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Assessment of ems induced genetic variability in *Foeniculum vulgare* mill. (Fennel)

Girjesh kumar[1], Moni Mishra[2*], Kaushal Tripathi[3]
Plant Genetics Laboratory, Department of Botany, University of Allahabad, Prayagraj, Uttar Pradesh, India

Abstract
Fennel is a major economical important crop, consumed as spices across the globally to fulfil the high traditional medicinal value. Because of its demands and Economical importance, it is required to induce changes in genotype and enhance the genetic variability. In this present investigation mutagenic treatment of EMS found to be potent and effective in enhancing quantitative and qualitative traits. Agronomic trait analysis showed inter and intra treatment phenotypic variations such as plant height, no of branches and seed yield. Cytological study revealed that higher frequency of chromosomal aberrations at higher doses in 5 h treatment as compared to 3h treatment and recovery rate was observed to be higher at lower doses which showed positive response in correspondence to duration. In this investigation, phenotypic categories of variants associated with the vegetative organs like plant size, leaf morphology and seed size were dominant in higher doses. Main objective of this experiments to select particular optimum mutagenic doses according to plant species for successful running of mutation breeding programs and also to optimize their adverse effect.

Keywords: *Foeniculum vulgare*, chlorophyll variants, cytological study, quantitative traits

Introduction

Foeniculum vulgare Mill. (2n=22) belongs to Apiaceae family, commonly known as sounf or fennel, is an important seed spices cum essential oil yielding crop. Due to presence of essential fatty acid, fennel has valuable nutritional composition [1]. Because of its nutritional and economic importance, it is imperative that adequate attention should be given to increase their production with high quality and also to increase the variability with other desirable attributes of *Foeniculum vulgare* Mill. Its seeds are aromatic, stimulant and carminative [2]. So it is good source in treatment of digestive endocrine and respiratory disorders, also act as galactagogue agent for lactating mothers. A brief study on fennel plant showed that it controlled the various type of infectious disorders caused by different type of bacterial, viral, mycobacterium, protozoan and fungal pathogens [3, 4]. that provide a note worthy basis in pharmaceutical biology for the developments formulation of new drugs and future clinical uses. In such situation, induced mutagenesis is very effective and powerful technique to create variability in a very short period of time [5, 6]**.** It can be used to generate variation in quantitatively inherited characters [7]. it is easy to handle by plant breeders especially for those plants which have narrow genetic base and self-pollinating plants [8]. It provides beneficial variation for practical plant breeding practices, currently more than 2252 mutant varieties have been officially released by breeders in this scientific world, during the fast seven decades by using this mutation breeding programs [9].

Mutation breeding has been used to improve the various desirable characters in crop plants like earliness, dwarfness, yield, and quality. Mutagenesis also used to develop the resistance to biotic and a biotic stress in plant [10, 11]. Induced mutagenesis involves the different type of physical and chemical mutagens. Physical mutagen includes the different type of ionizing and non-ionizing radiations. Ionizing radiations normally causes the deletion and chromosomal rearrangements [12]. Whereas chemicals mutagens mainly cause the point mutations [13]. Due to its potency the alkylating agents like as mustard gas, methyl methane sulfonate (MMS), ethyl methane sulfonate (EMS), and nitrosoguanidine have caused many effects on DNA. EMS is a chemical mutagen which is most commonly used in plant as alkylating agent that alkylates guanine bases and causes to mis pairing in DNA passes like G pairs with T instead of C as a resulting in primarily causes or G/C- to-A/T transitions [14]. These chemical mutagens also cause large scale changes in morphological aspects and yield structure of crop plant as comparison to normal plants or control set. Ethyl methane sulphonate (EMS) causes various type of chemical modification of nucleotide sequences in DNA, that result in base changes and mispairing [13, 15].

