



MUTAGENIC IMPACT OF ETHYL METHANE SULPHONATE ON MORPHOLOGICAL AND CYTOLOGICAL EFFECTS IN BERSEEM (*Trifolium alexandrinum* L.)

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ABSTRACT

Induced mutagenesis has been effectively demonstrated as one of the best possible methodology for the genetic improvement of crop species. Egyptian clover (*Trifolium alexandrinum* L.), an important green fodder plant species of the family *Fabaceae* with immense agricultural value, was selected for the present study to improve its genetics by using the strategy of induced mutagenesis. Dry and healthy seeds of Egyptian clover cv. 'Wardan' were exposed to different doses (0.1, 0.3 and 0.5%) of Ethyl methane sulphonate (EMS) for variable time (3 and 5 h). The germination and survival percentages were recorded in M₁ generation and 0.3% EMS at 5 h was determined as LD₅₀ dose; whereas frequency of chlorophyll mutants and viable mutants along with cytological observations were screened in M₂ generation. A broad spectrum of chlorophyll and viable mutants with different frequencies were observed. Cytological abnormalities increased along with rising EMS concentrations and mutagen exposure time. The findings are encouraging and show that significant genetic variability had been induced by EMS. Thus, the comprehensive selection of the desirable mutants may bring about advancement of improved and high yielding mutants of *T. alexandrinum* in upcoming generation.

Keywords: Berseem, chlorophyll mutants, cytological abnormalities, ethyl methane sulphonate, mutagenesis, mutation breeding

INTRODUCTION

Egyptian clover or berseem (*Trifolium alexandrinum* L.) is an annual legume forage in northern India. This forage crop was introduced in India in 1904 (Mehta and Swaminathan, 1975) and presently covers more than 2 million ha area in India (Pathak *et al.*, 2015). Due to narrow genetic base, berseem is highly restricted for breeding. Certain breeding obstacles, such as very small size, complex flower structures, and lesser proportion of out crossing, self-fertility and low seed setting rates may be the other possible hurdles in genetic improvement of berseem. The genetic variation can be induced by mutation technology, and the traits possessed by plants can be significantly introduced (Nirmala and Rao, 1996). Mutation breeding serves as a milestone in inducing genetic variations in the crops having narrow genetic bases to enhance the desirable traits. The new method of agricultural mutation to improve crops is considered very promising in conventional breeding and is widely practice.

In conventional breeding methods, natural variability exists in the basic population, and small variations can be introduced by crossing, recombination and selection; which are carried out to enhance the frequency of favourable combinations of genes in the selected line. Mutation breeding helps to increase the chance of variation in traits under short-term mutagen exposure, but it can significantly

increase crop yields and possibility helps in inducing desired traits (Yaqoob and Rashid, 2001). In past century, several developed and developing countries have released more than 2543 mutants from 175 kinds of plants including fodder, cereals, pulses, fibre, vegetable, and ornamental plants (Roychowdhury and Tah, 2011). Ahloowalia *et al.* (2004) reported that the majority of mutant varieties (64%) were developed by the use of mutagens. Mutation breeding is an effective and powerful tool especially in plants which are autogamous and have narrow genetic base (Kharkwal, 1998).

In spite of the fact that gene mutation usually occurs as an error in DNA replication where most of the errors are recuperated, some of them pass to subsequent generations of cell division in order to become permanent in the plant offspring as spontaneous mutations. Kavina *et al.* (2020) has reported that mutagenic agents have been used to induce useful phenotypic variations in plants for more than seventy decades. Ethyl methane sulphonate (EMS) is a potent chemical mutagen of alkylating group, commonly used in plant breeding. High frequency of gene mutations as correlation with chromosomal distortion is fundamentally brought by this chemical mutagen. EMS alkylates guanine residues, delivering O⁶-ethyl guanine, pair with thymine instead of cytosine (Dhakshanamoorthy *et al.*, 2010). In various reports on mutagenic studies, replication of un-repaired alkylation harm in EMS mutagen, was found to successfully replace the G/C base pair with A/T (Dhakshanamoorthy *et al.*, 2010). These alterations emerging from mutagen treatment have the opportunities to be inherited from one generation to next so can establish successful viable mutant with some superior traits.

