Mutagenic Potential of Lead Nitrate and Cadmium Nitrate for Generating Genetic Variability in Grain Amaranth (Amaranthus hypochondriacus L.)

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Table 1 to 5 are available in the Supplementary Files section.

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Abstract: There is little evidence of heavy metals being used as mutagens to induce genetic variation in crops. This study was conducted to evaluate the relative effectiveness and the cyto-morphological and physiological effects of various doses of heavy metals [$Cd(NO_3)_2$ and $Pb(NO_3)_2$] in grain amaranth. A range of morphological and chlorophyll variations was observed in treated populations of two varieties of amaranth. Variable order of chlorophyll mutants were observed in the M_2 generation across both the heavy metals. The mutagenic efficacy and efficiency considerably decreased with increasing concentrations of the mutagens, with the order of mutagenic effectiveness being $Pb(NO_3)_2 > Cd(NO_3)_2$ in both the varieties. The yield and yield contributing traits were seen to improve at lower concentrations of the mutagens. Eleven mutant lines exhibiting improved agronomic traits were selected from the 10ppm, 20ppm, and 30ppm concentrations of both mutagens in the M_3 generation. The high heritability estimates and genetic advance (GAM) for agronomic traits in M_3 lines suggest that traits are governed by additive gene action and less affected by environment. The standardized concentrations of heavy metals can serve as a very effective and efficient mutagen in a lesser explored crop like grain amaranth with limited breeding advancements. The genetic and transcriptomic studies are advised between the mutant lines and wild types to identify specific genes associated with altered phenotypes. The large mutagenized populations (M_2) can serve as an ideal TILLING populations to decipher the specific genes linked with agronomic traits in grain amaranth.

Keyword: Amaranth, Cadmium, Lead, Genetic variability, Physiological parameters, Mutants

Introduction

Among the minor and underutilized crops, grain amaranth (*Amaranthus hypochondriacus*) has the huge potential as an alternative crop to sustain food production, enhance nutritional security and diversification of modern cropping systems. Grain amaranth is known for its high methionine and lysine content, enhance minerals, gluten free nature and low glycemic index, contrasting to major cereal crops (Malik et al. 2023). Moreover, bioactive compounds such as squalene, sterol, tocopherols, tocotrienols and lipohillic compounds are important constituents of amaranth and have significant therapeutic importance to reduce risk of the cancer, obesity, cardiovascular diseases and type 2 diabetes (Reyad-ul-Ferdous et al. 2015).

Despite its versatile adaptation and high relevance in climate resilient low input agriculture, efforts for genetic enhancement are lacking in grain amaranth. Due to its diminutive florets (< 1mm), limited genetic variability, and cumbersome crossing techniques, endeavors to augment the genetic base of the crop by recombination breeding remain speculative (Stetter, Joshi, and Singh 2025). Mutation breeding provides the opportunity over conventional breeding methods by addressing the genetic limitation in induce cultivars, offering a sustainable pathway for the improvement of crop species. Despite constrained efforts, cultivars and genotypes exhibiting enhanced grain yield, early maturity and nutritional quality have been developed through mutation breeding in amaranth (Yeshitila et al. 2023). The outcome of mutation breeding mainly depends on the mutagenic effectiveness, efficiency, and mutagen concentration (Goyal et al. 2019). Thus, at the start of a multi-year mutagenesis experiment, it is essential to assess the mutagenic efficacy and efficiency of used mutagens (Goyal et al. 2020). The optimal dose of mutagens would be revealed by determining mutagenic effectiveness and efficiency. Several hundred improved mutant varieties have been successfully developed and formally released using optimal doses of mutagens and determine mutagenic effectiveness and efficiency (Raina et al. 2022). In mutation breeding, effectiveness and efficiency are very important factors to determine the mutations induced by various concentrations of used mutagens (Dhanavel and Girija 2013). The mutagenic effectiveness determines the effective doses of mutagens to induce desirable mutations among treated populations and efficiency indicates mutation rate in order to seedling injury (I), sterility (S) and meiotic abnormalities (Me). The mutagenic effectiveness and efficiency were reported in various crops such as fenugreek (Naaz et al. 2023), chilli (Hasan et al. 2025) and grain amaranth (Joshi et al. 2026).