Material and methods

The dry and inbred seeds of *the Foeniculum vulgare* Mill. variety-AF-2 were obtained from ICAR-National research center on seed spices. Ajmer, Rajasthan. The present study on induced mutagenesis in Fennel plant was carried out in the plant genetics laboratory, Department of Botany, University of Allahabad during 2019-2020. Seeds of Fennel variety AF-2 were placed in petri plate, water was added. Seeds were soaked overnight at room temperature for 24 hours. Subsequently, the water was decanted and pre-soaked seeds were treated for two time periods *viz* 3 h and 5h with different concentrations of EMS solution. EMS solution *viz.* 0.1%, 0.3%and 0.5% prepared in buffer solution (potassium phosphate buffer) at pH.7.0. The treated seeds were washed thoroughly in running tap water to terminate the residual effect of the mutagenic chemicals. One set of seeds was kept in distilled water to use as control.

Cytological observation

Treated seeds along with their respective controls were germinated in seed germinator at 25 ± 20 °C with humidity

50-70% in petri plates with filter paper and distilled water was sprinkled regularly. When seeds germinate then these treated seeds along with control were transplanted in pots in 3 replicates, After few days at seedling stage root tip meristems obtained from seedlings were treated in 2 mM of 8 hydroxyl quinalin solution at 3 4°C for 6 hours then transfer the plant material in cornoys fix ative (1: 3: 6 glacial acitic acid chloroform ethanol) the root tip were hydrolysed in 1N HCL for 2-3 minute at 25°C After washing in distill water, root tips were placed in acetocarmine stain for 3hrs. Staining was done using 2% acetocarmine. 20 Slides were prepared for each dose of EMS and observed under electron microscope using Olympus PCTV Vision Software, Several microscopic views from each slide were recorded for scoring of Active Mitotic Index (AMI%) and Total Abnormality Percentage (TAB%). Data was calculated in replicates. The AMI and TAB percentage was calculated by using the following formulae

Active Mitotic index (AMI) % = Total no. of dividing cell / Total no. of cell observed×100

Total Abnormality percentage (TAB) % =No. of Abnormal cell / Total no. of cell observed × 100

Biochemical observation

Chlorophyll and Carotenoid contents were determined according to procedure described by Lichtenthaler (1987) [16] Leaf tissues (0.5 mg) were homogenized in 5ml of acetone 80%. The homogenate was centrifuged and the O.D. was taken at the wavelengths of 663, 646 and 470 nm in a spectrophotometer and calculated chl a, chl b and carotenoid content.

Chlorophyll variant observation

The chlorophyll mutants were scored on 20th day after sowing. Different kind of chlorophyll mutants that include Albino, Xantha, Chlorina, and Viridis were observed in treated plants with respect to control.

Statistical observation

For using Statistical analysis in the table, three replicates for each treatment were used. Statistical analysis was performed using the SPSS 16.0 software. ANOVA and Duncan‟s multiple range test was conducted for mean separation. By using Sigma plot 10.0 software Graphs were prepared. Actual mean and standard error were calculated and the data was subjected to analysis of variance

Results and discussion

Mutation breeding is an important part of the solution of world's food crises [17]. It is a very efficient tool to the global agriculture community to broaden the adaptability of the crops in the face of climatic change, increasing population, raising prices and soils that lack fertility or have other major problems [18]. Thus, to govern fruitful mutagenesis, selection of efficient mutagen and treatment is required as mutagens are the potent tool for direct improvement or bringing about certain qualitative and quantitative changes in crop plants. The impact of mutagenic treatments is usually measured by parameters like seed germination (%), seedling height (cm), plant survival, chromosomal aberrations in mitosis and by determination of quantitative characters.

Effect of EMS-Induced mutagenesis on germination and survivability percentage

Data analysis of number of seeds that germinated and survival showed an attendant decrease in germination and survivability along with increasing the concentrations of EMS were recorded (Figure.1, 2). The results obtained indicate that continuous reduction in seed germination as well as seedling survivability occurred with corresponding increase in EMS concentration ($P < 0.01$).Maximum germination was recorded at control (496.67±0.67^a) which decreases from 88.67±0.50^b (0.1%) to76.67±1.51^d(0.5%) at 3 h treatment and in case of 5h treatment it declined from 83.31±1.09^c to 60.33±1.20^e respectively, In present investigations, EMS causes the toxic effect on seed germination.

Germination percentage were suppressed by demolition of the action of gibberellic acid reason behind this is because of chromosomal lesion or distrubance in DNA synthesis or metabolic process [19].