Adverse impact of mutagen could lead to the decrease of germination and, survival rates and to a significant deviation in phenotypic characters, *i.e.* root to shoot length proportion, plant height, number of leaves per plants, leaf to stem ratio, trifoliolate/ tetrafoliate/ pentafoliolate leaves, small leaflets, elongated, bright green and slightly hairy (Rajoriya *et al.*, 2016). During this mutagenesis experiment, an optimum dose (LD₅₀) was screened, one which causes maximum mutations and minimum killings. LD₅₀ shifts with crop species and with mutagens utilized. The present work was aimed to evaluate the morphological and cytological effects of EMS in berseem through induced changes in genotype so as to improve the hereditary inconstancy in this plant and its hereditary basis for the determination of desirable genotypes to expand large-scale cultivation.

MATERIALS AND METHODS

Procurement of seeds

Fresh and dry seeds of berseem (*Trifolium alexandrinum* L.) cv. 'Wardan', released in 1981, were procured from Indian Grassland & Fodder Research Institute, Jhansi, Uttar Pradesh (ICAR-IGFRI), India. The present mutagenesis, experiment work has been executed in the province of Roxburgh Botanical Garden, Department of Botany, University of Allahabad, Prayagraj, Uttar Pradesh (India) during *rabi* 2019. The experiment was conducted up to two generations in *rabi* season (2019-2020 and 2020-2021).

Mutagenic treatments

In M₁ generation, healthy and viable seeds were selected for treatment with chemical mutagen ethyl methane sulfonate [EMS (CH₃OSO₂C₂H₅)], having a half-life period of 30 h with a molecular weight of 124.16 and density of 1.15 gcm⁻³. Berseem seeds were presoaked in distilled water for 3 h, followed by treatment in three graded concentration of EMS *i.e.* 0.1, 0.3, and 0.5% solution (v/v) for 3 and 5 h, respectively, to determine the LD₅₀ value. The treated seeds were gently washed in running tap water to look at the lingering impact of EMS. Then the treated seeds were sown in pots (10 litter pot, with top diameter 28 cm, base diameter 24 cm and height 22.5 cm) along with their respective controls in triplicates. All the agricultural practices, namely irrigation, weeding, and plant protection were carried out during crop growth period. The seed germination and plant survival at maturity were recorded in M₁ generation. At maturity, all the surviving M₁ fertile plants were harvested separately according to

doses; and the seeds sown in next season to raise M_2 generation. Different kinds of chlorophyll mutants were scored in M_2 generation and classified according to the method of Kharkwal (1998). The mutation frequencies were studied in M_2 progenies for both chlorophyll and morphological mutations in each treatment. The level of seed germination (SG) of every single treatment doses were recorded on 7th day as follow:

$$\text{Seed germination (SG\%)} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds sown}} \times 100$$

Plant survivability (PS) was recorded 30 days after sowing in triplicate pots and survival percentage was assessed as follows:

$$\text{Plant survivability (PS\%)} = \frac{\text{Number of seed survived}}{\text{Total number of seed sown}} \times 100$$

Screening of mutants

In mutagenic treatments, chlorophyll mutants were screened 10 days after planting. Significant variations for recurrence and spectrum of chlorophyll mutations were observed in M_2 plants population. Some chlorophyll mutations exhibited complete lethality which were scored in beginning but later declined; whereas viable chlorophyll and morphological mutations were scored throughout the growing season of plants.

