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Chemical Mutation Induced Variations On Quantitative Traits In *Linum Usitatissimum* L. Var. Shekhar.

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ABSTRACT

Linum usitatissimum L. (flaxseed/linseed) is an important medicinal crop of family linaceace grown for its edible seed, the oil from the seed and for the fibres obtained from the caffeine. Induce mutagenesis plays a vital role in crop improvement by inferring variations in germplasm and is preferred over hybridization and recombination because it improves a defect in an otherwise elite cultivar, without losing its agronomic and quality characteristics. In present investigation *Linum usitatissimum* var. Shekhar was exposed with five different mutagenic dose of four mutagens viz caffeine and Sodium azide (SA) i.e, 0.10%, 0.25%, 0.50%, 0.75% and 1.00% and heavy metals lead nitrate and cadmium nitrate i.e. 20, 40, 60, 80 and 100 ppm. Seed germination, plant survival and pollen fertility was found to be decreased in treated population than control in all the mutagens. The higher concentrations of mutagens significantly reduced plant height, branches per plant and various yield parameters. The present study reveals moderate concentrations of each mutagen induce mutagenic damage in *Linum usitatissimum* var. Shekhar which can facilitates screening of desirable mutant in M_2 and M_3 generations. On the basis of these results, it was concluded that lower concentrations of mutagen did not significantly affect the morphology of *Linum*, while higher concentrations of four mutagen were found to be more mutagenic.

Keywords: *Linum usitatissimum* L., linseed, chemical mutagen, caffeine, SA, $Pb(NO_3)_2$, $Cd(NO_3)_2$

1. INTRODUCTION

Linseed (*Linum usitatissimum* L., 2n = 30), also known as flaxseed is an important medicinal rabi crop, commercially cultivated for seed oil and fibres. The genus *Linum* is a violet-blue flowering annual herb belongs to family linaceae. Linseed and its derivate, linseed oil, contains about 36 to 48% oil content highly rich in unsaturated fatty acid called alpha-linolenic acid (ALA) (Kouba, 2006; Khan *et al.*, 2010), which is biological precursor of omega-3 fatty acids. Presently, flax is primarily cultivated in Asia (fiber

and linseed), Western Canada (linseed), the northern regions of China (fiber and linseed), north-central USA (linseed), and Western Europe and Russia (fiber and linseed).

According to FAOSTAT 2019, Kazakhstan is at top for linseed production (tonnes) in world followed by Russian Federation and Canada. India is at number 6th position and shares approximately 3.22% of world's total linseed production (tonnes).

Table 1 World's top 10 producers of linseed in 2019

Rank	Region (Country)	Production (tonnes)
	World	**3068254**
1	Kazakhstan	1007244
2	Russian Federation	658644
3	Canada	486100
4	China	340000
5	USA	162440
6	India	99070
7	Ethiopia	79695
8	Afghanistan	55000
9	France	45500
10	United Kingdom of Great Britain and Northern Ireland	27000
Source: FAOSTAT / FAO Statistics Division, 2019.		

Induced mutations pave a way to enhance genetic variability in self-pollinated crops where recombination and hybridization is quite difficult. For crop-improvement, chemical mutagenesis is a vital tool and caffeine and SA are effective mutagen that causes AT-GC base pair transition leading to point mutations in plant genome.

Mode of action of mutagens

In the present investigation, four chemical mutagens viz., caffeine, sodium azide, lead nitrate and cadmium nitrate have been applied on *Linum usitatissimum* L. Their mechanisms, mode of action and several significant studies related to these mutagenic agents are given below:

Caffeine

Caffeine, also known as 1,3,7-trimethylxanthine, is a naturally occurring methylxanthine compound found in coffee beans, tea leaves, and various other plants. Fries and Kihlman (1948) were the first to report the mutagenic effects of caffeine on *Ophiostoma multiannulatum*. Caffeine is a solubilizing agent, forming molecular complexes that facilitate chromosomal abnormalities. Caffeine inhibits photoreactivation at low concentrations in *E. coli* (Harm, 1970) and nucleotide excision repair in vivo (Witkin, 1961). Observations indicate that caffeine

inhibits the mechanism of photo reactivation by reducing the binding of photolyase to damaged DNA and inhibiting the nucleotide excision repair mechanism. Also, it interacts with DNA, modifying its physical properties, such as denaturation temperature, thereby increasing spontaneous mutation rates (Aslam *et al.*, 2017a). Furthermore, caffeine inhibits DNA repair mechanisms (Itoyama and Bicudo, 2000), promotes DNA-DNA or DNA-protein cross-linking (Amin, 2002), and acts as a DNA intercalator (Tornaletti *et al.*, 1989). Several researchers have reported caffeine-induced mutations in various crops, including *Silene* (Jiang and Dunn, 2016), *Trigonella* (Naaz *et al.*, 2023) and *Lens culinaris* (Yousuf *et al.*, 2023).

Sodium azide (SA)

Sodium azide (NaN_3) is among the most potent mutagenic agents employed for inducing mutations in crop plants. It was used for the first time as a mutagen by Nilan *et al.* (1973) in barley. Research on the genotoxic effects of sodium azide across various organisms has demonstrated its ability to induce gene mutations, AT→GC base pair transitions, and chromosomal aberrations through transversions (Gruszka *et al.*, 2012). The mutagenicity of SA is due to the development of an azide compound that enters into the nucleus, interacts with DNA, and causes point mutation in the genome (Ingle *et al.*, 2018; Chaudhary *et al.*, 2021).

