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Chemo-sensitive Responses of EMS and EBr on Physiological and Biochemical Traits of Cowpea [*Vigna unguiculata* (L) walp.]

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ABSTRACT

Background: Cowpea [*Vigna unguiculata* (L) walp.] productivity is limited by soil and environmental factors, along with its narrow genetic base which collectively reduces the photosynthetic efficiency by reducing key photosynthetic components and nitrogen assimilation. Therefore, improving productivity requires developing stress-resilient and genetically diverse varieties. Induced mutagenesis offers an effective approach to create genetic variability and enhance crop performance for sustainable agriculture.

Methods: The laboratory and pot culture experiments were conducted at Department of Genetics and Plant Breeding, SRM College of Agricultural Sciences, SRM Institute of Science and Technology, Chengalpattu, Tamil Nadu from August 2025 to December 2025 aimed to study the effects of chemical mutagens, Ethyl Methane Sulfonate (EMS) and Ethidium Bromide (EtBr), on seedling growth, physiological and biochemical traits in the M_1 generation of cowpea variety CO (CP) 7. Seeds were treated with five concentrations of EMS (20-40 mM) and EtBr (0.05-0.25 mM) along with a control and observations were recorded under a completely randomized design with three replications.

Result: Results revealed significant variation among treatments. Moderate doses of EMS (20 and 25 mM) and EtBr (0.05 and 0.10 mM) enhanced germination, root length, shoot length and seedling height, indicating a stimulatory hermetic effect. In contrast, higher doses reduced growth parameters and increased seedling injury. Photosynthetic traits such as total chlorophyll, carotenoids and soluble protein improved at lower doses but declined sharply at higher concentrations. Nitrate reductase activity and total soluble sugars increased under mild treatments but decreased under severe mutagen stress. Proline accumulation showed a dose-dependent increase, reflecting stress response. The LD_{50} range was identified between EMS 30-35 mM and EtBr 0.15-0.20 mM. Principal component analysis revealed that PC1 explained 88.4% of the total variation. PC1 showed a positive association with growth and metabolic traits and a negative association with seedling injury and proline. Overall, moderate mutagen doses were optimal for inducing useful variability and can serve as effective selection criteria in cowpea breeding programs.

Key words: Cowpea, EMS, Ethidium bromide, Mutation breeding, Seedling physiology.

INTRODUCTION

Cowpea [*Vigna unguiculata* (L) walp.] is an important legume crop commonly cultivated across tropical and subtropical areas. It is highly adapted to different environments and high ability to fix atmospheric nitrogen in the soil and rich nutritional profile. It contains major source of dietary protein, minerals and vitamins for millions of people around the world including Africa and Asia and also it plays a crucial role in food and nutritional security (Kim *et al.,* 2025). However, its productivity is frequently constrained by biotic and abiotic stresses such as pests, diseases, drought, heat and poor soil fertility (Kebede and Bekeko, 2020). Earlier breeding methods have significantly contributed to cowpea improvement even narrow genetic information and limited availability of natural variability often restrict progress in developing high-yielding and stress-resilient cultivars (Mekonnen *et al.,* 2022). Induced mutagenesis is developed as an important tool for producing novel genetic variability that is not available in existing germplasm. Chemical mutagens such as ethyl methane sulfonate (EMS) and ethidium bromide (EtBr) are broadly used to induce point mutations that smooth the development of new traits in self-pollinated crops. EMS is used as chemical mutagen and it producing stable single-base substitutions, thereby it increasing variability in growth, physiological and biochemical parameters (Shamshad *et al*., 2023; Singh and Kumar, 2024). EtBr is also commonly used as mutation chemical but it can efficiently stimulate

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mutations. To know the effect of these chemical mutagens on M_1 generation plants is crucial for expecting mutation efficiency and the inheritance of required traits in subsequent generations (Keadtidumrongkul *et al.,* 2018).