Meiotic analysis

At the onset of reproductive stage, the young flower buds of appropriate sizes were fixed in Carnoy's fixative (3:1, alcohol: glacial acetic acid) for 24 h, and then stored in 70% alcohol at 4°C till further use. By screening the pollen mother cells, these buds were used for cytological evaluation. The important stages were taken on Nikon phase contrast microscope (E200, Japan) using PCTV software. The pollen fertility (%) was calculated on the basis of its staining ability in 1 % glycerol-acetocarmine stain (10 g carmine dissolved (SRL C.I. No. 75470) in 1 L of 45% glacial acetic acid, add boileezers, and reflux for 24 h. Filter into dark bottles and store at 4°C). The fully stained pollen grains were considered fertile (Mark, 1954); whereas sparsely stained, enucleated pollen grains were considered as sterile. Five slides for each treatment set were prepared and ten microscopic views of each slide were examined.

Statistical evaluations

The data was analyzed by using SPSS 16.0 software. There were three replicates of plants for each treatment and one independent variation. A one way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT, $P < 0.05$) were performed for mean separation and the graph was plotted using Sigma Plot 10.0 software (Systat Software Inc.). Actual means and standard errors were calculated.

RESULTS AND DISCUSSION

Seed germination and plant survival percentage in M_1 generation

Seed germination (SG) and plant survival (PS) are important parameters to estimate the effects of mutagen on plants. Data analysis of the number of seeds that germinated and survival showed an attendant decrease in germination and survivability along with increasing the concentrations of EMS (Fig. 1A, B). The results revealed continuous reduction in seed germination as well as seedling survivability with corresponding increase in EMS concentration. The maximum germination was recorded in control (93.22 ± 0.27) which decreased from 86.67 ± 0.40 (0.1%) to 68.89 ± 0.68 (0.5%) at 3 h treatment and in case of 5 h treatment it declined from 79.32 ± 0.60 to 56.33 ± 0.84 . In present study, EMS showed toxic effect on seed germination. The highest survival was recorded in control (88.89 ± 0.39). It decreased to 80.00 ± 0.55 and 62.23 ± 0.86 (0.1-0.5%) at 3 h treatment and to 71.23 ± 0.49 and 44.28 ± 0.81 in case of 5 h treatment. Higher doses of EMS might be the probable cause of

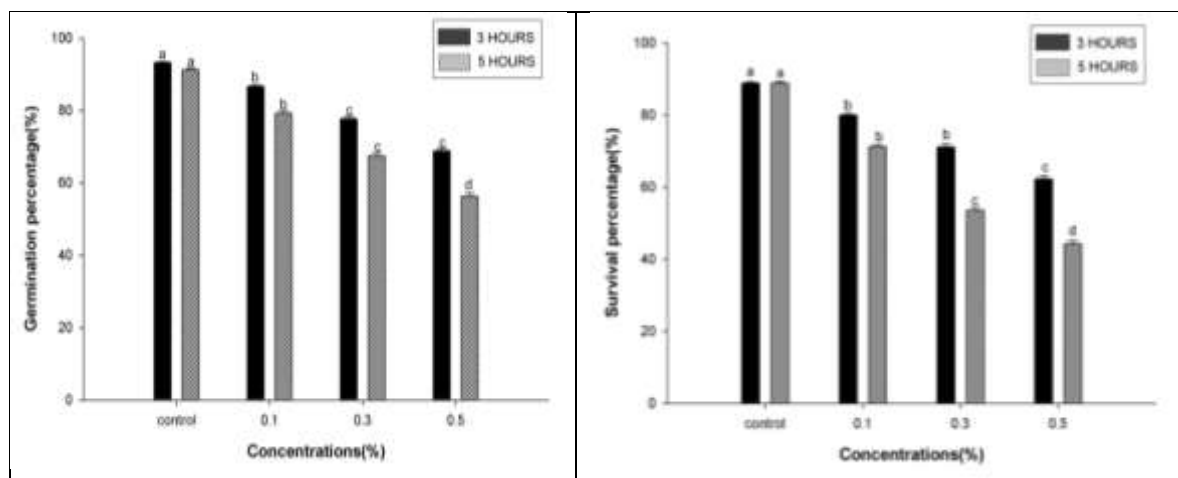


Fig. 1: Comparative germination percentage and survival percentage after EMS treatment in berseem cv. 'Wardan' for 3 h (A) and 5 h (B), respectively

