Original Article

J Genet Appl Biotechnol, 2026: *e2026002*
DOI: https://doi.org/10.66432/3sj9s175
Received: 07 Sep 2025; Revised: 10 Oct 2025; Accepted: 20 Oct 2025; Published online: 25 Feb 2026

Optimization of LD50 and Induction of Mutation Through EMS in *Dalbergia sissoo*

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Abstract

Dalbergia sissoo is an economically important tree of the Indian subcontinent. In this study, three ethyl methanesulfonate (EMS) concentrations with four exposure times were applied to identify the optimum combination for the induction of mutations. The lethal dose (LD) estimated using germination percentage was not a suitable criterion, as the survival percentages reduced drastically. The survival percentage after 30 days to estimate LD. Among the LD25, LD50, and LD75, the LD50 was identified as the optimal criterion for the survival of mutants. The LD50 was observed with two combinations of treatments: 0.48% EMS dose for 12 hours and 1.39% EMS dose for 6 hours. The progressive increase in EMS concentrations reduced the survival percentage, indicating residual toxicity. 0.48% MS for 12 hours and 1.39% EMS for 6 hours were used to develop a mutagenized population. Among the affected traits, height, stem morphology, leaf shapes and leaf size was. The chlorophyll content was negatively affected due to EMS; a decrease to 9.7 was observed compared to the control of 42.01. When the collected data were analyzed using analysis of variance significant differences were found among all the traits studied. A decrease in survival rate was also observed after six months and again after one year. The study would improve our understanding of EMS-induced mutagenesis and its use in tree plants.

Keywords: *Dalbergia sissoo*; Mutagenesis; EMS; LD50; leaf morphology

How to cite this article: Ahmed AI, Tayyab M, Shakeel MA, Rehman HM, Khan SHU and Rana IA. Optimization of LD50 and Induction of Mutation Through EMS in *Dalbergia sissoo*. *J. Genet. Appl. Biotechnol.* 2026: e2026002. https://doi.org/10.66432/3sj9s175

Introduction

D. sissoo belongs to the family Fabaceae (1) and the genus *Dalbergia.* This genus contains 300 trees, shrubs, and woody climbers (2). *D. sissoo* is a deciduous tree native to the Indian subcontinent (3). Its leaves are pinnately compound, and the trunk is greyish-yellow, which can attain up to 25 meters in height and 2-3 meters in diameter (1, 4). Its plant bears whitish to pinkish self-pollinated flowers, but cross-pollination is also reported. Its wood has the quality to withstand high pressure, resulting in its abundant usage for furniture, timber, decorative carvings, shelter belts, and windbreaks (1). Its wood is also used as firewood due to its excellent calorific value, i.e. 4,908 Kcals/kg and 5,181 Kcals/kg of sapwood and heartwood, respectively (1, 5).

The prevalence of diseases and environmental factors is narrowing genetic diversity. *D. sissoo* is also facing numerous biotic and abiotic such asdrought (6) an salinity (7) threats reducing its population significantly. Among them, wilt and Dieback (Shisham decline) are popular for their decline (8). Sufficient genetic diversity is a prerequisite to developing biotic and abiotic stress-resistant germplasm. However, in trees, creating genetic diversity through hybridization is not easy, as trees have long life cycles, difficult to access plant architecture, and long juvenile phases (9, 10). In trees, mutagenesis has been reported to cause stable trait modifications due to their ability to vegetatively propagate, unlike most crop plants (11).

Genetic diversity could be generated via mutagenesis in a shorter time as compared to hybridization. A mutation is an alteration in genetic information that is heritable to the next generation. Mutations could be induced through various biological, chemical, and physical agents (12, 13). Nowadays, EMS (Ethyl Methanesulphonate) ($C_3H_8O_3S$) is the most popular and effective chemical mutagen. It has a molecular weight of 124 g/mol and is a colorless liquid that is 8% soluble in water. EMS is a member of the class of

alkylating agents. These substances include one or more reactive alkyl groups that can transfer to other molecules at a site with a greater electron density (13). Reports showed that it mostly causes point mutations in DNA by replacing the G/C pair with the A/T pair, but insertions, deletions, and duplications have also been reported. EMS could also create transversions of G/C to C/G or G/C to T/A by errors in 7-ethyl guanine hydrolysis or 3-ethyl adenine repair during A/T to G/C transitions (14).