Growth, biochemical and physiological traits play an important indicator of plant growth and metabolic function in mutation studies. Plant height, leaf area, total chlorophyll content, photosynthetic enzyme activity and antioxidant activity give ways into plant adaptation to mutagenic stress (Singh and Kumar, 2024). Chemical mutations can change cellular homeostasis, increasing photosynthetic efficiency, oxidative balance and enzyme activity. The resulting variation in growth and physiological traits can be exploited for selecting improved mutants (Li *et al.,* 2025). Despite the growing interest in mutation breeding for legumes, comprehensive studies reporting the physiological response variability in chemically mutagenized cowpea remain limited. Therefore, assessing the impact of chemical mutagens on growth and physiological parameters in the M_1 generation is essential to identify promising mutants for further evaluation (Khursheed *et al.,* 2019). Hence, the present study aims to characterize the variability induced by EMS and EtBr treatments in cowpea by evaluating important growth and physiological parameters. These findings are expected to contribute to the development of improved genetic resources and enhance the efficiency of mutation breeding programs for cowpea improvement.

MATERIALS AND METHODS

The present laboratory and pot culture study were conducted at Department of Genetics and Plant Breeding, SRM College of Agricultural Sciences, SRM Institute of Science and Technology, Chengalpattu, Tamil Nadu from August 2025 to December 2026 aimed to evaluate the effects of chemical mutagens, Ethyl Methane Sulfonate (EMS) and Ethidium Bromide (EtBr), on seedling growth, physiological and biochemical traits in the M_1 generation of cowpea variety CO (CP) 7. Healthy and uniform seeds of *cowpea* (*Vigna unguiculata* L.) variety CO (CP) 7 were selected for this experiment. Seeds were pre-soaked in distilled water for 6 hrs prior to the treatments. Ten different treatments *viz*., ethyl methane sulfonate (EMS) at 20, 25, 30, 35 and 40 mM and Ethidium Bromide (EtBr) at 0.05, 0.10, 0.15, 0.20 and 0.25 mM with control in completely randomized design with three replications. The treatments were applied to 100 seeds for each treatment for 6 hours with intermittent shaking. After treatment, treated seeds were washed thoroughly under running tap water for 1 hour to remove mutagen residues. Then it was transferred to petri dishes containing moist filter paper to observe the number of seeds germinated at 7^{th} day and seedling variations for each treatment were subsequently determined. The untreated seeds soaked in distilled water used as control.

Determination of lethal dose (LD_{50})

LD_{50} is the concentration of a mutagenic chemical that results in a 50% survival of the plants. Also, it is used to found the ideal dose for a specific crop and a specific dose of each mutagenic chemical. To record the seed germination and the seedling height on the seventh day, petri dishes with three replications, each containing ten seeds, were kept using seed germination paper.

Growth parameters

Root length, shoot length and seedling height were recorded from uniformly germinated seedlings using a millimetre scale by measuring the primary root, the shoot from the root-shoot junction and the total seedling length from root tip to shoot apex, respectively. Seed germination percentage was calculated using the following formula:

$$\text{Germination percentage} = \frac{\text{No. of seeds germinated}}{\text{No. of seeds sown}} \times 100$$

Mutagen induced seedling injury was measured using the following formula:

Seedling injury percentage (Injury in terms of height) =

$$\frac{\text{Seedling height in control - Seedling height in treatment}}{\text{Seedling height in control}} \times 100$$

Physiological and biochemical parameters

Total chlorophyll and carotenoid contents were estimated using the acetone extraction method described by Arnon (1949). Soluble proteins were extracted from fresh leaf tissue using phosphate extraction buffer and centrifuged to obtain the supernatant solution. Measurements were taken using spectrophotometer following the procedure of Bradford (1976). Proline was extracted using acid-ninhydrin reagent and the reaction mixture was incubated in a water bath. The chromophore was extracted with toluene and absorbance was recorded spectrophotometrically (Bates *et al.,* 1973). Total soluble sugars were extracted from homogenized plant tissue using an appropriate aqueous solvent. The extract was reacted with phenol and concentrated sulphuric acid to develop a stable chromogenic complex. Sugar content was quantified spectrophotometrically following the phenol-sulphuric acid method of Dubois *et al.* (1956).

