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Kill Curve Analysis and Response of Ethyl Methanesulfonate and γ-rays in Diploid and Tetraploid Cotton

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Abstract

Mutagenesis has been used to a good extent to induce genetic variability in plant species to achieve the desired genetic variability. To attain maximum useful mutation density per unit genome and comparative effectiveness of γ-rays and Ethyl methanesulfonate (EMS), optimal dose for treatment is the key to success. This study focuses on the development of kill curve in three cotton species (*Gossypium arboreum*, *G. barbadense* and *G. hirsutum*). Four genotypes from each of these *Gossypium* species were treated with eight varying levels of EMS (0.1 to 0.8%) and two doses of γ-rays (100 to 800 Gy) for kill curve analysis. The data collected on germination percentage, plant height, number of bolls/plant, boll weight, lint yield and its percentage were analyzed and significant interaction among species, genotypes, mutagens and levels of mutagens was observed for all traits under study. Morphologically unusual mutants (rudimentary sparse leaves pattern and broad leaf shape) were also identified from M_1 generations of cotton genotypes. Optimal mutagenic doses were calculated based on survival rate and seed viability, which were considered useful in developing cotton mutagenized populations for forward and reverse genetic studies. EMS observed to be more effective than γ-rays as it generated overall more number of mutants, while later caused higher physical injury in all cotton species.

Keywords: Mutagenesis; LD_{50}; Cotton; Variability; Seedling traits; Forward and reverse genetics

Introduction

Improved crop plants in relation to yield and disease resistance are the prime objectives of a typical breeding program. Naturally existing genetic variability in germplasm is the main source of evolving new genotypes having desirable economic characters. Since time cotton breeders and geneticists have been using different strategies and methods to manipulate these naturally existing variations for the development of new ideotypes. However, for the last several years seed cotton yield has remained stagnant due to narrow genetic base of the existing germplasm (Zhang *et al*., 2011). Mutagenesis is considered an effective and potential method to create genetic variation especially in crop plants (Hussain *et al*., 1982; Auld *et al*., 2000). The mutation breeding started with the use of physical mutagen i.e., gamma (γ) irradiation (Stadler, 1928) and continued at slower pace. The increased understanding of genomics allowed devising new tools of mutation analysis providing an impetus to potential and effectiveness to the use of mutation in plant breeding.

A number of physical and chemical mutagens have been used to induce genetic variations in crop plants. The mutagens are primarily grouped into two broad categories; i) physical mutagens like α, β, γ and x-rays, thermal neutrons and UV radiations, which act on DNA by breaking hydrogen bonds and sugar-phosphate moiety and ii) chemical mutagens which are sub grouped into alkylating agents like Ethyl Methane Sulphonate (EMS), acridine dyes, base analogues, hydroxylamine and nitrous acid. The nature of alteration of genetic makeup (deletions, insertions, rearrangements and point mutations) depends on the specific mode of action of a particular mutagen (Feldmann *et al.*, 1994; Meinke *et al.*, 1998). They usually generate mispairing, base pair substitutions and small deletions and insertions in genomes. Larger deletions, insertions and rearrangements of DNA fragments may also occur based on dose level and treatment time that can result in the loss-of-functional alleles. The point mutations can create series of effects, which may be hypomorphic (decreased gene function), hypermorphic (increased gene function) and neomorphic (novel gene function) effects (Alonso and Ecker, 2006). These sorts of point mutations are desirable in functional genomics experiments to characterize genes.

Mutagenesis has been useful in manipulating the cotton genome for enhancement of quantitative and qualitative traits (Bhat and Dani, 1990). The dominant mutations in M_1 generations can be estimated from leaf shapes, branching pattern and plant height. An okra leaf mutation (L_2O) in cotton (*Gossypium barbadanse*) was identified, the broad leaf shape changed into narrow type with increased lobe length, number and decreased lamina

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width (Dolan and Poethig, 1998). Useful variations induced by EMS mutagenesis are considered helpful in bypassing the lengthy process of insertion of foreign genes to improve fibre quality traits in cotton (Andy *et al.,* 2004).

