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Establishing an Optimized EMS Mutagenesis Protocol for Tagetes erecta cv. Pusa Narangi Gainda: A Dose-Response Analysis in the M 1 Generation

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Abstract

The improvement of novel marigold (*Tagetes erecta* L.) cultivars with enhanced ornamental value is constrained by limited genetic variability. Induced mutagenesis using ethyl methanesulfonate (EMS) provide a strong tool to produce new attribute, but its employment in marigold remains hampered by the lack of standardized, protocols. For widely cultivated African marigold, consistent methods to balance mutation induction with standard plant viability are critically needed to enable efficient mutant screening. This study demonstrates an refined EMS mutagenesis protocol for the commercially important cultivar i.e. 'Pusa Narangi Gainda'. The effects of six EMS concentrations (10–60 mM) and two treatment durations (6 and 8 h) was studied on M_1 generation (germination, growth, flowering, lethality, and mutagenic parameters). Results showed a significant ($P \leq 0.05$), effects on all vegetative and floral traits. Germination and plant architecture (height, spread) decreased gradually with increasing the concentration of EMS. The higher doses strongly restrained vegetative growth, an intermediate dose of 20–30 mM EMS for 6 hours was identified as optimal, creating a favorable mutation window. This treatment increased number of flower per plant to 86.33 (at 30 mM) over 81.33 in the control, while maintaining > 80% germination and acceptable plant architecture. The 6 hour duration was generally more effective than 8 hours for key traits such as flower number. Probit analysis showed an extrapolated LD_{50} of 261.27 mM, while mutagenic effectiveness and efficiency were highest at lower concentrations (10–20 mM). We conclude that EMS mutagenesis in marigold is highly dose specific, with intermediate doses (20–30 mM) optimally balancing floral enhancement against vegetative reduction. The known protocol gave a valid, reproducible framework for creating mutant populations in this cultivar, establishing a critical foundation for forward genetic screening and applied mutation breeding in marigold.

Introduction

Marigold, a commercially important and popular flower, is widely grown worldwide. It is a member of the family Asteraceae and genus Tagetes. The genus Tagetes is grouped into 3 main types i.e. 'Wild Marigold' (*Tagetes minuta*), 'French Marigold; (*Tagetes patula*) and 'African Marigold' (*Tagetes erecta*), of which *Tagetes erecta* (African marigold) and *Tagetes patula* (French marigold) are under commercial cultivation in India [1]. Marigold (Tagetes *erecta*) is universally recognized as an ornamental plant for its bright and delightful appearance and is used as a loose flower, bedding plant, and pot plant. Apart from this, its commercial applications include cosmetics, food, and pharmaceuticals. South India is the major contributor (~ 75–80%) to the global output of marigold (~ over 6 lakh tons annually), with Andhra Pradesh, Uttar Pradesh, and Tamil Nadu as the leading states, followed by Maharashtra, Orissa, Karnataka, and Kerala. Among the loose-flower crops in India, marigold ranks first, followed by chrysanthemum, jasmine, tuberose, crossandra, and barleria.

In recent years, the ornamental plant industry has been growing at a very fast pace, and the demand for the development of new and novel cultivars is infinite. Mutation breeding can be used to increase genetic diversity and generate novel traits, such as unique shapes and beautiful flower colors, in a short span of

time [2]. In previous years, many efforts have been made to improve ornamental plants using conventional breeding methods, which have resulted in the generation of novel cultivars; however, very little work has been done on mutation breeding.

Induced mutagenesis has become wide-spread in the biological sciences as a tool for functional genomics. In many ornamental plants such as chrysanthemum, gerbera and gladiolus, induced mutagenesis remains successful with improvements in flower and leaf characters (color, shape, size) and physiological traits (flowering time and tolerance to stresses) [3]. Gene mutagenesis changes nucleotide sequences by four methods: (1) physical mutagens viz. UV, X-ray and gamma rays' radiation and fast neutron [3], (2) chemical agents viz. dimethyl sulphate (DMS), ethyl methane sulfonate (EMS), ethyl nitroso-urea (ENU), N-nitroso-N-methylurea (NMU) etc. [3, 4], (3) biological agents viz. T-DNA and transposons [5] and (4) transgenic technologies such as CRISPR-Cas9, TALENs, gene knockdown using RNAi [6, 7]. Out of these, first two methods i.e. physical (Gamma rays) and chemical mutagens (EMS) have been employed commonly for improving required flower characteristics in several ornamental crops. In developing countries, 64% of crop varieties are developed using gamma-ray irradiation [1]. With respect to the chemical mutagen EMS, reduced heights of sunflower plants were observed with an increase in its concentration [8] reported the production of a yellow marigold putative and a chimeric mutant with 0.6% and 0.9% EMS immersion, respectively. Recently, putative mutants have been reported in marigolds on a wide scale.

In comparison to physical mutagens, chemical mutagens proven to be more effective as high penetrating gamma radiation results in random DNA damage whereas EMS is causing high frequency mutations in genetic material by nucleotide substitution with base pair transition (G:C to A:T), thereby producing only point mutations without killing the plants [9] and some of these changes lead to a novel stop codon for different genes [10, 2, 3]. Further, EMS as a mutagen, depending on its concentration has been reported to affect the physiology, anatomy, biochemistry and morphology of plants differently [11]. It is also reported to be the most effective and powerful mutagen. Apart from this, EMS handling and disposal is relatively easy than other chemical mutagens [3]. While EMS mutagenesis is a recognized tool in ornamental breeding, standardized protocols for *Tagetes erecta* and an understanding of the physiological cost of mutagenesis at sub-lethal doses remain underexplored, creating a barrier to efficient mutant recovery [12]. In the present investigation, aim was to improve morphological characteristics of T. erecta L. cv. 'Pusa Narangi Gainda' using EMS as a tool to induce mutation; as morphological mutants play a crucial role in development of new varieties of plants [3]. Further, to determine the mutagen concentration is also very important; as high concentrations may increase phenotypic variant frequency but it could also end up with unwanted mutations (sterility or lethality); alternatively, lower concentrations may result in a lower phenotypic variant frequency. Several factors affect the effectiveness of EMS concentration, viz. method and timing of pre-soaking, pH, temperature etc. So, keeping all these factors in mind, the aim of this investigation was to identify the optimal EMS dose and duration for the commercial cultivar 'Pusa Narangi Gainda' by evaluating not only growth and flowering but also establishing the mutagenesis efficiency threshold, providing a reproducible framework for future mutant screening and breeding programs.

Materials and methods

2.1. Plant Material and Mutagen

The experimental material consisted of certified, commercially available seeds of African marigold (*Tagetes erecta* L.) cv. 'Pusa Narangi Gainda'. Seeds were procured from Benary Seed Company, Germany, to ensure genetic uniformity and high initial viability. The chemical mutagen used was Ethyl Methanesulfonate (EMS) (CAS No. 62-50-0, Sigma-Aldrich, ≥ 99% purity).

2.2. Experimental Design and Mutagenic Treatment

A factorial completely randomized design (CRD) was employed with two factors: EMS concentration and treatment duration. The experiment comprised six EMS concentrations (0 (Control), 10, 20, 30, 40, 50, and 60 mM) and two soaking durations (6 and 8 hours), resulting in 7 treatment combinations. Each treatment combination was replicated three times, with each replicate consisting of 100 seeds.

2.3. Pre-treatment and Treatment Protocol:

2.3.1 Pre-soaking

Seeds were first surface-sterilized with a 2% (v/v) sodium hypochlorite solution for 5 minutes, followed by three rinses with sterile distilled water. They were then pre-soaked in sterile distilled water for 12 hours at 25 ± 2°C to initiate imbibition and sensitize the embryonic cells.