genetic injuries leading to the decreased survivability. EMS causes random point mutations, and as much as the concentration of EMS increases the probability of point mutation induction would increase (Sikora *et al.*, 2011). These mutations may lead to the defects in the synthesis of essential compounds for plants. Higher doses probably lead to more genetic disturbance in treated plants which may explain why SG and PS rate are lower. Although 50% germination was observed at higher doses (>0.5% EMS, 5 h), limiting the study to this data may lead to incorrect conclusions. Because, the deadly disturbance induced by genetic damage would appear on treated seedling after germination and emergence stage. It is, therefore, recommended to use 0.3% EMS at 5 h dose (with 50% survival rate) as the optimal dose (LD₅₀), especially for fodder field researches.

Chlorophyll content and viable mutant frequency

In this mutagenic study, chlorophyll and viable mutations were observed in M₂ generation. Albino, xantha, chlorina, viridis, yellow viridis, bi-colored variation and aurea (half leaflet, full leaflet mutants) were the various chlorophyll mutants; whereas viable mutant parameters such as tall, dwarf, early maturity, late maturity, seed variabilities, and high yield mutant were screened in all the mutagenic treatments (Table 2). In case of chlorophyll mutants, the intermediate dose was found more potent in producing high mutation frequencies for 4.81% at 5 h and 4.36% at 3 h, whereas the lowest frequency of chlorophyll mutation 1.81% was recorded at 0.5% EMS for 5 h (Table 1). Among these chlorophyll mutants, xantha (Fig. 2D) was most frequent in both treatment sets. Singh *et al.* (2000) have previously reported that similar changes in urdbean (*Vigna mungo*) plants. Albina (Fig. 2E) was the least frequent chlorophyll mutant in both treatment sets. Such type of chlorophyll and viable

Table 1: Effect of EMS on frequency of chlorophyll mutants in M₂ generation of berseem cv. 'Wardan'

Conc. (EMS)	Treat- ment (h)	No. of plants studied	Percentage of chlorophyll mutants							No. of chlorophyll mutants	Mutation frequency (%)
			Albina	Xantha	Chlorina	Viridis	Yellow Viridis	Bicoloured variation	Aurea		
Control	-	295	-	-	-	-	-	-	-	-	-
0.1%	-	294	-	1.02	0.34	0.34	1.02	0.68	0.34	11	3.74
0.3%	3	298	0.34	0.67	0.34	0.67	0.67	0.67	1.01	13	4.36
0.5%	-	282	0.35	0.71	0.35	0.71	-	0.35	0.35	8	2.84
Control	-	288	-	-	-	-	-	-	-	-	-
0.1%	-	292	-	0.68	1.37	0.34	0.68	0.34	0.68	12	4.10
0.3%	5	291	0.34	1.37	-	0.69	0.69	0.69	1.03	14	4.81
0.5%	-	276	0.36	1.09	-	0.36	-	-	-	5	1.81

Table 2: Effect of EMS on the frequency of viable mutants in M₂ generation of berseem cv. 'Wardan'

Dose of mutagens	EMS (3 h)			EMS (5 h)		
	0.1%	0.3%	0.5%	0.1%	0.3%	0.5%
Number of plants studied	294	298	282	292	291	276
Tall	1	3	-	2	4	-
Dwarf	-	1	2	-	-	3
Leaf mutants	1	-	-	1	3	2
Tetra-foliolate	1	3	2	1	4	1
Penta- foliate	3	5	2	1	3	2
Stipule mutant	-	1	1	1	1	2
Early flowering	-	1	-	1	3	-
Inflorescence mutants	-	1	1	-	-	-
Flower color mutant	-	1	-	-	1	-
Complete sterile plants	-	-	1	-	2	2
Early maturity	1	1	-	-	3	-
Late maturity	-	-	2	-	1	1
Light green seeds	1	1	1	-	1	2
Red seeds	0	2	2	1	1	2
Bold size seeds	2	2	-	3	2	-
High yield plant	2	3	-	4	2	-
Total	12	25	14	15	31	17
Frequency%	4.08	8.39	4.96	5.13	10.65	6.16