Similar results were also reported in *Lycopersicon esculentum* and *Solanum lycopersicum* by Nusrat *et al.* (2002) [20] Watanabe *et al*. (2007) [21]. Reduction in seed germination in treated sets may be due to delay or inhibition in physiological as well as biological processes which is very essential for seed germination**.** whereas, in case of survivability percentage maximum survivability was recorded at control (77.83±1.15) which decreases from 75.44±1.06 (0.1%) to 58.83±.1.52 (0.5%) at 3 h treatment and in case of 5h treatment it declined from 71.80±1.12 to 58.83±1.52. Higher doses of EMS would causes genetic injuries which may reason for decreasing survivability percentage, why survival rates are lower among them along with increasing concentration and duration of EMS.

EMS is attributed to alter the enzyme activities and causes disturbance in their physiological, cytological and biochemical activity.

The decrease in survival of plants when concentration of EMS and duration of treatment increased was reported by Das *et al*. (2010) [22] in *Withania*, Kumar and Pandey (2018) [23] in *Coriandrum sativum* L, Girija and Dhanavel (2009) [24] in many other crop plant.

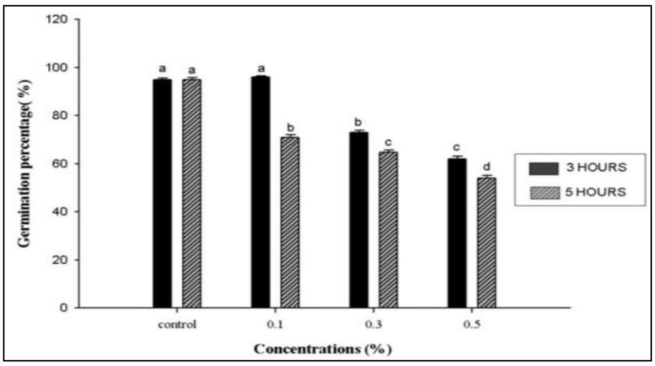

Fig 1: Comparative account of Germination Percentage after EMS treatment in *Foeniculum vulgare* Mill. For 3 h and 5 h, respectively.

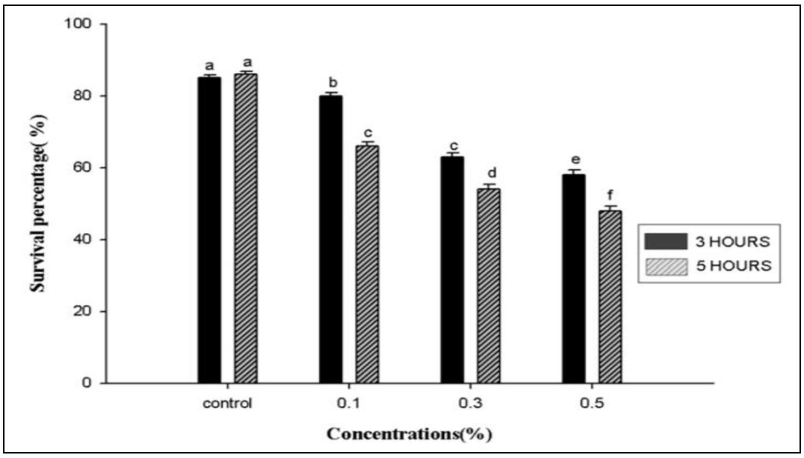

Fig 2: Comparative account of Survival Percentage after EMS treatment in *Foeniculum vulgare* Mill. For 3 h and 5 h, respectively.

Effect of EMS mutagenesis on no. of primary branches, no of umbel/plant and no, of umblet /umbel

The Number of primary branches, Number of umbel/plant and Number of umbellates /umbel were decreases in dose dependent manner but it shows the significant effect at lower doses 3 h treated plant shows more beneficial effects as compared to 5 h treated plant which shows the harmful effect on plant (Figure.3, 4, 5). In lower doses the Number of primary branches, Number of umbel/plant and Number of umbellates /umbel were increase with respect to control. Kumar and Pandey (2018) [23] observed the similar results in *Coriandrum sativum* L. and also reported a significant decrease these morphological parameters along with increasing concentration and duration of EMS.