EMS and sodium azide (SA) are widely used chemical mutagens that mainly induce GC → AT point mutations through alkylation of guanine, producing stable and heritable mutations (Gruszka, Szarejko, and Maluszynski 2012; Sikora et al. 2011). In contrast, heavy metals cause genotoxicity indirectly through oxidative stress, DNA repair inhibition, and chromosomal instability (Hartwig 2013). Although less explored as mutagens, cadmium and lead have been reported to induce chromosomal aberrations, micronuclei, and morphological mutations in crop plants, indicating their potential to cause heritable genetic changes (Aslam et al. 2023; Hasan, Choudhary, Laskar, et al. 2022; Jaiswal, Dey, and Bag 2021).

Grain amaranth is gaining recognition as a possible alternative to traditional cereal crops. However, natural genetic diversity for economic traits in crop gene pool is limited. Therefore, induction of novel variability through mutagenesis is essential to address the current and future issues of grain amaranth breeding. Consequently, this study aimed to employ induced mutagenesis in grain amaranth to generate genetic variation for economically significant traits using lesser explored heavy metals (lead nitrate and cadmium nitrate). Given the genotypic specificity of mutagen response, initial doses of lead and cadmium nitrate were standardized for field mutation experiments using two cultivars of grain amaranth (*A. hypochondriacus*). The primary purpose of this study was to determine the effective concentration and ascertain the mutagenic effectiveness and efficiency of lead nitrate and cadmium nitrate to induce mutations in grain amaranth cultivars.

Material and methods

Grain amaranth (*Amaranthus hypochondriacus* L.) varieties VL 44 and VL 110 developed by Indian Council of Agricultural Research-Vivekananda Institute of Hill Agriculture, Almora, Uttarakhand, India, constituted the biological material for mutagenesis experiments in the present study. The varieties were selected due to its wide adaptability to rainfed agro-ecology of North Western Himalayan region of the country. These varieties are popular for its high yield potential and creamish colour grains. However, it has some inherent drawbacks, like small grain size and susceptibility to lodging. Therefore, comparative effectiveness and efficiency of two heavy metals as mutagens were tested with the objective to isolate desirable mutants in heavy metal treated populations of grain amaranth.

Mutagenic treatments of seeds

Working solutions (10–50 ppm) of cadmium nitrate [$Cd(NO_3)_2$] and lead nitrate [$Pb(NO_3)_2$] were prepared from a 1000 ppm stock solution inside a certified fume hood. Heavy metal treatments were conducted at minimal effective concentrations with proper safety measures to prevent environmental and occupational exposure. For each treatment, 250 seeds pre-soaked in water for 18 h were immersed in the mutagen solution for 6 h. A separate batch of 250 water-soaked seeds without mutagen treatment was maintained as the control for comparative evaluation.

The treated seeds were sown in mid-June during rainy season with the spacing of 50 × 15 cm in RCBD (randomized complete block design) design. The experiments were conducted at experimental farm, Hawalbagh (79.39° E longitude and 25.35° N latitude, mean rainfall-1000 mm and 1250 m above msl) of ICAR- Vivekananda Institute of Hill Agriculture, Almora. Total five replicates were made and 50 seeds sown in each replication (total 250 seeds were taken for each treatment). Total five plants were selected from each replicate to perform the statistical analysis for the biological, physiological and quantitative data, and to evaluate the mutagenic effects of heavy metals.

Description of seed sowing and seed germination from M_1 to M_3 generation in Var. VL 44 and Var. VL 110 varieties was listed in supplementary table 1. Total 70,206 and 71,917 seeds were harvested from M_1 generation of both VL 44 and VL 110 and only 66,107 and 68,790 seeds were germinated in M_2 generation, respectively. Total 10,338 seeds were collected from selected superior progenies of both VL 44 and VL110 and sown to raise M_3 generation. Only, 10,101 seeds were germinated in M_3 generation and morphological mutations were observed in selected mutant lines from seedling stage to maturity. The pictorial representation of heavy metals induced mutagenesis and development of mutant lines during successive generations in grain amaranth is depicted in Fig. 1.