In this study, we have estimated the lethal dose (LD) of the EMS by using different doses and time duration as treatment and developed a mutant population showing significant alterations in various morphological and physiological traits. The said population is going to act as a novel gene pool for future *D. sissoo* improvement programs.

Materials and Methods

Seeds sterilization

Seeds were collected from an identified healthy source of *D. sissoo* from the University of Agriculture, Faisalabad. The collected seeds were surface sterilized with Tween-20 for 1 minute, followed by 50% sodium hypochlorite washing for 2 minutes. Then, 70% ethanol washing was performed for 1 minute, and seeds were rinsed twice with sterile water to remove the ethanol residues. Afterwards, seeds were soaked for 24 hours in distilled water to improve seed germination. There were two experiments were conducted first experiment was conducted to calculate the lethal dose for EMS. The second experiment was conducted to generate a diverse germplasm of *D. sissoo.* In the first experiment, there were 50 seeds for each combination (Time*Dose). For the second, 1200 seeds were treated to generate the diverse germplasm.

EMS treatment

The treatment with EMS was taken out in a fume hood to avoid the fumes. The 0.5%, 1.0%, and 1.5% v/v EMS were prepared using distilled water as solvent. The seeds were dipped in each concentration and incubated for 6 hours, 12 hours, 18 hours, and 24 hours, at 30°C, and 70 rpm. The untreated seeds were taken as a control and dipped in distilled water. Treated seeds of all combinations were washed under tap water for 6 hours to remove any EMS residues. The seeds were then sown in trays filled with compost for 30 days under growth room conditions (25 ± 2 °C, and 3600 ≈ lx). The germination rate can be increased by the use of compost.

Data recording and statistical analysis

The experiment was carried out in a completely randomized design with factorial combinations having three replications, and variability was assessed through a combined analysis of variance (ANOVA). The EMS concentrations and the time of its exposure were taken as separate factors. The germination percentage was calculated after seed emergence, and the survival percentage was calculated for the plants that survived after 30 days using the formula described by Omosun, Ekundayo (15). Subsequently, the LD25 (Lethal dose 25%), LD50 (Lethal dose 50%), and LD75 (Lethal dose 75%) were estimated based on the survival percentage.

$$\text{LD50 (\%)} = \frac{\text{Total number of survival plants after 30 days}}{\text{Total number of germinated seeds}} \times 100$$

The germination data were taken after 12 days of sowing because we wanted to evaluate the effect on physical characteristics at various time intervals. The number of leaves per plantlet was manually counted, and plant height was measured from the plant base to the top leaf, whereas chlorophyll contents were measured with a chlorophyll meter SPAD-502 plus after 45 days and after six months of sowing. The ANOVA, standard deviation, and means were calculated in Python using the states models package. The graphs were prepared in Python using Seaborn for data visualization.

Results

EMS effect on germination and survival percentage: The germination percentage accounts for one of the most important characteristics of the EMS mutagenesis experiment. The use of fresh seeds had a positive effect on germination, as germination increased from 85% to 95%. In this experiment, the germination percentage of the control increased gradually with the increase in soaking time (Figure 1a). The 89.8%, 90.5%, 95.7%, and 100% germination percentage was observed at 6hrs, 12hrs, 18hrs, and 24hrs soaking period respectively. This increase indicated that the germination percentage could be improved by increasing the soaking time up to 48 hours.

Normally, in *D. sissoo*, germination is completed in 5-7 days. However, after the treatment of EMS, the germination time was delayed, and germination was completed in 12 days after sowing. The germination was observed fashion, no seeds germinated during the first 5 days and then completed in the next five days in most of the doses except 12 hours with 1.5% EMS concentration (completed in 12 days). The germination process was delayed further when EMS was given at higher dosages. This shows a dose-dependent connection in which longer germination periods were connected with greater EMS doses. These results highlighted how EMS interferes with the typical germination of *D. sissoo* seeds, which indicates the possible detrimental effects of this chemical mutagen on seed viability and germination efficiency.

