Mutagenic Effectiveness and Efficiency of Chemical Mutagens in Developing Novel Variability in M_2 Population of Sunn Hemp (*Crotalaria juncea* L.)

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Abstract

This study assessed the effectiveness and efficiency of three chemical mutagens, ethyl methane sulphonates (EMS), diethyl sulfate (DES), and sodium azide (SA), in producing genetic diversity in the M_2 generation of sunn hemp (Crotalaria juncea L.). Seeds were exposed to varying concentrations of EMS (30, 35, and 40 mM), DES (25, 30, and 35 mM), and SA (20, 25, and 30 mM). A diverse range of chlorophyll and viable morphological mutants influencing leaf shape, growth habit, and seed properties were found. EMS at 35mM caused the most chlorophyll and morphological changes, but SA at 20-25mM demonstrated higher mutagenesis effectiveness and efficiency with less biological damage. DES caused significantly less mutations. The findings revealed that intermediate mutagen dosages were more effective than higher levels at producing useful genetic diversity. The identified viable mutants represent excellent genetic resources for mutation breeding and future crop enhancement projects in sunn hemp.

Keywords: *Crotalaria juncea*, chemical mutagenesis, ethyl methane sulphonate (EMS), diethyl sulfate (DES), sodium azide (SA), chlorophyll mutants, morphological mutants, mutagenic effectiveness, mutagenic efficiency, mutation breeding.

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INTRODUCTION

Sunn hemp (*Crotalaria juncea* L.) is a tropical Asian plant that is a member of the Fabaceae family of legumes. It is sometimes called brown hemp, Madras hemp, or Indian hemp. *Crotalaria juncea* is a native of India, where it is grown for its fiber (Sengupta and Debnath, 2018). Because of its high nitrogen content, crotalaria is also being used more frequently as green manure, which improves soil quality and crop-system sustainability by lowering nitrogen inputs, N_2O emissions, and nutrient leaching (Kumar and Bordoloi, 2024). Breeders have favoured mutation breeding as an alternative method because it makes it possible to create traits that are either lost during evolution or do not exist in nature. Using the mutation breeding method, new variations can be produced quickly (Radwan *et al.,* 2025). Mutagenesis has proven effective in generating genetic diversity across various crops. This technique has allowed researchers to identify mutants with beneficial characteristics, including higher seed yields, earlier maturation (Wongyai *et al.,* 2001), altered plant structures, closed seed capsules, disease resistance, improved seed retention, larger seed size, desirable seed color, and increased oil content(Cagirgan, 1996). Induced mutagenesis may result in traits that are inherited. The use of radiation and the application of chemicals are the two axes of anthropogenic mutagenesis. While chemical mutagenesis primarily uses alkylating chemicals like ethyl methane sulfonate, methyl methane sulfonate, colchicine, and sodium azide, physical mutagenesis uses radiation such as gamma rays, X-rays, ions, etc. The results showed that chemical mutagens worked better in *Vicia faba* L. more than actual mutagens (Agarwal *et al.,* 2025). Effective doses and mutation frequencies are typically determined using the rates of germinated or surviving plants (Ahumada-Flores *et al.,* 2020), plant developmental retardation (kodym *et al.,* 2012), and chlorophyll mutations (Ciftci and Senay, 2005). In plant breeding programs, chlorophyll mutations are successfully employed as genetic markers to show the effects of mutagens and the response of the target genotype to the mutagen (Wani *et al.,* 2011). Chlorophyll mutations are also used as genetic markers in basic and applied research and are considered the most reliable indices for evaluating the efficiency of different mutagens in inducing the genetic variability for crop improvement. Chlorophyll mutations have been reported in several crops after treatments with physical and chemical mutagens (Kolar *et al.,* 2011).

Chlorophylls are vital pigments that plants use for photosynthesis (Yu *et al.* (2014). Chlorophyll mutations are frequently fatal to plants because they inhibit photosynthesis (Wani *et al.* (2011). The M_2 seedlings chlorophyll mutants were screened between days 45th and 50th. The *Kalmegh* was the location of the different chlorophyll mutants (Kasthuri and Dhanavel, 2020). The nomenclature used by (Gustafesson, 1940) was used to identify the chlorophyll mutants. Since the turn of the century, mutation breeding has proven to be one of the main forces behind evolution. In the current study, the frequency and spectrum of morphological and chlorophyll mutants in the M_2 generation of *Crotalaria juncea* were examined in relation to the effects of EMS, DES, and SA.

MATERIAL AND METHODS

Plant material

Sun hemp (*Crotalaria juncea* L.) The Tamil Nadu Agricultural Research Institute in Coimbatore is the source of the healthy, dry seeds. Chemical mutagens such as Sodium azide, Diethyl sulfate, and Ethyl methane sulphonate were used at different concentrations to cause mutagenesis.

Mutagenic Treatment (Chemical mutagen)

Crotalaria juncea L. seeds, which were healthy and completely pure, were treated with Ethyl methane sulphonate, Diethyl sulfate and Sodium azide. Treatments (50gm seeds for treatments) consist of ten different concentrations of EMS (30mM, 35mM, 40mM), DES (25mM, 30mM, 35mM), and SA (20mM, 25mM, 30mM),. The EMS, DES and SA treated seeds were pre-soaked in distilled water for six hours at room temperature and covered by a moist germination paper. After that, the treated seeds were washed in a running tap water. The untreated seeds were respectively considered as a control. The seeds were sown in the field by following the Randomized Block Design (RBD) method along with control to raise M_2 generation.