The observations made in M_2 generation are expressed as a percent reduction in biological (*viz.,* germination reduction, sterility, and seedling injury) and quantitative (*viz.,* plant height, flowering, 1000-seed weight, etc.) parameters over their respective control. The floral buds (10-15 buds) were collected randomly from each treatment for the analysis of pollen fertility. The mature and undehisced anthers were dusted on glass slide and stained with 1% acetocarmine solution. Pollen grains with regular shape (round shape) and irregular shape (shrunken pollen) were considered as viable pollens and sterile pollen, respectively. The percent pollen fertility and sterility were measured by using following formulae.

Percent pollen fertility = $\frac{\text{Number of fertile pollent grains}}{\text{Total number of pollen grains}} \times 100$

Percent pollen sterility (reduction) = $\frac{\text{Number of sterile pollen grains}}{\text{Total number of pollen grains}} \times 100$

The number of progeny in M_1 and progeny segregated in M_2 generation characterized as chlorophyll mutants are represented in supplementary table 2 & 3. The biological parameters *viz.,* seedling injury, percent germination reduction were recorded at the time of seedling emergence and pollen fertility and sterility measured at the time of 50% flowering.

Chlorophyll mutants

A range of chlorophyll mutations were observed after 25 days of seed sowing and characterized into distinct mutants *viz., chlorina, xantha, variegated, viridis, albino, etc.* based on color appearance at seedling stage. Chlorophyll mutations were categorized into following chlorophyll mutants by using the protocol proposed by Gustafsson, (1940). The mutation frequency (*F*) of distinct chlorophyll mutants was measured by using the following formula:

$$Mutation\ frequency\ (\%) = \frac{Number\ of\ particular\ chlorophyll\ mutants\ in\ M2}{Total\ number\ of\ chlorophyll\ mutants\ studied} \times 100$$

Mutant phenotyping

Morphological mutations were observed critically in treated populations and mutant lines of M_2 and M_3 generations. The major morphological traits such as plant height, panicle morphology, leaf morphology, etc. were characterized into sub-categories viz., tall/dwarf mutant, red/green panicle, distinct leaf morphology etc. Morphological mutations in various parts of treated plants were observed, recorded and analyzed to understand the mutagenic role of heavy metals in underutilized crops. The mutation frequency (*F*) in morphological traits was measured by using the given formula:

$$F\ (\%) = \frac{Number\ of\ mutant\ plants}{Total\ number\ of\ studied\ plants} \times 100$$

Effectiveness and Efficiency

Mutagenic effectiveness was determined by calculating the frequency of chlorophyll mutants in M_2 per unit dose of mutagen, while efficiency was assessed by relating mutation frequency to parameters of biological damages such as seedling injury (I), pollen sterility (S) and meiotic abnormalities (Me). Mutagenic effectiveness and efficiency were determined in M_2 generation by the formula given by (Konzak et al. 1965).

$$Mutagenic\ Effectiveness = \frac{Rate\ of\ mutation\ (Mp)\ \text{in M2 generation}}{Concentration\ (ppm) \times duration\ of\ treatment}$$

$$Mutagenic\ Efficiency = \frac{Rate\ of\ mutation\ (Mp) in\ M2\ generation}{Biological\ damage\ in\ M1\ generation}$$

Mutagenic efficiency for biological damage = $\frac{\text{Mp}}{\text{I}}$ or $\frac{\text{Mp}}{\text{S}}$ or $\frac{\text{Mp}}{\text{Me}}$

$$Mutation\ rate\ (\%) = \frac{Number\ of\ mutant\ plant\ in\ M2}{Total\ number\ of\ plants\ observed} \times 100$$

Mp = Rate of mutation expressed in percent of chlorophyll mutation in M_2 generation, estimated on M_1 plant basis, **I** = Percent seedling injury, **S** = Percent pollen sterility, **Me** = Percent of meiotic aberrations.