Principal component analysis

Principal component analysis (PCA) was performed to assess variability among seedling, physiological and biochemical traits in mutagenized cowpea. The data were standardized to reduce scale of variability and performed using the correlation matrix. Eigenvalues followed by percentage variance were used to know the significant components with components having eigenvalues>1 retained. The biplots were prepared using first two principal components to visualize trait and treatment relationships.

PCA also helps in identifying key traits contributing to variability and differentiating tolerant and susceptible treatments under mutagenic stress.

Statistical analysis

The experimental data were statistically analysed using R programme. This study was laid out in a completely randomized design (CRD) with three replications. Analysis of variance (ANOVA) was performed to reveal the significant differences among treatments. Critical difference at 5% significant level was used to compare the treatment means. Standard error (SE) values were considered to assess variability among treatments (Gopinath *et al.,* 2021).

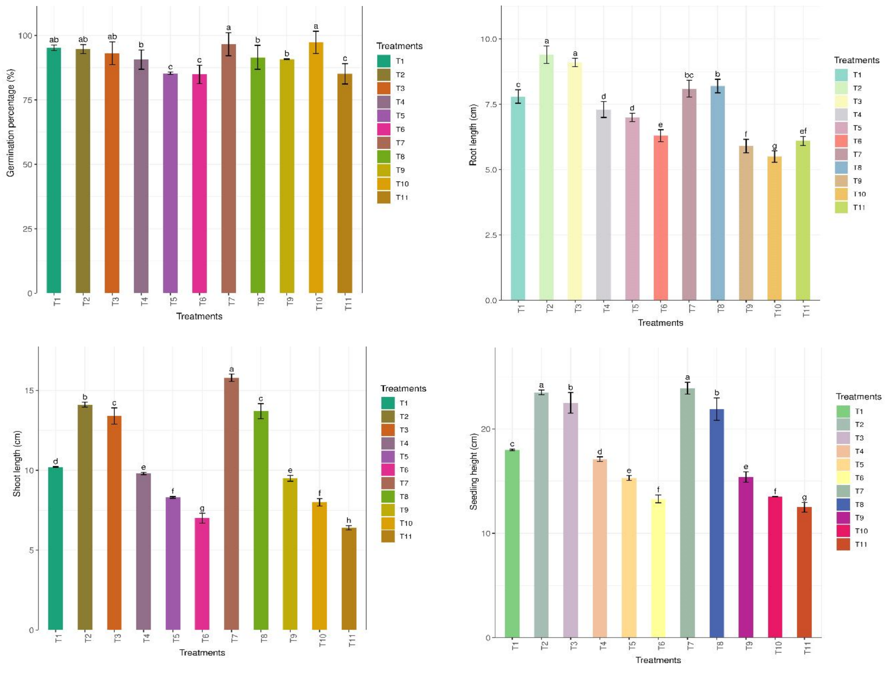
RESULTS AND DISCUSSION

The effect of EMS and EtBr on seedling growth, physiological and biochemical parameters of cowpea at M_1 generation were showed significant variations among different treatments and it was clearly indicating the mutagenic influence on early seedling development and metabolism.