The mutation based reverse genetic approaches like Targeting Induced Local Lesions IN Genomes (TILLING) can identify plants carrying mutations at molecular level. TILLING also provides a means of functional analysis of target genes by exploiting available genomic information (Ostergaard and Yanofsky, 2004; Alonso and Ecker, 2006). Several crop plants including wheat (Bahar and Akkaya, 2009; Borzouei, *et al.,* 2010), rice (Cheema and Atta, 2003; Ashraf *et al.,* 2003), oats (Arias and Frey, 1972), barley (Engvild and Rasmussen, 2004), pea (Dhulgande, *et al.,* 2011), chickpea (Karimi *et al.,* 2008), soybean (Wang *et al.,* 1993), mungbean (Khan *et al.,* 2004), pigeonpea (Giri and Aprao, 2011), lentil (Gaikwad and Kothekar, 2004), sunflower (Selvaraj and Jaykumar, 2004), sweet potato (Tabares and Perez, 2003), okra (Dhankhar and Dhankhar, 2003) and also the tetraploid cotton (Bhat and Dani, 1990; Auld *et al.,* 2000) have been successfully improved for various quantitative and qualitative traits through EMS and γ-rays induced mutagenesis. In comparative response studies of physical and chemical mutagens, EMS was observed to be more effective and efficient than physical mutagens like γ-rays (Gaikwad and Kothekar, 2004; Giri and Aprao, 2011). The EMS induced mutagenic effectiveness and efficiency was observed to be genotype and mutagen dependent (Giri and Aprao, 2011). No linear relationship between DNA content and mutation frequency per single locus existed (Koornneeff *et al.,* 1982). It has been observed that variable concentrations of particular mutagens are helpful in improving particular traits in several crop plants (Karimi *et al.,* 2008; Anitha and Sabesan, 2011).

To use a mutagen for inducing desired variability, determination of the optimum dose is the most important factor. If the dose is properly optimized, it would increase the chances of producing useful mutants. Therefore, to achieve successful induction of mutagenesis in crop improvement, kill curve analysis of a mutagen is vital (Badere and Chaudhary, 2007). We surmise that the use of different species and different genotypes within species may help determine species and genotype specific mutagen responses. The optimum doses thus determined may help to develop TILLING populations for functional analysis of target genes. This experiment was conducted to evaluate the comparative response of physical (γ-rays) and chemical (EMS) mutagens on diploid and tetraploid cotton species.

Materials and Methods

Plant Material

The cotton seed of one diploid species (*G. arboretum*, L.) and two tetraploid species (*G. hirsutum* L. and *G. barbadense* L.) were collected from University of Agriculture, Faisalabad and Central Cotton Research Institute, Multan (Pakistan) and used for mutagenesis.

Four genotypes from each of three species were selected and used for γ-irradiation (ionizing source) and EMS (chemical source) treatments (Table 1).

Mutagens Treatment

To determine an optimum dose of the mutagens for creation of maximum mutation density and variability in three species of cotton, 8 different doses of γ-rays (100, 200, 300, 400, 500, 600, 700, 800 Gy) were used. Dry seeds were γ-irradiated through cobalt-60 source. For EMS mutagenesis, ginned cotton seeds (30) each of the 12 varieties were de-linted with conc. H_2SO_4 and pre-soaked in sterile de-ionized water for at least 10 h in the dark at room temperature. The pre-imbibed 30 seeds of each of 12 genotypes were treated with EMS at 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8% (v/v) concentration along with untreated control treatment. The seeds were further incubated at room temperature for 3 h in a shaker with mild shaking (45 rpm). After EMS treatment, the seeds were thoroughly washed three times with running tap water and air-dried.

The treated and untreated cotton seeds were grown in polythene bags (10 seeds/bag) in randomized three replicates of 10 seeds (n = 30). Data were collected on germination percentage after 10 days. When M1 plants became 20 days old, they were transferred to soil pots and routinely irrigated. Data were collected for: seed germination, plant height, number of monopodial branches, number of sympodial branches, number of bolls/plant, boll weight, seed cotton weight and lint percentage.

Phenotypic Evaluation of Lethal Dose

DNA damage was indirectly evaluated by visual observations of changes induced by the mutagen treatments during developmental time course from germinations to maturity for the traits under study. The fertility of the plants from the M1 population was also evaluated by the rate of successful treatments. The data were statistically analyzed by ANOVA and nested analyses. The nested analysis was used to calculate the lethal mutagen dosage for within 12 genotypes, among three cotton species and within species among 4 genotypes. The results were elaborated by graphical representation of evaluated data sets. The plotted values represent the mean and the standard deviation for each treatment obtained from data of 20 plants randomly distributed in three independent replicates.