2.3.2 Mutagene Treatment

Pre-soaked seeds were treated with the respective EMS solutions prepared in 0.1 M phosphate buffer (pH 7.0) to maintain stable pH during treatment. For each replicate, 100 seeds were immersed in 50 ml of EMS solution in a 250 ml beaker. The beakers were sealed and placed on a rotary shaker at 80 rpm to ensure uniform exposure. All procedures were conducted in a fume hood with appropriate personal protective equipment. EMS concentrations were selected based on preliminary trials and literature reports on Tagetes and related ornamental species to ensure sub-lethal mutagenic effects while maintaining adequate plant survival.

2.3.3 Post-treatment Washing

After the designated soaking period, the EMS solution was carefully decanted. Seeds were washed thoroughly six times with copious amounts of sterile distilled water (for a minimum of 30 minutes total) to ensure complete removal of mutagen residues, a critical step to halt alkylation.

2.3.4 Control Treatment

Control seeds underwent an identical process but were immersed in 0.1 M phosphate buffer (pH 7.0) without EMS.

2.4. Germination, Cultivation, and Agronomic Management

Treated and control seeds were sown immediately after washing.

2.4.1 Germination Phase: Seeds were sown in sterile potting mixture (Peat moss:Perlite:Vermiculite, 3:1:1) in 8 inch plastic pots. Pots were maintained in a greenhouse under controlled conditions: temperature of 25/18°C (day/night), ~ 70% relative humidity, and a 16/8 hour photoperiod provided by natural sunlight supplemented with LED grow lights (Photosynthetic Photon Flux Density of 300 µmol m^{-2} s^{-1}).

2.4.2 Seedling Transplanting: At the 4–5 true leaf stage, uniform seedlings were transplanted into a prepared experimental field with a spacing of 40 cm × 40 cm, following a randomized layout.

2.4.3 Crop Management: The field soil was a sandy loam (pH 6.8, EC 0.8 dS m^{-1}). Standard agronomic practices for marigold were followed uniformly: drip irrigation, application of NPK fertilizer at 120:60:60 kg ha^{-1}, integrated pest management, and manual weeding.

2.5. Data Collection

Data were recorded on the M_1 generation plants for the following vegetative and reproductive traits:

1. Germination Percentage: Recorded at 14 days after sowing (DAS).

2. Vegetative Growth Parameters (at 50% flowering):

Plant Height (cm): From soil surface to the apical meristem.

Plant Spread (cm): Average of the north-south and east-west canopy diameters.

Leaf Morphology: Length and width (cm) of the fifth fully expanded leaf from the apex.

Number of Primary Branches: Branches originating directly from the main stem.

3. Floral Characteristics (at full bloom):

Peduncle Length (cm): Length of the flower stalk for the first five primary flowers.

Number of Flowers per Plant: Total number of fully opened flowers per plant over the peak flowering period (4 weeks).

Flower Diameter (cm): Vertical diameter of five randomly selected flowers per plant using a digital caliper.

2.6. Estimation of LD_{50} of EMS by Probit Analysis

LD_{50} was estimated using probit regression; however, since mortality did not reach 50% within the tested concentrations, the LD_{50} value represents an extrapolated estimate beyond the experimental range.

2.6.1 Dose-Response Data Preparation

Germination percentage was recorded for each EMS concentration. Mortality percentage was calculated as:

$$Mortality\,(\%) = 100 - Germination\,(\%)$$ (I)

2.6.2 Log Transformation of EMS Dose

EMS concentrations were log-transformed to normalize the dose–response relationship:

$$X = Log_{10}\,(EMSconcentration)$$ (II)

2.6.3 Probit Transformation

Mortality percentages were converted to probit values using standard probit tables or statistical software.

Regression Model

A linear regression model was fitted:

$$Probit = Log_{10}\,(Dose)$$ (III)

where

a = intercept

b = slope

LD_{50} Estimation

The LD_{50} was calculated using:

$$LD50 = 10^{\frac{5-a}{b}}$$ (IV)

2.7. Estimation of mutagenic parameters

2.7.1 Lethality (Biological Damage)

Lethality was calculated based on reduction in seed germination relative to control:

$$L\,(\%) = \frac{G_c - G_t}{G_c}$$ (V)

Where:

G_c = Germination percentage in control

G_t = Germination percentage in treated seeds

2.7.2. Phenotypic variation frequency in M_1 generation

phenotypic variant frequencywas calculated as:

$$M\,(\%) = \frac{N_m}{N_t} \times 100$$ (VI)

Where:

N_m = Number of putative variant plants showing observable phenotypic deviations from the control (e.g., altered plant height, leaf morphology, chlorophyll mutants, flowering time, flower color, or growth abnormalities)

N_t = Total number of plants screened

Note

In the M_1 generation, observed variants were considered putative phenotypic variants because true heritable mutations can only be confirmed in the M2 generation.

2.7.3. Mutagenic Effectiveness

Mutagenic effectiveness was calculated as the percentage of mutations per unit dose of mutagen, according to the method of Konzak et al. (1984).

$$\text{Effectiveness} = \frac{M}{C \times T}$$ (VII)

Where:

M = phenotypic variant frequency (%)

C = Mutagen concentration (mM)

T = Treatment duration (h)

2.7.4. Mutagenic Efficiency

(a) Based on Lethality:

Efficiency$= \frac{M}{L}$

Where:

M = phenotypic variant frequency (%)

L = Lethality (%)

2.8. Statistical Analysis

All the data were subjected to statistical analysis. The significance of the main effects (EMS concentration, soaking duration) and their interaction was determined using two-way Analysis of Variance (ANOVA). The assumptions of ANOVA (normality, homogeneity of variance) were checked prior to analysis. Where the ANOVA indicated significant differences ($p < 0.05$), means were separated using Tukey's Honest Significant Difference (HSD) post-hoc test at a 5% probability level. All analyses were performed using R statistical software (v4.1.2) with the $agricolae$ and 'car' packages and IDLE (Python 3.13.64-bit). Data are presented as the mean ± standard error (SE) of three biological replicates.

Results

Test material comprising M_1 generation of marigold varied significantly ($P \leq 0.05$) for all the vegetative and flowering characters at six doses of EMS application (M_{10}-M_{60}). With respect to treatment duration and mutagen dose; seed germination, plant height, plant spread, peduncle length and number of flowers per plant were significantly varied (Table 1–4). Flower diameter showed significant variation in dosage time interaction.

Table 1
Mean values of morphological parameters in M1 plants of marigold cv. "Pusa Narangi Gainda" following exposure to EMS mutagen.

Traits	Seed germination (%)			Plant height (cm)		
Treatments	**6 hr**	**8 hr**	**Mean**	**6 hr**	**8 hr**	**Mean**
M0	88.67	86.50	84.20[a]	60.57	57.67	59.12 [a]
M10	84.33	80.25	81.40[a]	58.33	57.27	57.80 [a]
M20	81.25	79.85	79.60[a]	56.53	56.00	56.27 [ab]
M30	74.80	71.30	72.75[b]	55.20	54.13	54.67[bc]
M40	73.20	68.35	70.45[b]	52.53	51.43	51.98 [cd]
M50	71.50	67.45	69.60[bc]	51.00	49.83	50.42 [de]
M60	68.50	62.25	64.55[c]	49.57	47.43	48.50 [e]
Mean	77.46	73.71	74.65	54.82	53.40	54.11*[$]

Table 2
Mean values of morphological parameters in M_1 plants of marigold cv. 'Pusa Narangi Gainda' following exposure to EMS mutagen.