Chlorophyll mutation

Following treatment, the treated and control seeds were sown right away. (Gustaffson, 1940) description of chlorophyll mutations was scored during the M_2 generation plant growth period. At the time of maturation, data on chlorophyll mutants were gathered, documented, and expressed as a percentage of the control. Seeds were collected according to the dose and concentration of the mutagen after the M_1 generation emerged. The M_2 generation, which was raised from the M_1 generation, produced morphological mutants of chlorophyll on the 45th day.

$$\text{Mutation frequency} = \frac{\text{Mutated plants observed}}{\text{Plants observed in M2}} \times 100$$

Morphological mutations

The frequency of morphological mutations was calculated on the basis of M_2 plants. While counting total number of leaves per plant (leaf index) and measuring leaf dimension, we observed certain modification in leaf morphology, particularly in leaf shape in the mutagenized population. Initially different forms (shapes) of leaves in control plant were identified first and then number of plants with modification in lead, in term of leaf apex, base, margin and lamina, was counted. Data on percent frequency of number of plant with leaf variation as well as number of variant leaves was recorded, averaged and presented in tabular form.

$$\text{Mutation frequency} = \frac{\text{Number of mutant seedlings}}{\text{Total number of M2 seedlings}} \times 100$$

Isolation of mutants

The following chlorophyll mutants and morphology mutants, including striata, Xantha, Albina green, Viridis, tinch effect, semi-aurea, semi-chlorina, and semi-viridis were found in the M_2 generation from EMS, DES, and SA concentration at the 45th day, opposite leaves, linear shape, falcate (sickle shaped), curling leaf, trifoliate leaf with oblong lanceolate leaf, asymmetrical lamina with incised leaf, asymmetrical lamina with incised leaf, asymmetrical lamina with pointed apex, bi-lobed or emarginated apex, rectangular lamina with pointed apex, bi-foliate compound leaf or adnation of petiole or broad stem mutant, altered leaf shape and venation pattern. The M_2 seed was used to calculate the mutation frequency. The mutation frequency was estimated on M_2 seedling basis. In the M_2 generation, morphological and physiological mutants observed were classified based on the stature, duration and leaf shape. Mutation frequency was estimated on M_2 plant basis. Mutagenic effectiveness is a measure of the frequency of mutation induced by unit mutagen, whereas mutagenic efficiency gives an indication of the proportion of mutation in relation to undesirable changes like lethality and injury. The effectiveness and efficiency of the mutagens namely, EMS, DES and sodium azide were worked out by using the formulae suggested by (Konzak *et al.,* 1965).

$$\text{Mutagenic effectiveness} = MF \times 100 / C \times T$$

$$\text{Mutagenic efficiency (Lethal)} = MF \times 100 / L$$

$$\text{Mutagenic efficiency (Injury)} = MF \times 100 / I$$

Where

MF = Mutation frequency for 100 M_2 plants

T = Period of treatment with chemical mutagens in hours

C = Concentration of mutagen in mM in percent
L = Percentage of lethality or survival reduction
I = Percentage of injury or reduction in seedling size

RESULT

Both micro and macro mutants are crucial for determining mutagen concentrations, with nearly all mutagenic treatments resulting in varying degrees of mutants at their respective concentrations. EMS was found to generate a greater number of chlorophyll and morphological mutants compared to DES and SA. Compared to DES and SA, EMS resulted in a greater variety of mutations, including chlorophyll mutants and viable mutants affecting leaf morphology, growth habit, and seeds mutants. The study aimed to determine the economic potential of viable mutants and the impact of induced variability on quantitative features in the M_2 generation. Overall, parameters have been decreased in M_1 generation at maturity time. (Jana, 1964) found that mutagens and dry seeds reduced development in *Phaseolus mungo*. According to (Fehr, 1987), sensitivity to mutagen treatments varies between species and genotypes within each species. Increasing the concentration of chemical mutagens reduced development and other quantitative features.

Chlorophyll mutants

The frequency of chlorophyll mutants in M_2 generation is primarily employed as a reliable indicator of mutagen-induced genetic consequences (Nilan and Konzak, 1961). Chlorophyll mutations typically arise within 15th days of seed germination, resulting in chlorophyll deficiency in the main leaves (Stummann and Henningsen, 1980).The most crucial component of the cell system that gives plants the ability to synthesize food on their own is chlorophyll. Even after a generation of treatment, a deficiency in the production of chlorophyll in leaf cells indicates a decrease in the synthesis of various chlorophyll components as a result of the action of mutagens. Similar to this, these mutagenic treatments were discovered to prevent plants from developing their typical morphological dimensions, resulting in variation (Deshpande and Malode, 2021). Our experiment generated different kinds of chlorophyll mutants were found in this investigation. These include striata, Xantha, Albina green, Viridis, tinch effect, semi-aurea, semi-chlorina, and semi-viridis. Frequencies of chlorophyll mutations were determined as a percentage of M_2 plants (Table 1; Figure 1; Plate 1). According to (Lalitha *et al.,* 2020), mutations in the genes responsible for chlorophyll synthesis result in the production of various kinds of chlorophyll mutants. While analysing the results, it emerged that the frequency of mutations climbed as mutagen concentrations increased. EMS was the most successful mutagen, with the highest frequency of total chlorophyll mutations. As 35mM EMS, the highest frequency (7.06%) was recorded indicating an ideal mutagenic dosage. As 30mM (3.97%) and 40mM (3.03%), lower frequencies were observed, suggesting a decrease at higher concentrations as a result of increasing mortality or physiological damage. These findings are consistent with the previous observation of (Auti, 2012; Vairam *et al.,* 2017; Sakar and Kundagrami, 2018). DES treatments caused modest induction of chlorophyll mutations, with the highest frequency (3.51%) at 25mM, subsequent to 30mM (3.19%), and a significant reduction at 35mM (1.92%), implying dose-dependent suppression at higher concentrations. These results agree with earlier reports in black gram and barley showing higher chlorophyll mutation frequency at intermediate doses and reduction at higher concentrations (Arulbalachandran and Mullainathan, 2009; Rao, 1972). SA treatments also caused significant chlorophyll changes, with the highest frequency (5.25%) at 25mM, followed by 20mM (3.17%) and 30mM (3.01%), indicating an ideal intermediate concentration. (Gowtham *et al.,* 2018) investigated the mutagenesis efficiency of sodium azide in Indian mustard. Across all treatments, the most common mutants were Viridis, Xantha, Semi-viridis, and Semi-chlorina, with Albina and Striata appearing less frequently. The data clearly show that moderate mutagen concentrations were more successful at generating chlorophyll mutations than higher levels (Table 1; Plate 1).