Determining seed germination percentage is crucial in a mutagenesis experiment. The progressive increase in EMS concentration from 0.5% to 1.5% and exposure time from 6 hours to 24 hours drastically decreased seed germination from 88.3% to 0.0% (Figure 1(a)). The 24hrs exposure at both 1% and 1.5% EMS, resulting in the death of all seeds, highlights the lethality of EMS exposure. The lethality of EMS is not restricted to the germination rate, but it also affects the plant survival rate, and the plant could die after germination as well. Like the germination rate survival rate also decreased in the same fashion (Figure 1(a)). After comparing the germination percentage with the survival percentage, an inverse relation was observed, which could be the result of the toxicity of EMS residuals after germination.

EMS treatment

The EMS also affects the mortality time of germinated plants. In higher dosages of EMS, the mortality rate was observed to occur immediately, typically within 2-9 days

after germination. It is interesting to note that as the dosage of EMS decreased, the time to mortality increased. For example, when subjected to a 0.5% EMS concentration with a 6-hour treatment, the plants died approximately 23-28 days after germination. In contrast, when exposed to higher dosages, such as all treatments with a 1.5% EMS concentration, the plants died more rapidly, between 3 and 11 days after germination. These results highlight the significant impact of EMS dosage on plant survival, with higher dosages resulting in higher mortality rates. The survival was also reduced after six months. There was the same pattern of survival observed, increasing the dose of EMS, reduced survival (Figure 1(b)). This suggests that the detrimental effects of increasing the EMS dose on plant survival remained for a considerable amount of time. As a result, even while the general pattern of declining survival with increased EMS dose continued, the extent of the survival rate decline was considerably less after the first month. The survival percentage was also decreased after one year, but its percentage was very low (Figure 1(c)) ().

ANOVA showed highly significant differences among mutants for germination, chlorophyll contents, survival rate, leaves per plant, and shoot length, indicating the effectiveness of EMS to create variability (Table 1). These results provide strong proof of the efficiency of EMS as a tool for inducing genetic variation. The differences in several attributes between the mutants demonstrated the effective production of varied phenotypes by EMS mutagenesis, further highlighting its potential as an important tool in genetic research and agricultural development programs.

Table 1: Mean squares of Germination percentage, Survival percentage, Shoot length, Chlorophyll contents, and Number of leaves after 45 days of sowing. Shoot length, Number of leaves, and Number of branches after six months of sowing. (SOV: Source of variation, and DF: Degree of Freedom)

SOV	Dose	Time	Dose*Time	Error
D.F	3	3	9	32
Germination %	11547.15 ***	4702.67 ***	797.28 ***	1.26
Survival %	12518.29 ***	4646.47 ***	744.22 ***	0.66
Shoot length	166.32 ***	84.60 ***	36.89 ***	7.49
No. of leaves	437.05 ***	206.55 ***	50.53 *	21.12
Chlorophyll contents	4246.56 ***	222.34 ***	48.52 ***	6.43
Shoot length after 6m	3398.24 ***	572.07 ***	143	2.81
No. of leaves after 6m	19836.46 ***	4597.29 ***	1381.16 ***	43.08
Number of branches after 6m	14.72***	42.61***	21.77***	2.02

*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001

Estimation of the optimum lethal dose (LD) and exposure time

The dose of a substance (rays or chemicals) that is fatal to 50% of the test population is known as the LD50 (lethal dose 50). The LD50 value is used in mutagenesis research to determine the dose at which 50% of the organisms exposed to a mutagenic agent, such as EMS, will survive. It helps in figuring out the ideal dosage for promoting genetic diversity while retaining an adequate survival rate. LD50 values change based on the species, environment, and research aims. It helps in developing dose recommendations and safety standards for mutagenesis investigations by providing important information about the toxicity characteristics of EMS. The efficiency of mutagenesis experiments is mostly estimated through LD50.