The mutagenic effect of sodium azide is attributed to the formation of an organic metabolite, the β-azidoalanine moiety [N3–CH2–CH(–NH2)–COOH], identified as the amino acid analogue L-azidoalanine in both bacteria and barley (Gruszka *et al.*, 2012). This metabolite penetrates the nucleus, interacts with DNA, and induces point mutations in the genome (Khan *et al.*, 2009b). It has been observed to reduce cellular levels of calmodulin, a calcium-binding protein involved in signal transduction and cell division. A significant reduction in cell division was observed in barley anthers treated with SA (Castillo *et al.*, 2001). SA has been documented to induce a diverse array of morphological, physiological, cytological, agronomic, and colour mutations in various crop species including *Brassica napus* (Hussain *et al.*, 2017), *Triticum* (Türkoğlu *et al.*, 2022), *Capsicum* (Udogu *et al.*, 2023), *Coffea arabica* (Rojas-Chacón *et al.*, 2024) and *Arachis hypogaea* (Parmar and Parveen, 2025)

Lead nitrate ($Pb(NO_3)_2$) and Cadmium nitrate ($Cd(NO_3)_2$)

Heavy metals are naturally found in the environment and play an important role in various physiological functions of living organisms, but only in trace amounts. Lead (Pb), cadmium (Cd), and zinc (Zn) are among the most abundant heavy metals, accumulating alongside other pollutants in the environment and exhibiting toxic effects on plants, animals, and humans. These metallic elements are systemic toxicants even at low exposure levels (Kaur *et al.*, 2019).

Exposure to high levels of Pb and Cd leads to the production of reactive oxygen species (ROS) in plants (Huihui *et al.*, 2020). The accumulation of reactive oxygen species (ROS) triggers oxidative stress, leading to cell membrane damage and disruptions in cellular metabolism and physiological processes. Under these conditions, key cellular components, including nucleic acids, soluble sugar levels, and chloroplast pigments, may be altered (Ali *et al.*, 2014; Khan *et al.*, 2021).

Lead (Pb) is a highly toxic heavy metal and a growing environmental contaminant, posing a serious threat to agroecosystems (Rao *et al.*, 2018). It enters the environment primarily through human activities and industrial waste, including the use of chemical fertilizers, herbicides, pesticides, and polluted irrigation water (Khan *et al.*, 2018). In plants, Pb accumulates in the roots and may also be transported to the shoots (Dalyan *et al.*, 2018), disrupting metabolic functions. Its presence leads to excessive production of reactive oxygen species (ROS), reduces photosynthetic efficiency, and induces oxidative stress, ultimately causing delays in plant growth and germination (Ye *et al.*, 2018). Its mutagenic potential has been reported by several workers such as Choudhary *et al.* (2012), Shahwar *et al.* (2019), Hasan *et al.* (2022) and Yousuf *et al.* (2023).

Cadmium (Cd) is another highly toxic heavy metal that even at low concentrations, exerts harmful effects on plants (Paunov *et al.*, 2018), including disruptions in water and nutrient uptake, reduced photosynthesis, chlorosis, root tip browning, and, in severe cases, plant death (Mohanpuria *et al.*, 2007). Similar to lead (Pb), cadmium (Cd) disrupts the cell's redox balance by promoting the excessive generation of reactive oxygen species (ROS), leading to oxidative damage to cell membranes and other biomolecules (Shanmugaraj *et al.*, 2019). Apart from inducing ROS generation, cadmium also inhibits DNA repair processes, increasing genetic instability and consequently raising the increased probability of mutations (Filipič *et al.*, 2006). Its mutagenic potential has been documented by several researchers, including Shahwar *et al.* (2023), Verma *et al.* (2024) and Sharma *et al.* (2024).

Objectives

The conventional approaches of plant breeding have exploited the available genetic variability in *Linum usitatissimum* L. As a result of the use of these conventional approaches for longer periods, there has been a significant decline in genetic variability which has led to a narrow genetic base of this crop. Mutagens have remarkable possibilities of improving plants with regard to their qualitative and quantitative characters. Therefore, an attempt has been made to explore the possibilities of inducing alteration in the genotype to enhance the variability and screen the plants in M_1 generation, keeping the following objective in view for this study and future study:

1. To explore the possibility of inducing genetic variability with the help of chemical mutagens and to select the desirable variants through screening and selfing in M_1 generation.
2. To investigate the effect of different mutagenic treatments on various biological parameters and quantitative traits in M_1 generations.
3. To investigate the effect of mutagenic treatments on the meiotic behavior of chromosomes in M_1, M_2, and M_3 generations.
4. To isolate variants on the basis of their cyto-morphological features.
5. To find out the effectiveness and efficiency of chemical agents (caffeine, sodium azide) in inducing mutations in M_2 generation.
6. To induce maximum variations, with minimum damage of the plants for the selection of mutants in M_2 and M_3 generations.

7. To study genotypic variability in the mutant lines using Start Codon Targeted (SCoT) markers in M_3 generation.
8. To assess variation in stomatal morphology using Scanning Electron Microscopy (SEM) in isolated high yielding mutants in M_3 generation.
9. To analyze micromorphological seed variations using Scanning Electron Microscopy (SEM) in isolated high yielding mutants in M_3 generation.
10. To estimate seed oil, protein and mineral (iron) content in selected high yielding mutants in M_3 generation.
11. To investigate variations in phytochemical compounds through Gas Chromatography-Mass Spectrometry (GC-MS) in the methanolic extract of seeds from isolated high yielding mutants in M_3 generation.

2. MATERIAL AND METHODS

Healthy and certified seeds of *Linum usitatissimum* L. var. Shekhar were procured from ICAR- National Bureau of Plant Genetic Resources, New Delhi. Seeds were presoaked and subjected to five different mutagenic concentrations of caffeine and SA (0.10%, 0.25%, 0.50%, 0.75% and 1.00%) and heavy metals lead nitrate and cadmium nitrate i.e. 20, 40, 60, 80 and 100 ppm for 12 hrs at room temperature and pH 7.0. After treatment, treated seeds were washed under running tap water to remove excess mutagen. 100 seeds were sown in earthen pots in 4 replicates of each mutagenic concentration along with control seeds. M_1 generation was raised and germination (%), plant survival (%) and pollen fertility were studied. Various agronomic traits viz., plant height, number of branches/plant, capsules/plant, seeds/capsules, 1000 seed weight and total yield were also evaluated for treated and control plants.