Effect of EMS and EtBr mutagens on seedling growth parameters

The data given in Fig 1 clearly demonstrated that mutagen chemical treatments were induced significant changes in germination percentage and seedling growth. These two parameters are play a key role in crop improvement. Higher doses of both mutagens normally resulted in reduced germination percentage compared to moderate doses. The control treatment noted 95.2% of germination while EMS treatments recorded from 94.7% (20 mM) to 84.9% (40 mM). Similarly, EtBr treatments showed significant variation with the highest germination rate at 0.05 mM (96.6%) and a declined at 0.25 mM (85.1%). This results suggested that while lower doses may improve germination, higher concentrations induce inhibitory effects due to increased cellular damage of seeds (Yu *et al.,* 2025). Interestingly, moderate doses of EMS (20 and 25 mM) and EtBr (0.05 and 0.10 mM) enhanced root length, shoot length and seedling height compared to the control plants. For instance, EMS 20 mM recorded maximum seedling height of 23.5 cm and EtBr 0.05 mM observed the highest shoot length of 15.8 cm. This enhancement effect at lower doses of both mutagens may be due to changes in the hormonal balance, where mild stress enhances metabolic activity and cell division. However, a progressive decline of root length, shoot length and seedling height was observed at higher doses of both mutagens (EMS 35 and 40 mM and EtBr ≥0.15 mM), indicating inhibition of mitotic activity of cells and cell elongation (Rafiq *et al.,* 2017; Perumal *et al.,* 2025).

In Fig 2, it was clearly shown that lower doses of EMS 25 mM (25.0%) and EtBr 0.10 mM (21.7%) revealed moderate seedling injury levels, whereas higher doses showed highly significant deviation which indicating physiological stress of seedlings due to induced mutation. These results confirm that excessive mutagen treatment disrupts cellular organization, enzyme activity and hormonal balance, leading to reduced growth of seedling (Bhat *et al.,* 2026).



a) Germination percentage (%); b) Root length (cm); c) Shoot length (cm); d) Seedling height (cm).

Fig 1: Effect of EMS and EtBr mutagens on seedling traits of cowpea.

LD_{50} determination

The LD_{50} concentration is as the concentration of mutagen which affecting approximately 50% reduction in growth and development. It is highly useful in mutation studies. And it was observed between EMS 30-40 mM and EtBr 0.15-0.25 mM based on the germination rate and seedling growth parameters. Treatments below LD_{50} including EMS 20 and 25 mM and EtBr 0.05 and 0.10 mM were maintained higher germination and better growth performance, suggesting their suitability for mutation breeding aimed at generating viable M_2 populations with sufficient variability (Jayaveer *et al.,* 2026).

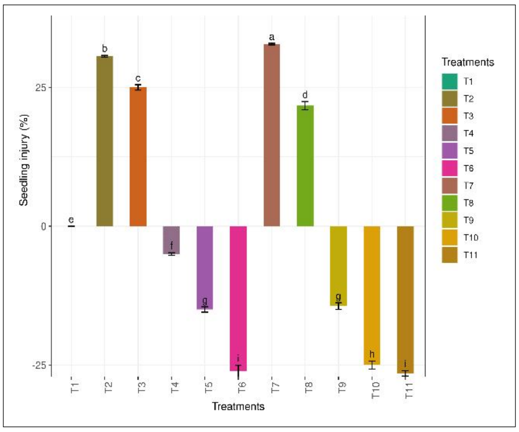


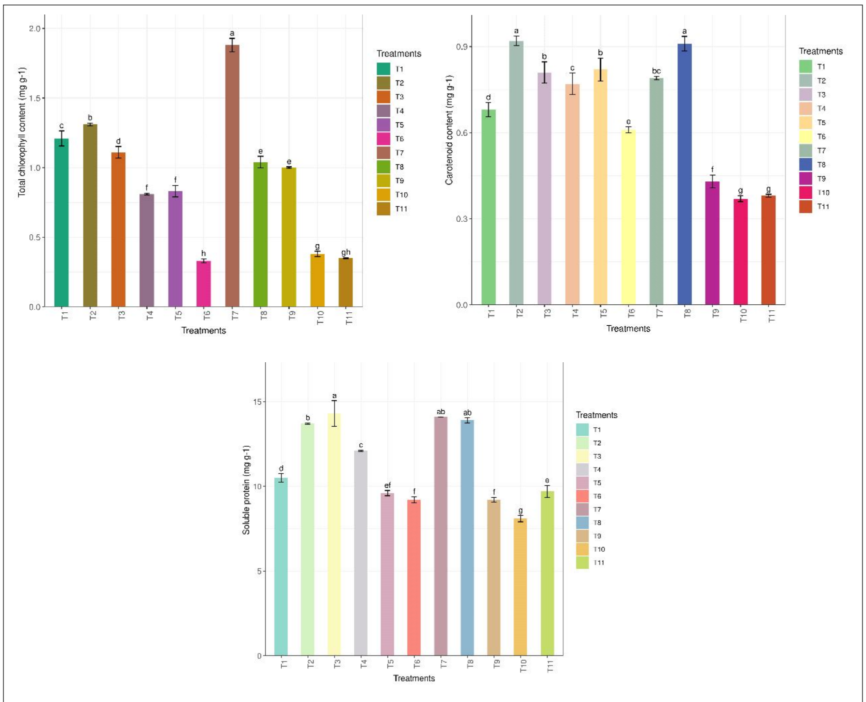
Fig 2: Effect of EMS and EtBr mutagens on seedling injury (%) of cowpea.