Traits	Plant spread (cm)			Leaf length (cm)			Leaf width (cm)		
Treatments	**6 hr**	**8 hr**	**Mean**	**6 hr**	**8 hr**	**Mean**	**6 hr**	**8 hr**	**Mean**
M0	49.53	47.97	48.75[a]	10.57	10.00	10.28 [a]	7.07	6.87	6.97 [a]
M10	45.90	45.30	45.60 [a]	9.97	9.53	9.75 [ab]	6.90	6.57	6.73 [a]
M20	42.70	41.67	42.18 [b]	9.17	8.93	9.05 [ab]	6.13	5.93	6.03 [b]
M30	40.00	37.53	38.77 [c]	8.47	8.20	8.33[bc]	5.67	5.40	5.53[bc]
M40	37.87	36.27	37.07 [cd]	7.37	7.27	7.32 [cd]	5.47	5.27	5.37 [c]
M50	36.23	34.33	35.28 [de]	7.00	6.33	6.67 [d]	5.13	4.93	5.03 [cd]
M60	33.67	31.70	32.68 [e]	6.53	6.13	6.33 [d]	4.63	4.73	4.68 [d]
Mean	40.84	39.25	40.05*[$]	8.44	8.06	8.25*	5.86	5.67	5.76*

Table 4
Mean values of morphological parameters in M1 plants of marigold cv. “Pusa Narangi Gainda” following exposure to EMS mutagen.

Traits	No of flowers per plant			Flower diameter (cm)		
Treatments	**6 hr**	**8 hr**	**Mean**	**6 hr**	**8 hr**	**Mean**
M0	80.33	82.33	81.33 ^{ab}	4.50	4.50	4.50 ^{a}
M10	73.00	88.00	80.50 ^{ab}	4.53	3.97	4.25^{abc}
M20	61.00	79.33	70.17 ^{b}	4.30	4.37	4.33 ^{ab}
M30	81.00	89.33	85.17 ^{a}	3.77	4.23	4.00^{abc}
M40	75.33	74.33	74.83 ^{ab}	3.97	3.57	3.77 ^{c}
M50	78.67	66.33	72.50 ^{ab}	4.00	3.63	3.82^{bc}
M60	82.67	90.00	86.33 ^{a}	3.83	4.20	4.02^{abc}
Mean	76.00	81.38	78.69*$^{\$}$	4.13	4.07	4.10*$^{!}$

3.1 Seed Germination (%)

A strong decrease in seed germination is the sign of induced mutagenesis. The seed germination percentage at 6 hrs treatment was found maximum (88.67%) under control conditions (M_0), with the increase of mutagen dose from M_{10}-M_{60}, the germination decreased from 84.33% to 68.50% which could be attributed to seed germination without wholly developed embryo (Table 1). In context of treatment duration, seed germination was decreased under 8 hours (80.25% to 62.25%). The interaction (dosage time) was non-significant. For induced mutagenesis, mutagen should be absorbed by the germinating embryo so that it can reach the meristematic region; only then it can affect percent germination (Serrat et al., 2014).

3.2 Plant Height

Plant height was reciprocally related to mutagen dose i.e. with the increase in dose, it decreases from 57.80 cm to 48.50 cm (Table 1). Again, it was maximum under control (59.12 cm). Further, the plants have attained lesser height on long term treatment in mutagen. Both treatment duration and EMS dosage affected plant height significantly in marigold whereas, the interaction effect was non-significant. An optimal plant height is a preferred agronomic trait that contributes to a high yield (Dutta, 2023), but mutagen treatment affects this even at low concentrations.

3.3 Plant Spread

Plant spread varying from 45.60 cm to 32.68 cm with the application of different doses of EMS. Over control, the non significant effect was observed in plant spread at M_{10} dose. A linear decrease was observed in marigold with increase in mutagen dosage. There effect was non significant from M_0 to M_{10}, then at M_{20}; it was a significant increase and thereafter it decreased non-significantly upto M_{50} and further a significant decrease was observed at M_{60}. In marigold, both the treatments (soaking time and EMS dosage) affected the plant spread significantly.

3.4 Leaf Length and Width

Mutagen dose effect was different for leaf length and width in marigold. Soaking time has shown non significant effect on length and width of leaf. In marigold, a linear and significant decrease was observed with increase in EMS dosage for both leaf length and width.

3.5 Penduncle Lenght

In marigold, both mutagen dose as well as soaking time have a significant effect in peduncle length. With both the treatments (from M_{10} to M_{60}: 8.67 cm to 6.90 cm and from 6 to 8 hour soaking 8.21 cm to 7.93 cm), a decrease in peduncle length was observed.

3.6 Number of Primary Branches

In marigold, number of primary branches per plant decreased significantly from 8.90 to 7.07 with increase in EMS dosage from M_{10} to M_{60}. Maximum primary branches were reported under control. Soaking time (from 6 to 8 hours) has also resulted in decreased number of primary branches.

3.7 Number of Flowers per plant

The change in treatment duration's from 6 to 8 hours resulted into significant increase in the number of flowers per plant from 76 to 81.38. Whereas, mutant doses have also resulted a significant increase in number of flowers per plant (86.33) over the control (81.33) but the increase in no of flowers was not linear. The interaction of treatments has no effect on number of flowers per plant.

3.8 Flower Diameter

Diameter of the marigold flowers decreased significantly with change in the concentration of mutagen dose, however, it was not influenced by treatment duration (Fig. 1, Table 4).

3.9 Determination of LD_{50} of EMS

Since mortality did not reach 50% within the tested range, the LD_{50} value of 261.27 mM is an 'extrapolated estimate' based on the linear probit model. This indicates a high tolerance threshold for this cultivar, but the value should be interpreted carefully as it lies outside the experimental dose range (Fig. 2). This LD_{50} value can be used as an optimal mutagen dose for mutation breeding experiments to balance mutation induction and plant survival.

3.10 Mutagenic Effectiveness of EMS

Mutagenic effectiveness showed a decreasing trend with increasing EMS concentration (Fig. 3). The highest effect was observed under M_{10} (3.00), followed by M_{20} (2.50), whereas the minimum effect was observed under M_{60} (0.98). This shows that lower EMS doses were more effective in inducing mutations per unit dose, while higher concentrations resulted into excessive damage without proportionally increasing phenotypic variant frequency. Therefore, lower EMS doses were comparatively more potent in inducing mutations.

3.11 Effect of EMS Dose on Seed Germination (Lethality)

Seed germination decreased gradually with increase in EMS concentration, showing a dose dependent inhibitory effect of the mutagen (Fig. 4). The control (M_0) recorded the maximum germination (84.20%), while the lowest germination (64.55%) was observed under highest EMS concentration (M_{60}). The sudden decline in germination indicate increasing physiological and cellular damage induced by EMS, demonstrating its lethality and phytotoxicity at higher doses.

3.12 Mutagenic Efficiency of EMS

Mutagenic efficiency, calculated as the ratio of phenotypic variant frequency to lethality, was observed maximum under M_{20} (0.64) and M_{10} (0.63), and gradually declined at higher EMS concentrations, reaching the lowest value at M_{60} (0.18) as shown in Fig. 5. This suggests that moderate EMS doses induced mutations with comparatively less biological damage, whereas higher concentrations resulted in greater lethality with reduced efficiency. Thus, M_{10}-M_{20} EMS concentrations were identified as the most prompt treatments for mutation induction.

Discussion

- The application of Ethyl methanesulfonate (EMS) as a chemical mutagen in marigold (*Tagetes* spp.) elicited significant phenotypic changes in the M_1 generation across all studied vegetative and floral traits. This exhibits the potent effect of alkylating agents in creating genetic and physiological perturbations, even in a chimeric generation where mutations are not yet fixed. The dose dependent responses discovered, ranging from stimulatory effects at lower concentrations to severe inhibition at higher doses provide critical insights into the trade-offs between mutation induction and plant fitness, essential for optimizing mutation breeding protocols.