In this experiment, the LD50 was estimated at two experimental combinations: (1) 0.48% EMS for 12 hrs, and (2) 1.39% EMS for 6 hrs. The comparison of LD25, LD50, and LD75 indicated that LD50 is the better parameter while attempting a mutagenesis experiment in tree plants. The combination (1% EMS × 6 hrs) having LD25 showed less morphological variation compared to the control (Figure 1(d,e,f)). While at LD75 (1% EMS × 12 hrs) lower number of plants survived (Figure 1(d,e,f)), but produced plants with extreme morphological variants compared to LD50 and LD25 (EMS 1% × 12 hrs) (Figure 1 (d,e,f). Similarly, the combinations having LD75 to LD100 (EMS 1.5% × 12 hrs, EMS 1% × 18 hrs, and EMS 0.5% × 24 hrs) were found to be identified producing extreme phenotypes (Figure 1(d,e,f)). Among the LD50 combinations, 1.5% EMS × 6 hrs caused a 16.7% reduction in survival rate and yielded maximum morphological variation, compared to the 0.5% EMS × 12 hrs combination caused 3% reduction in survival rate but lower morphological variation was observed (Figure 1(d,e,f). In this experiment, we have observed that an increase in the EMS concentration results in lower plant survival. This observation has led us to opt for the suitable combination characterized by lower mortality coupled with sufficient morphological variation.

Morphological characterisation of mutants

A mutation could affect plant morphology by affecting its nucleotide combinations. After identifying the treatment combination having LD50, we have developed a mutant population comprised of 586 mutagenic plants, which were morphologically characterised. Shoot length is an important characteristic of tree plants, as a long shoot is always a desirable trait, being a direct source of wood. The mutant population's shoot length gradually reduced as EMS

concentrations and exposure duration increased. This indicates that EMS treatment's mutagenic effects affected the genetic control of shoot elongation, resulting in shorter shoots than the control, but some irregularities were also identified (Figure 1(e)). In control mean shoot length was 11.52 cm, which was reduced to 5.75 cm in the mutagenic population after 45 days of sowing Figure 2(a). These findings suggested that EMS disrupted the normal growth process, induced the genetic variation, and reduced the shoot length. Some irregular behavior such as some plants had a height more than the control. The growth of some plants stopped after germination. Their shoot length did not grow, but their root growth continued. EMS resulted in a block in cellular DNA, which stopped or slowed down plant growth. In the case of shoot length, an EMS-induced mutation may interfere with the normal operation of genes involved in shoot growth, leading to stunted or diminished shoot growth. EMS disrupts genes involved in cell division, cell growth, or the synthesis of hormones that control shoot elongation, which may happen. It may be due to alkylation of DNA, which can cause base pair substitution or alter the DNA sequence, resulting in alteration in protein production. These affect the growth and development of plants.