Results

To estimate kill curve, two characters are of prime importance, (i) germination percentage or mortality rate and (ii) production of viable descendents. Based on these two traits physical and chemical mutagen doses were calculated

Table 1: Calculated optimum doses of EMS and gamma rays for genotypes of 3 *Gossypium* species

Species name	Genotype Name	Calculated optimum dose	
		EMS	γ-rays
G. arboreum	ANB-P	0.2%	200Gy
	GOA-18	0.2%	200Gy
	GOA-2	0.2%	200Gy
	C-118	0.2%	200Gy
G. hirsutum	TADLA-16	0.2%	300Gy
	TADLA-32	0.2%	100Gy
	VPE-2	0.1%	200Gy
	PIMA-S2	0.3%	100Gy
G. barbadense	PB-899	0.3%	300Gy
	PB-900	0.2%	200Gy
	CIM-496	0.2%	200Gy
	FH-113	0.2%	200Gy

for each genotype of three cotton species (Table 1). Eight treatments of both mutagens (0.5, 0.6, 0.7 and 0.8% EMS, and 500, 600, 700 and 800 Gy) either did not flower or were unable to produce fruit due to lethality up to 100%. On the basis of these results four treatments of EMS (0.1, 0.2, 0.3 and 0.4%) and four treatments of γ-irradiation (100, 200, 300 and 400 Gy) were selected for further data collection and analysis. Significant variations for seed germination (%), plant height (cm), number of monopodial branches, number of sympodial branches, number of bolls/plant, boll weight (g), seed cotton weight (g) and lint percentage were observed at the selected levels of both EMS and γ-rays. Cumulatively, physical mutagen (γ-rays) demonstrated higher physical injury than chemical mutagen (EMS) (Fig. 1). Some unique mutants like rudimentary sparse leaves pattern (RSLP) and broad leaf shape (BLS) were observed in different genotypes of *G. arboreum* at 0.3% EMS level (Fig. 2 and 3). Significant interactions among genotypes, species, mutagens and mutagenic levels were observed. EMS showed highly significant interaction among genotypes of a particular species for plant height, sympodias, number of bolls/plant, seed cotton yield and lint percentage, whereas γ-rays express less significant interaction for most of the traits except plant height (Table 2).

In tetraploid cotton species sterility increased with the increase in dose concentration of physical and chemical mutagens, while diploid cultivars of cotton showed variable behavior in response to increase in mutagen dose of both EMS and γ-rays (Fig. 1E). All the traits showed considerable variation at various doses of the two mutagens. Trait specific optimum doses for the improvement of particular traits were determined by analyzing the data collected for variations in these traits (Table 3).

The comparison of data at species level showed that genotypes belonging to *G. arboreum* revealed parabolic growth nature with increase in dose rate in some traits (plant height, monopodia, sympodia, boll weight, and lint percentage), mostly in case of EMS (Fig. 1A-H). Some traits also showed similar behavior in case of γ-irradiation (Fig. 1C).

Morphological mutations (variations in leaf shape, plant height, branching pattern and any physical injury) observed in all the 4 doses of EMS and γ-rays were recorded in M1 populations of the twelve genotypes (Table 4). In total EMS showed 54 and γ-rays showed 67 mutations in observed morphological traits. The 0.3% EMS and 300Gy γ-rays showed highest number of morphological variations.

Discussion

The LD_{50} is usually determined by examining 50% survival rate with having viable seed production. In the present studies, most of the genotypes produce viable seeds at 50% survival rate that was considered optimum dose; conversely some genotypes were unable to produce fertile seeds or did not fruit at this survival rate. Therefore, for such genotypes with prerequisite of fertile seed production, 60 or 70% survival rate was also considered an optimum dose. The results showed that the specific doses were responsible for desirable variations in particular traits. For improvement in a particular trait of cotton plant, trait specific doses were determined by studying the interaction of traits with doses (Table 3). The calculated trait specific optimum doses of physical and chemical mutagens (Fig. 1) were effective for improvement of particular traits in cotton like other crops (Karimi *et al.,* 2008; Anitha and Sabesan, 2011) (Fig. 1A-H).

