 2002). Efforts were made for widening the genetic window using recombinant breeding, introgression breeding, inducing mutations, changing the ploidy level, editing genomes, etc. (Hussain *et al.*, 2018, Ahmad *et al.*, 2019, Rahmat *et al.*, 2019).

In the present study, mutagenesis experiment was conducted for enhancing the extent of genetic diversity in *G. hirsutum* var FH-Lalazar, and were gauged on various phenotypic traits. There are several artificial means to induce mutations in cotton genome. Chemically induced mutations bring changes throughout the genome more frequently than the naturally occurring mutations (Hussain *et al.*, 2018, Hussain *et al.*, 2021).

A total of 131 M_6 lines were selected for studying the phenotypic variations in different traits including plant height, GOT percentage, UHML, fiber strength and MIC value. In multiple reports, limited number of mutant lines were selected based on some important traits, for

example, fiber quality traits, plant height, etc. (Muthusamy & Narayanasamy, 2011; Aslam *et al.*, 2013). In our study, we selected the mutant lines based on their high resistance to cotton leaf curl disease and number of bolls as both significantly impact the final yield per unit area. In these reports, significant phenotypic variations were reported. Similarly, we observed variations for all the phenotypic traits but did not notice for UHML. Possibly that the variety used in the present study has already improved fiber traits especially UHML. Other possible reason is that we selected disease resistant mutant plants bearing high number of bolls followed by developing their mutant progenies. Selection for these desirable traits would have limited the scope for improvement in UHML. Lastly, historically, breeders in Pakistan kept on improving UHML which may have fixed the genes responsible for UHML.

In other mutagenesis experiments, wide range of variations in different phenotypic traits were reported in cotton (Patel *et al.*, 2016, Bechere *et al.*, 2017, Witt *et al.*, 2018). However, these induced variations were not reached to the extent as reported the naturally occurring variations in cotton germplasm (Chandra & Sreenivasan, 2011; Iqbal & Rahman, 2017; Rathinavel, 2018).

We established that mutants exhibiting phenotypic variations are due to changes in genetics of mutants by conducting replicated trials. It was observed that variations in traits exhibited by few mutants over the wild type are consistently smaller or larger which are not occurred by chance or due to environmental artifacts. Thus, the observed phenotypic variations have genetic basis. Further work is required either these variations are due to the creation of new alleles. This can be undertaken by developing a mapping population by crossing each of the mutant lines expressing extreme phenotypes with the wild type (FH-Lalazar). Initially, the parent genotypes can be surveyed for SNPs through deploying GBS or re-sequencing the whole cotton genome. Lines selected for one fiber trait sometimes conferred other attributes.

Information regarding association among traits helps in making indirect selection for some other traits simultaneously, which can facilitate the cotton breeding programs (Ali *et al.*, 2009). If the correlation among the traits is significantly positive, then the other traits will be improved simultaneously. Also, the extent of correlation among the traits is considered as one the parameters for measuring the genetic stability in several environments.

The plant height showed non-significant negative association with GOT percentage, however, found positively correlated with UHML, fiber strength and MIC value. These findings are in agreement with multiple reports demonstrated positive association between plant height, UHML and MIC value (Nizamani *et al.*, 2017, Nawaz *et al.*, 2019). Similar pattern was reported for the diploid cultivated cotton species (Khan *et al.*, 2017). Positive association between plant height and fiber strength was reported (Zhang *et al.*, 2019). The GOT showed negative correlation with UHML and fiber strength. Negative association between GOT and UHML was reported earlier in several studies (Iqbal *et al.*, 2015, Saeed *et al.*, 2015). Negative association between GOT and fiber strength which was in accordance with the previous findings (Zeng *et al.*, 2007, Iqbal *et al.*, 2015). The GOT showed positive correlation with MIC value as was demonstrated earlier (Iqbal *et al.*, 2015, Saeed *et al.*, 2015). The UHML expressed highly significant positive correlation with fiber strength which is also in accordance with the earlier studies (Zhang *et al.*, 2020, Song *et al.*, 2021). Similar results were reported for diploid cultivated cotton species (Iqbal *et al.*, 2015, Khan *et al.*, 2017). The UHML and MIC values are the most important fiber features which determine the lint quality. Both the traits are negatively correlated especially in *G. hirsutum* (Sun *et al.*, 2017, Ma *et al.*, 2018, Liu *et al.*, 2020) as was found in the present study. In spite of the fact, breeders released cotton varieties by selecting plants showing high UHML as well as acceptable MIC value from a large F_2 population. The task of accumulating favor genes for high quality lint is extremely difficult when cotton is growing under extreme weather conditions especially high temperature. Fiber strength showed highly significant negative correlation with MIC value. Negative correlation was found between fiber strength and MIC value that was in accordance with the previous findings (LingLing *et al.*, 2020, Liu *et al.*, 2020). Similar pattern (negative correlation between fiber strength and MIC value) was reported for diploid cultivated cotton species (Iqbal *et al.*, 2015, Khan *et al.*, 2017). Also, correlation studies suggest that allelic variations occur in the same locus, and no new allele in new genes was induced which can impact the trait. Variation in extent of correlation among the traits might be due to the varying accumulation of genes impacting the same trait in various mutant lines or possibly due to fluctuation in prevailing environmental conditions, especially locations. However, the correlations driven by genes are important for using in breeding programs.