Mutagenic effectiveness and efficiency

The results indicate that mutagenic effectiveness and efficiency were greater at low doses to mild doses of mutagens. EMS 20-25 mM and EtBr 0.05-0.10 mM treatments created enhanced seedling growth when compare to control along with less levels of seedling injury. These treatments induced genetic alterations without severely affecting physiological and biochemical processes. In other hand, higher doses of mutagens (EMS 40 mM and EtBr 0.25 mM) resulted in reduced germination, poor seedling growth and increased physiological stress indicating that excessive mutagen exposure leads to deleterious mutations and reduced mutagen efficiency. Thus, moderate doses are more suitable for inducing useful genetic variations while maintaining plant viability (Shamshad *et al.,* 2023; Jayaveer and Sathya, 2025). The similar results were recorded by Shukla *et al.* (2025) who found that a lower concentration of mutagens were more effective in generating viable mutants.

Effect of mutagens on physiological parameters

Significant improvement were recorded in physiological parameters under mutagen treatments (Fig 3). The total chlorophyll content increased at lower concentration of EtBr 0.05 mM (1.88 mg g^{-1}) and EMS 20 mM (1.31 mg g^{-1}) when compared to the control plants (1.21 mg g^{-1}). This enhancement may be due to stimulated chloroplast activity under mild stress conditions (Chandrasekaran *et al.,* 2024). However, at higher doses of EMS 40 mM and EtBr 0.20-0.25 mM, total chlorophyll content declined sharply (0.33-0.38 mg g^{-1}) which



a) Total chlorophyll content (mg g^{-1}); b) Carotenoid content (mg g^{-1}); c) Soluble protein content (mg g^{-1}).

Fig 3: Effect of EMS and EtBr mutagens on photosynthetic characteristics of cowpea.

indicating damage to chloroplast structure and inhibition of photosynthetic pigment biosynthesis. Carotenoid content showed a similar trend with increased levels of mutagen chemicals (EMS 20 mM: 0.92 mg g^{-1}; EtBr 0.10 mM: 0.91 mg g^{-1}) and a marked decline at higher concentrations. Since carotenoids are key protectors against oxidative stress and their reduction at higher doses suggests compromised photoprotection and increased susceptibility to oxidative damage. Soluble protein content increased at lower doses reaching a maximum at EMS 25 mM (14.3 mg g^{-1}) and EtBr 0.05 mM (14.1 mg g^{-1}) when compared to the control plants (10.5 mg g^{-1}). This increase may reflect enhanced metabolic activity and synthesis of stress-related proteins in plant system. However, higher doses led to a decline in protein content, indicating inhibition of protein synthesis and increased degradation due to mutagen-induced damage to genetic and translational machinery (Choudhury and Behera, 2001).