The pigment (total chlorophyll and carotenoid) contents of leaves were estimated by method of MacKinney (1941). About 1g fresh leaves were grinded in 20ml acetone. The fine mixture was centrifuged at 5,000 rpm for 5 minutes and the supernatant was extracted. The residue was washed three times, using 80% acetone. The absorbance was recorded at 645 nm and 663 nm for chlorophyll content and 480 and 510 nm for carotenoid content. The pigment contents of leaves extracted were calculated using formulae:

Total chlorophyll (mg/g fw) = $\{20.2\ (OD_{645}) + 8.02\ (OD_{663})\} \times \frac{V}{1000 \times W}$

Carotenoid (mg/g fw) = $\{7.6\ (OD_{480}) - 1.49\ (OD_{510})\} \times \frac{V}{d \times 1000 \times W}$

Where, OD_{645}, OD_{663}, OD_{480}, OD_{510} = Optical densities at 645nm, 663nm, 480nm and 510nm wavelength respectively

V= Volume of extract

W= Mass of leaf tissues

d= Length of light path (=1.4cm)

Statistical analysis was done using SPSS 16.0 for Window (SPSS, Chicago, IL, USA). One way Analysis of variance (ANOVA) was calculated through DMRT to determine significant difference between the treatments at $p<0.05$ level of significance.

3. RESULT

The mutagenic effect of chemical mutagens, such as caffeine, sodium azide, lead nitrate and cadmium nitrate were investigated in *Linum usitatissimum* L. variety shekhar. Various parameters such as biological damage, morphology and plant yield were evaluated in terms of seed germination, inhibition in seed germination, plant survival, variation/mutation frequency, pollen fertility, reduction in pollen fertility, height of mature plant, number of branches per plants, number of capsules per plant, number of seeds per capsule, 1000 seed weight and seed yield per plant were studied in M_1 generations. The chromosomal aberrations such as univalents, bivalents, multivalents, stickiness, laggards, bridges, precocious movement of chromosomes, stray chromosomes, disturbed polarity, unequal separation of chromosomes, cytomixis, micronucleate and multinucleate condition, were examined for cytological study in M_1 generations.

To identify mutations, plants grown from treated seeds were compared with those from untreated (control) seeds throughout their development. Variants were selected from the treated populations in the M_1 generation based on cytomorphological variations compared to the control. Flowers from the M_1 generation were self-pollinated, and the resulting seeds were collected to sown for the M_2 generation, where selected mutants were studied in detail for further studies. Mutants were named on the basis of morphological and cytological analysis in M_2 generation.

3.1 Biological damage in M_1 generation

Seed germination (%)

The seed germination was counted on alternate days after sowing in control as well as untreated populations. A gradual decrease in seed germination was found with increasing concentrations of mutagens. The maximum germination in control was 95.00%, while it decreased from 89.00-56.00% and 79.00-51.00% in 0.10-1.00% caffeine and SA, and from 72.00-43.00% and 69.00-40.00% in 20-100 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$ respectively.

Inhibition in germination (%)

The germination recorded in control (untreated) was found to be maximum and it was considered as standard for calculating the percent inhibition in treated populations. The percentage of inhibition increased from 8.53-43.68% and 13.84-44.87% in 0.10-1.00% caffeine and SA; 20.23-50.30% and 22.47-53.39% in 20-100 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$ respectively. The maximum inhibition was recorded in $Cd(NO_3)_2$ followed by $Pb(NO_3)_2$, SA and caffeine

Plant survival and lethality (%)

The survival of plants at maturity was 94.62% in control, while it decreased from 85.63-76.77% in caffeine, 83.53-75.33% in SA, 80.33-65.09% in $Pb(NO_3)_2$ and 67.46-63.64% in $Cd(NO_3)_2$ with their increasing concentrations. The maximum survival of plants was recorded in caffeine followed by SA, $Pb(NO_3)_2$ and $Cd(NO_3)_2$.

Simultaneously, the lethality due to the toxic effect of mutagens increased from 4.34-16.83% and 5.37-18.38% in caffeine and SA and from 15.11-27.28% and 17.22-34.03% in $Pb(NO_3)_2$ and $Cd(NO_3)_2$ respectively.

Variation frequency (%)

Morphological variation was observed at seedling stage in treated populations. The variation frequency increased from 15.11-37.33% and 16.40-46.95% in 0.10-1.0% caffeine and SA; 21.31-43.13% and 25.55-37.14% in 20-100 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$ respectively. The variation frequency was highest in $Cd(NO_3)_2$ followed by $Pb(NO_3)_2$, SA and caffeine.

Pollen fertility and sterility (%)

Pollen fertility is an important parameter in breeding programme. Study on pollen fertility was done in mutagenized population which forms an authentic index to evaluate variation in plants as well as to investigate the efficiency of mutagens. The pollen fertility in control was 92.51% but decreased from 84.42-61.51% and 81.11-61.31% in caffeine and SA; 72.43-53.23% and 74.65-55.74% in $Pb(NO_3)_2$ and $Cd(NO_3)_2$ respectively. Pollen sterility was dose dependent and increased from 7.45-24.80% in caffeine and 12.07-28.32% in SA; 16.30-39.24% in $Pb(NO_3)_2$ and 20.40-41.88% in $Cd(NO_3)_2$. The highest reduction in pollen fertility was recorded in $Cd(NO_3)_2$ followed by $Pb(NO_3)_2$, SA and caffeine.

3.2 Types of morphological variations

The effect of mutagens appeared at the seedling stage up to the formation of cotyledonary and a few pairs of vegetative leaves in M_1 generation.

Cotyledonary leaves variations

Control seedlings bear two normal, opposite, green, entire, obtuse, smooth, and equal sized cotyledonary leaves.