The higher physical injury by physical mutagen (Table 5) may be due to chromosomal aberrations caused by γ-rays as compared to point mutations induced by EMS mutagenesis. Some unique mutations in *G. arboreum* were also observed at 0.3% EMS level (Fig. 2 and 3), which suggested its effectiveness γ-gamma rays. The γ-rays reveal higher efficiency than EMS as it had more morphological mutations and physical injuries (Table 4 and 5).

The germination percentage (Fig. 1A) indicated increase in plant mortality with the raise in dose level of both EMS and γ-rays as reported in various crop plants (Toker *et al.,* 2005; Giri and Aprao, 2011). Reduced growth may be attributed to auxin destruction, newly created lethal alleles for particular traits, changes in ascorbic acid content and physiological and biochemical abnormalities (Gunchel and Sparrow, 1954; Usuf and Nair, 1974). Novel results were found for genotypes of *G. arboreum* showing desired variability at lower doses and vice versa (Fig. 2 and 3). The RLSP mutation is undesirable due to undersized leaves having less photosynthesis activity unable to fulfill the plant nutritional requirement. The BLS mutation is desirable in *G. arboreum* since broader leaves account for increased photosynthetic activity and substrate for appropriate fiber development.

Table 2: Primary and nested ANOVAs for estimating interactions among genotypes, species, mutagens and levels of mutagens

Serial No.	SOV	df	Plant height (cm)	Monopodial branches	Sympodial branches	No. of bolls/plant	Boll wt./plant (g)	Seed cotton wt. (g)	Lint (%)
			MS	MS	MS	MS	MS	MS	MS
Trait wise primary ANOVA									
1	Replication	2	45.8	1.3194	5.66	6.013	0.4053	9.36	59.50
2	Treatment (T)	9	5638.7	10.3198	417.69	231.466	42.9262	1548.02	6976.96
3	Genotype (G)	11	5933.5	1.8232	478.55	38.630	7.9278	411.51	377.49
4	Gen. x Treat.	99	1083.5**	0.1723$^{N.S}$	22.86*	8.775*	2.2645*	68.25**	236.11*
5	Error	238	3.8	0.0954	2.52	0.473	0.1698	0.60	6.88
6	Total	359							
Nested ANOVA for species and genotypes									
7	Replication	2	4.58	0.13194	0.566	0.6013	0.4053	0.936	5.950
8	Species	2	2389.82	0.23528	177.766	10.9078	2.61062	126.754	0.202
9	Genotypes within Species	9	194.13**	0.17056*	18.986**	2.2975**	0.38882*	22.128**	46.092**
10	Error	22	0.77	0.02194	0.380	0.0453	0.02497	0.061	0.917
11	Total	35							
Nested ANOVA for EMS									
12	Replication	2	2.2	0.08778	1.710	0.2058	0.0100	0.897	1.752
13	Species	2	6189.1	0.22111	305.890	12.6925	2.6240	207.768	58.476
14	Genotypes within species	9	282.7**	0.27222*	21.370**	4.0714**	0.9345*	19.415**	102.291**
15	Error	22	1.0	0.2960	0.165	0.0458	0.0289	0.088	1.298
17	Total	35							
Nested ANOVA for Gamma rays									
18	Replication	2	14.41	0.21000	6.103	1.2953	0.09510	1.058	12.998
19	Species	2	1037.39	0.27000	84.202	11.8503	2.87810	69.054	47.361
20	Genotypes within species	9	363.01**	0.10333$^{N.S}$	23.309*	2.4336*	0.41575$^{N.S}$	34.051**	64.755*
21	Error	22	1.39	0.3545	1.008	0.1153	0.05397	0.169	2.004
22	Total	35							
Nested ANOVA for Mutagen levels									
23	Replication	2	3.81	0.10995	0.472	0.501	0.0338	0.78	4.96
24	Mutagen	1	175.01	0.18148	1.102	4.537	0.0557	17.48	5.22
25	Levels within mutagen	8	506.75**	0.94479**	39.021**	21.133**	4.0174**	142.94**	653.44**
26	Error	18	0.69	0.01608	0.596	0.086	0.0209	0.13	0.86
27	Total	29							

*Significant ($p \leq 0.05$); **highly significant ($p \leq 0.01$); NS: non-significant, MS: Mean Square; SOV: Source of Variation; df: Degree of freedom