Estimation of chlorophyll and physiological parameters

Fresh leaves were collected from each treatment and transferred 1.g fresh leaves to motor and pestle. Leaf sample was crushed by adding 20ml of 80% acetone solution for homogenization. The homogenized solution was transferred to centrifuge tubes for centrifugation at 5000 rpm for 10 minutes. The supernatants were collected into 100ml volumetric flasks to make the final volume by adding 80% acetone solution. These solutions were subjected to spectrophotometry for the absorbance at 663nm and 645nm wavelengths to analyze the total chlorophyll content. The total chlorophyll content was calculated by the following formula suggested by (Arnon 1949).

Total chlorophyll per gram fresh leaves = $\frac{[20.2\ (OD645 + 8.02\ (OD663)]}{W \times 1000 \times a} \times V$

Where,

OD_{645} and OD_{663} = Optical density at 645nm and 663nm wavelength, respectively.

W = Weight of fresh leaf sample (g)

V = Volume of sample solution (ml)

a = Length of light path in cell (1cm)

The physiological parameters *viz.,* stomata conductance, transpiration rate and photosynthetic rate were analyzed in each treatment. Infrared Gas Analyzer (IRGA) was performed for the assessment of physiological parameters during day time from 10:00 to 11:00 am.

Statistical Analysis

Statistical analysis was conducted for seven quantitative traits viz., flowering & maturity (days), number of spikelet per panicle (n), panicle length (cm), 1000-seed weight (g) and grain yield (g). Trait comparisons were specified in the experimental design and statistical model (e.g., ANOVA), along with the mean separation test were used (LSD/DMRT)at significance level (α = 0.05 or 0.01). Heritability estimates was calculated, including the variance components and estimation method (ANOVA) by using SPSS, OriginPro and R studio.

Genetic Components

Genetic component of variance (GCV)

Genetic variance for quantitative traits was calculated by using the formula given (Burton and DeVane 1953) as follows:

$$\text{Genotypic coefficient variance (GCV)} = \frac{\sqrt{\sigma 2G}}{\bar{X}} \times 100$$

Where,

σ^2G = genetic variance = MSG – MSE/r (MSG is estimation of mean square of observations, MSE is estimation of mean square of error and r is replication number).

Broad sense heritability (h^2bs)

Broad sense heritability represents the variations arise due genetic elements and calculated by using given formula (SURPRENANT et al. 2011).

$$h^2bs = \sigma^2G/\sigma^2P$$

Where,

σ^2G/σ^2P = Genetic variance/Phenotypic variance

Range of heritability has been classified and suggested by (SURPRENANT et al. 2011).

Low < 40%, Medium:High 60 – 80%, Very high > 80%

Genetic Advance as Percent of Mean (GAM)

Selection intensity (k) of a trait is mainly depend on genetic advance as percent mean (GAM) and estimated by using the formula (Johnson, Robinson, and Comstock 1955).

$$\text{GAM} = \frac{k.\sigma p.h^2\ bs}{\bar{\text{X}}} \text{ x } 100$$

Where,

σh = phenotypic standard deviation, h^2bs = broad sense heritability and k = selection differential (k = 2.06 at 5% selection intensity).

Range of genetic advance has been suggested by (Johnson, Robinson, and Comstock 1955).

Low < 10%; Moderate 10 – 20%; High > 20%

Results

Morphological characterization

The eleven mutant lines selected from effective concentrations in M_3 generation reflected the significant modification in inflorescences characteristics such as spike length, density, color, panicle architecture, panicle morphology and plant height compared to the untreated control (Fig. 2 and S1).These morphological variants were evaluated for heritability, phenotypic stability across generations, agronomic superiority and absence of deleterious pleiotropic effects. The high estimates of heritability for all the traits indicated less environmental influence in their expression.