Spectrum and Frequency of chlorophyll Mutation

Albina Green

Plants with the albina mutation have completely white leaves, stems, and other tissues because they produce no green pigment. The frequency of albina green mutations varied depending on the mutagen and concentration, with DES at 30mM (0.87%) having the highest frequency, followed by SA at 25mM (0.75%). Moderate frequencies were obtained in DES at 25mM, EMS at 40mM, and SA at 30mM, while lower values were observed in EMS at 30-35mM and SA at 20mM. There were no albina green mutants found in DES at 35mM.

Viridis

Seedlings begin dark green and transition to regular green as they mature. Mutants develop normal-looking blooms and set seeds. The frequency of Viridis mutations varied by mutagen and concentration, with the highest frequency found in EMS at 35mM (1.57%), followed by EMS at 40mM (0.95%) and DES at 30mM (0.87%). Moderate frequencies were recorded in EMS at 30mM (0.72%) and SA at 20mM (0.71%), while

lower values were observed in SA at 30mM (0.43%), 25mM (0.37%), and 35mM (0.32%). There were no viridis mutants detected in DES at 25mM.

Tinch effect

Yellow and green spots were seen on the entire leaf. The seedling lived until maturity. The frequency of Tinch effect mutations varied with mutagen concentration, with DES at 30mM (1.16%) having the highest frequency, followed by SA at 30mM (0.86%) and EMS at 35mM (0.78%). Moderate frequency were recorded in EMS at 30mM (0.36%), SA at 20mM (0.35%), and DES at 25mM (0.27%), however no tinch effect mutants were detected in EMS at 40mM, DES at 35mM, and SA at 25mM.

Semi-Aurea

Characterized by yellow golden leaves due to deficiency of chlorophyll content. In beginning half of the leaves are golden yellow in colour. The incidence of semi-aurea mutations varied according to mutagen and concentration, with the maximum frequency found in SA at 25mM. This was followed by 25mM DES (0.54%) and 40mM EMS (0.47%). Moderate rates were found in EMS at 35mM (0.39%), EMS at 30mM (0.36%), SA at 30mM (0.43%), and SA at 20mM (0.35%), whereas DES at 35mM had a lower frequency (0.32%). No semi-aurea mutants were found in DES at 30mM.

Striata

Leaves display clear stripes, streaks, or bands of lighter (yellow, white) and normal green colors running lengthwise. A mutation in genes responsible for chlorophyll synthesis or chloroplast development. Striata mutation frequency was only found in EMS and SA treatments, with the greatest value at 40mM (0.47%), followed by 35mM (0.39%) and 30mM (0.36%). SA at 20mM had a low frequency (0.35%), but no striata mutants were detected in either DES treatments or SA at 25 and 30mM.

Xanthan

Mutants have colors ranging from deep yellow to yellowish white. Mutants grow slowly and frequently die within 17-20 days of emergence.The highest xanthan mutation frequency was seen in EMS at 35mM (1.18%) and SA at 25mM (1.50%). Lower frequencies were detected in EMS at 30mM (0.36%), DES at 25mM (0.81%), and 35mM (0.64%), however no xanthan mutants were found at EMS 40mM, DES 30mM, or SA 20mM.

Semi-Chlorina

At early stage leaves are half part of the leaves are ligtht yellow in colour called as semi-chlorina. The frequency of semi-chlorina mutations differed depending on the mutagen and concentration. The highest frequency of EMS was seen at 35mM (1.18%), which was followed by 40mM (0.47%). At 30mM, no mutations were found. At 25mM (1.08%), 35mM (0.64%), and 30mM (0.29%), DES treatments showed moderate frequencies. Semi-chlorina mutants were found in SA at 20mM (0.71%) and 30mM (0.43%), but not at 25mM.

Semi-Viridis

The initial light green (viridine green) color is due to impaired chlorophyll biosynthesis or accumulation in the early growth stages. EMS had the highest frequency of semi-viridis mutations at 30mM (1.45%), followed by 35mM (1.18%) and 40mM (0.47%). Only at 25mM (0.27%) did DES exhibit a low frequency; at greater dosages, there were no mutants. Semi-viridis mutants were found in SA treatments at 20mM (0.35%) and 25mM (0.75%), but no mutants were found at 30mM.

The proportion of total chlorophyll mutations varied clearly between mutagens and dosages. With the highest frequency at 35mM (7.06%), EMS treatments were the most successful; lower values were noted at 30mM (3.97%) and 40mM (3.03%). While DES treatments showed somewhat lower frequency, with a maximum at 25mM (3.51%) and a minimum at 35mM (1.92%), SA also caused significant mutations, especially at 25mM (5.25%). The mutants reproduce true for the altered characteristics. Similar observation were by (Fridose Kolar *et al.,* 2011) in *Delphinium malabaricum*, (Gnanamurthy *et al.,* 2011), (Rajani Singh, 2011) in *Artemisia annua,* (Girija and Dhanavel, 2009) in cowpea; (Thilagavathi and Mullainathan, 2009; Arulbalachandran, 2006) in black gram; (Solanki, 2005) in lentil; (Jayakumar and Selvaraj, 2003) in sunflower; (Pavadai, 2006) in soybean; (Mensah and Obadoni, 2007) in *Arachis hypogaea.*