The common variations observed in the treated populations of *Linum usitatissimum* L. were as follows: Both cotyledonary leaves were opposite, shiny, thick, and curved toward inner side. Another seedling had two cotyledonary leaves, opposite, one slightly large with notching at tip. The seedlings in caffeine showed three unequal cotyledonary leaves, green, entire, smooth, and thick with one slightly curved leaf. Another seedling in same concentration of caffeine, both cotyledonary leaves were deformed, bigger, green, and slightly shifted towards one side. In the third variant of the same concentration, two cotyledonary leaves were

elongated, dark green, shiny, and thick with undulated margins. The seedlings also had four unequal green, elongated, curved overlapped cotyledonary leaves. Seedlings showed deformed green, rough cotyledonary leaves, out of which one was larger with side notch while other was comparatively smaller with slightly curved and undulated margin. Another seedling showed two elongated cotyledonary leaves, with chlorina type of chlorophyll variation. In the third variant of the same concentration, two opposite, green, thick, slightly curved kidney-shaped cotyledonary leaves with side notching. Seedlings had two unequal, light green, rough cotyledonary leaves having undulated margin with leaf tip burn. Another seedling in same concentration had two small, light green cotyledonary leaves with elongated hypocotyl. Also cotyledonary leaves with xantha chlorophyll mutation also observed.

3.3 Quantitative traits in M_1 generation

Height of mature plants

Average height of control plant was 96.05 cm. It increased over control from 104.23-96.61 cm in caffeine, but in higher concentrations plant height decreased from 92.95-84.78 cm in 0.75-1.00% concentrations respectively. In case of SA, it increased from 102.05-94.71 cm in 0.10-0.25% SA respectively, while in the higher concentrations the height decreased from 91.22-84.16 cm in 0.50-1.00% concentrations respectively. In $Pb(NO_3)_2$ and $Cd(NO_3)_2$ populations, plant height decreased from 90.66-80.79 cm and 85.22-80.53 cm in 20-100 ppm concentrations of $Pb(NO_3)_2$ and $Cd(NO_3)_2$ respectively.

The increase in height was significant at 5% level in 0.25% SA and at 1% level in 0.10% caffeine, 0.25% caffeine and 0.10% SA, whereas reduction in height was significant at 5% level in 0.75% SA and at 1% level in 1.00% caffeine, 1.00% SA, 60-100 ppm $Pb(NO_3)_2$ and 20-100 ppm $Cd(NO_3)_2$. The CV increased in all mutagens along with increasing concentrations showing enhanced variability in the treated populations.

Number of branches per plant

The average number of branches per plant in control was 4.60. It increased over control from 5.21-4.31 in 0.10-0.50% caffeine and decreased from 4.34-3.16 in 0.75-1.00% caffeine. In SA also it increased from 5.11-4.28 in 0.10-0.50% but decreased from 4.22-3.34 in 0.75-1.00%. In case of $Pb(NO_3)_2$ and $Cd(NO_3)_2$, it decreased gradually from 4.25-3.38 and 4.18-2.17 in 20-100 ppm respectively.

The increase in number of branches was significant at 5% level in 0.25% SA and at 1% level in 0.10-0.25% caffeine and 0.10% SA. On the other hand, the reduction in number of branches was significant at 5% level in 20 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$ and at 1% level in 1.00% caffeine, 0.75-1.00% SA, 40-100 ppm of $Pb(NO_3)_2$ and $Cd(NO_3)_2$. The CV in $Cd(NO_3)_2$ treated populations were generally higher followed by $Pb(NO_3)_2$, SA and caffeine.

Yield parameters

All yield parameters were determined and assessed in terms of number of capsules per plant, number of seeds per capsule, 1000 seeds weight (g) and seed yield per plant (g) in M_1 generation.

Number of capsules per plant

Average number of capsules per plant was 49.19 in control. It increased significantly over control from 53.11-51.16 in 0.10-0.50% caffeine while decreased from 49.12-42.73 in 0.75-1.00% caffeine. In case of SA, it increased from 51.26-56.08 in 0.10-0.25% while decreased from 49.51-42.66 in 0.50-1.00%. Number of capsules per plant decreased gradually from 48.32-43.97 and 46.31-36.28 in 20-100 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$ respectively (Tables, 19-20; Graphs 10, C-D).

The increase in number of capsules per plant was significant at 5% level in 0.25-0.50% caffeine and 0.10% SA whereas at 1% level in 0.10% caffeine. The reduction was significant at 5% level in 0.75% SA, 40 ppm $Pb(NO_3)_2$ and 20 ppm $Cd(NO_3)_2$ and at 1% level in 1.00% SA, 60-100 ppm $Pb(NO_3)_2$ and 40-100 ppm $Cd(NO_3)_2$. The coefficient of variation increased along with the increasing concentrations of all mutagens and it was generally higher than control.

4.10.3.2 Number of seeds per capsule

The average number of seeds per capsule was 8.11 in control. It increased over control from 8.27-8.01 in 0.10-0.50%, but in the higher concentrations it decreased to 7.31-6.74 in 0.75-1.00% caffeine. In SA also, it increased from 8.14-8.05 in 0.10-0.25% and decreased to 7.45-6.17 in 0.50-1.00%. In case of $Pb(NO_3)_2$ and $Cd(NO_3)_2$ it decreased successively from 7.77-6.86 and 7.18-6.24 in 20-100 ppm respectively.

The significant decrease in number of seeds per capsule was observed at 5% level in 0.75% SA and 60 ppm $Pb(NO_3)_2$ and at 1% level in 1.00% caffeine, 1.00% SA, 80-100 ppm $Pb(NO_3)_2$ and 60-100 ppm Cd(NO3). The coefficient of variation was dose dependent in all the mutagens and maximum values of CV were obtained in the highest concentrations of mutagens with a maximum of 13.22% in 100 ppm $Cd(NO_3)_2$.

1000 seeds weight (g)

The average weight of 1000 seeds were 7.28 g in control. It increased significantly to 7.74 in 0.10% caffeine and insignificantly from 7.51-7.38 g in 0.25-0.50% caffeine and 7.61-7.42 g 0.10-0.25% SA. The average weight decreased from 7.20-7.09 g in 0.75-1.00% caffeine and to 7.24-6.99 g in 0.50-1.00% SA, whereas it decreased gradually to 7.15-6.78 g and 7.11-6.61 g in 20-100ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$.