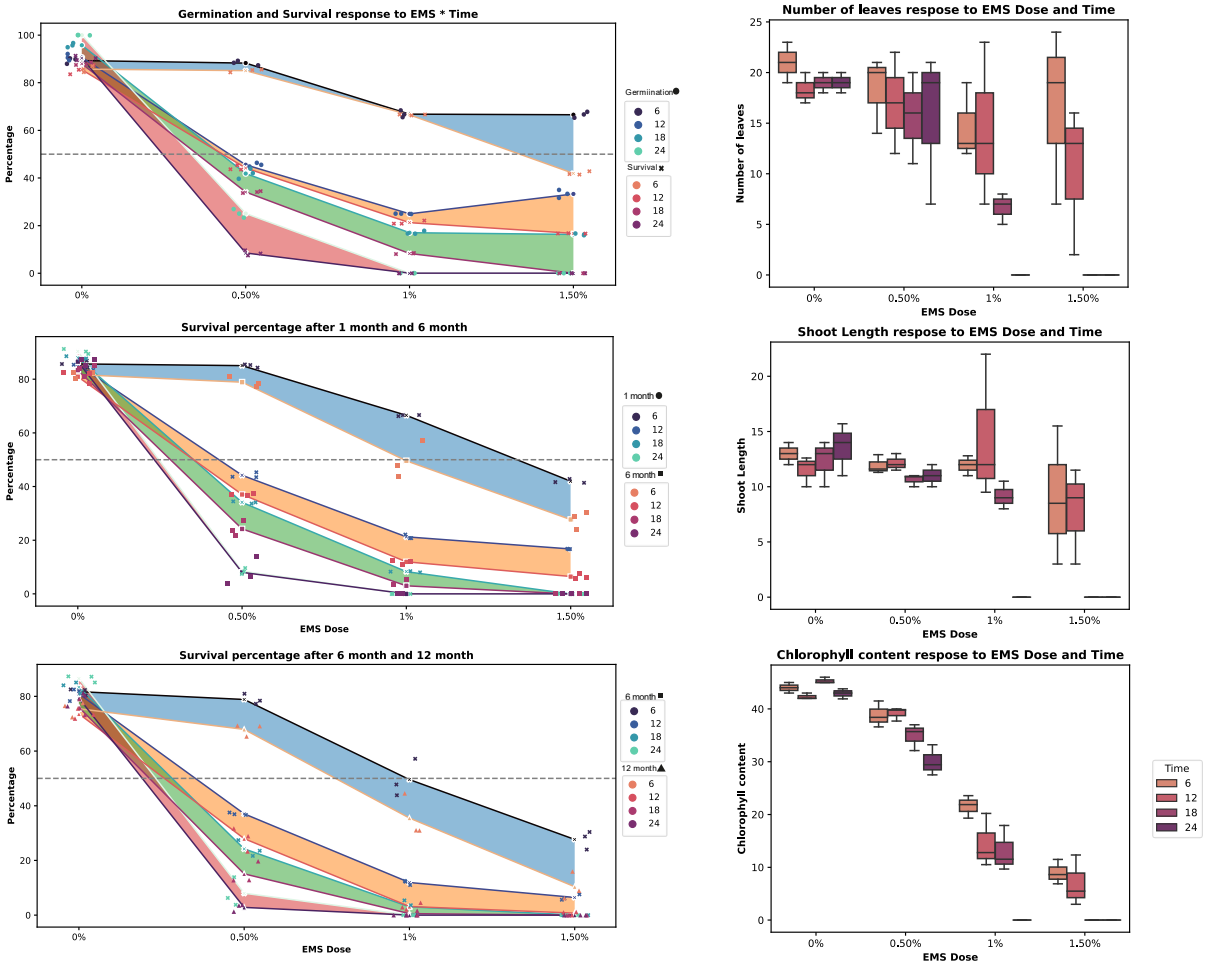

Figure 1: (a) Difference between Germination and Survival percentages as the survival rate is decreasing due to EMS effect, (b) Trend in Survival percentage at six months after sowing, (c) Trend in Survival percentage at one year after sowing, (d) Number of leaves against EMS treatments, (e) Shoot length against EMS treatments, (f) Chlorophyll contents against

The chlorophyll content was also decreased in the population, as at control mean chlorophyll content was 42.01, which reduced to 9.7 when the EMS dose increased to 1.5% (Figure 1(f)). In one treatment (1% EMS dose with 18 hrs of treatment), the chlorophyll contents were also increased with the EMS treatment. By alkylating guanine bases, EMS causes point mutations in DNA that may disrupt the normal functioning of genes involved in chlorophyll production. Chromosome rearrangements and DNA strand breaks are two additional effects of EMS that reduce chlorophyll synthesis. In addition, plants that have less chlorophyll may be more vulnerable to environmental challenges, including drought, diseases, and pests. EMS also affects plant architecture like stem types (erect and curved types) (Figure 2b). All the plants in control had erect stem. Curved stems were mostly observed in higher doses. This behavior can be related to EMS-induced mutations that interfere with the growth and development of stem cells. These mutations may have an impact on the genetic makeup, gene expression, and signaling systems

that control stem cell differentiation. From economical point view, erect stem is desirable.