Table 3: Estimates of trait specific doses in three species of cotton

Name of Trait	Species Name					
	G. arboreum		*G. barbadense*		*G. hirsutum*	
	γ-rays	EMS	γ-rays	EMS	γ-rays	EMS
Plant height (cm)	100Gy	0.2 %	100Gy	0.1%	200Gy	0.2%
No. of Monopodial branches	200Gy	0.1%	200Gy	0.1%	200Gy	0.1%
No. of Sympodial branches	100Gy	0.1%	200Gy	0.1%	100Gy	0.1%
No. of bolls/plant	100Gy	0.2%	100Gy	0.1%	200Gy	0.2%
Boll weight/plant (g)	100Gy	0.2%	100Gy	0.1%	100Gy	0.2%
Lint %age	100Gy	0.1%	100Gy	0.1%	200Gy	0.1%

Table 4: Morphological mutations (leaf shape, height, branching pattern and any physical injury) observed in EMS treated and gamma irradiated populations

Name of Genotype	EMS					Gamma rays					Total obs./Gen.
	Dose 1	Dose 2	Dose 3	Dose 4	Sub-Total	Dose 1	Dose 2	Dose 3	Dose 4	Sub-Total	
ANB-P	0	1	2	1	4	1	3	2	2	8	12
GOA-18	0	2	2	0	4	1	2	3	3	9	13
GOA-2	0	1	4	2	7	1	1	2	2	6	13
C-118	0	1	2	0	3	0	2	2	2	6	9
TADLA-16	1	0	2	1	4	0	0	2	3	5	9
TADLA-32	0	1	1	1	4	1	1	2	1	5	9
VPE-2	1	0	2	2	4	1	1	1	2	5	9
PIMA-S2	0	1	1	2	4	1	0	1	1	3	7
PB-899	1	1	1	1	4	0	1	1	3	5	9
PB-900	0	2	1	1	4	0	0	2	2	4	8
CIM-496	1	2	2	3	8	0	2	2	2	6	14
FH-113	0	1	2	1	4	1	0	2	2	5	9
Total obs./dose	4	13	22	15	54	7	13	22	25	67	121