Viable mutants

Morphological mutations - Leaf Morphology, Growth Habit and Seed mutant

As a photosynthetic organ that is involved in food synthesis, the number of leaves per plant and the surface area of the leaf lamina are directly correlated with plant growth and development, which in turn controls biomass production (Aney and Choudhary, 2018). Various morphological mutations were identified in the Sun hemp M_2 population. These mutations include characteristics that leaf morphology (Linear shape, Falcate (Sickle shaped), Opposite leaves, Incised leaf, Crumpled leaf, Curling leaf, Trifoliate leaf with Oblong lanceolate leaf, Asymmetrical lamina with incised leaf, Asymmetrical lamina-2, Lobed apex, Lobed apex, Curved lamina apex, Curved apex, Fused leaflet or Acme apex, Leaf with emarginated apex, Rectangular lamina with pointed apex, Bi-lobed apex or Emarginated apex, Bi-foliate compound leaf or Adnation of petiole or Broad stem mutant, Altered leaf shaped and venation pattern of mutant), growth habit (Broad stem mutant, Entire plant with Notches leaf, Dwarf mutant), Seed mutant (Light

brown and black coated, Dark brown with black color and small sized, Bold black color, Yellowish brown color, Wrinkle surfaced with black color and white patches, Yellowish green color, Smooth and white). Mutation frequency was estimated on an M_2 plant basis (Table 1; Plate 2-6).

Linear shape mutant

The linear leaf form mutant had narrow, elongated leaves with a smaller lamina width. It appeared mainly in EMS, DES, and SA treatments, with the highest prevalence in EMS at 35mM and absence at SA 20mM. This characteristic is indicative of mutagen-induced changes in genes that influence leaf growth.

Falcate (Sickle-shaped) mutant

The falcate (sickle-shaped) leaf mutant had curled leaves with a distinctive sickle-like morphology. It happened seldom with EMS and DES treatments, but more frequently in SA at 25mM. This mutant reflects mutagen-induced alterations in genes that control leaf curvature and lamina symmetry.

Opposite leaves mutant

The opposite leaf mutant has paired leaves at a single node rather than the regular alternate pattern. It appeared infrequently under EMS and DES, with considerably increased expression at lower concentrations, particularly EMS 30mM and SA 20mM, indicating mutagen-induced disruption of phyllotaxy control.

Incised leaf mutant

The incised leaf mutant, which has deeply sliced or serrated leaf margins, appeared at a very low frequency across all mutagen treatments. EMS and DES only produced a few incised leaf mutants, but SA produced slightly more, especially at 20 and 30mM. This mutant reflects mutagen-induced changes in genes that regulate leaf margin growth and lamina differentiation.

Crumpled leaf mutant

The crumpled leaf mutant, which has wrinkled and warped leaf surfaces, was observed at a low frequency. It emerged exclusively at higher EMS concentrations (40mM) and in SA treatments at 25 and 30mM, but not in any DES treatments, showing concentration-specific mutagenesis effects on leaf growth and lamina formation.

Curling leaf mutant

The curling leaf mutant, which is distinguished by inward or outward rolling of leaf margins, occurred at a very low frequency in all treatments. It was only detected at EMS 35mM, DES 25mM, and SA 20-25mM, indicating a random and concentration-dependent mutagenesis effect on leaf development and polarity.

Trifoliate leaf with Oblong lanceolate leaf mutant

The trifoliate leaf with oblong-lanceolate leaflet mutant was found infrequently in all mutagen treatments. EMS at 30mM and SA at 20mM were more common, but DES only produced a few mutants at intermediate doses. This variance shows that mutagens altered genes that control leaflet quantity and shape.

Asymmetrical lamina with incised leaf mutant

The asymmetrical lamina with incised leaf mutant, which is defined by unequal leaf halves with cut borders, emerged infrequently throughout treatments. It was more common at EMS 35mM and DES 30mM, with occasional occurrences in SA, indicating mutagen-induced disruptions in lamina symmetry and marginal development.

Asymmetrical lamina-2, mutant

The asymmetrical lamina-2 mutant, which has irregular and unequal leaf lamina, appeared at a low frequency. It was only found at concentrations of EMS 30mM, DES 25mM, and SA 20mM, demonstrating that mutagenesis effects on leaf symmetry are rare and concentration dependent.

Lobed apex mutant

The lobed apex mutant, which has a noticeably lobed leaf tip, was seen in all mutagen treatments with varying frequency. EMS at 35mM had the highest occurrence, followed by DES at 30mM and SA at 20mM, whereas higher concentrations generally lowered expression. This pattern indicates dose-dependent mutagenesis effects on genes involved in leaf apex development.

Lobed apex mutant

The lobed apex mutant occurred infrequently across all mutagen treatments. DES 30mM and SA 30mM revealed somewhat increased expression, whereas EMS showed consistent but modest incidence across concentrations, demonstrating a mild and dose-independent mutagenesis effect on leaf apex development.

Curved lamina apex mutant

The curved lamina apex mutant, which is distinguished by the bending of the leaf tip, was documented at low frequency. It was more common under EMS 35mM, with occasional occurrence in DES treatments, but completely absent at all SA concentrations, demonstrating mutagen-specific effects on lamina apex formation.

Curved apex mutant

The curved apex mutant, which is distinguished by bending of the leaf tip, was found at low to moderate frequency across mutagen treatments. Higher incidence was observed in DES at 30mM, followed by EMS at 35mM, but SA displayed only sporadic expression, showing mutagen- and concentration-dependent effects on leaf apex development.