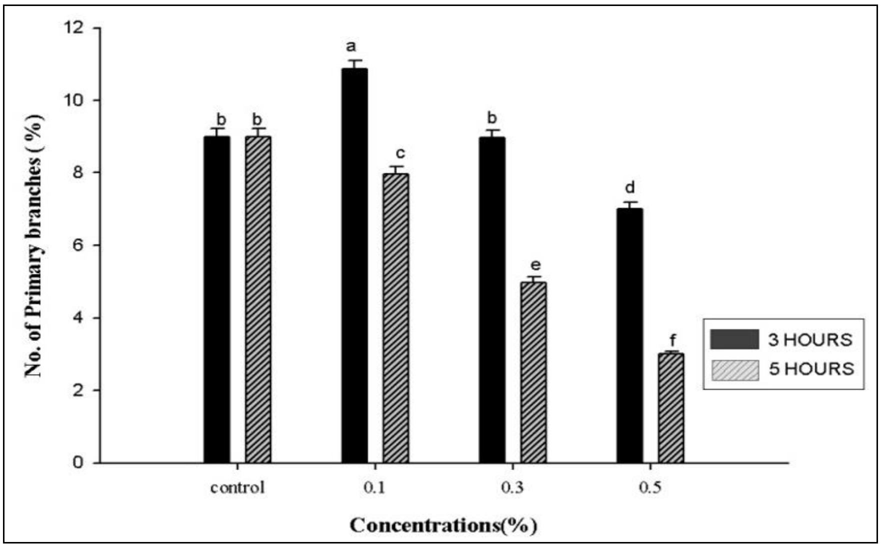

Fig 3: Comparative account of No of primary branches after EMS treatment in *Foeniculum vulgare* Mill. For 3 h and 5 h, respectively.

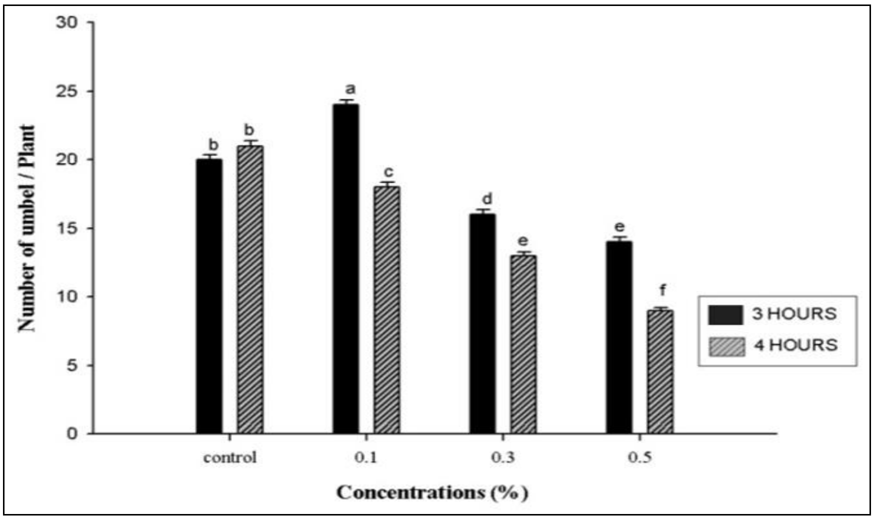

Fig 4: Comparative account of number of umbel / plant after EMS treatment in *Foeniculum vulgare* Mill. for 3 h and 5 h, respectively.

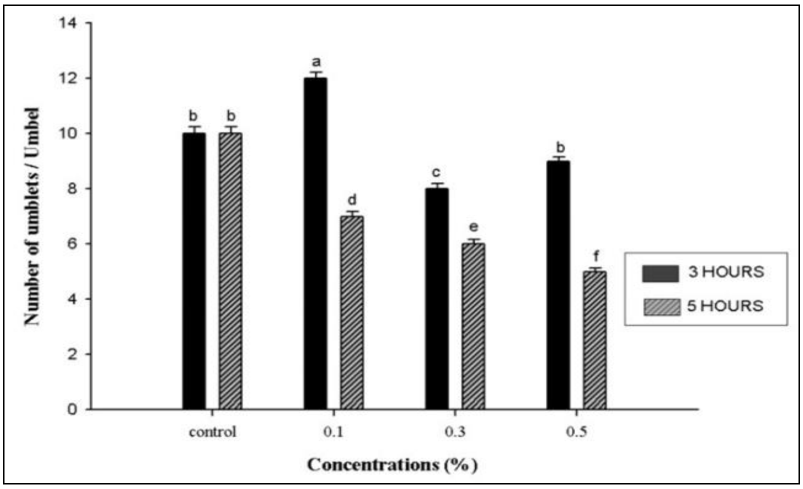

Fig 5: Comparative account of number of umblets / umble after EMS treatment in *Foeniculum vulgare* Mill. for 3 h and 5 h, respectively.