3.1. Germination Inhibition and Lethality: A Mark of Mutagenic Stress

- The strong, dose dependent decrease in seed germination (from 88.67% in M_0 to 68.50% at M_{60}) is a classic indicator of successful mutagen uptake and induced physiological stress [13] as shown in Table 1. The results are in line with findings across species, where EMS check germination by interrupting key processes such as enzyme activation, hormone balance (i.e. GA/ABA ratios), and

meristematic cell division [14, 15]. The extrapolated LD_{50} of 261.27 mM, lying beyond our experimental range, indicates a relatively high tolerance threshold for marigold cv. 'Pusa Narangi Gainda'. However, the forward decline in germination shows that even sub lethal doses subject significant metabolic cost. Recent work on *Tagetes patula* suggests this cost is partly mediated by EMS induced oxidative stress, marked by elevated malondialdehyde (MDA) and altered antioxidant enzyme (SOD, POD) activities, leading to cellular damage and compromised vigor (Meng et al. 2024). The previous studies reported the supporting results on the decrease in seed germination with a similar increase in EMS dose by Unan et al. [16], Shamshad et al. [17] and Tonthong et al. [18] in rice.

3.2. Vegetative Growth Retardation

- The direct relationship between EMS dose and key vegetative parameters, plant height, plant spread, leaf dimensions and primary branch number, points to a systemic growth inhibition (Table 1–3). This is conformable with results in chrysanthemum [19] and other ornamentals exposed with chemical and physical mutagens [20, 3]. The underlying mechanisms are multifaceted due to meristematic arrest as EMS induced DNA alkylation and chromosomal damage directly damage cell division in lateral and apical meristems [21]. the physio-biochemical shifts resulted, mutagenesis which can change auxin metabolism, reduce amylase activity, increase peroxidase activity, and diminish photosynthetic efficiency, collectively restricting assimilate production and elongation growth [2]. the resource re-allocation decreased vegetative growth co-occur with an increase in number of flower at intermediate doses (M_{20}-M_{30}), confirms a stress induced shift in resource partitioning. Plants may prioritize reproductive output under mild mutagenic stress, a potential hormetic response or a compensatory survival strategy. The similar results on the effect of EMS on reduced vegetative growth has been reported by Lee et al. [22] in rapeseed, and Wu et al. [23]in watermelon.
- This phenomenon correspond with the resource allocation trade off theory, which poses that organisms have finite metabolic resources which must be divided among growth and reproduction [24, 25]. Under mutagenic emphasis, plants may deviate assimilates from vegetative biomass accumulation toward reproductive growth as a balancing strategy, resulting into reduced plant height and leaf area but increased flower production. Such trade offs have been reported in stress physiology and mutation breeding studies, where reproductive traits are favored under moderate stress to increase fitness [26].

Table 3
Mean values of morphological parameters in M1 plants of marigold cv. “Pusa Narangi Gainda” following exposure to EMS mutagen.

Traits	**Peduncle length (cm)**			**No. of primary branches per plant**		
Treatments	6 hr	8 hr	Mean	6 hr	8 hr	Mean
M0	10.03	10.03	10.03 ^{a}	11.00	10.80	10.90^{a}
M10	8.80	8.53	8.67 ^{b}	9.07	8.73	8.90 ^{b}
M20	8.50	8.17	8.33 ^{b}	8.93	8.60	8.77^{bc}
M30	8.00	7.80	7.90^{bc}	8.33	7.93	8.13^{bcd}
M40	7.73	7.30	7.52 ^{cd}	8.00	7.67	7.83^{cde}
M50	7.33	6.93	7.13 ^{cd}	7.60	7.33	7.47 ^{de}
M60	7.07	6.73	6.90 ^{d}	7.20	6.93	7.07 ^{e}
Mean	8.21	7.93	8.07*$	8.59	8.29	8.44*

3.3. Floral Trait Modulation: Identifying the Optimal ‘Mutation Window’

- The response of floral traits to EMS was more nuanced, highlighting the potential for selective breeding. Peduncle Length and Flower Diameter decreased linearly with dose, likely a direct morphological consequence of reduced cell expansion and generalized growth inhibition, as seen with other growth regulators [27](.
- Flower Number per Plant showed a significant and non-linear increase, particularly at intermediate doses (M_{20}-M_{30}) and longer soaking times (Fig. 1, Table 4). This critical finding suggests a disruption in the genetic or hormonal pathways governing the vegetative-to-reproductive transition. Similar non-linear, stimulatory effects on flowering have been reported in dahlia and calendula [28, 29]. This could result from hormonal Trade-offs as EMS may alter the auxin-cytokinin-gibberellin network, repressing apical dominance (reducing branches/height) while promoting floral initiation. Stress-Induced Flowering may result into mild mutagenic stress might trigger early or prolific flowering as a drought or survival response. This defines a valuable ‘mutation window’ (M_{10}-M_{30}) where genetic diversity is induced alongside a potentially desirable commercial trait, increased flower yield without catastrophic physiological damage.
- This dual phase effect design is accordant with the hormesis theory, where low to moderate doses of a stressor affect biological action while higher doses are inhibitory or toxic [30]. In these circumstance of mutagenesis, sub-lethal EMS doses can trigger DNA repair pathways, antioxidant defense and stress responsive signaling networks which transiently heighten growth or reproductive

traits [31]. Hormetic effect have been widely observed in plants exposed to chemical and physical mutagens, where mild genotoxic stress results in redeeming growth stimulation and increase reproductive characters before harmful effects predominate at higher doses [32, 33].

- Alternatively, the accrued flower production at average EMS doses may show a stress induced flowering effect, a well referenced adaptive action where plants intensify reproductive modification under sub-lethal stress conditions to ensure progeny survival [34, 35]. Chemical mutagen induced oxidative stress and hormonal unhinge can activate early or prolific flowering by modulating flowering integrator genes and stress amenable transcription factors, thereby shifting developmental priorities from vegetative growth to reproduction [36, 37].

Mutagenic Effectiveness and Efficiency

- The calculated mutagenic parameters offer a quantitative framework for protocol optimization. The highest effectiveness (mutations per unit dose) at M_{10} (3.00) confirms that lower concentrations are more efficient at inducing genetic changes. Conversely, the highest efficiency (phenotypic variant frequency relative to lethality) at M_{20} (0.64) identifies this dose as the optimal balance between creating usable genetic variation and maintaining plant viability. The steep decline in both parameters at M_{60} underscores the counterproductive nature of high doses, where excessive cellular damage and lethality overshadow mutation induction. This aligns with the consensus that moderate EMS doses (20–60 mM) are generally optimal for balancing phenotypic variant frequency and survival in various crops [38,39,3].

3.4. Future Perspectives

- This study establishes that EMS mutagenesis in marigold effectively generates significant phenotypic variation in the M_1 generation, with clear dose-response relationships. The inhibition of vegetative growth and the stimulation of flower production at intermediate doses reveal a stress-induced resource re-allocation that could be exploited in breeding for compact, high-yielding cultivars.

Key recommendations for future work include:

- To transition EMS mutagenesis from a blunt tool for generating random variation to a precise strategy for functional genomics and trait development in marigold, a multi-pronged approach in subsequent research is essential. First, rigorous screening of the M_2 generation is imperative to segregate and identify fixed, heritable mutations, moving beyond the chimeric M_1 phenotypes to determine true mutation frequencies. Concurrently, mechanistic studies profiling oxidative stress markers (e.g., ROS, MDA, antioxidant enzymes) and phytohormonal dynamics (auxins, cytokinins, gibberellins) in treated plants are needed to elucidate the physiological basis of the observed trade-

offs between vegetative inhibition and reproductive enhancement. Finally, molecular validation through techniques like TILLING (Targeting Induced Local Lesions in Genomes) on established M_2 populations will enable the precise linkage of observed phenotypic variants to specific genetic alterations, thereby advancing the study from phenomenological description to causal genetic understanding and facilitating the development of novel marigold phenotypes with commercial appeal.