Effect of mutagens on biochemical parameters

The mutagenic treatments increased the biochemical parameters of cowpea plants under lower to moderate concentrations (Fig 4). Nitrate reductase activity increased at lower doses and recorded the maximum activity in EtBr 0.05 mM (32.3 µmol NO_2^- g^{-1} h^{-1}) and EMS 25 mM (30.1 µmol) when compared to the control treatments (20.6 µmol). This suggests that enhanced nitrogen metabolism under mild stress. However, the enzyme activity declined significantly (17.9 µmol in EMS 40 mM and 17.3 µmol in EtBr 0.25 mM) at higher concentrations indicating enzyme inhibition and impaired nitrogen assimilation (Chandrasekaran *et al.,* 2023). Proline content showed a consistent increase with increasing mutagen dose rising from 22.8 µmol g^{-1} in control cowpea plants to 31.1 µmol at EMS 40 mM and 39.6 µmol at EtBr 0.25 mM. This accumulation reflects a typical stress response as proline act as good osmoprotector of plants which stabilize the proteins and membranes and scavenging the reactive oxygen species. The higher accumulation of proline content under EtBr treatments suggests relatively greater stress intensity when compare to other treatments. Total soluble sugars increased at lower concentration of EtBr 0.05 mM (19.2 mg g^{-1}) and EMS 20 mM (18.1 mg g^{-1}) compared to the control plants (15.2 mg g^{-1}). It possibly due to enhanced photosynthetic activity and carbohydrate accumulation under mutagen treatments in cowpea. However, sugar content decreased significantly at higher doses (9.4-10.1 mg g^{-1}), indicating impaired photosynthesis and increased utilization of carbohydrates for stress mitigation and repair processes (Kitao *et al.,* 2022; Kaaviya *et al.,* 2025). A similar result was given by Raina *et al.* (2016) who recorded that moderate mutagen treatments improve key plant metabolic activities whereas higher concentration of chemical lead to

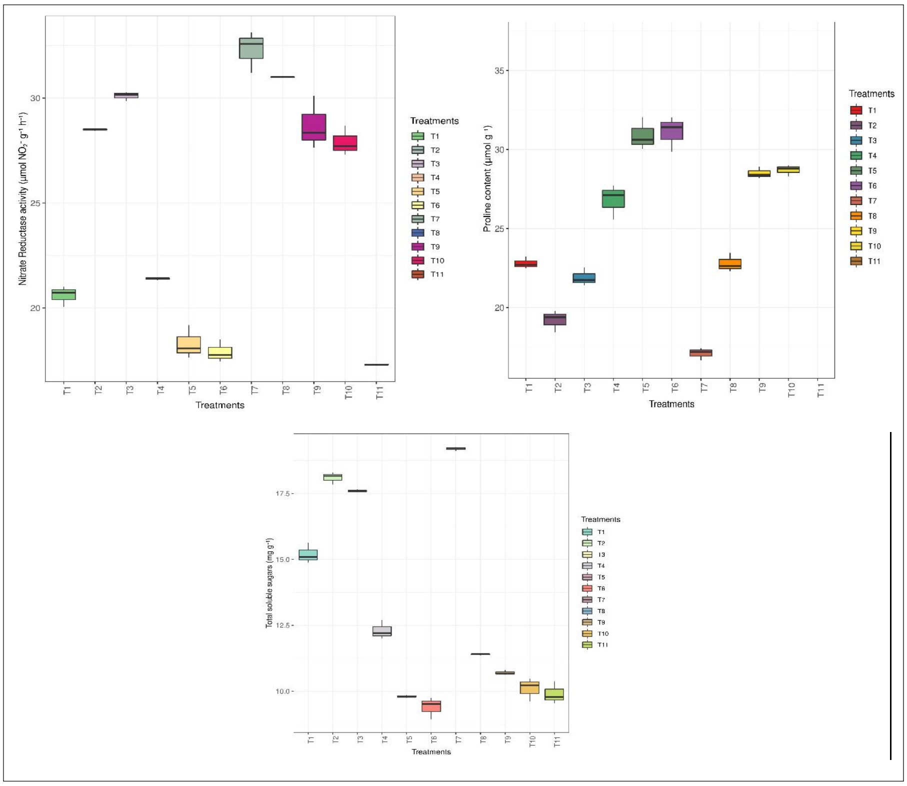


Fig 4: Effect of EMS and EtBr mutagens on nitrate reductase activity (µmol NO_2^- g^{-1} h^{-1}), proline content (µmol g^{-1}) and total soluble sugars (mg g^{-1}) of cowpea.

oxidative stress, enzyme suppression and disruption of primary metabolism.