Effect of EMS mutagenesis on 50% flowering

Slightly delay in 50% flowering was observed along with increasing the concentration of EMS with respect to their control sets. It increases 114±1.54 in 3h sets where as in 5h set it reaches to 118±1.34 (Fig 6). Flowering time increased with an increase in dose/concentration of EMS treatment. However, lower dose of mutagens showed significant effect and shows earliness. Kaul (1980b) [25] suggested that the mutation of two dominant genes to their recessive forms makes for an early flowering in pea. Early flowering observed in present study might be due to physiological changes in production of hormones responsible for the flowering and late flowering/maturity in present study. it was observed that higher concentrations might be causing low production of hormones required for flowering and maturity This is in favors of earlier work by Jaykumar and Selvaraj (2003) [26] in sunflower, Manjaya and Nandanvar (2007) [27] and Tambe (2009) [28] in soybean.

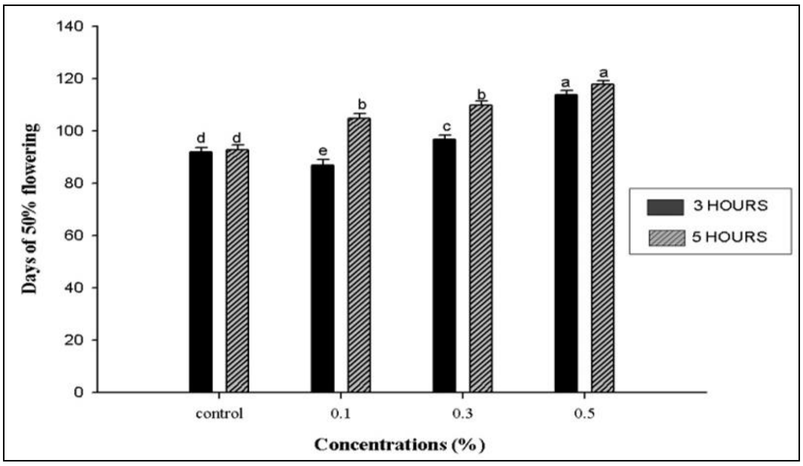

Fig 6: Comparative account of days of 50% flowering after EMS treatment in *Foeniculum vulgare* Mill. for 3 h and 5 h, respectively

Frequency of chlorophyll variant

In the present appraisal, the frequency of chlorophyll variants calculated as percent of plant progenies. The spectrum of chlorophyll variants was observed at seedling stage (Figure. 7) It was observed that the frequency of induced chlorophyll variant was increased with an increase in the concentration of EMS (Table. 1). Chlorophyll variant is act as genetic marker in applied research and also use to calculating the efficiency of mutagens in increasing the genetic variability for crop improvement. Large number of chlorophyll variants were recorded at high dose of EMS treatment. The spectrum of chlorophyll mutations *viz*., albino, xantha and virescence were observed at all mutagenic treatments (Figure.7). The seedlings variant were recorded periodically. Albino mutant/variant, colour of leaves, caused due to absence of all pigments like chlorophyll, carotenoid and other pigments. These mutant leaves were white in colour, albino seedling are smaller in height and survive only 18-20 days after germination and then die. In Xantha, variant the leaves turned yellow in colour due to the absence of xanthophylls. Growth of variants is retarded and most of them die within 17 to 25 day 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 stage. These mutants produce normal flower and also set seeds. One or two mutants were observed at all mutagenic treatments. Several authors have reported the occurrence of different type of chlorophyll mutations such as xantha, albina, viridis, chlorina etc. [29, 30]. Chlorophyll variants and mutant frequency are an indication in assessing effectiveness of a mutagen and estimation of mutational events in crop plants. The scoring of chlorophyll variant is one of the most important method for calculating the mutagenic efficiency and effectiveness in the plants The reason behind occurrence of chlorophyll mutants in various crop could be attributed to impaired chlorophyll biosynthesis which leads to degradation of chlorophyll and bleaching occurring because of deficit carotenoids [31].