Seed germination (%):

Percent seed germination was recorded to decrease significantly with enhanced concentrations of the mutagens (Fig. 3 & 4). The highest significant reduction in percent seed germination was recorded to be 55.20% and 53.64% in var.VL 44 at higher concentration (50ppm) of cadmium nitrate and lead nitrate, respectively. In var. VL 110, the highest significant reduction was recorded to be 53.45% in cadmium nitrate treated populations, while it was 51.24% in lead nitrate treated populations (Fig. 3 & 4). The order of percent reduction of seed germination was $Cd(NO_3)_2$> $Pb(NO_3)_2$] for the mutagens, while it was var. VL 44 >var. VL 110.

Pollen fertility (%):

In pollen fertility, a significant reduction was recorded with increasing doses of the mutagens in both VL 44 and VL 110. The lowest pollen fertility was recorded to be 58.65% and 59.05% in var. VL 44, while it was 59.14% and 62.70% at higher concentration (500ppm) of $Cd(NO_3)_2$ and $Pb(NO_3)_2$ in var. VL 110, respectively. The highest significant reduction was recorded to 39.35% and 38.40% in var. VL 44, while it was 37.30% and 33.28% in var. VL 110 at higher concentration/dose (Fig. 3 & 4). The order of mutagenic effects was $Cd(NO_3)_2$ > $Pb(NO_3)_2$ and var. VL 44 affected more by the usage of mutagens than var. VL 110 (var. VL 44 >var. VL 110).

Quantitative traits:

Quantitative traits such as plant height, number of spikelets per panicle, panicle length, 1000-seed weight, and grain yield showed a positive response at lower concentrations (10–20 ppm) of $Cd(NO_3)_2$ and $Pb(NO_3)_2$ in both varieties (Fig. 3 & 4). Flowering and maturity duration increased with increasing mutagen concentration, with the maximum delay observed at 50 ppm in both varieties. Plant height, panicle length, spikelets per panicle, 1000-seed weight, and grain yield increased slightly at lower doses but declined significantly at higher concentrations (Fig. 3 & 4).

The highest reduction in plant height at 50 ppm was recorded in var. VL 44 under $Cd(NO_3)_2$ (48.34%) followed by $Pb(NO_3)_2$ (45.11%), while in var. VL 110 the reductions were 44.00% and 34.26%, respectively. Similarly, the number of spikelets per panicle and panicle length declined markedly at higher concentrations in both varieties. The 1000-seed weight and grain yield per plant also showed significant reductions at 50 ppm, with $Cd(NO_3)_2$ exerting a stronger inhibitory effect than $Pb(NO_3)_2$.

Overall, var. VL 110 was more affected than var. VL 44, and $Cd(NO_3)_2$ proved to be more effective than $Pb(NO_3)_2$ in reducing quantitative traits (Fig. 3 & 4).

Physiological parameters

Physiological traits, such as total chlorophyll content, stomata conductance, transpiration rate, and photosynthetic rate, were assessed in treated populations of grain amaranth. A lower concentration (10–20 ppm) of $Cd(NO_3)_2$ and $Pb(NO_3)_2$ enhanced these parameters in both varieties, but higher concentrations (50 ppm) resulted in considerable declines (Fig. 3 & 4). The total chlorophyll content exhibited a little rise at lower concentrations but observed a significant loss at elevated concentrations, with the most substantial reduction noted in var. VL 44 under $Cd(NO_3)_2$ (48.77%), followed by $Pb(NO_3)_2$ (39.09%). In variety VL 110, the reductions were 39.46% and 35.16%, respectively.