Principle component analysis

Principal component analysis (PCA) exposed significant variation among the different chemical mutagen treatments (T1-T11) based on seedling growth, physiological and biochemical parameters (Fig 5). The first two principal components of PC1 and PC2 accounted for 88.4% and 5.8% of the total variation respectively which cumulatively explaining 94.2% of the variation among treatments. The PCA biplot analysis (Fig 6) showed that germination percentage, root length, shoot length, seedling height, total chlorophyll content, carotenoid content, soluble protein, nitrate reductase activity and total soluble sugars demonstrated strong positive loadings on PC1. In contrast, seedling injury percentage and proline content were negatively associated with PC1. This suggests that PC1 primarily represents overall seedling vigor and metabolic efficiency under mutagenic stress conditions in cowpea. Treatments located on the positive side of PC1 demonstrated superior performance, higher germination percentage, enhanced growth parameters and improved biochemical activity which indicating better stress tolerance under mutagen treatments. The lower concentrations of EMS (20-25 mM) and ethidium bromide (0.05-0.10 mM) were closely associated with these positive characters reflecting their effectiveness in inducing beneficial variability with minimal physiological damage. Conversely, treatments located on the negative side of PC1 especially higher mutagen doses (EMS 40 mM and EtBr 0.25 mM) were connected with increased seedling injury and proline accumulation along with reduced growth and biochemical responses. This indicates stress-induced metabolic impairment at higher mutagen concentrations. The scree plot further supported these findings showing that PC1 alone contributed the majority of the variation (88.4%) followed by PC2 (5.8%) and PC3 (2.9%) while the remaining parameters contributed negligibly. The sharp decline in variance after PC1 highlights its dominant role in explaining trait variability under mutation treatments. Overall, the PCA results demonstrate that moderate doses of mutagens

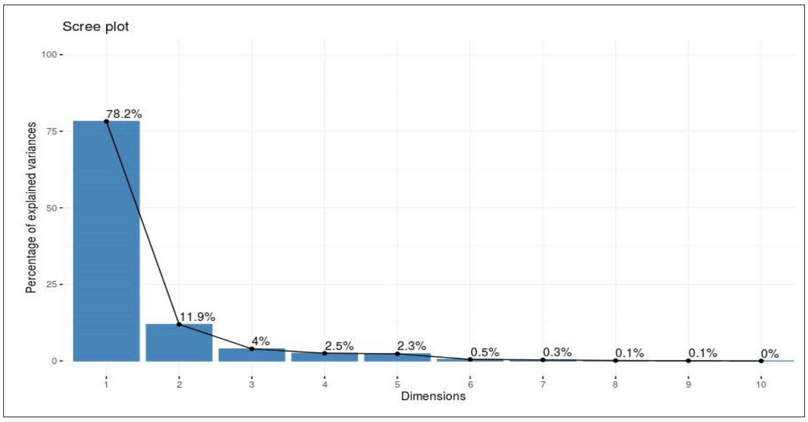


Fig 5: Scree plot analysis of mutagen effects on cowpea.