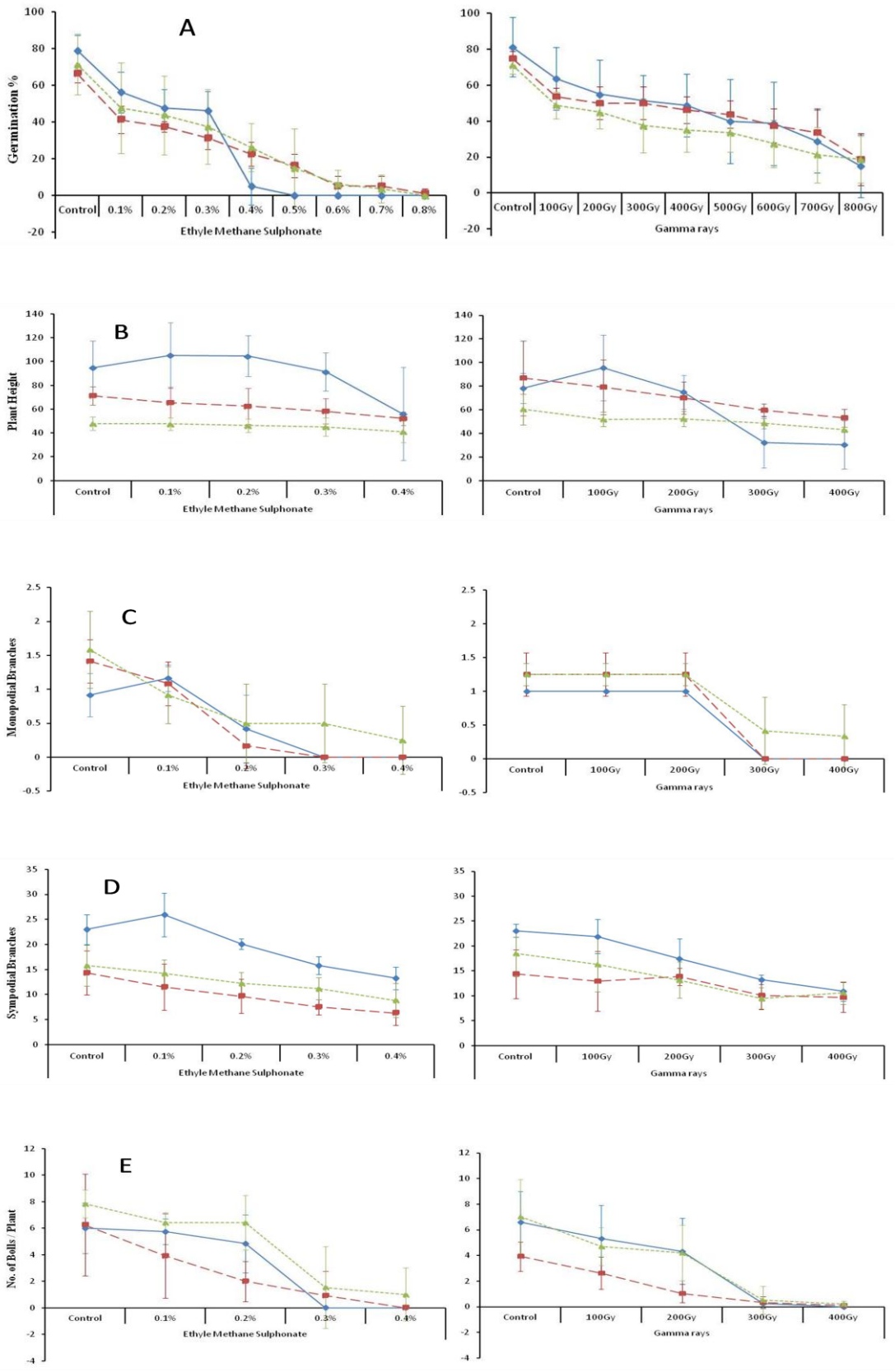

Fig. 1: Continued

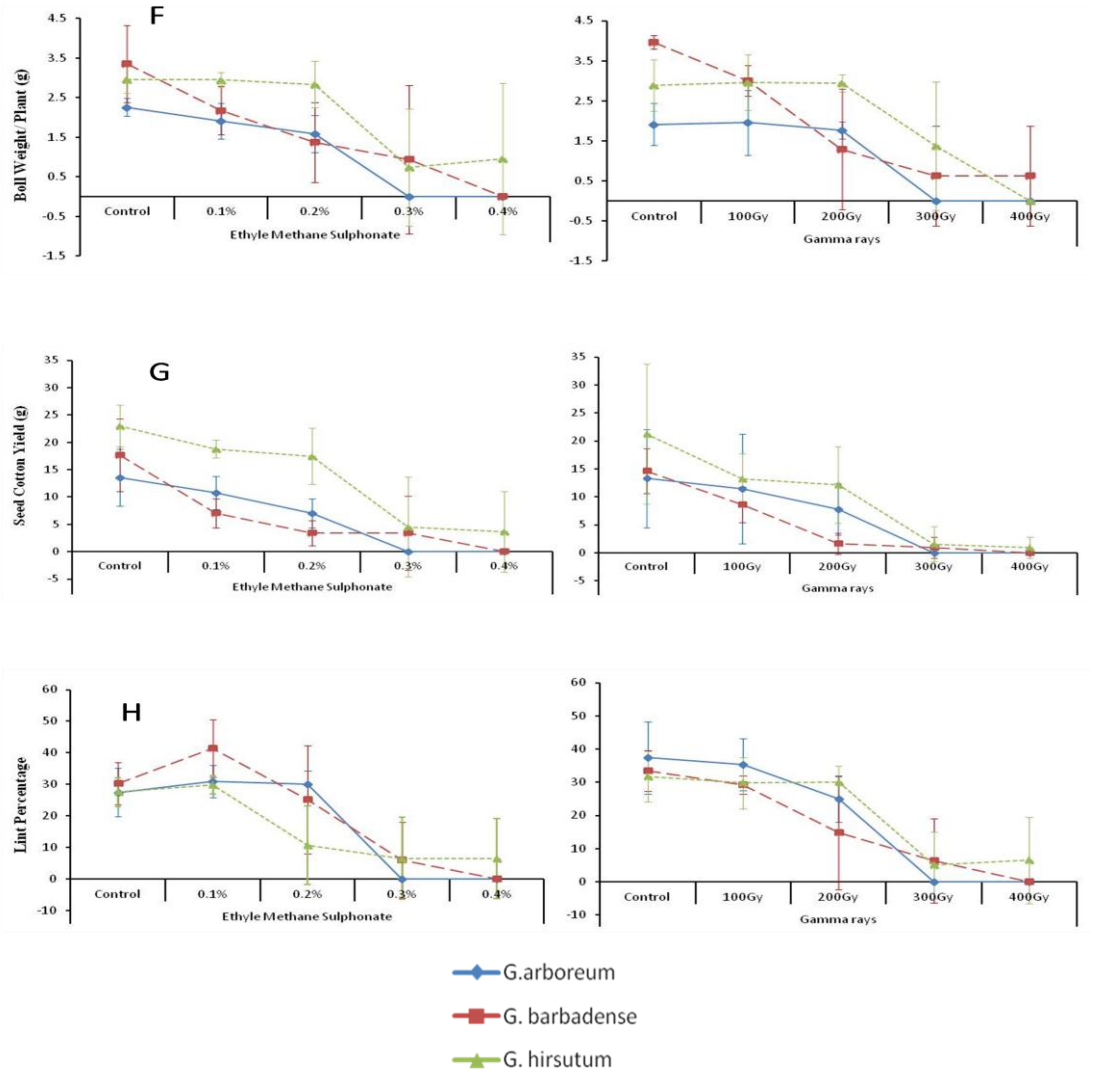

Fig. 1(A-H): 1. Inter-species comparison of gamma rays and EMS mutagenesis on (**A)** germination percentage, (**B)** plant height, (**C)** No. of monopodial branches, (**D)** No. of sympodial branches, (**E)** No. of bolls/plant, (**F)** boll wt./plant, (**G)** seed cotton yield and (**H)** lint percentage in 3 cotton species (*G. arboreum*, *G. barbadense* and *G. hirsutum*)

The enhancement of yield traits at lower mutagenic doses in genotypes of *G. arboreum* may be the result of cumulative effect of less number of lethal genes of diploid AA genome. The ultimate decline in the yield traits at higher mutagenic doses was possibly the result of combined effect of already existed and newly created lethal genes by mutagenesis. In case of tetraploid genotypes having AADD genomes, cumulative effect of double number of lethal genes (a phenomena of doubling of lethal genes in polyploids as compared to diploids) demonstrate decline even at lower doses as compared to diploid species (Fig. 1A-H) and it continue at higher doses of mutagens by creation of more lethal genes (Comai, 2005).

Throughout plant growth stages mutagenic lethal effects are usually more prominent on plant height as it is easily noticeable trait (Micke and Wohrmann, 1960). The percent data of morphological mutations revealed maximum