Conclusion

Our findings demonstrate that EMS mutagenesis elicits strong, dose-dependent effects on marigold, revealing a clear physiological trade-off between mutation induction and plant viability. While higher doses (40–60 mM) progressively inhibited vegetative growth and reduced germination, intermediate doses (20–30 mM) generated a practical mutation window suitable for population development. Specifically, a treatment of 20–30 mM EMS for 6 hours is recommended as the optimal starting point for creating a mutant population in the cultivar ‘Pusa Narangi Gainda’. This treatment maintained germination above 80% and acceptable plant architecture while inducing significant phenotypic variation, including an increase in flower number, in the M_1 generation. This dose range aligns with the highest mutagenic efficiency (M_{10}-M_{20}), where the induction of genetic variation is balanced against minimal biological damage, despite the highest mutagenic effectiveness occurring at the lowest concentration. The extrapolated LD_{50} of 261.27 mM confirms that the recommended dose is well below the lethal threshold, ensuring a sufficiently large M_1 population for downstream screening.

This study provides a validated, genotype-specific protocol that emphasizes the importance of characterizing phenotypic responses and mutagenic parameters (lethality, effectiveness, efficiency) when establishing mutant populations. The identified dose (20–30 mM EMS, 6 h) offers a reproducible framework for generating a genetically diverse M_2 population in this commercially important marigold cultivar, forming a critical foundation for forward genetic screens and trait discovery in marigold improvement programs. The recorded hormetic stimulus of flowering and the vegetative reproductive trade-off under average EMS doses emphasize the importance of exploiting stress induced developmental plasticity in mutation breeding programs.

Declarations

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare no conflicts of interest.

Note

*Values are significantly different at P ≤ 0.05 w.r.t. mutagen dosage; $ Values are significantly different at P ≤ 0.05 w.r.t. soaking time; ! Values are significantly different at P ≤ 0.05 w.r.t. interaction (dosage*time).

Note

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Author Contribution

AKS and PK: Conceptualization. AHS, RK and NK: data curation. AS, AHS, SG, and NK: formal analysis. AKS and PK: Funding acquisition. AKS, SG and PK: investigation. AKS, PK, NK, RK and AHS: Methodology. AKS, and PK: Project Administration. NK, AHS, AKS, RK and SG: Software. AKS, and PK: Resources. AKS, and PK: Supervision. AKS, SG, RK and PK: Validation. AKS, SG, RK, NK, AHS and PK: Visualization. AHS, NK, Hk, AK and RK: Writing-original draft preparation. NK, AHS, HK, AK, RK and AKS: writing-review and editing.

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Data Availability

The datasets used and analyzed in the current study are available from the corresponding author on reasonable request.

References

1. Sarhan AZ, Nasr AA, Elsheinhab NAA, ElSawy MA. Studies on the effect of radiation mutagens on the growth and flowering of *Tagetes erecta* plants. Middle East J Agric. 2019;8(3):954–8.
2. Pathania S, Sahare H. Effect of chemical mutagens (Ethyl methane sulphonate and colchicine) on African marigold (*Tagetes erecta*). J Appl Nat Sci. 2025;17:697–701. https://doi.org/10.31018/jans.v17i2.6167.
3. Datta SK. Induced mutations: technological advancement for development of new ornamental varieties. Nucleus. 2020;63:119–29. https://doi.org/10.1007/s13237-020-00310-7.
4. Jyothi R, Singh KP, Kumar NK, Chand P. Induced mutation breeding in tuberose (*Polianthes tuberosa*) – A review. Curr Hortic. 2024;12:12–21. https://doi.org/10.5958/2455-7560.2024.00017.1.
5. Tao X-Y, Feng S-L, Li X-J, Li Y-J, Wang W, Gilliham M, et al. TTLOC: A Tn5 transposase-based approach to localize T-DNA integration sites. Plant Physiol. 2025;197. https://doi.org/10.1093/plphys/kiaf102.
6. Ye R, Yang X, Rao Y. Genetic Engineering Technologies for Improving Crop Yield and Quality. Agronomy. 2022;12:759. https://doi.org/10.3390/agronomy12040759.
7. Cong Gan W, Ling PK. CRISPR/Cas9 in plant biotechnology: applications and challenges. BioTechnologia. 2022;103:81–93. https://doi.org/10.5114/bta.2022.113919.
8. Habib SH, Akanda MAL, Roy P, Kausar H. Effect of different dosage of EMS on germination, survivability and morpho-physiological characteristics of sunflower seedling. Helia. 2021;44:167–80. https://doi.org/10.1515/helia-2021-0008.
9. Glickman BW, Ripley LS. Structural intermediates of deletion mutagenesis: a role for palindromic DNA. Proceedings of the National Academy of Sciences. 1984;81:512–6. https://doi.org/10.1073/pnas.81.2.512
10. Singh H, Khar A, Verma P. Induced mutagenesis for genetic improvement of Allium genetic resources: a comprehensive review. Genet Resour Crop Evol. 2021;68:2669–90. https://doi.org/10.1007/s10722-021-01210-8.

11. Ramadan K, Nader S, Mokrani L, Al Aswd G, Abou-Isba S, Dakah A. The effect of ethyl methanesulfonate (EMS) and environmental factors on soybean traits. BMC Plant Biol. 2025;26. https://doi.org/10.1186/s12870-025-07979-5.
12. Meng C, Ullah I, Wu W, Zhang Y, Shi R, Luo S, et al. EMS-Mediated Mutagenesis in Marigold Seeds and Its Effects on Seedling Growth and Physiology. Phyton. 2024;93:3029–38. https://doi.org/10.32604/phyton.2024.057857.
13. Serrat X, Esteban R, Guibourt N, Moysset L, Nogués S, Lalanne E. EMS mutagenesis in mature seed-derived rice calli as a new method for rapidly obtaining TILLING mutant populations. Plant Methods. 2014;10:5. https://doi.org/10.1186/1746-4811-10-5.
14. Dhange PR, Gowda APM, Kandpal K, Smitha GR. Exploring of Lethal Dose (LD50) Using Gamma Rays and Its Impact on Seed Germination and Seedling Survival in *Stevia rebaudiana* Bertoni cv. CIM-Madhu Int J Plant Soil Sci. 2023;35:407–12. https://doi.org/10.9734/ijpss/2023/v35i234256.
15. Borovsky Y, Tadmor Y, Bar E, Meir A, Lewinsohn E, Paran I. Induced mutation in β-CAROTENE HYDROXYLASE results in accumulation of β-carotene and conversion of red to orange color in pepper fruit. Theor Appl Genet. 2012;126:557–65. https://doi.org/10.1007/s00122-012-2001-9.
16. Unan R, Deligoz I, Al-Khatib K, Mennan H. Protocol for ethyl methanesulphonate (EMS) mutagenesis application in rice. Open Res Europe. 2022;1:19. https://doi.org/10.12688/openreseurope.13317.3.
17. Shamshad A, Rashid M, Jankuloski L, Ashraf K, Sultan K, Alamri S, et al. Effect of ethyl methanesulfonate mediated mutation for enhancing morpho-physio-biochemical and yield contributing traits of fragrant rice. PeerJ. 2023;11:e15821. https://doi.org/10.7717/peerj.15821.
18. Tonthong Y, Chanprasert W, Romkaew J, Kaewsorn P. Germinability and storability of pre-germinated rice (Oryza sativa L.) seeds. Seed Sci Technol. 2018;46:119–29. https://doi.org/10.15258/sst.2018.46.1.12.
19. Dalda-Sekerci A. Comprehensive assessment of genetic diversity in chrysanthemum germplasm using morphological, biochemical and retrotransposon-based molecular markers. Genet Resour Crop Evol. 2023;70:2321–36. https://doi.org/10.1007/s10722-023-01634-4.
20. Shamshad A, Rashid M, Jankuloski L, Ashraf K, Sultan K, Alamri S, et al. Effect of ethyl methanesulfonate mediated mutation for enhancing morpho-physio-biochemical and yield contributing traits of fragrant rice. PeerJ. 2023;11:e15821. https://doi.org/10.7717/peerj.15821.
21. Li D, Wang Y, Li Y, Wang W, Wang C, Wang X, et al. Maize ZmDLR2/BRU1 Is Required for Lateral Root Primordium Emergence by Participating in DNA Repair. Plant Cell Environ. 2025;48:7377–91. https://doi.org/10.1111/pce.70037.
22. Lee Y-H, Park W, Kim K-S, Jang Y-S, Lee J-E, Cha Y-L, et al. EMS-induced mutation of an endoplasmic reticulum oleate desaturase gene (FAD2-2) results in elevated oleic acid content in rapeseed (Brassica napus L). Euphytica. 2018;214. https://doi.org/10.1007/s10681-017-2106-y.
23. Wu Z, Fu X, Zhan Q, Lin Z, Zhu W. An Efficient Strategy for Developing Fusarium Wilt-Resistant Watermelon Germplasm Using EMS Mutagenesis and Toxin Screening. Plant Dis. 2025. https://doi.org/10.1094/pdis-12-24-2713-re.