The significant increased in 1000 seeds weight at 1% level was observed in 0.10% caffeine while reduction was significant at 5% level in 80-100 ppm $Pb(NO_3)_2$ and 60-80 ppm $Cd(NO_3)_2$ and at 1% level in 100 ppm $Cd(NO_3)_2$. CV in all mutagens increased linearly in higher concentrations and it was maximum at highest concentrations of $Cd(NO_3)_2$ followed by $Pb(NO_3)_2$, SA and caffeine.

Seed yield per plant (g)

The average seed yield per plant in control was 2.66 g, but it increased over control significantly from 3.33-3.12 g in 0.10-0.25% and insignificantly to 3.078 g in 0.50% caffeine, whereas in SA, it increased significantly to 3.31 g in 0.10% and insignificantly to 3.50 in 0.25% concentration. Moreover, it decreased from 2.62-2.18 g in 0.75-1.00% caffeine and 2.11-1.39 g in 0.50-1.00% SA (Tables, 17-18; Graphs 13, A-

B). Total yield per plant decreased gradually from 2.15-1.32 g and 2.10-1.11 g in 20-100 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$ respectively.

The significant increase in total yield per plant was observed at 5% level in 0.25% caffeine and 0.10% SA whereas at 1% level in 0.10% caffeine. Moreover, significant reduction was recorded at 5% level in 40 ppm $Pb(NO_3)_2$ and at 1% level in 1.00% caffeine, 0.75-1.00% SA, 60-100 ppm $Pb(NO_3)_2$, 40-100 ppm $Cd(NO_3)_2$. The coefficient of variation increased with the increasing concentrations of all mutagen and it was generally higher than control.

Physiological parameters

Chlorophyll a (Chl a) (mg g^{-1})

The average chlorophyll a in control was 0.76 mg g^{-1}. It increased over control from 0.92-0.83 mg g^{-1} in 0.10-0.50% caffeine and 0.83-0.80 mg g^{-1} in 0.10-0.25% SA but decreased from 0.77-0.71 mg g^{-1} in 0.75-1.00% caffeine and 0.73-0.65 mg g^{-1} in 0.50-1.00% SA, whereas in heavy metals it decreased from 0.71-0.51 mg g^{-1} and 0.68-0.54 mg g^{-1} in 20-100 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$ respectively.

The increase in chlorophyll a was significant at 5% level in 0.10% SA and at 1% level in 0.10% caffeine. The significant reduction in chlorophyll a was observed at 5% level in 80 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$ and at 1% level in 100 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$.

The coefficient of variation increased linearly and was higher than control in all the mutagen treated populations.

Chlorophyll b (Chl b) (mg g^{-1})

The average chlorophyll b in control was 0.51 mg g^{-1}. It increased over control from 0.62-0.54 mg g^{-1} in 0.10-0.50% caffeine and decreased to 0.51-0.48 mg g^{-1} in 0.75% and 1.00% caffeine. In SA treated population it increased from 0.65-0.61 mg g^{-1} in 0.10-0.25% SA and decreased from 0.53-0.48 mg g^{-1} in 0.50-1.00% SA respectively, whereas in heavy metals chlorophyll b decreased from 0.45-0.25 mg g^{-1} and 0.47-0.33 mg g^{-1} in 20-100 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$ respectively.

The coefficient of variations increased with the increasing concentrations of all mutagens. The increase in chlorophyll b was significant at 5% level in 0.25% caffeine and 0.10% SA and at 1% level in 0.10% caffeine. The significant reduction in chlorophyll b was observed at 5% level in 40 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$ and 1% level in 60-100 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$ (Table, 21-24).

Total chlorophyll content (mg g^{-1})

The average total chlorophyll in control was 1.34 mg g^{-1}. It increased over control from 1.51-1.44 mg g^{-1} in 0.10-0.50% caffeine and 1.50-1.47 mg g^{-1} in 0.10-0.25% SA but decreased from 1.29-1.19 mg g^{-1} in 0.75-1.00% caffeine and 1.18-1.14 mg g^{-1} in 0.50-1.00% SA respectively, whereas in case of heavy metals it were

found to gradually decreased from 1.10-0.91 mg g^{-1} and 1.06-0.81 mg g^{-1} in 20- 100 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$ respectively.

The increase in total chlorophyll was significant at 5% level in 0.25% caffeine and 0.10% SA and at 1% level in 0.10% caffeine. The significant reduction in total chlorophyll was observed at 5% level in 60 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$ and 1% level in 80-100 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$.

The coefficient of variation increased linearly and was higher than control in all the mutagen treated populations.

4.11.4 Carotenoids (mg g^{-1})

The average carotenoid content in control was 0.86 mg g^{-1}. It increased over control from 0.97-0.88 mg g^{-1} in 0.10-0.50% caffeine and decreased to 0.80-0.77 mg g^{-1} in 0.75-1.00% caffeine respectively. In SA it increased from 0.94-0.88 mg g^{-1} in 0.10-0.25% SA and decreased from 0.80-0.72 mg g^{-1} in 0.50-1.00% SA, whereas carotenoid content decreased from 0.64-0.59 mg g^{-1} and 0.70-0.52 mg g^{-1} in 20-100 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$ respectively.

The increase in carotenoid content was significant at 5% level in 0.10% SA and at 1% level in 0.10% caffeine. The significant reduction was recorded at 5% level in 60-80 ppm $Pb(NO_3)_2$ and 60 ppm $Cd(NO_3)_2$ and at 1% level in 100 ppm $Pb(NO_3)_2$ and 80-100 ppm $Cd(NO_3)_2$ (Table, 21-24). The coefficient of variations increased with the increasing concentrations of all mutagens.

4.12 Biochemical parameter

4.12.1 Proline content (µg g^{-1})

The average proline content in control was 10.65 µg g^{-1}. It increased over control from 11.34-19.33 µg g^{-1} and 12.21-22.55 µg g^{-1} in 0.10-1.00% caffeine and SA respectively and from 14.34-25.73 µg g^{-1} and 16.02-26.14 µg g^{-1} in 20-100 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$ respectively.