number of mutations in plant height i.e., 62% as compared to other corresponding traits (Table 5). Linear reduction was observed in the plant height and number of surviving plants with the increase in dose in almost all genotypes for both γ-rays and EMS treated populations (Sarawgi and Soni, 1993). This reduction in plant height may be due to inhibition of DNA synthesis causing deleterious effects to production of plant growth factors. It may further inhibit nutrient transport and increase the concentration of diffusible growth-retarding substances (Sjodin, 1962).

The appearance of bolls on cotton plant is a primary sign of productive fruiting. Improvement in this trait is economically very desirable in cotton crop.

Table 5: Cumulative and percent estimates of morphological mutations observed in mutagenic cotton populations

Trait Name	EMS	Gamma rays	% age
Plant height	37	39	62%
Branching pattern	10	15	20%
Leaf shape	2	0	1.6%
Physical injury	5	13	15%
Total	54	67	

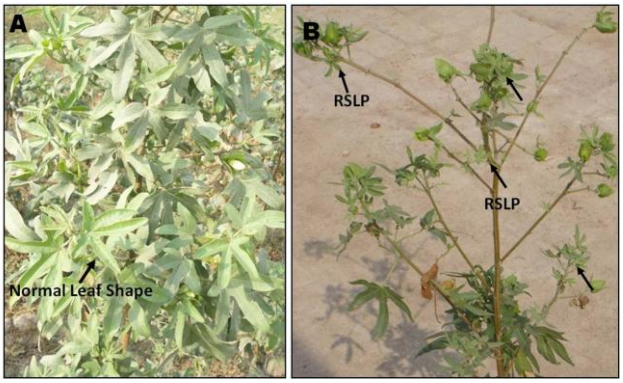

Fig. 2: Rudimentary scanty leaf patterned mutation. **A:** Control plant *G. arboreum* spp. (Var. GOA-2) at 0.0% EMS level showing normal leaf shape. **B:** Mutant plant showing rudimentary scanty leaf patterned (RSLP) at 0.3% EMS level, arrows pointing observable mutations. This mutant has tiny leaves which are unable to carry out sufficient photosynthesis required for proper fertility of plant