In control, all the plants had three leaves per node. But in some mutants, there are different number of leaves per node like 2, 4, and 5. This type of behavior was not observed on all the nodes but on some nodes of mutants. (Figure 2f). The number of leaves per plant was also affected by EMS. The number of leaves did not follow any trend. With EMS treatment, the number of leaves increased in some cases but also vice versa. The number of leaves decreased or increased due to the disruption of the normal regulation of the normal leaf development which resulted in the change in leaf initiation and growth. (Figure 2c).

The leaf size was greatly affected by EMS treatment (Figure 2(e)). The increase in leaf size was due to chromosomal aberration; DNA replication disturbance resulted in palisade and spongy mesophyll cell enlargement. The leaf size decreased in most cases due to cell division suppressed and auxin biosynthesis inhibition. There was a phenomenon observed in leaf size and number. The more the number of leaves, less the size of the leaves and vice versa.

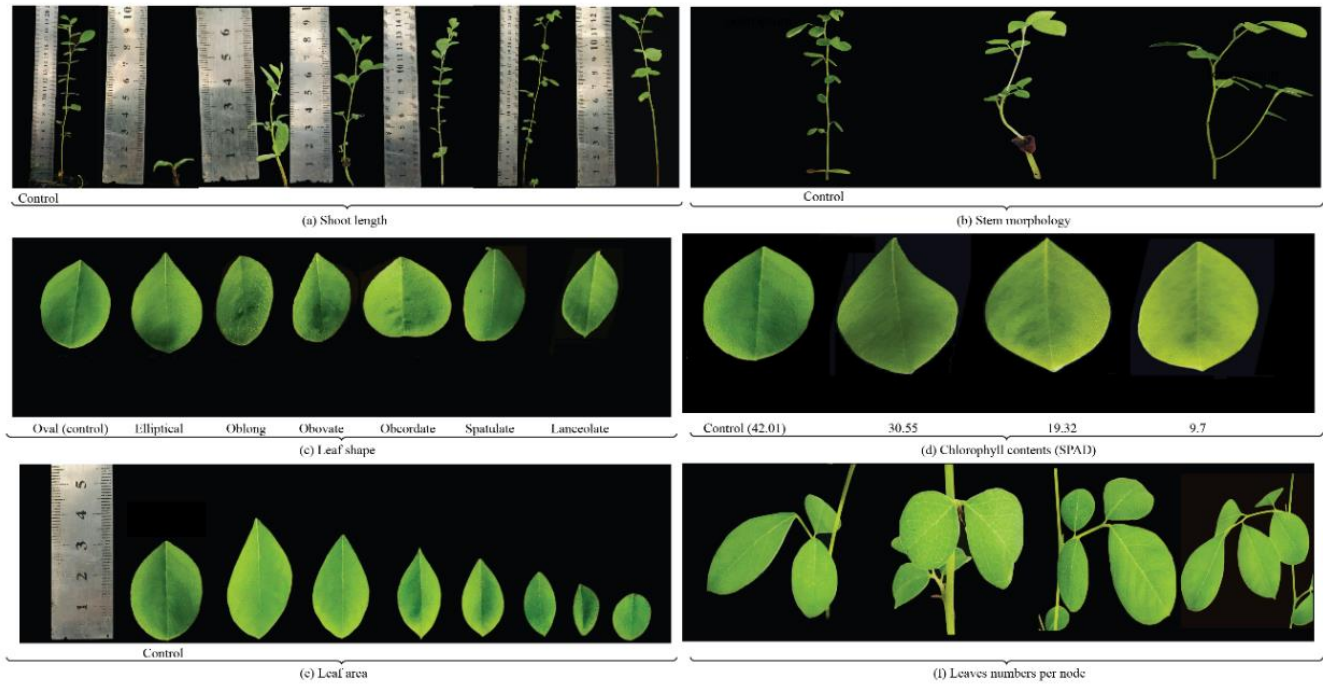

Figure 2: The altered phenotypes observed in this experiment. (a) The control length was 19.5 cm which could be seen reducing but the second plant from the left was observed to have more plant length (24 cm), (b) Stem morphology of the control was the erect type but curved and multiple branching stem morphology was also observed, (c) Leaf shapes with a range of the phenotypes were observed while the control had the oval type leaves, (d) The chlorophyll content in the control was 42.01 but it reduced to 9.7, (e) Leaf area was also seen to have reduced as from the left the right a progressive decrease is visible, (f) Leaf number per node were also identified to have altered as the control were identified with 3 leaves but in the mutant plants 2, 4, and 5 (Left to right) leaves were identified.

The number of branches had a large number of phenotypic variations. In control, the plants had only a single stem, but, in some mutants, there were two main primary branches from the root base which could be a good addition to producing *D. sissoo* having more biomass (Figure 2b). Mutants also produced the large number of secondary branches, but these morphological variations appeared after 6 months of germination. This may be due to the disturbance in the genes involved apical dominance, axillary bud growth, and hormones signaling. Different morphological changes were observed over time. But these changes were stable, the most visual changes were the extensive branches. The growth was also slowed in EMS-treated plants.

Moreover, EMS greatly affected the leaf morphology and different variants of leaf phenotype were obtained which include elliptical, oblong, obovate, obcordate, spatulate, and ovate compared to oval-shaped leaves in control (Figure 2c). Oblong leaves have parallel sides that are longer and narrower than elliptical leaves, which are long and rounded like an ellipse. The form of obovate leaves is inversely egg-shaped, with a wide top and a tapering base. Obcordate leaves exhibit a heart-like shape, with the base acting as the apex and the tapering tip at the other end. Similar to oblong leaves, spatulate leaves have a broader, rounded tip that resembles a spatula. Oval shape, with rounded edges, symmetrical sides, and tapering points at both ends, leaves have an oval form.