A significant increase in proline content was observed at 5% level in 0.25% caffeine and 20 ppm $Pb(NO_3)_2$, at 1% level in 0.50-1.00% caffeine, 0.25-1.00% SA, 40-100 ppm $Pb(NO_3)_2$ and 20-100 ppm $Cd(NO_3)_2$..

The coefficient of variation increased linearly and was higher than control in all the mutagen treated populations with maximum increase in 100 ppm $Cd(NO_3)_2$.

Meiotic studies in M_1 generation

Cytological studies of chromosomal behavior during meiosis are regarded as one of the most reliable indices for assessing the potential of mutagens and the response of a plant's genotype. In the present experiments, various types of chromosomal abnormalities and their frequencies at different stages of meiosis were analyzed.

Linum usitatissimum L. has 15 bivalents (2n=30) showing normal meiotic division in control population. The diakinesis showed 15 pairs of ring bivalents. At metaphase-I, all 15 bivalents were normally arranged at equator, followed by the separation of 15-15 chromosomes (univalents) to their respective poles at anaphase-I. Telophase-I showed two groups of 15 chromosomes at each pole. Metaphase-II exhibited two groups of normal chromosomes at two equatorial planes, followed by four groups of chromosomes moving towards opposite poles at anaphase-II. At telophase-II, four groups of chromosomes were present on their respective poles. These meiotic stages in control were generally normal.

Various chromosomal aberrations have been recorded at different stages of meiosis in treated populations. The parameters of meiotic studies were univalents, multivalents, precocious separation, stray chromosomes, stickiness, laggards, bridges, unequal separation of chromosomes, multinucleate condition, disturbed polarity and cytomixis.

Abnormalities at Prophase-I stage

The number of PMCs with meiotic aberrations increased with increasing concentrations of mutagens, but their frequencies were comparatively lower than their respective concentrations in M_1 generation.

At the prophase stage, the number of cells with meiotic aberrations, such as frequency of univalents and multivalents, increased with increasing concentrations of all mutagens. The univalents and multivalents were absent in control but increased from 0.01-1.51% and 0.33-1.91% in 0.10-1.00% caffeine and SA; 0.73-2.66% and 1.66-3.56% in 20-100 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$ respectively.

Abnormalities at Metaphase-I/II stage

Some of the abnormalities observed at metaphase-I and II stages were univalents, multivalents, precocious separation of chromosomes, stray chromosomes and stickiness.

The chromosomal abnormalities were generally absent or minimum in lower concentrations of all mutagenic treatments, but increased in higher concentrations. The overall percentage of abnormal cells at metaphase-I/II stages ranged between 0.77-5.84% and 1.27-6.22% in 0.10-1.00% caffeine and SA; 1.88-7.89% and 3.35-9.37% in 20-100 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$ respectively.

Abnormalities at Anaphase-I/II stages

At the anaphase-I/II stage, the frequency of abnormalities like laggards, bridges, stickiness and unequal separation of chromosomes were observed, and their frequency was dose-dependent and increased with the increasing concentrations of mutagenic treatments. The overall percentage of cells showing anaphasic anomalies ranged between 0.71-5.22% and 0.71-5.91% in 0.10-1.00% caffeine and SA; 1.56-5.96% and 2.81-7.91% in 20-100 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$ respectively.

4.14.3 Abnormalities at Telophase-I/II stages

The abnormalities, particularly laggards, bridges, unequal separation, micronuclei and disturbed polarity, were higher in the increasing concentrations of all mutagens but either absent or present in very low frequency in control and lower concentrations. The frequency of abnormal cells at telophase ranged between 1.11-7.22% and 1.27-10.45% in 0.10-1.00% caffeine and SA; 3.65-12.12% and 4.23-12.38% in 20-100 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$ respectively.

The above result showed that the percentage of total abnormal cells was directly proportional to the increasing concentrations of mutagens and increased from 2.67-19.78% and 3.00-24.88% in 0.10-1.00% caffeine and SA; 6.92-28.99% and 11.81-34.77% in 20-100 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$ respectively.

These induced chromosomal abnormalities resulted in the higher frequency of morphological changes in treated populations providing the greater chances for the selection of desirable mutants in *Linum usitatissimum* L.

Variation in stomatal behaviour

Variation in leaf stomatal morphology was observed in the form of length and width of stomatal pores in the treated populations of all the mutagens. Structural variation in leaf stomata was observed using SEM at x3000 magnification and microphotographs.

Stomatal length

The average length of stomata in control were 9.46 µM. In caffeine, it increased significantly from 21.19-11.23 µM in 0.10-0.75% caffeine, but it decreased to 8.44 µM in 1.00% caffeine and in SA it increased from 18.45-11.34 µM in 0.10-0.50% SA and decreased from 9.21-7.92 µM in 0.75-1.00% SA. It decreased from 9.14-6.48 µM and from 8.77-5.79 µM in $Pb(NO_3)_2$ and $Cd(NO_3)_2$ respectively (Table, 14).

A significant increase in stomata length was observed at 5% level in 0.75% caffeine, while at 1% level in 0.10-0.50% caffeine and 0.10-0.25% SA. The reduction was significant at 5% level in 60 ppm $Cd(NO_3)_2$ and at 1% level in 80-100 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$.

The coefficient of variation increased linearly and was higher than control in all the mutagen treated populations with maximum increase in 100 ppm $Cd(NO_3)_2$.

Stomatal width

The average width of stomata in control were 2.24 µM. In caffeine, it increased from 3.41-2.43 µM in 0.10-0.50% caffeine, but it decreased from 1.91-1.68 µM in 0.75-1.00% caffeine and in SA it increased from 3.12-2.57 µM in 0.10-0.25% SA and decreased from 2.18-1.51 µM in 0.75-1.00% SA (Table, 13). It decreased from 2.07-1.16 µM and from 1.91-0.92 µM in $Pb(NO_3)_2$ and $Cd(NO_3)_2$ respectively.