24. Bloom AJ, Chapin FS III, Mooney HA. Resource Limitation in Plants-An Economic Analogy. Annu Rev Ecol Syst. 1985;16:363–92. https://doi.org/10.1146/annurev.es.16.110185.002051.
25. Bazzaz FA, Chiariello NR, Coley PD, Pitelka LF. Allocating Resources to Reproduction and Defense. Bioscience. 1987;37:58–67. https://doi.org/10.2307/1310178.
26. van der Weele CM, Spollen WG, Sharp RE, Baskin TI. Growth of Arabidopsis thaliana seedlings under water deficit studied by control of water potential in nutrient-agar media. J Exp Bot. 2000;51:1555–62. https://doi.org/10.1093/jexbot/51.350.1555.
27. Janowska B, Stanecki M. Effect of benzyladenine on the abundance and quality of flower yield in the calla lily (*Zantedeschia spreng*). Acta Agrobotanica. 2012;65:109–16. https://doi.org/10.5586/aa.2012.027.
28. Samadi N, Naghavi MR, Moratalla-López N, Alonso GL, Shokrpour M. Morphological, molecular and phytochemical variations induced by colchicine and EMS chemical mutagens in Crocus sativus L. Food Chemistry: Mol Sci. 2022;4:100086. https://doi.org/10.1016/j.fochms.2022.100086.
29. Samatadze TE, Yurkevich OYu, Khazieva FM, Basalaeva IV, Konyaeva EA, Burova AE, et al. Agro-Morphological and Cytogenetic Characterization of Colchicine-Induced Tetraploid Plants of *Polemonium caeruleum* L. (Polemoniaceae). Plants. 2022;11:2585. https://doi.org/10.3390/plants11192585.
30. Calabrese EJ, Baldwin LA, Hormesis. The Dose-Response Revolution. Annu Rev Pharmacol Toxicol. 2003;43:175–97. https://doi.org/10.1146/annurev.pharmtox.43.100901.140223.
31. Kovalchuk I, Molinier J, Yao Y, Arkhipov A, Kovalchuk O. Transcriptome analysis reveals fundamental differences in plant response to acute and chronic exposure to ionizing radiation. Mutat Res - Fundamental Mol Mech Mutagen. 2007;624:101–13. https://doi.org/10.1016/j.mrfmmm.2007.04.009.
32. Velini ED, Alves E, Godoy MC, Meschede DK, Souza RT, Duke SO. Glyphosate applied at low doses can stimulate plant growth. Pest Manag Sci. 2008;64:489–96. https://doi.org/10.1002/ps.1562.
33. Møller AP, Mousseau TA. Strong effects of ionizing radiation from Chernobyl on mutation rates. Sci Rep. 2015;5. https://doi.org/10.1038/srep08363.
34. Takeno K. Stress-Induced Flowering. In: Abiotic Stress Responses in Plants. New, York. NY: Springer New York; 2011. pp. 331–45. Available at: https://doi.org/10.1007/978-1-4614-0634-1_17 (Accessed February 11, 2026).
35. Kazan K, Lyons R. The link between flowering time and stress tolerance. J Exp Bot. 2015;67:47–60. https://doi.org/10.1093/jxb/erv441.
36. Cho L, Yoon J, An G. The control of flowering time by environmental factors. Plant J. 2017;90:708–19. https://doi.org/10.1111/tpj.13461.
37. Porch TG, Blair MW, Lariguet P, Galeano C, Pankhurst CE, Broughton WJ. Generation of a Mutant Population for TILLING Common Bean Genotype BAT 93. J Am Soc Hortic Sci. 2009;134:348–55. https://doi.org/10.21273/jashs.134.3.348.

38. Rawat AS, Bhuj BD, Srivastava R, Chand S, Singh NK, Bisht YS, et al. Determination of lethal and mutation induction doses of gamma rays for gladiolus (*Gladiolus grandifloras* Hort.) genotypes. Heliyon. 2024;10:e37387. https://doi.org/10.1016/j.heliyon.2024.e37387.