Different morphological differences in plant architecture were observed with time such as the number of branches, leaf size, plant height, and stem shapes). Data was recorded of different traits such as survival rate, shoot length,

number of leaves, and number of branches after six months of sowing (Figure 2S).

Discussion

The tree breeding has many constraints such as a long lifespan, crop final size, difficult physical phenotyping, and quality traits appeared at maturity (16). Like other trees, *D. sissoo* lacks genetic diversity and mutation could generate genetic diversity rapidly compared to hybridisation (17) The EMS causes stable point mutations that result in a range of phenotypic variations. The rate of germination and survival percentage has been reported as the most affected trait in mutagenesis studies (18, 19). The germination was also delayed due to EMS treatment, this behavior was also reported in rapeseed-mustard (20), Zea mays (21), and *Sesamum indicum*(22), The germination process was also slowed by mutagenic treatments. It was discovered that the start of metabolism after germination was delayed, which uniformly delayed mitotic activity, seedling development, and the production of ATP and DNA (21).

The reduced germination percentage could be due to cellular disturbance (23) or due to the inhibitory effect of mutagen on important physiological or genetic processes (24). The early death of plants after germination was may be due to severe DNA breakage which could not be repaired resulted in death (25). The LD value depends on the plant species, targeted tissues, mutagen type, and doses of mutagen (15). Here we have identified LD50 to be the optimal criterion as the survival reduction and variability were identified as optimal at its two combinations. The LD50 was estimated at two combinations, 0.48% EMS for 12hr, and 1.39% EMS for 6hr. The LD50 was 1.39% EMS with 6 hours was more suitable because maximum variations were observed on this treatment. With the increasing the EMS dose increase the mutation but decreased the survival (13).

In this study, the survival rate decreased against the higher dose of EMS regardless of exposure time which could be due to the presence of EMS residues in the plant. Based on these observations, we have used 0.48% EMS for 12hr as LD50 to develop a mutagenic population. Estimation of LD50 always depends on crop and nature of seed like in *Vigna unguiculata* LD50 was identified at 0.4% EMS for 6hr (26), but for *A. esculentus* and *T. foenum-graecum* LD50 was identified with 0.5% EMS for 18hr and 3hr respectively (27, 28). The growth was also slowed down with EMS treatment; similar behavior was also observed in *Cucumis sativus* (29) and *Vigna mungo* (30). Some plants observed where the plant stopped growing after germination.