Fused leaflet or acme apex mutant

The fused leaflet or acme apex mutant, which is characterized by partial leaflet fusion or a pointed apex, was observed seldom across all treatments. EMS 35mM and SA 30mM revealed greater expression, whereas DES produced only a

few mutants, demonstrating that mutagens disturb leaflet separation and apex differentiation.

Leaf with emarginated apex mutant

The leaf with emarginated apex mutant, defined by a notched leaf tip, emerged seldom throughout treatments. It was more common at EMS 35mM and DES 30mM, with occasional occurrence in SA, suggesting mutagen-induced changes in leaf apex differentiation.

Rectangular lamina with pointed apex mutant

The rectangular lamina with pointed apex mutant was observed infrequently across mutagen treatments. EMS had a larger prevalence, particularly at 40mM, but DES and SA produced only a few mutants, demonstrating mutagen- and dose-specific effects on lamina shape and apex development.

Bi-lobed apex or Emarginated apex mutant

The bi-lobed or emarginated apex mutant, with a leaf tip divided into two lobes or notched, occurs seldom across treatments. EMS 30&35mM showed considerably higher expression, whereas DES caused few or no mutants, and SA showed rare occurrence, showing mutagen- and concentration-dependent effects on leaf apex shape.

Bi-foliate compound leaf or Adnation of petiole mutant

The bi-foliate complex leaf, petiole adnation, and broad stem mutants all appeared seldom across treatments. EMS at 30 and 40mM, as well as SA at 20&25mM, produced only infrequent expression, whereas DES produced none, demonstrating that mutagen-induced changes in leaf complexity and stem morphology are rare.

Altered leaf shaped and venation pattern of mutant

The altered leaf form and venation pattern mutant, which changed the overall shape of the leaf and the arrangement of the veins, was rare. EMS 35mM caused the most mutations, while DES and SA caused only a few, demonstrating that mutagens and concentrations had different impacts on leaf morphology and vascular patterning.

Broad stem mutant

The broad stem mutant, distinguished by an exceptionally thickened stem, emerged seldom throughout treatments. EMS 35mM had the highest prevalence, with infrequent expression in DES and SA, suggesting concentration- and mutagen-specific impacts on stem morphology.

Entire plant with Notches leaf mutant

The complete plant with notched leaves mutant, which is distinguished by leaves with irregular notches throughout the plant, occurred at low to moderate frequency across treatments. EMS at 40mM and SA at 30mM exhibited increased expression, while DES only produced a few mutations, demonstrating mutagen- and concentration-dependent effects on overall leaf shape.

Dwarf mutant

The dwarf mutant, which causes plants to grow shorter, occurred seldom across treatments. EMS at 40mM and DES at 35mM exhibited increased expression, while SA and other dosages produced only infrequent mutants, demonstrating dose-dependent effects on plant growth.

Light brown and black coated mutant

The light brown and black seed coat mutants, which cause altered seed color, occurred infrequently across all treatments. EMS (40mM) and DES (25mM) had slightly higher expression, while SA caused infrequent mutants, demonstrating mutagen- and concentration-specific effects on seed coat color.

Dark brown with black color and small sized mutant

The dark brown with black and small-sized seed mutant, which has reduced seed size and changed pigmentation, occurred at a low to moderate frequency in all treatments. EMS at 30mM and SA at 20 and 25mM exhibited increased expression, but DES caused infrequent mutants, demonstrating mutagen- and concentration-specific effects on seed size and color.

Bold black color mutant

The bold black seed color mutant, which is distinguished by deep black pigmentation of seeds, occurred at a low to moderate frequency across treatments. EMS at 40mM and SA at 25 and 30mM resulted in greater expression, while DES only produced infrequent mutants, demonstrating mutagen- and concentration-specific effects on seed coat coloration.

Yellowish brown color mutant

The yellowish-brown seed color mutant, which has light brown to yellowish pigmentation, appeared at low to moderate frequency across all treatments. EMS at 30mM and SA at 20mM exhibited increased expression, while DES generated fewer mutations, showing mutagen- and concentration-dependent effects on seed coat color.

Wrinkle surfaced with black color and white patches mutant

The wrinkle-surfaced seed with black tone and white patches mutant, which has an uneven seed surface and variegated pigmentation, occurred at low to moderate frequency in all treatments. SA and EMS at 25 and 30mM demonstrated increased expression, while DES generated fewer mutations, suggesting mutagen- and concentration-specific effects on seed surface and coat patterning.

Yellowish green color mutant

The yellowish-green seed color mutant, distinguished by a faint greenish hue, occurred seldom across treatments. EMS at 30mM and DES at 25mM increased expression marginally, but other doses and SA treatments resulted in occasional mutants, demonstrating mutagen- and dose-specific effects on seed pigmentation.

Smooth and white seed mutant

The smooth and white seed mutant, with a homogeneous, light seed coat, appeared at low to moderate frequency across treatments. EMS at 35mM and DES at 25mM exhibited increased expression, while SA produced fewer mutants, showing mutagen- and concentration-specific effects on seed coat texture and color.

EMS was the most efficient mutagen tested, resulting in the highest mutation frequency (25.19%) at 35mM, indicating an optimal dose. SA treatments were moderate to very effective, with the highest mutation frequency at 30mM (22.60%), followed closely by 25mM (21.88%). DES was less successful, with mutation rates ranging from 11.44% to 16.95% with a peak at 30mM. Overall, mutation frequency showed a dose-dependent response, increasing up to an optimal concentration before dropping at higher dosages, probably due to increased physiological damage and mortality. A similar observation was made by (Gaul, 1964; Swaminathan, 1964; (Aney Avinash and Choudhary Arvind, 2018 in *Rivinia humilis*) (Rajani Singh, 2011 in *Artemisia annua* (Roshan Jahan *et al.,* 2020 in *Linum usitatissiumum*).