A significant increase in stomata width was observed at 5% level in 0.25% SA, while at 1% level in 0.10%, 0.25% caffeine and 0.10% SA. The reduction was significant at 5% level in 0.75% SA at 1% level in 1.00% caffeine, 1% SA, 40-100 ppm $Pb(NO_3)_2$ and $Cd(NO_3)_2$.

The coefficient of variation (CV) increased with increasing concentrations of all the mutagens.

DISCUSSION

Biological damage (Seed germination, plant survival, pollen fertility)

This study observed a gradual decline in seed germination, plant survival and pollen fertility with increasing doses of all tested mutagens, including caffeine, sodium azide (SA), lead nitrate [$Pb(NO_3)_2$], and cadmium nitrate [$Cd(NO_3)_2$], with the maximum decrease observed in $Cd(NO_3)_2$ followed by $Pb(NO_3)_2$, SA and caffeine. The inhibition of germination, lethality and pollen sterility was directly proportional to the increasing concentrations of mutagens, while the germination percentage showed an inverse correlation, indicating a dose-dependent effect of mutagenic treatments. The inhibition of germination in *Linum usitatissimum* observed due to the effects of mutagens aligns with the findings of other researchers in the same crop, such as Rowland *et al.* (2003). The reduction in seed germination has been attributed to delays or inhibition in essential physiological processes, including enzyme activity (Kurborne *et al.*, 1979; Kumar, 2005), hormonal imbalance (Chrispeeds and Varner, 1976), and suppression of the meiotic process (Ananthaswamy *et al.*, 1971), chromosomal deletions (Khan *et al.*, 2007), molecular damage to cellular components, and disruptions in enzymatic functions (Khan and Goyal, 2009) and increased chromosomal aberrations due to mutagens (Alka *et al.* 2012). Girija *et al.* (2013) reported that mutagenic effects can lead to increased chromosomal aberrations, which may cause mitotic arrest and ultimately result in cell death.

Cotyledonary and vegetative leaves variations

Mutagens were observed to be significantly effective in causing morphological variations and mutations in both cotyledonary and vegetative leaves. The present investigation revealed that the highest frequency of variations was recorded in the M_1 generation. The morphological deviations recorded in M_1 were primarily adoptive, giving rise to recessive mutations and a few stable homozygous dominant mutations. A wide range of variations was observed in cotyledonary and vegetative leaves, including alterations in the number, shape and size of leaves, variations in leaf margin and thickness, fusion of margins and modifications in leaf apices such as blunt, obtuse, or notched forms. Additionally, a decrease in the angle between the two cotyledonary leaves was observed under varying concentrations of caffeine, SA, $Pb(NO_3)_2$, and $Cd(NO_3)_2$. Similar variations in leaf morphology have been reported by several researchers in different plant species using both physical and chemical mutagens, including *Linum usitatissimum* (Akhtar *et al.*, 2012; Jahan *et al.*, 2019; Jahan *et al.*, 2024), *Trigonella foenum-graecum* (Choudhary, 2012; Naaz *et al.*, 2020), *Capsicum annuum* (Arisha *et al.*, 2015), *Glycine max* (Lande *et al.*, 2018), *Phaseolus vulgaris* (El-Lithy *et al.*, 2023), and *Vigna unguiculata* (Dhanasekar and Souframanien, 2024).

Lea (1955) reported that the emergence of abnormal leaf forms in plants exposed to mutagens could be due to alterations in internal growth regulators such as indole-3-acetic acid (IAA), influenced by mutagenic activity, or as a consequence of the primary or secondary impact of free radicals produced by mutagens. Hagen and Gunckel (1958) stated that elevated amino acid levels in leaves might be associated with the formation of abnormal leaf structures. Such abnormalities could also arise from disturbances in metabolic functions triggered by mutagenic treatments (Devreux and Mugnozza, 1964), or due to chromosomal abnormalities, as reported by Bhat *et al.* (2012b).

Quantitative traits

Quantitative traits such as, plant height, number of fertile branches, number of capsules per plant, number od seeds per capsule, 1000 seed weight and total seed yield per plant, generally showed a decreasing trend with increasing concentrations of all mutagens. However, at lower concentrations of caffeine and SA, an increase in quantitative traits was observed over the control in M_1. These lower and moderate doses of caffeine and SA appeared to be more effective in inducing beneficial mutations. Overall, the most severe reduction in quantitative traits was caused by $Cd(NO_3)_2$, followed by $Pb(NO_3)_2$ and the higher concentrations of caffeine and SA. Several researchers have reported an increase in quantitative trait induced by various chemical mutagens across different crops, such as in *Helianthus annuus* by caffeine (Khursheed *et al.*, 2009), *Sesamum indicum* by EMS (Kumar and Yadav, 2010), *Digitaria exilis* by colchicine (Nura *et al.*, 2017), *Vigna radiata* by gamma rays, EMS, and NG (N-nitrosoguanidine) (Swain *et al.*, 2019), and *Trigonella* by caffeine and SA (Naaz *et al.*, 2023). On the other hand, higher concentrations of mutagens demonstrated a suppressive effect, leading to a decrease in plant height compared to the control. This observation is consistent with findings in several crops, including *Glycine max* (Ahire and Auti, 2015), *Capsicum annuum* (Aslam *et al.*, 2017), *Trigonella* (Hassan *et al.*, 2018), wheat (*Triticum aestivum*) (Nazarenko *et al.*, 2019), *Linum usitatissimum* (Bhat *et al.*, 2016; Jahan *et al.*, 2024), and *Chrysanthemum* (Nasri *et al.*, 2021).

Different researchers have explained that the decline in quantitative trait following mutagenic treatments could be attributed to chromosomal irregularities and disruptions in cell division, ultimately leading to a general decline in plant or seedling growth (Yang *et al.*, 2018; El Rasafi *et al.*, 2021). Hedden (2003) attributed the reduction in quantitative trait to a decrease in the number and length of internodes, which may result from reduced cell size, diminished cell number, and changes in gibberellic acid levels. The reduced growth has been attributed to the destruction of auxins, changes in ascorbic acid content, and disturbances in physiological and biochemical processes (Ussuf and Nair, 1974; Sharma *et al.*, 2020). Additionally, chromosomal injury during mitotic cell division, inhibition of DNA synthesis, and damage to meristematic cells have been identified as further contributors to reduced plant growth (Ramya *et al.*, 2014).