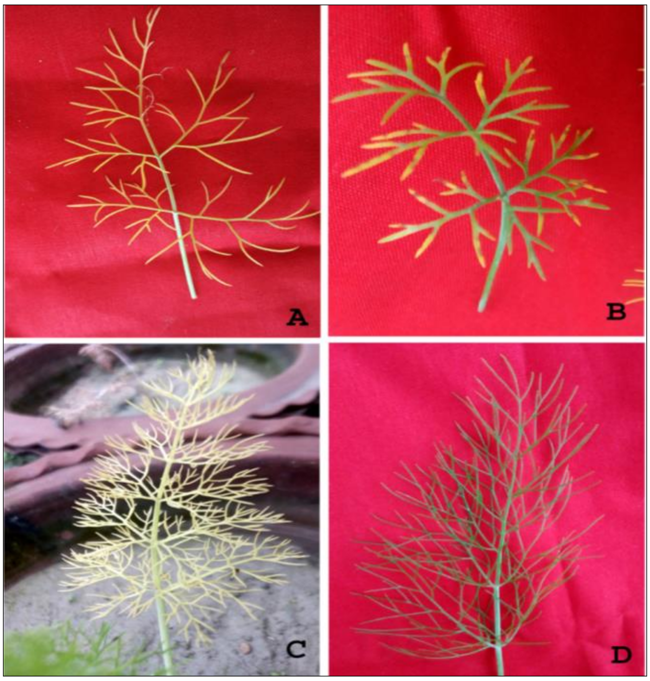

Fig 7: Chlorophyll variant in *Foeniculum vulgare* Mill A-Xentha variant, B- Semi-xantha mutant at seedling stage C-Albina variant D-Viridis variant

Table 1: Frequency of chlorophyll variant in different concentration and duration of chemical mutagen EMS

Duration	Concentration	Total no of plant observed	Total no of chlorophyll variant	Frequency of chlorophyll variants				
				Albino	Xantha	Viridis	Chlorina	Total frequency of chlorophyll variant
3Hours	0.1	60	22	5	6	6	5	36.67
	0.3	60	18	5	3	4	6	30.00
	0.5	60	10	5	2	2	1	16.67
5 Hours	0.1	60	21	3	7	5	6	35.00
	0.2	60	14	4	5	1	4	23.34
	0.3	60	8	2	3	1	2	13.34

Effect of EMS on photosynthetic pigment

Estimation of photosynthetic pigment shows variations among control and treated sets. A dose dependent decrease in photosynthetic pigments was registered in all the treated sets. Chlorophyll a and chlorophyll b are main components of photosynthesis which represent mostly half of the total quantity of green and yellow pigment in leaf. Carotenoids are also essential constituents of Chl-binding proteins in plants, that absorb the light energy for photosynthesis and also protect the plants from photo inhibition. The chlorophyll a and b were recorded to be 0.98±0.08^{b} mg/gFW and 0.68±0.09 ^{b} mg/gFW in control sets whereas the estimated carotenoid content was recorded 0.47±0.09 ^{b} mg/gFW (Table 2.). At lower concentration of EMS (0.1 %) and their treatment duration 3h shows very stimulatory and effective action where as higher concentration and maximum duration 5 h shows negative responses in respect to photosynthetic pigments. The maximum inhibition observed at the maximum dose of treatment with minimum Chlorophyll (Chl) contents. Similar result were reported by Kumar and Srivastava 2018 [32]. The decreasing in photo synthesis contents clearly indicate that the mutagenic impact of EMS on *Foeniculum vulgare* Mill. and maximum inhibition of these pigments were observed at the highest concentration (0.5%) which having minimum photosynthetic pigment.

Table 2: Effect of EMS treatment on photosynthetic pigments contents of *Foeniculum vulgare* Mill. After 3 and 5 h durations, respectively.