Principal component analysis (PCA) has been applied in multiple crop species including cotton for grouping the genotypes based on their phenotypic data (Jarwar *et al.*, 2019, Rizwan *et al.*, 2021). In the present study, variability among phenotypic traits of mutant lines was measured by calculating eigenvalue of PC. High eigenvalue is an indicator of high variability for a particular trait. Thus, the eigenvalue represents variation accounted for the PCs while eigenvectors demonstrate correlation among PCs and original data sets (Table 5). In the present study, PCA grouped the total variations into five PCs. Out of these, two PCs showed more than one eigenvalue with a significant amount of variability (56%). Similarly in another report, two PCs showed 50.13% variability (Rizwan *et al.*, 2021). Also, two PCs were reported while evaluating 185 genotypes of *G. hirsutum* and *G. arboreum* for six different traits, however, genetic variability was 97% (Iqbal & Rahman, 2017). In another study, 94.83% of the total variation was reported for first two components (Zhengwen *et al.*, 2019). It is obvious that mutant lines were developed from one cultivated variety which might be genetically closer than the accessions originated from two different cotton species. This phenomenon can be explained by re-sequencing mutant lines followed by identifying the SNPs induced through mutagenesis experiment. The SNP frequency can be compared with naturally occurring SNPs found in

cotton germplasm. The PCA further validated that ample number of variations for all the studied traits exist in the mutant lines that could be utilized for designing a successful cotton breeding program (Table 5). Multiple researchers deployed this technique for validating the extent of genetic divergence found in cotton germplasm.

The mutant lines in PC1 exhibited positive effects for fiber strength (0.672) and UHML (0.514). These studies are in accordance with the earlier findings. For example, in PC1 positive effects for fiber strength and UHML were noticed (Iqbal *et al.*, 2015, Rathinavel 2018, Jarwar *et al.*, 2019). In PC1 negative effects were shown for MIC value (-0.464) and GOT percentage (-0.236) as were shown in earlier reports (Iqbal & Rahman, 2017). In the present study, mutant lines in PC2 exhibited positive values for plant height (0.752), UHML (0.434) and MIC (0.480) while negative value for fiber strength (-0.119). Positive values for plant height and MIC were reported in previous findings (Iqbal & Rahman, 2017; Rathinavel, 2018) while negative values for fiber strength were also shown (Shabbir *et al.*, 2016, Shakeel *et al.*, 2018). The mentioned traits which load either highly negative or highly positive contributes more towards genetic diversity and those are the ones that most differentiated the clusters.

Biplot analysis showed the extent of genetic variation found in a variety or derived line. For instance, if a variety is more distantly away from the origin, it shows that the variety is more genetically diverse. Such lines can be used in future breeding programs (Firincioglu *et al.*, 2009). Mutant lines present in four quadrants of the graph showed that these lines are genetically diverse and also contained desirable combination of phenotypic traits. Biplot analysis is dependent on the first two principal components. Scattering of all mutant lines in biplot showed the presence of considerable genetic variation. This analysis was also used to study the extent of genetic variation among the germplasm of various crop species including cotton and chickpea (Mafakheri *et al.*, 2010, Rana *et al.*, 2013). The extent of variability in each trait in mutant lines showed high divergence. In the present study, all mutant lines were grouped into eight clusters. Cluster analysis illustrates grouping of genetically similar lines/accessions/genotypes (Rahman *et al.*, 2008, K. Bayyapu Reddy, 2015). Classification of genotypes into different clusters helps for the selection of varieties for using in future breeding program.

Conclusions

Our results clearly demonstrate that induction of mutations in an improved variety through chemical means are extremely useful in creating genetic variations for different traits. Thus, if the genetic variability is exhausted or low in a germplasm, mutagenesis with chemical means is an alternative for widening the genetic window of a cultivated varieties or germplasm. Also, the derived mutant lines can be used for bringing the novel alleles created through mutagenesis experiments in breeding programs thus cotton production can be sustained. These mutants can also be exploited for studying the function of genes using TILLING together with re-sequencing followed by validating these novel mutations by developing segregating population derived from crossing mutants with the wild type.

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