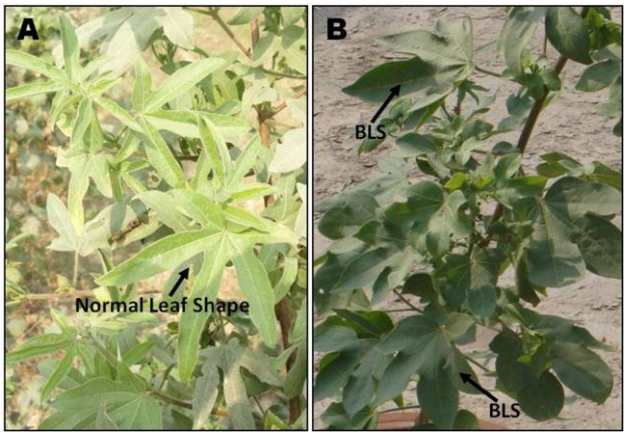

Fig. 3: Broad leaf shape mutation. **A:** Control plant *G. arboreum* spp. (Var. C-118) at 0.0% EMS level showing normal okra leaf shape. **B:** Mutant plant showing modification of okra leaf shape into upland cotton type broad leaf shape (BLS) at 0.3% EMS

The number of bolls per plant increases at lower doses of γ-rays in case of GOA-18 and ANB-P and for GOA-18 in case of EMS. The PIMA-S2 of *G. barbadense* and FH-113 belonging to *G. hirsutum* revealed increase in number of bolls per plant at higher doses (Fig. 1E). This may be due to enhancing effect of favorable alleles arises at respective doses of mutagens. These results showed the potential of mutagenesis in cotton yield improvement.

Ascending mutagenic doses revealed higher rate of growth retardation for genotypes of Egyptian cotton (*G. barbadense*) in contrast to that of upland cotton (*G. hirsutum*). The genotypes of American tetraploid cotton showed lesser decline in trait values with increase in dose level than Egyptian tetraploid cotton (Fig. 1A-H). It indicated the higher sensitivity of Egyptian cotton to artificial mutagens adding with this the limited adaptability of *Barbadense spp*. in environments of cotton growing regions like South East Asia (Wu *et al*., 2005).

Significant interaction for genotypes within species revealed that genotypes belonging to a particular species behave differently against particular mutagen and even to a specific level of mutagen. Higher interaction by EMS to genotypes within a species for most of the traits indicated that lethal effects of EMS dose are genotype and species specific for particular traits only. In contrast, less interaction by γ-rays for most of the traits except plant height represent that it caused physical damage independent of the genotypes of a particular species. The higher interaction for levels within a mutagen for all the traits revealed that optimum level/dose of a mutagen is reliant upon type and nature of mutagen. Likewise greater interaction for genotypes within species, levels within mutagens particularly in case of EMS explained that optimum dose of mutagen is species dependent, even genotypes within species behaved differently for a particular mutagen and mutagen levels (Table 2). The morphological differences in mutagen treating parts of cotton spp. and genotypes like width of seed coat, lint intensity, and size of seed might affect the penetration of mutagen to the embryonic structures of the seed. Though mutations are induced at random in the genome regardless of the method of mutagenesis and type of mutagen used, the mutation spectrum is greatly based on the genetic makeup of the organism, type and dose of the selected mutagen (Shaikh and Begum, 1991).

In conclusion, the upland and Egyptian cotton showed similar response to both mutagens as compared to desi cotton showing typical parabolic behavior with rising dose levels. It may be due to the genome similarity in former tetraploid species as both having AADD genome. The later desi cotton (diploid spp.) own AA genome only and revealed some characteristics mutations as compared to tetraploid species. This may be due to higher buffering capacity of tetraploid genome that is why mutations are more pronounced in diploid genotypes.

Particular levels of particular mutagens can be used to improve particular traits. EMS had higher effectiveness than γ-rays in achieving useful mutations, while γ-rays possess higher efficiency as it cause easily observable morphological alterations. The induced alterations of γ-rays

usually comprise chromosomal deletions and rearrangements and hence cause higher frequency of physical injuries. In comparison EMS induce point mutations that lead to broader range of effects (hypomorphic, hypermorphic, neomorphic) and hence generate some unusual mutations.

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