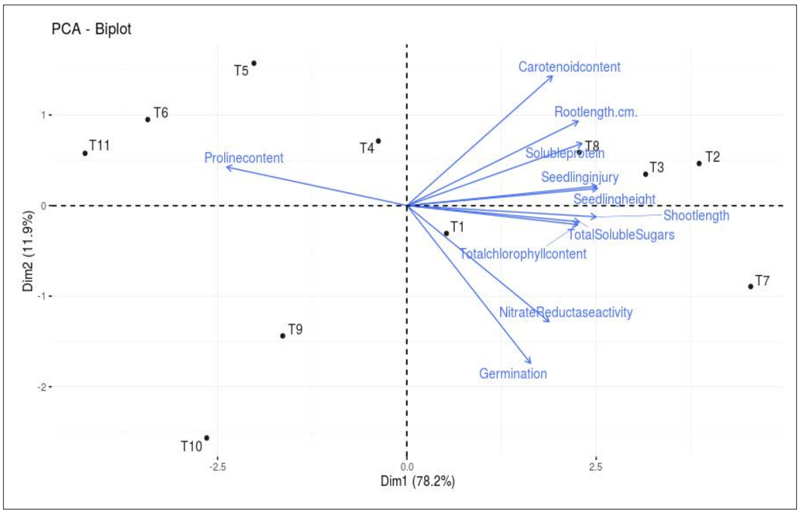


Fig 6: PCA biplot showing relationship among treatments and traits.

Table 1: Summary of growth, physiological and biochemical characters of cowpea under EMS and EtBr treatments.

Characters	Control	Moderate doses (EMS 20-25 mM/ EtBr 0.05-0.10 mM)	Positive responses by mutagens
Germination (%)	95.2%	Increased up to 96.6%	Improved germination at low doses of mutagens.
Root length	Moderate	Increased	Enhanced root growth.
Shoot length	Moderate	Increased	Improved shoot elongation.
Seedling height	Moderate	Enhanced	Increased vigor.
Seedling injury (%)	Low	Moderate	Minimal injury at optimal dose.
Total chlorophyll (mg g^{-1})	1.21	Improved up to 1.88	Enhanced photosynthetic efficiency.
Carotenoids (mg g^{-1})	Moderate	Increased	Improved photoprotection of leaves.
Soluble protein (mg g^{-1})	10.5	Increased up to 14.3	Enhanced metabolic activity and photosynthetic efficiency.
Nitrate reductase activity	20.6	Increased up to 32.3	Improvement of nitrogen metabolism.
Proline (µmol g^{-1})	22.8	Increased	Induction of plant stress tolerance mechanism .
Total soluble sugars (mg g^{-1})	15.2	Increased up to 19.2	Better carbohydrate accumulation.
Mutagenic efficiency	-	High	Optimal mutation induction for positive responses.

are optimal for generating useful variability for maintaining physiological and biochemical stability. Traits positively associated with PC1 can serve as reliable selection criteria for identifying superior genotypes in cowpea mutation breeding whereas proline content and seedling injury act as indicators of stress severity.

The positive responses of various growth, physiological and biochemical characters under moderate mutagenic treatments are summarized in Table 1.

CONCLUSION

The present study demonstrated that optimal level of EMS and Ethidium Bromide significantly improved seedling growth, physiological and biochemical parameters in cowpea plants. Lower to moderate concentrations of EMS (20 and 25 mM) and EtBr (0.05 and 0.10 mM) enhanced germination rate, root and shoot growth and seedling height, indicating a stimulatory effect of induced mutation. These treatments also improved total chlorophyll content, carotenoid content, soluble protein, nitrate reductase activity and total soluble sugars reflecting better metabolic activity of cowpea plants. In contrast, higher concentrations (EMS 35 and 40 mM and EtBr 0.20 and 0.25 mM) caused significant decline in growth and physiological traits due to increased cellular damage by mutation stress. Proline content and seedling injury percentage increased with increasing mutagen dose indicating stress response and metabolic imbalance. The LD_{50} concentration was identified between EMS 30-35 mM and EtBr 0.15-0.20 mM suggesting the threshold for optimal mutagenic effect. Principal component analysis revealed that PC1 explained the majority of variation and was positively associated with growth and biochemical traits while negatively associated with stress indicators. Overall, the study highlights that moderate mutagen doses are ideal for inducing useful variability while maintaining plant vigor. These findings provide a strong basis for selecting suitable mutagen doses in cowpea improvement programs.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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