CONCLUSION

In the present investigation, it is concluded that caffeine generates variety of phenotypic variants in *Linum usitatissimum* var. Shekhar. Lower concentrations (0.10% - 0.50%) of caffeine and SA did not significantly affect the morphology of *Linum*, while higher concentrations (0.75% - 1.00%) of caffeine and SA and heavy metals significantly affect the morphology and reduce various yield parameters. The present study reveals moderate concentrations of caffeine and SA induce mutagenic damage in *Linum usitatissimum* var. Shekhar which can facilitates screening of desirable mutant in M_2 and M_3 generations.

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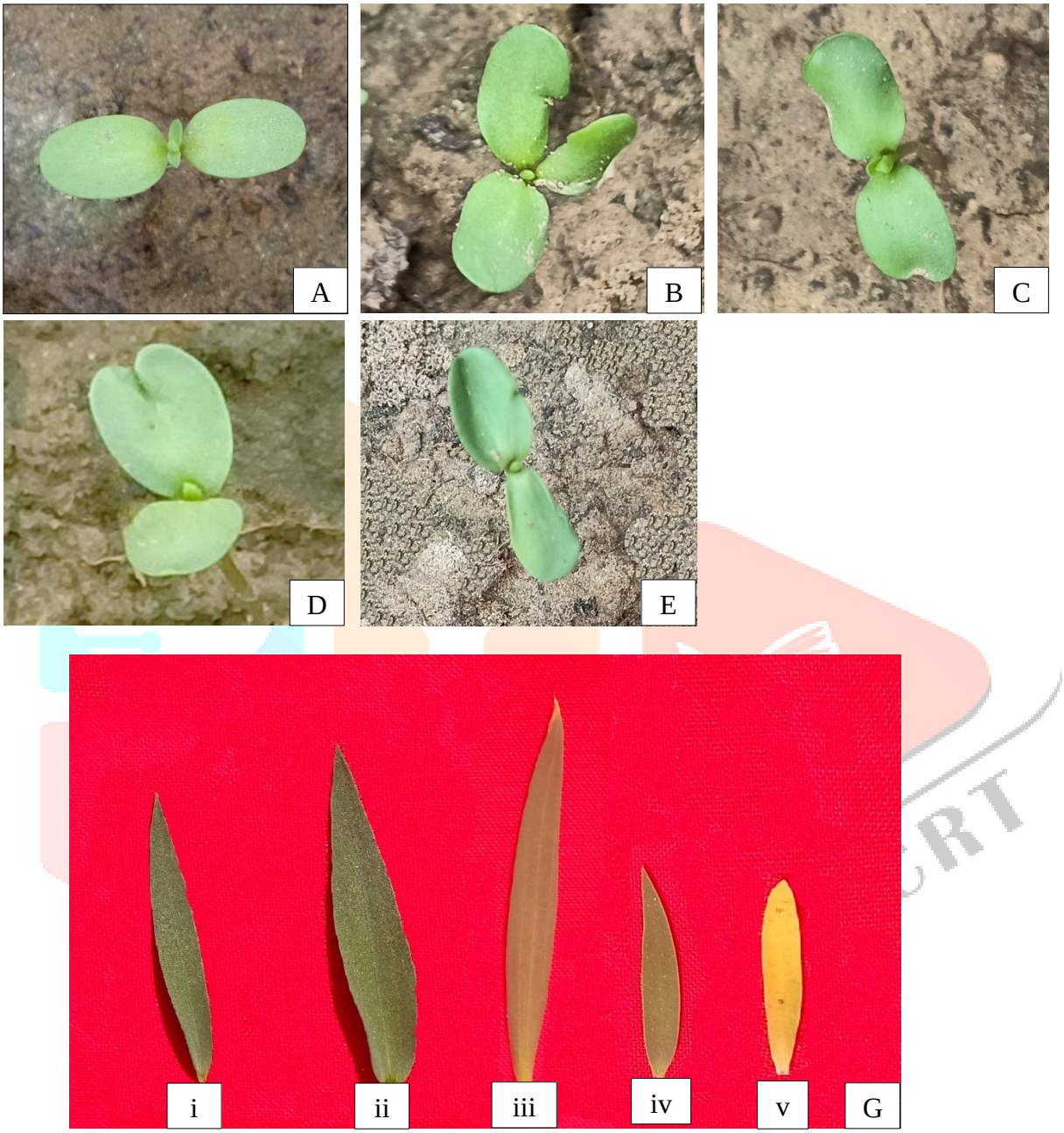

Figure 1- Different leaves variants- (A) control cotyledonary leaves with two opposite, elliptical, entire and smooth margin, (B)- Three cotyledonary leaves, one normal and two with wavy margin, (C)- Broad leaves with notch in margin, (D)- Broad heart shape leaf, (E)- curved cotyledonary leaves, (G)- i. Control vegetative leaf, lanceolate and with entire margin, ii. Long broad with slightly curved vegetative leaf, iii. Long, narrow lanceolate with smooth margin, iv. Small vegetative leaf, v. Small vegetative leaf showing chlorophyll mutation.

Plate- 2

Figure 2- (A)- control plant with normal height and yield, (B)- Tall and bushy variant with increased yield, (C)- Tall variant, (D)- dwarf variant with no fruit setting

Figure 3- (A) - Control flower with violet blue, free equal sized 5 petals (pentapetalous), (B), Hexapetalous flower variant, (C), variant flower with distorted symmetry, (D)- variant flower with white colored petals, (E)- variant flower with dark purple colored petals

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Figure 4- (A)- i. Control capsules, ii. Large sized capsules, iii. Small size capsules, (B)- i. control glossy brown seeds, ii. Large size seeds, iii. Small size seeds

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