Figures

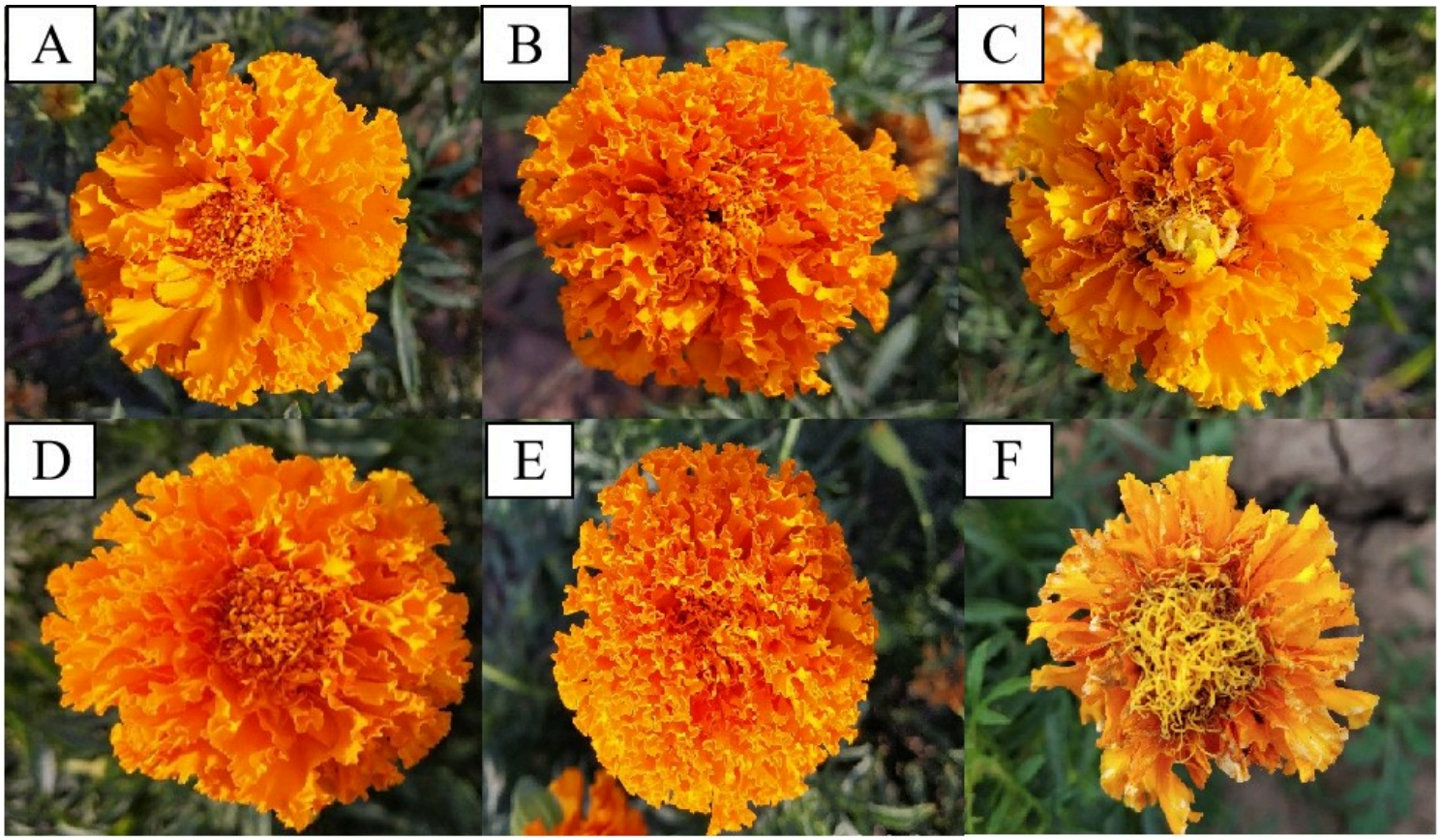


Fig. 1 Effect of different concentration of mutagen on flower morphology

Figure 1

See image above for figure legend

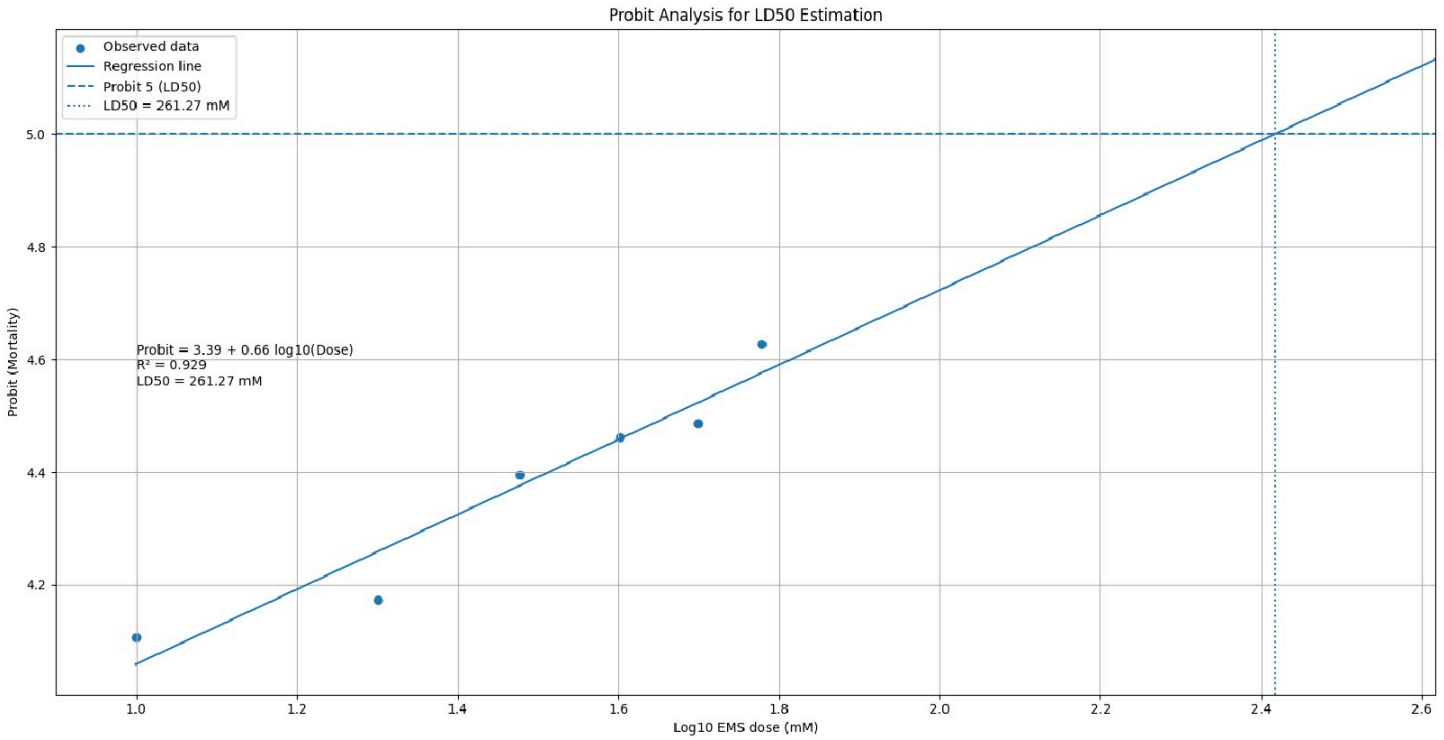


Fig. 2 Probit Analysis for LD50 Estimation

Figure 2

See image above for figure legend

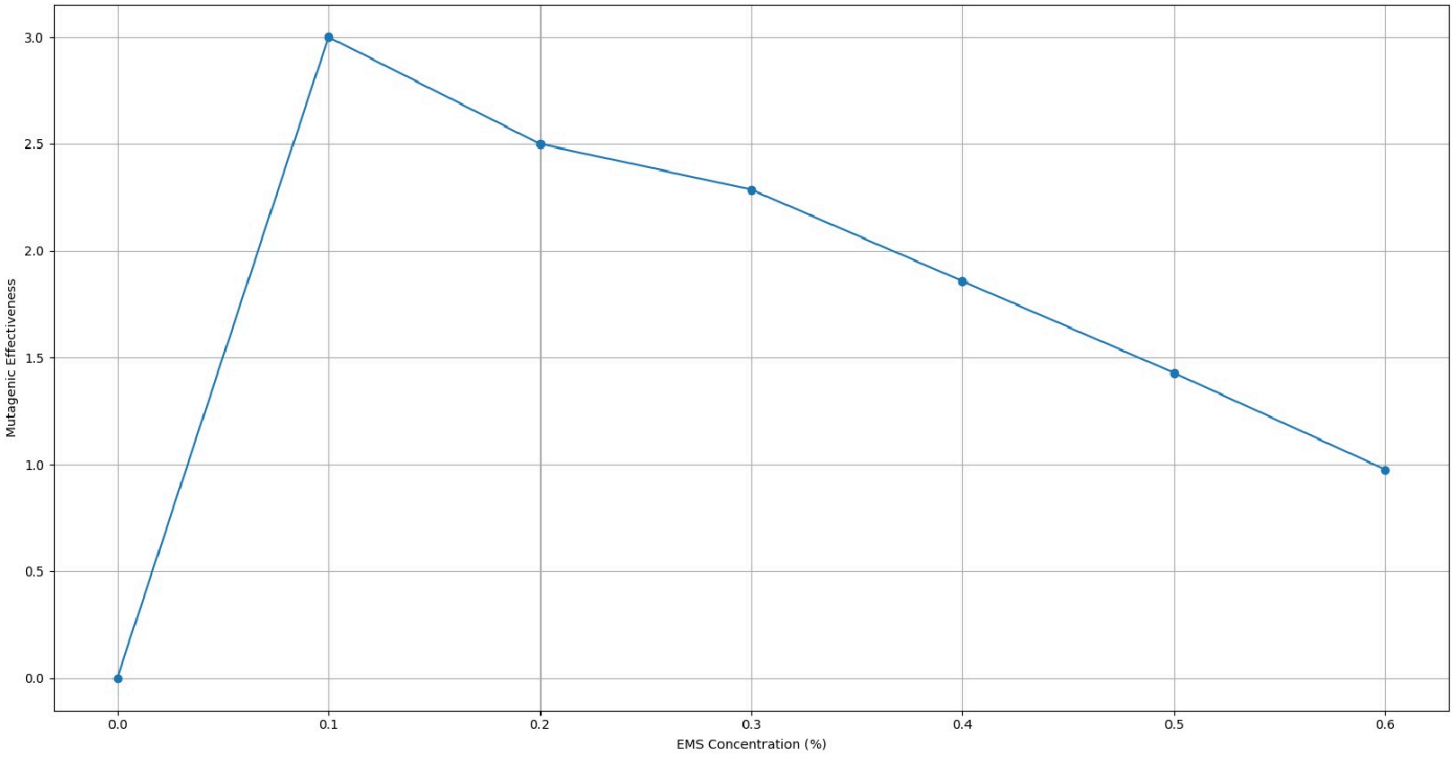


Fig. 3 Mutagenic Effectiveness of EMS