Fig. 2: Some chlorophyll and viable mutants in berseem cv. 'Wardan' A: Control; B: (Aurea) half leaf; C: Aurea; D: Xantha; E: Abina; F: Bi-coloured variation; G: Tetrafoliate leaf; H: Penta-foliolate leaf; I: Curved tomentous leaf; J: Sagittate leaf apex; K: Normal stipule; L: Coloured and hairy stipule; M: Reduced inflorescence with sterile flowers; N: Normal inflorescence; O: Branched inflorescence; P: Bold size seeds; Q: Green seeds; and R: red seeds.

mutants were also observed as mutagen markers in lentil (Solanki and Sharma, 2001), chickpea (Ambarkar, 1997); and the frequency of these markers is mainly used as reliable measure of genetic effects for mutagens (physical/chemical). High mutation frequency of viable mutant (10.65 and 8.39%) was observed in intermediate doses (0.3%) of EMS for 5 and 3 h, respectively whereas less effective dose for viable mutants (4.08%) was observed at 0.1% of EMS at 3 h (Table 2). The plant

height was highly increased with higher number of branches leaves as compared to control. These mutants were observed in almost lower concentration of mutagenic treatments, especially 0.3% EMS. The mutants showed plant height which was very much reduced with less number of branches and leaves as compared to control; these mutants were observed at higher concentration of the mutagenic treatments for especially 0.5% EMS at 5 h.

A maximum of three mutant plants, exhibiting 8-12 days earlier maturity than control plants, was observed at 0.3% EMS for 5 h treatment; whereas a maximum of two mutant plants exhibited 5-10 days late maturity than control plants with 0.5% EMS for 3 h treatment (Table 2). The mutants showed tetrafoliate (Fig. 2G) and pentafoleate (Fig. 2H) leaflets which were observed at all mutagenic treatments, especially 0.3% EMS for 5 h. The mutants showed for flower colour and inflorescence (Fig. 2N and O) as well as floral sterility (Fig. 2M) at higher doses of EMS treatments (Table 2). Among these treatment sets, seed mutants show a significant role for upcoming generation. Bold size seed mutants (Fig. 2P) were observed frequently at lower doses *viz.*, 0.1% and 0.3% EMS (Table 2) whereas green seed (Fig. 2Q) and red seed (Fig. 2R) mutants were very often observed due to stress at higher doses of EMS. Stipules (Fig. 2K and L) were more hairy in all the treatment sets (Table 2). Chemical mutagen induced cellular damages resulting in alteration of metabolism which ultimately leads to leaf abnormalities (Fig. 2I, J). Frequency of mutants (*F*) either chlorophyll or viable, estimated by using the following formula:

$$\text{Frequency of mutants (F)} = \frac{\text{Number of mutants}}{\text{Total number of plants}} \times 100$$

The origin of chlorophyll deficiencies and variation in pigments might be due to mutations in genes, which are created by mutagens and, are responsible for synthesis of photosynthetic pigments. Albino mutants/variants, characterized by colour of leaves, are caused by the absence of all pigments like chlorophylls, carotenoids and other pigments. These mutant leaves were white in colour, albino seedlings are smaller in height and survive only 18-20 days after germination and then died. In Xantha, variant the leaves turned yellow in colour due to the absence of xanthophylls. Growth of variants was retarded and most of them died within 17-25 days after emergence. Viridis, variants showed leaf margin more segregated as compared to control. Young leaves were dark green in the early stages of development and turn green in later stages. These mutants produced normal flower and also set seeds. Several authors have reported the occurrence of different type of chlorophyll mutations such as xantha, albina, viridis, chlorine, etc. (Kolar *et al.*, 2011; Arisha *et al.*, 2015; Verma *et al.*, 2018). It is also possible to induce new features which do not exist in the available range of variability in a high yielding and well adapted variety.