EMS treatment (Hours)	Conc.* (%)	Chl a** (mg/gm f.w.)	Chl b*** (mg/gm f.w.)	Carotenoid (mg/gm f.w.)
3h	Control	0.98±0.08^{b}	0.68±0.09^{b}	0.47±0.09^{b}
	0.1	1.06±0.03^{a}	0.56±0.08^{b}	0.63±0.06^{b}
	0.3	0.81±0.09^{c}	0.52±0.09^{b}	0.59±0.07^{b}
	0.5	0.51±0.09^{d}	0.49±0.09^{b}	0.44±0.06^{b}
5h	Control	0.93±0.05^{a}	0.61±0.08^{b}	0.36±0.06^{b}
	0.1	0.85±0.06^{b}	0.52±0.09^{b}	0.33±0.06^{b}
	0.3	0.75±0.09^{c}	0.41±0.06^{b}	0.56±0.02^{b}
	0.5	0.46±0.06^{d}	0.38±0.06^{b}	0.41±0.08^{b}

Effect of EMS on AMI%, TAB% and chromosomal structure

Cytological studies of treated sets provide information regarding the response of fennel genotypes to a chemical mutagen and also provide greater chances for the selection of desired traits. EMS shows a noteworthy impact on the cytology of root meristems of *Foeniculum vulgare* Mill. It causes a significant changes on the mitotic activity and chromosomal morphology. Table.3 represent complete data of AMI% and TAB%. The root tips are directly exposed to the mutagenic treatments, the effects of known concentrations can be studied. A highly significant decrease in AMI (%) as compared to the control was evident at all the doses. The reduction rate was higher in case of 5 h treatment duration. AMI declined from $12.75{\pm}0.10^{a}$ % (control) to $11.47{\pm}0.14^{b}$ (0.1%) and up to $9.63{\pm}08^{c}$ (0.5%) in 3 h treatment dose whereas in 5 h treatment set the reduction was up to $7.34{\pm}0.20^{d}$ (0.5%). However, in case of lower doses, EMS is not cases much more mito-inhibitory effect on this plant but the rate of AMI and TAB % decline is accordingly in a dose dependent manner. Whereas TAB (%) increases in dose dependent manner, thus AMI and TAB percentages show inverse relationship. Percentage of various chromosomal abnormalities induced by EMS in the abnormal cells of root meristem of fennel plant is documented in Table 3. Chromosomal abnormality such as stickiness, scattering, bridge, laggards, unorientation at metaphase and anaphase and precocious movement were also observed. (Figure.8) cytological analysis is best way to study the mitotic behavior of chromosome which is most dependable indices to calculate the potency of mutagens Ems treatment causes the mis pairing or mismatch pairing in DNA by alkylation of guanine base. Under this conditions, EMS causing a G/C to A/T transition in the backbone of the DNA. EMS treatments can cause allelic mutations, small deletions and other chromosomal rearrangements [33] such as-stickiness, scattering, precocious, chromosomal aberrations or anomalies are a good indicator of deviations in the normal mechanism of cell cycle. The most dominant chromosome abnormality observed was scattering which is due to the loss of microtubules of spindles fibers. Which retards the movement of spindle fibers, towards the opposite poles. The precocious movement occurs due to disturbed homology for chromosomal pairing, disruption of spindle fibres or inactivation of spindle mechanism. Predominant abnormalities such as clumping and stickiness is characterized by clustering of chromosomes at any phase of cell cycle [34]. Unorientation may occur due to disruption of spindle structure, leading to the spindle fibre imbalance on both the sides of centromere traction power or chromosome acentric fracture cannot cause normal movement of chromosome [35]. Stickiness was found in both metaphase and anaphase of mitosis. Chromosome stickiness leads to inactivation of DNA replication, increased chromosomal contraction and condensation or nucleoproteins probably leading to cell death [36].