Mutagenic Effectiveness and Efficiency

The mutagenic effectiveness and efficiency were assessed using chlorophyll and viable mutants. Plant survival and height were reduced when exposed to EMS, DES, and sodium azide mutagens (Table -3; Figure 3; Plate 3). The mutagenic efficiency measures the proportion of mutations caused by a mutagen compared to other undesired biological outcomes as damage, death, and sterility (Konzak *et al.,* 1965).The study concluded that EMS outperformed DES and sodium azide in terms of effectiveness. Mutagenic effectiveness and efficiency diminish with increased mutagen concentrations, up to a specific level. EMS was more successful than DES and sodium azide in generating mutations (Table 2).

At optimal dose, EMS exhibited a moderate to high mutation frequency while causing significantly less biological harm. The 35mM EMS treatment had the highest mutation frequency (32.28%) with balanced survival and height loss, resulting in a high injury efficiency (80.78) and good efficacy. At 40mM, greater lethality and damage reduced both efficacy and efficiency, indicating an overdose. DES treatments resulted in fewer mutations than EMS and SA. Although survival and height decrease were high, mutation induction remained moderate, resulting in reduced effectiveness and efficiency. Among DES treatments, 30mM outperformed 25 and 35mM, indicating that sunn hemp has little mutagenesis potential. SA displayed extremely high mutagenic efficacy and efficiency, particularly at 20 and 25mM. Despite the decreased height reduction, mutation frequency remained high, yielding maximum effectiveness (119.73 at 20mM) and extraordinarily high efficiency values for both mortality and damage. However, at 30mM, increased biological damage limited effectiveness. SA was the most effective and efficient of the three mutagens, followed by EMS, with DES being the least effective. SA (20 and 25mM) and EMS (35mM) produced higher mutation frequencies while causing less biological damage, making them appropriate dosages for mutation breeding in *Crotalaria juncea* L. (Arulbalachandran and Mullainathan, 2009; Wani *et al.,* 2011).

Disscussion

Induced mutations create more variety in agricultural plants than spontaneous mutations (Chopra, 2005). Screening and selecting morphogenic mutants based on phenotypic characteristics is a basic rule for a successful breeding plan. Most mutant characters are determined by recessive genes, which can be sorted through selection. In segregating populations, physical characteristics and visual screening are the most effective ways to find and select mutations. The mutation frequency percentage indicates the amount of mutation in plants. The M_2 generation comprises chlorophyll and viable mutants, which are crucial in determining the genetic impact of mutagens (Kulkarni and Mogle, 2013). During M_2 generation, mutations are segregated to identify homozygotes for recessive or dominant alleles (Page and Grossniklaus, 2002). (Nerker, 1973) explains the evolutionary implications of leaf mutations. To accurately identify intra- and inter-specific variances in order to efficiently collect and preserve genetic resources. Leaf shape is an important aspect that must be assessed (Misra *et al.,* 1994). Chlorophyll synthesis in plants is the end consequence of an extended chain of metabolic processes involving several loci. According to (Von Wettsein *et al.,* 1971), nuclear genes influence plastid biogenesis. The author discovered that chlorophyll synthesis in high plants is controlled by nuclear genes through regulatory products. Other scientists (Van Harten, 1998) believe that chlorophyll production is controlled by nucleus and non-nuclear (cytoplasmic) genes. Chlorophyll mutations are employed to assess the genetic activity of mutagenic agents (Svetleva, 2005). They are the most often reported and easily identifiable factorial mutations in M_2 generation so far (Sanjai Gandhi *et al.,* 2014). In mutagenesis, inducing chlorophyll mutants helps examine the genetic impact and sensitivity of various mutagens (Neto *et al.,* 2011). The frequency of chlorophyll mutants is a solid indicator of a mutagen's power, capability, influence, efficacy, and potency due to its high scoring precision (Girija and Dhanavel, 2009; Maluszynski *et al.,* 2009; Gustafsson, 1940; Mackay,1951). Combining mutagens increased the