Cytological observation

Cytological study is one of the proper approaches for mapping genetic diversity and unraveling the mysteries of inheritance. This study was focused on the effect of chemical mutagen, *i.e.* EMS on the pollen mother cell of clover plants. The study displayed that meiotic chromosomes of control in *Trifolium alexandrinum* ($2n = 16$) having 8 bivalents at diakinesis along with nucleus (Fig. 3A). However, the treated sets showed a considerable mutagenic effect on the chromosomal architecture. A range of different chromosomal abnormalities *viz.*, stickiness, scattering, unorientation, asynchronus anaphase, laggard and precocious movement were recorded. Among them, stickiness at metaphase (Fig. 3B, E and F) and anaphase (Fig. 3C) were of serious concern. During 3 h treatment of EMS at doses 0.1, 0.3 and 0.5% the frequency of metaphasic stickiness recorded was 0.78 ± 0.21 , 1.0 ± 0.23 and $1.18 \pm 0.13\%$, respectively. Maximum metaphasic stickiness of $1.98 \pm 0.21\%$ was observed at 0.5% EMS for 5 h (Table 3). Along with this, anaphasic stickiness was recorded to be maximum $1.27 \pm 0.25\%$ at 0.5% EMS for 5 h.

Unorientation at metaphase (Fig. 3D) was maximal of $0.81 \pm 0.08\%$ at 0.5% EMS for 3 h and minimal $0.12 \pm 0.13\%$ at 0.1% EMS for 3 h. Precocious movement in chromosome due to EMS was found as another abnormality in this treatment, disorientation at metaphase II with one precocious movement (Fig. 3D), stickiness at metaphase II with 1 precocious movement (Fig. 3E) and stickiness

Table 3: Spectrum and %age of chromosomal abnormalities induced by EMS in M₂ generation of berseem cv. 'Wardan'

Conc. (EMS)	Time	<u>Metaphasic abnormalities (%)</u>				<u>Anaphasic abnormalities (%)</u>				Other (%)	TAB%	Pollen fertility (%)
		Sc	St	Un	Pr	St	Sc	St	Un			
Control	-	-	-	-	-	-	-	-	-	-	-	98.21±0.13 ^a
0.1%	3 h	0.27±0.13	0.78±0.21	0.12±0.12	0.12±0.12	0.51±0.25	0.13±0.13	0.14±0.13	-	0.13±0.13	2.21±0.31	87.22±0.28 ^b
0.3%		0.50±0.12	1.00±0.23	0.25±0.12	0.25±0.12	0.62±0.12	0.38±0.23	0.25±0.12	0.38±0.01	0.49±0.24	4.16±0.15	78.84±0.64 ^c
0.5%		0.71±0.21	1.18±0.13	0.81±0.08	0.71±0.08	1.17±0.23	0.35±0.01	0.48±0.31	0.24±0.12	0.46±0.10	6.14±0.27	68.22±0.74 ^d
Control	-	-	-	-	-	-	-	-	-	-	-	97.12±0.24 ^a
0.1%	5 h	0.58±0.16	0.82±0.09	0.24±0.12	0.13±0.13	0.22±0.11	0.34±0.20	0.37±0.23	0.13±0.13	-	2.83±0.22	84.43±0.33 ^b
0.3%		0.34±0.33	1.16±0.08	0.71±0.2	0.47±0.12	0.59±.24	0.70±0.02	0.34±20	-	0.58±0.11	4.90±0.30	71.23±0.68 ^c
0.5%		0.53±0.46	1.98±0.21	0.52±0.34	0.73±0.34	1.27±0.25	0.95±0.24	0.71±0.17	0.56±0.12	0.56±0.13	7.85±0.33	65.28±0.80 ^d

Abbreviations: Sc - scattering; St - stickiness; Un - unorientation; Pr - precocious movement of chromosomes association; Asy - asynchronous division; Lg - laggards; Oth - other abnormalities; TAB - total abnormality percentage.

Means followed by lowercase letter are statistically significant at $p < 0.05$ in Duncan's Multiple Range Test. The values are mean $\pm$ SE.