Along with variation in germination and survival percentage EMS also affects plant phenotype and physiology as well. The mutant population produced variable shoot length, leaves per plant, leaves per node, and chlorophyll contents as EMS could alter the open reading frame resulting in altered phenotype or physiology. The shoot length was greatly affected by the EMS doses, an increase in EMS doses reduced shoot length but some plants were also identified to have increased in length. Reduction in shoot length against EMS dose has been reported due to the reduction of seedling vigor caused by the increase in physiological damage (31), and the increase has also been reported due to unequal injury to the meristematic cells (32). The shoot length decreased with the increased in EMS concentration was also observed in *Oryza sativa* (33). Similarly, the number of leaves and chlorophyll contents were also affected due to EMS as reported in *S. indicum* (31, 34), *Guizotia abyssinica* (35) and *Garcinia mangostana* (36). This might have been due to a change in cell division rate or due to growth hormone activation (37). In the past variation in the leaf shape has also been reported in *O. sativa* (38), *Chrysanthemum morifolium* (39), *C. sativus* (40), *Brassica campestris ssp. pekinensis* (41), and *Solanum lycopersicum* (42) due to EMS. Interestingly range of leaf shapes in mutagenized plants was also observed. Several abnormalities like phytochrome disruptions, chromosomal abnormalities, mitotic inhibition, disturbed auxin synthesis, mineral shortages, disturbance in DNA synthesis, and expansion of palisade, spongy, and mesophyll cells could cause variation in leaf shapes (43). The EMS treatment also had an impact on the stem phenotype, which caused some plants to develop bent and multiple stems. This indicates that the aberrant morphological traits resulted from the mutagenic effects of EMS altering the growth and development of the stems. The curved stems suggest a loss of structural integrity or a change in orientation, which may have an impact on the stability of the plant and its general structure. Similar observations were also reported in *Chrysanthemum indicum* (44). Having many stems indicates increased branching or the beginning of new shoot structures. A link between lignin and cellulose concentrations and mechanical strength the bent-stem mutant, which had lower lignin and cellulose content. This indicated that cell wall metabolism may be impacted by EMS mutations. This demonstrated that the lignin and cellulose found in cell walls are not only a simple combination but are linked together chemically. This further suggests that EMS might control the amount of lignin and cellulose in the stem (44). EMS causes degradation of the chlorophyll contents due to which we have observed light yellow to dark green leaves and leaves having lower chlorophyll depicted light yellowish leaves (45-47). In contrast to lower chlorophyll contents, an increase in chlorophyll contents has also been reported at higher doses of EMS in *Capsicum frutescens* (48).

Conclusion

D. sissoo has low genetic diversity and mutation is a powerful and easiest source for creating variability as compared to hybridization. Three EMS concentrations along with four exposure times have been used to identify the most suitable dose and time and LD50 was identified at 0.48% and 1.39% EMS for 12 hours and 6 hours respectively. Mutants showed a variety of morpho-physiological variations including reduced and increased shoot length, and a variable number of leaves, and branches per plant which could be the result of random alterations

caused by the EMS. Mutants produced in this study could be used in functional genomic studies to identify novel genes.

Acknowledgement: This study was supported by Food Punjab Agriculture Research Board grant number PARB 883. We also acknowledge infrastructural support from the Centre for Advanced Studies in Agriculture and Security (CAS-AFS), at the University of Agriculture Faisalabad, Punjab, Pakistan.

Disclaimer: None.

Conflicts of Interest: The authors declare no conflict of interest.

Source of Funding: None

Ethics approval statement: Not Applicable

Authors' Contribution Statements: AIA: Execution of experiment, Statistical analysis, data collection: MT, AS: Execution of experiment, data collection, HMR: Proofread the manuscript, SHK: Final revision and editing, and IAR supervised the experiment and provided the resources.

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