frequency of Albina and Chlorina mutants compared to separate dosages, possibly due to synergistic effects. Previous studies in *Cicer arietinum* (Kozgar, 2014) and *Linum usitatissimum* (Bhat *et al.,* 2016) have shown synergistic effects of multiple mutagen dosages. Synergism may occur when the first mutagen exposes protected mutable sites to the second mutagen, rendering repair enzymes ineffective and indirectly facilitating mutation fixation (Sharma, 1970; Aamir Raina *et al.,* 2022). Plants with albino, xantha, and chlorina type mutations typically die two to four weeks after germination due to their inability to photosynthesize, although plants with other kinds can maintain their vitality until harvest maturity and the amount of chlorophyll approaches normal in time (Motoyoshi, 1967; Ahumada-Flores *et al.,* 2021; Ergün *et al.,* 2023). Because chlorophyll mutations are typically fatal, they are not economically significant (Wani, 2017), are readily recognized and analyzed in M_2 generation and serve as genetic, physiological, and biochemical markers (Patial *et al.,* 2017). Chlorophyll-deficient mutants might be readily identified as recessive alleles during germination (Ilhan, 2014). According to Sakin and Sencar (2001) and Çiftçi and Şenay (2005), chlorophyll mutations are a crucial factor in determining the frequency and efficiency of mutations in M_2 production. These include Striata with normal growth EMS mutants in the M_2 generation, xantha with straw-colored yellow leaves, and viridis with whitish leaf tips that have a deadly impact (Arulbalachandra and Mullainathan, 2009). The type of chlorophyll mutation reported in pearl millet, black gram Mungbean (Singh and Singh, 1989), chickpea (Wani and Anis, 2004), and cow pea (Hood *et al.,* 2004) was shown to be regulated by genes on several chromosomes that may be found close to the centrosome and proximal portion of chromosome (Chandola *et al.,* 1963) whose frequencies decreased with the mutagen dose, and male sterile mutant frequency was later discovered to follow a same distribution trend (Ojiewo *et al.,* 2006). Chlorophyll mutations were most common at 40-50mM of EMS and 25mM of DES (Sheeba *et al.,* 2013) found similar results. According to Sjodin (1962), as reported by Prakash and Shambulingappa (1999) and Prakash and Khanure (2000), viridis types out number albina, xantha, and chlorina types in rice beans, regardless of cultivar. (Haq,1990) found the highest number of xantha mutants in three kabuli chickpea genotypes, while (Lal *et al.*, 2009) obtained similar results in black gram and (Khan *et al.,* 2005) in chickpea. (Charry and Bhalla ,1988) found that EMS induces more chlorophyll mutations and has a higher frequency of viridis mutants in pigeon pea, while (Khan and Tyagi, 2009) found similar results in soybean. Aurea (golden yellow leaves) survived for a few days due to a blockage in chlorophyll production (Blixt., 1961). The variance could be attributed to different treatment circumstances for the mutants. SA treatments may reduce the incidence of chlorophyll mutations by inhibiting enzymes such as catalase and peroxidase, as well as increasing peroxide content in cells (Kleinhofs *et al.,* 1978). (Arora and Kaul 1989) found a higher incidence of chlorophyll mutations in *Pisum sativum* and (Kharkwal, 1998) in *Cicer arietinum* at medium to low mutagen dosages. The frequency of chlorophyll and viable mutants found in M_2 generation is primarily utilized as a reliable measure of the genetic influence of mutations (Gautam *et al.,* 1998). Isolating and characterizing mutant chlorophyll genomes could shed light on the nature of the mutation at the molecular level. Desirable morphological mutations are crucial for improving crop plants through breeding programs. Macro mutations can be caused by minor deficits, duplications, chromosomal abnormalities, or gene mutations, according to several studies (Singh *et al.,* 1980). Cotyledon and morphological variation in *Brassica napus* L. cv. Excel, including fused cup-shaped cotyledons, three cotyledons with bilobed leaves, abnormal buds, and siliqua with three replums, were reported by (More and Malode 2016). They proposed using these morphological anomalies as a marker system for various genomic characters. Numerous workers have previously documented that the administration of various mutagens has resulted in the development of morphological defects in leaf and stem structure. (Kochanova *et al.,* 2012) documented how sodium azide affected *Diospyros* lotus morphological traits as leaf blade length and petiole length. In *Sesamum indicum*, colchicine and sodium azide have been shown to cause morphological changes such as shorter internodes and malformed leaves (Mensah *et al.,* 2007). It is non-linear even while every treatment exhibits an increase in the rate of mutation frequency with an increase in dose. Numerous researchers, including (Shirsat *et al.,* 2010), found a non-linear association between the amount of mutagen in horsegram and the rise in chlorophyll mutation. (Verma *et al.,* 2018) and (Ambavane *et al.,* 2015) have offered similar reports for finger millet and fennel, respectively. Numerous researchers tried to assess the efficacy and efficiency of various mutagens using the frequency of chlorophyll mutations. Mutations in plant height, bloom number and color, growth habit, leaf and pod shape were observed in the second generation of both kinds. Although most morphological mutants are uneconomical, they can be beneficial for cross-breeding, improving quantitative traits, determining crop evolution, and gene mapping studies (Khan *et al.,* 2011; Wani *et al.,* 2011; Toker, 2009; Gaur and Gaur, 2003). According to (Nilan, 1967), the proportion of distinct mutant types may vary depending on the mutagen used and the treatment