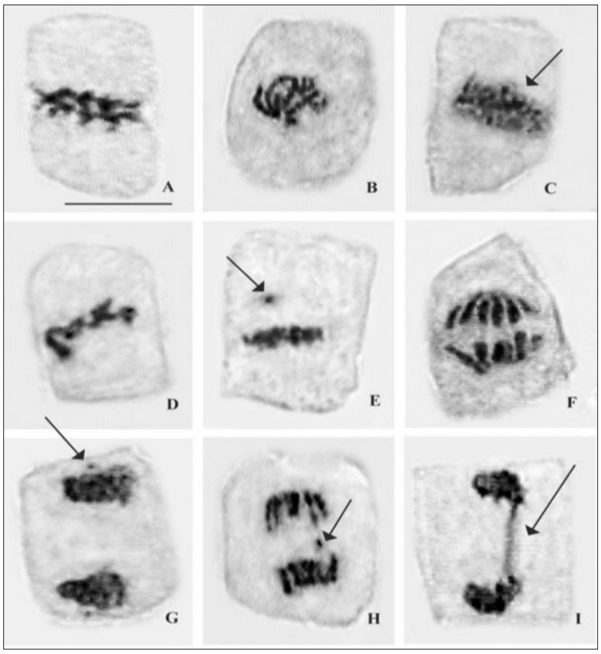

Fig 8: Different types of chromosomal aberrations induced by EMS on *Foeniculum vulgare* Mill.

A-Normal Metaphase(2n=22), B -scattaring at metaphase, C-unorientation at metaphase D-loop formation at metaphase, E- Precocious at metaphase, F-Normal Anaphase(22:22 separation), G- Forward movement of chromosome, H- Laggard at anaphase, I-Bridge formation at anaphase . Scale bar- Length -6.30 mm Width – 5.40 mm

Table 3: Effect of EMS on the chromosomal morphology and Total Abnormality Percentage (TAB %) in *Foeniculum vulgare* Mill.

Treatment	Cocentration	AMI (%) (Mean ± SE)	Metaphasic abnormalities % (Mean ± SE)				Anaphasic abnormalities % (Mean ± SE)					Oth % (Mean ± SE)	TAB (%) (Mean ± SE)
			Sc	St	Un	pr	Sc	St	Lg	Br	un		
Control		$12.75{\pm}0.10^{a}$											
3h	0.1	$11.47{\pm}0.14^{b}$	0.37±0.18	0.43±0.23	-	0.36±0.18	0.47±0.24	-	0	0.63±0.08	-	-	2.43±0.12
	0.3	$10.49{\pm}0.16^{b}$	0.60±0.03	0.38±0.19	-	0.24±0.19	0.58±0.35	0.42±0.21	0.42±0.21	-	0.39±0.20	-	3z.37±0.21
	0.5	$9.63{\pm}08^{c}$	0.80±0.41	0.76±0.46	-	0.67±0.08	0.60±0.30	-	0	0.83±0.08	0.40±0.20	-	4.20±0.11
5h	0.1	$8.40{\pm}0.11^{c}$	0.52±0.02	0.68±0.15	0.52±0.29	0.85±0.32	0.52±0.02	0.85±0.14	0.69±0.27	-	-	0.54±0.32	4.97±0.55
	0.3	$7.41{\pm}0.15^{d}$	0.80±0.41	0.73±0.36	-	0.43±0.23	0.63±0.33	-	0.33±0.17	0.90±00.47	0.53±0.32	0.43±0.40	5.30±0.25
	0.5	$7.34{\pm}0.20^{d}$	1.0±0.52	0.78±0.13	0.52±0.29	0.7±0.47	-	1.07±0.55	0.43±0.40	0.70±0.35	0.45±0.23	0.45±0.42	6.17±0.12

Abbreviations- Sc- Scattering; St- Stickiness; Un- Unorientation; Pr- Precocious movement; Lg- Laggard; Br-Bridge formation, Oth- Others; TAB. –Total abnormalities, Means followed by lowercase letters are statistically significant at $P \leq 0.05$

Conclusion

The present study showed that EMS mutagenesis was found to be potent and effective for inducing novel variability in some important agronomic traits in Fennel plant. In this investigation the frequency of chlorophyll variant, morphological parameters and cytological study found in fennel (AF-2) act as an indicator in analyzing the response of the chemical mutagen EMS. The morphological changes obtained were essential and useful in breeding technology to obtain suitable gene coding character for more yield in the

Fennel plant. It is very essential to select the concentration of particular mutagen according to plant species and minimize the adverse effects.

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Conflict of interest statement
The authors declare no conflict of interest.

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