Figure 3

See image above for figure legend

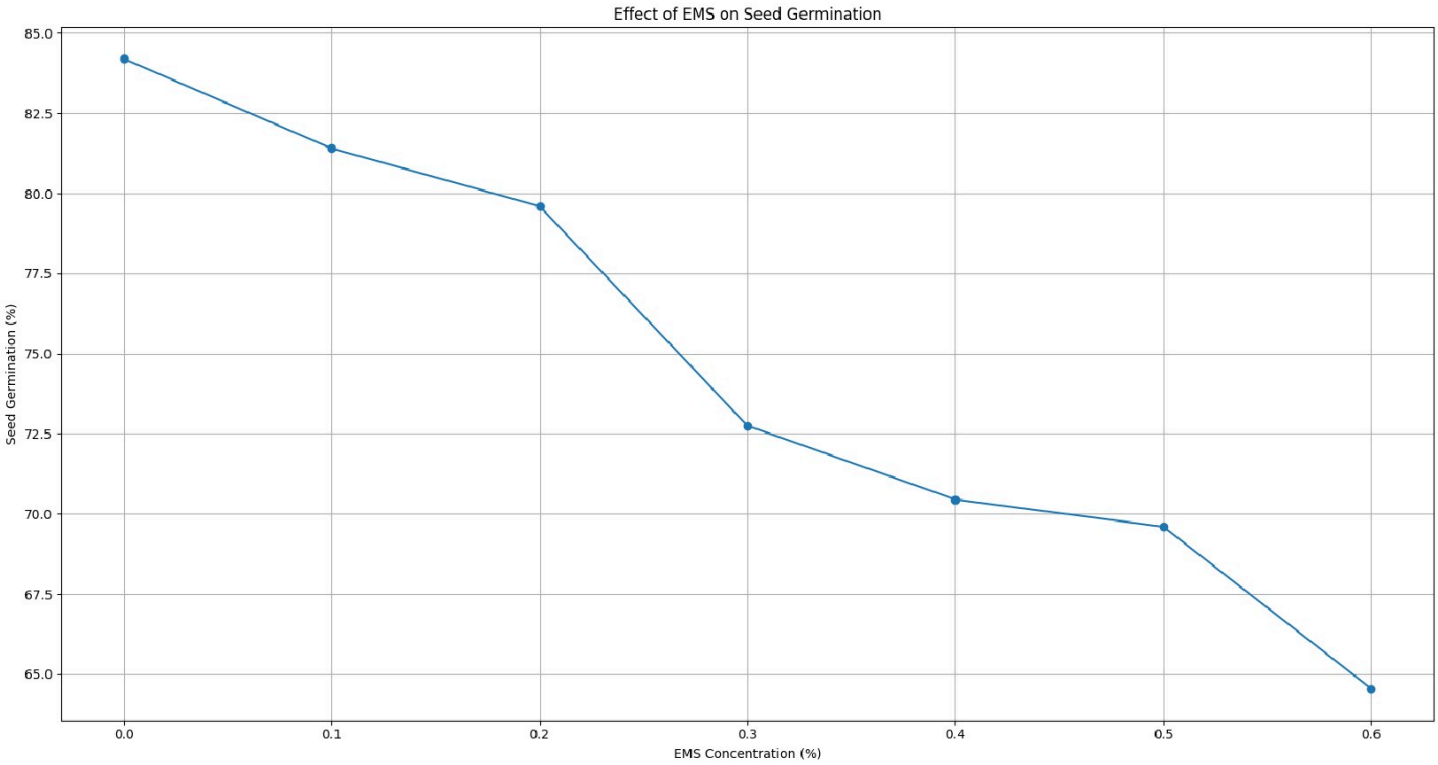


Fig. 4 Effect of EMS on Seed Germination

Figure 4

See image above for figure legend

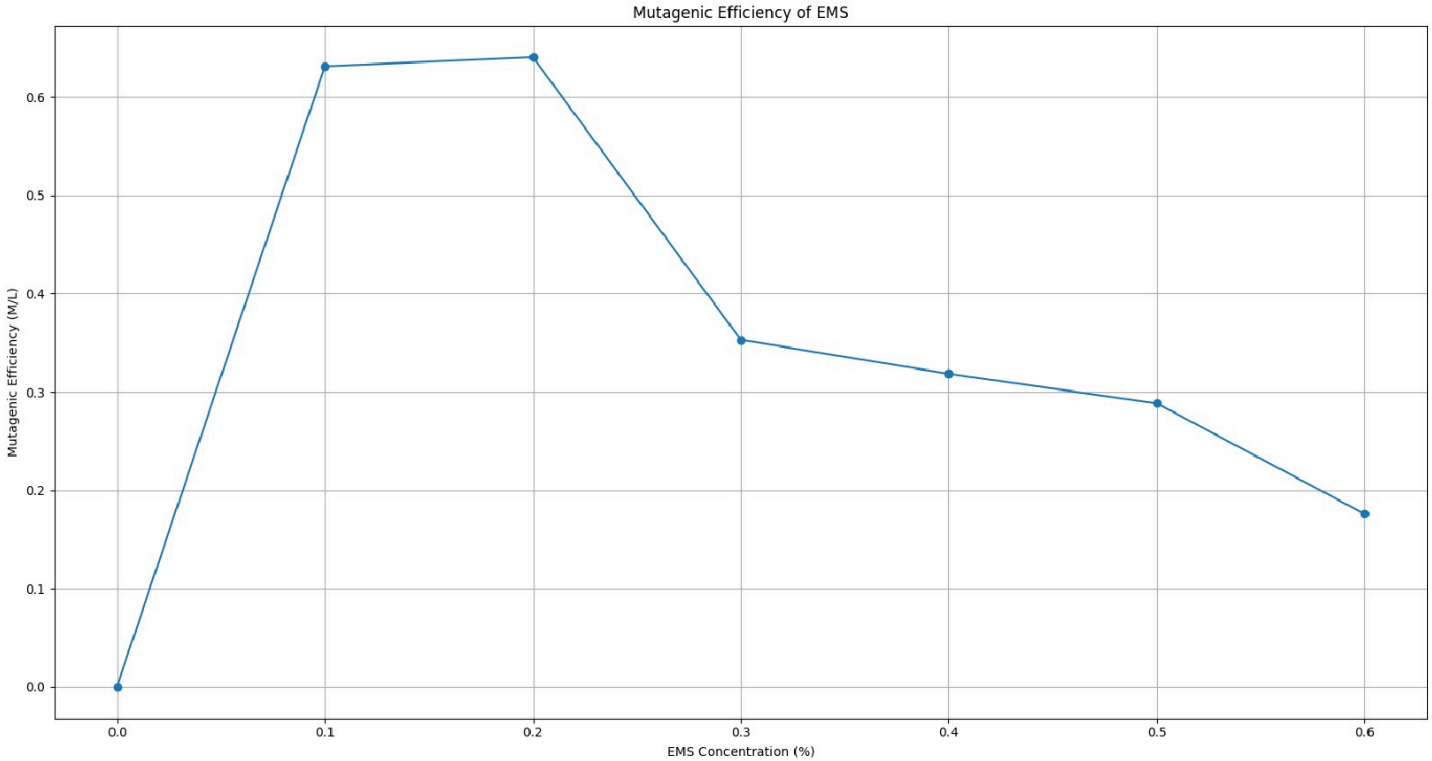


Fig. 5 Mutagenic Efficacy of EMS

Figure 5

See image above for figure legend

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