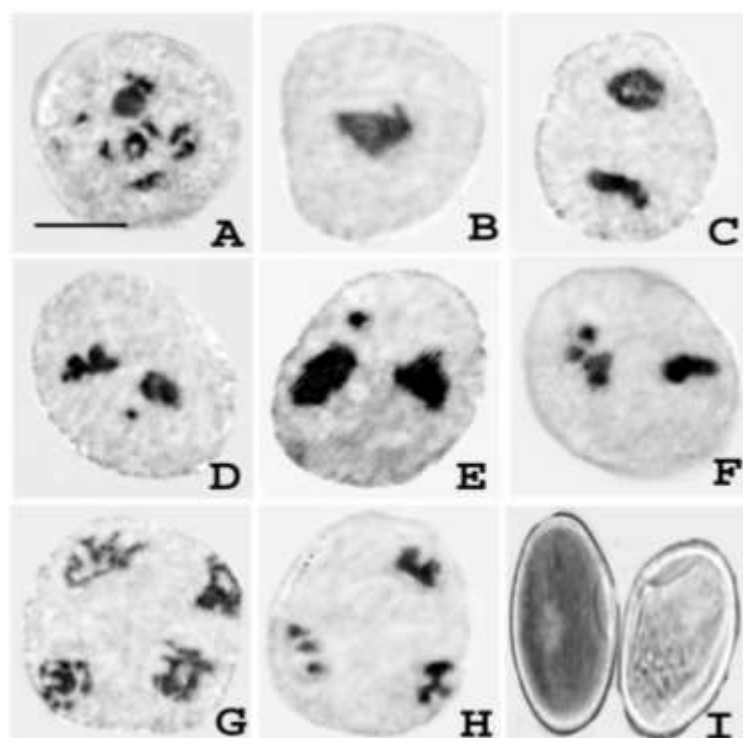


Fig. 3: Various chromosomal abnormalities observed in M₂ generation of berseem cv. 'Wardan'; A - Diplotene stage (8 bivalents), B - Stickiness at metaphase I; C - Stickiness at anaphase I; D - Unorientation at metaphase II with one precocious movement; E - Stickiness at metaphase II with one precocious movements; F - Stickiness at metaphase II with two precocious movements; G - Anaphase II; H - Asynchronous division at Anaphase II; and I - Sterile and fertile pollen

at metaphase II with 2 precocious movements (Fig. 3F) cause even more deleterious effects. Maximal precocious movement of $0.73 \pm 0.34\%$ at 0.5% EMS at 5 h and minimal 0.12 ± 0.12 at 0.1% EMS for 3 h was detected. It was gradually increased with increasing EMS concentration and the time of mutagen exposition.

Scattering at metaphase was maximum ($0.71 \pm 0.21\%$) at 0.5% EMS for 3 h and minimum ($0.27 \pm 0.13\%$) at 0.1% EMS for 3 h. The scattering at anaphase plays a significant role in chromosomal abnormality, which was maximum ($0.95 \pm 0.24\%$) at 0.5% EMS 5 h and minimum ($0.13 \pm 0.13\%$) at 0.1% EMS for 3 h (Table 3). Along with these abnormalities, asynchronous division at anaphase (Fig. 3H) increased gradually by increasing concentration as well as exposition time of EMS showing maximum of $0.71 \pm 0.17\%$ at 0.5% EMS for 5 h and minimum of $0.14 \pm 0.13\%$ at 0.1% EMS for 3 h. Moreover, the highest concentration of EMS treatment induced the highest

frequency of PMCs with unorientation of bivalents in this plant. Laggard was also one of the chromatic abrasions which were frequent in higher EMS concentration, showing the maximum of $0.56 \pm 0.12\%$ at 0.5% EMS 5 h. Total abnormality percentage was recorded maximum $7.85 \pm 0.33\%$ at 0.5% EMS 5 h and minimum $2.21 \pm 0.31\%$ at 0.1% EMS 3 h showed that increasing the concentration and mutagen exposition time the TAB% were also gradually increased. Enhancement in the frequency of TAB% was wide and includes a high proportion of stickiness and precocious movement along with laggards. It

implies that the chemical mutagen may have brought some alterations in the pattern of organization of chromosomes (Shah *et al.*, 2006).