process. Changes in individual genes (monogenic) are responsible for the morphological mutations that affect a single character (Van Harten, 1998). Morphological mutations that influence multiple characters are typically caused by pleiotropic gene mutations, altered gene clusters, or chromosomal breaks (Tyagi and Gupta, 1991; Wi *et al.,* 2005; Waghmare and Mehra, 2001; Dhole *et al.,*2003). Our analysis followed (Gnanamurthy *et al.,* 2012) mutation classification. Macro mutations are qualitatively hereditary and physically unique, such as color changes in flower and seed coats. Micro mutations, which impact plant height, growth habits, and pod characteristics, are quantitatively inherited but phenotypically unnoticeable. Mutations in genes that control organ development can cause morphological mutations. The study found that gamma rays caused DNA breakage, chromosomal aberrations, changed auxin metabolism, mineral, amino acid, and physio-morphogenetic differences in cells, possibly due to morphological mutations (Wani *et al.,* 2011; Sharma and Sharma, 1979; Gunckel and Sparrow, 1961). Lower and intermediate dosages of γ rays and SA resulted in a wider range of leaf mutations, including significant changes in size, shape, quantity, and arrangement. Mutations in leaf qualities caused by mutagens included broad-leaved, narrow-leaved, lengthened rachis, and changed architecture. Similar leaf mutants have been observed in many crops, including chickpea (Wani, 2011), lentil (Nair and Mehta, 2014), and faba bean (Khursheed *et al.,* 2019). Broad-leaved mutants have a higher leaf area index and receive more solar radiation, potentially increasing photosynthetic rate compared to the original variety (IITA, Annual report). Increased multi-foliation caused by mutagens may boost photosynthetic activity and biomass production, leading to improved seed output in cowpea breeding programs. Mutations in leaves may result from mutagen-induced cellular damage, chromosomal breakage, altered mineral metabolism, and disturbed auxin synthesis and transport (Anjana and Thimmaiah, 2002)**.** Dwarf mutants in this study have short internodes, possibly due to reduced cell quantity and length. These dwarf mutants have also been reported in barley (Sethi, 1974), grasspea (Talukdar and Biswas, 2006) and Vigna spp. (Wani *et al.,* 2011). The dwarf mutant resulted from shorter or fewer internodes. (Hedens, 2003) found that alterations in gibberellic acids led to a loss in plant height, while (Kleinhofs *et al.,* 1978) and (Suganthy *et al.,* 1994) reported irregular mitotic divisions. According to (Shakoor *et al.,* 1978) in triticale and (Konzak *et al.,* 1969) in wheat, polygenes regulate the nature of semi-dwarf mutants. According to (Qin *et al.,* 2008), the dominant dwarf mutation in rice is driven by a single dominant mutation. In accordance with (Satyanarayana *et al.,* 1989) and (Talukdar, 2009), several morphological mutants, such as tall, dwarf, and bushy, are monogenic and recessive. Because short genotypes are more resistant to lodging than standard kinds, reduced plant height is a crucial feature in plant breeding (Austin *et al.,* 1980; Fick and Miller, 1997). Many plant species have dwarf genotypes, which have been employed in a number of crops to improve crop management and boost production (Abel, 1976). It was discovered that using mutants and their descendants in cross-breeding produced better black gram cultivars (Pawar *et al.,* 2000). Furthermore, molecular studies that describe the structure and function of linked genes have increasingly focused on induced mutations. Therefore, mutated genes have become useful information for molecular biologists and plant breeders to understand not only the function but also to isolate and rearrange the genes amongst kinds (Souframanien *et al.,* 2002). Gene technology may take a new turn with the discovery and study of mutants utilizing molecular techniques like DNA fingerprinting, mapping with PCR-based markers like RAPD, AFLP, and STMS, and mutant tagging (Ahloowalia and Maluszynki, 2001). The frequency of mutations caused by a unit dose or concentration of mutagen is indicated by the mutagenic efficacy. In connection to biological harm like death, injury, sterility, and chromosomal abnormalities brought on by the mutagenic therapies, the mutagenic efficiency gives an understanding of the frequency of mutations (Konzak *et al.,* 1965). The selection of mutants with desirable features and the usefulness of mutagens in causing advantageous mutations depend on the assessment of efficacy and efficiency (Girija and Dhanavel, 2009). The mutation treatment shouldn't cause biological damages that reduce cell survival and ultimately eliminate the mutation, such as chromosomal abnormalities and biophysiological and genotoxic consequences. Effectiveness measures the number of mutations brought on by a single mutagen dose (Konzak *et al.,* 1965; Bhosale and Kothekar, 2010). Higher efficiency may not always follow from a highly potent mutagen, and vice versa. The degree of biological harm caused, the material's suitability for the biological system, and its mutagenic reactivity all affect mutagenic efficiency (Shah *et al.,* 2008). (Bhosale and Kothekar, 2010) showed that the efficiency and effectiveness of the mutagen decreased as the dose increased after using EMS, SA, and gamma rays to promote diversity among two types of cluster beans. (Mangaiyarkarasi *et al.,* 2014) found a linear rise in efficacy and efficiency in *Catharanthus roseus* with an increase in mutagen dosage. In the case of (Bolbhat *et al.,* 2012), frequency rises up to a specific mutagen dose before declining at greater doses. According to the current research, middle doses are more efficient and effective than lower or higher amounts. The similar point was highlighted by (Julia *et al.,* 2018) and (Ambavane et al., 2015),

intermediate doses are more effective and powerful in causing morphological abnormalities. Chlorophyll and viable mutants were used to determine the mutagenesis efficacy and efficiency. Plant height and survival were found to be reduced when EMS, DES, and sodium azide mutagens were compared. DES and sodium azide were shown to be less effective and efficient than EMS in the current study. However, up to a certain point, the mutagenic efficacy and efficiency generally declined as the concentration of mutagens increased. It was discovered that EMS was more successful at causing mutation than DES and sodium azide. The lowest mutagenic efficacy was found at 30mM DES (11.20), whereas the highest was found at 25mM SA (21.30). (Solanki, 2005), (Sassikumar *et al.,* 2003), (Thilagavathi and Mullainathan, 2009), and (Arulbalachandran, 2006) reported similar outcomes for lentils, Lima beans, and black gram. Based on injury and harm, the mutagenic efficiency was calculated.The highest mutagenesis efficiency was found at 25mM of SA (263.68) based on damage, whereas the lowest was found at 30mM of DES (30.22). On the basis of lethality, the highest mutagenic efficiency was recorded at SA (119.73), while the lowest mutagenic efficiency was observed at DES (42.68). As a result, the current investigation showed that EMS is far more effective than sodium azide and DES. Similar findings were reported by (Girija and Dhanavel, 2009) in cowpea, (Jabee and Ansari, 2005) in chickpea, and (Deepalakshmi and Anandakumar, 2003) and (Sharma et al., 2005) in urdbean.

Conclusion

As demonstrated by the presence of several chlorophyll and viable morphological mutants in the M_2 generation, the current study verified that chemical mutagens are successful in causing genetic variability in *Crotalaria juncea* L. Up to an optimal level, mutation frequency and spectrum rose with increasing mutagen concentration; at greater dosages, they decreased because of increased physiological damage. Mutations in chlorophyll have been shown to be accurate markers of genetic changes and mutagenesis activity. With the greatest frequency and broadest range of mutations, EMS was the most powerful and effective mutagen among those studied. Sodium azide came in second, while DES was relatively less effective. It was discovered that intermediate dosages, especially EMS at 35mM and sodium azide at 20 and 25mM, produced higher mutation frequencies with comparatively less biological damage.Future breeding programs can benefit greatly from the genetic resources provided by the viable morphological and seed mutations found in this study. Overall, the results demonstrate the value of induced mutagenesis in expanding sunn hemp's genetic base and provide a solid foundation for choosing appropriate mutagen kinds and dosages for crop improvement and mutation breeding.

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