Pollen fertility standards were likewise changed in accordance with evaluating the effects of EMS on viability of pollens. In the case of control (fertile), pollen was found to be a conspicuously stained spherical nuclear substance, while sterile pollen was insufficiently stained and contracted. Most elevated pollen fertility was recorded in control set that went for a consistent decay with increasing concentration and time of mutagen exposition. This was determined to be $98.21 \pm 0.13\%$ and $97.12 \pm 0.24\%$ in case of control which decrease up to $68.22 \pm 0.74\%$ (0.5% EMS, 3 h) while in 5 h treatment declination was accounted for $65.28 \pm 0.80\%$ at higher dose 0.5% EMS (Table 3). Whereas, Figure. 4I show fertile and sterile pollen of berseem plants. Morphological abnormalities were attributed to the chromosomal breaks, disturbed auxin synthesis, and disruption of mineral metabolism and accumulation of free amino acids (Gnanamurthy and Dhanavel, 2014). For EMS treatment, these mutagens appear to be liable for stickiness, which is brought about by the chromosomal condensation during dynamic cell division stages by target proteins. Jabee and Ansari (2005) proposed that chromosomal breakage may cause stickiness among the chromosomes. It may also be due to genetic and environmental factors (Rao *et al.*, 1990; Baptista-Giacomelli *et al.*, 2000). According to Jabee *et al.*, (2008), stickiness could be caused by depolymerisation of nucleic acid brought about by mutagenic treatment or because of partial separation of the nucleoproteins and change in their pattern of association. Precocious movement of chromosomes may have been brought about by the early terminalisation or stickiness of chromosomes and movement of chromosomes ahead of the rest during anaphase Srivastava and Kapoor (2008). It might be because of the abnormal homology for chromosome pairing and spindle mechanism (Agarwal and Ansari 2001), either in light of the unusual spindle activity (Kumar and Gupta 2009), or because of the reunion of chromatids during meiotic prophase. Some previous research reports stated that, precocious separation of univalent might be responsible for desynapsis or asynapsis (Kumar and Rai 2007a). Thus, EMS may have triggered genetic abnormalities because of mutagenic action and subsequently the aggravations in homology and blending of homologous chromosomes. Reddy & Munirajappa (2012) was opinion that due to the effects of mutagens, the spindle fibers failed to carry the respective chromosome to the polar regions, and resultantly the lagging chromosomes appeared at anaphase I. Bhat *et al.* (2006) reported that laggards may be the result of late chiasma terminalisation. Kumar and Rai (2007a, 2009) also support the opinion that laggards might have appeared due to improper spindle functioning.

Conclusion: In order to discover optimum dose of EMS, germination and survivable rates in M_1 were determined in berseem cv. 'Wardan'. Based on percentage survival, LD_{50} was determined in EMS concentration of 0.3% for 5 h. Several prominent chlorophyll mutants noticed were albino, xantha, chlorine, viridis, yellow viridis, bi-coloured variation, and aurea. The results demonstrated that increasing doses and exposition time of EMS extreme impacted plant development. The intermediate doses (0.3% EMS) at 3 and 5 h gave more mutagen (chlorophyll and viable) frequencies. Cytological studies provide information on the response of berseem to a particular mutagen and provide greater chances for the selection of desired characteristics. The study revealed that the isolation of vigorous, high yielding, tall plant mutants are possible at lower doses of EMS. The work needs continuous amplification by analysing molecular and phytophysiological changes due to EMS in berseem.

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Authors' contribution: Conceptualization of research (G. Kumar, Kaushal Tripathi); designing of the experiments (Moni Mishra and Kaushal Tripathi); analysis of data and interpretation (Kaushal Tripathi, Moni Mishra); preparation of the manuscript (Kaushal Tripathi and Moni Mishra).

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