Stomata conductance enhanced at lower concentrations but declined markedly at 50 ppm. The most significant reduction occurred in var. VL 44, exhibiting declines of 61.76% and 51.76% under $Cd(NO_3)_2$ and $Pb(NO_3)_2$ treatments, respectively, whilst var. VL 110 demonstrated reductions of 61.53% and 57.16% (Fig. 3 & 4). Likewise, the rates of transpiration and photosynthesis rose at lower dosages but decreased markedly at elevated concentrations. The maximum decrease in transpiration rate was observed at 52.76% and 51.76% for var. VL 44, and 52.58% and 39.94% for var. VL 110 under $Cd(NO_3)_2$ and $Pb(NO_3)_2$ treatments, respectively. The photosynthetic rate was significantly impaired by $Cd(NO_3)_2$, with reductions of 61.20% in var. VL 44 and 59.93% in var. VL 110, demonstrating that $Cd(NO_3)_2$ had a more pronounced inhibitory effect than $Pb(NO_3)_2$ on physiological parameters in both varieties .

Chlorophyll mutant characterization

Chlorophyll mutations were characterized on the basis of color appearance in leaves at seedling stage. Chlorina mutants with light green or pale green foliage with reduce chlorophyll and often viable were observed in 10ppm 30ppm treatments (Fig. 5G) and viridis mutant showing dark green leaves with altered chlorophyll metabolism generally viable was observed in 20ppm treatment of lead nitrate (Fig. 5D). Aurea mutants showing yellow to pale-green foliage with reduce chlorophyll synthesis often viable were observed in 30ppm and 40ppm (fig. 5B), while variegate mutant (white patches partially chlorophyll deficient and viable, Fig. 5F), and Albino mutant (seedling with non-pigmented leaves and lethal 1-3 weeks, Fig. 5H) were observed 10ppm and 50 ppm treatments of cadmium nitrate, respectively. In all the treatments, the highest frequency was observed for chlorina mutants, and in var. VL 110 it was recorded to be 27.02%

and 24.57% in cadmium nitrate and lead nitrate, respectively. While frequency of chlorina in var. VL 44 was recorded to be 24.47% and 20.08% in lead nitrate and cadmium nitrate, respectively (Tables 1, 2).. The frequency of chlorophyll mutations in var. VL 110 was found to more than var. VL 44 (var. VL 110 > var. VL 44). The overall chlorophyll mutation frequency in chemical treated populations was found to be more in lead nitrate than cadmium nitrate [$Pb(NO_3)_2$ > $Cd(NO_3)_2$] (Tables 1 & 2).

Mutagenic effectiveness and efficiency

Mutagenic effectiveness showed declining order with enhanced concentration of the mutagens (Table 3). In var. VL 44, the highest mutagenic effectiveness was recorded to be 1.57 and 1.72, and in var. VL 110, it was1.63 and 1.76 at lower concentration (10ppm) of $Cd(NO_3)_2$ and $Pb(NO_3)_2$, respectively (Table 3). The order of mutagenic effectiveness was $Pb(NO_3)_2$> $Cd(NO_3)_2$ in both varieties of amaranth, and var. VL 110 showed more mutagenic effectiveness than var. VL 44 (Table 3). Similarly, mutagenic efficiency was recorded to decrease with increasing concentrations of the mutagens. The highest mutagenic efficiency in inducing sterility and seedling injury was recorded to be 0.42/0.57 and 0.71/0.93 at lower concentration (10ppm) of $Cd(NO_3)_2$/$Pb(NO_3)_2$, while it was 0.52 and 0.58 at 20ppm and 10ppm of $Cd(NO_3)_2$ and $Pb(NO_3)_2$ for meiotic abnormalities in VL 44, respectively. In var. VL 110, the highest mutagenic efficiency for the following biological damages was recorded to 0.30/0.34, and 0.96/1.07 at lower concentration (10ppm) of $Cd(NO_3)_2$/$Pb(NO_3)_2$, while it was 0.53 and 0.65 for meiotic abnormalities in 10ppm and 20ppm of $Cd(NO_3)_2$/$Pb(NO_3)_2$, respectively (Table 3). The order of mutagenic efficiency in both varieties of amaranth was $Pb(NO_3)_2$> $Cd(NO_3)_2$ and var. VL 110 showed more mutagenic efficiency than var. VL 44.

Characteristics of high yielding mutant lines

Total eleven mutant lines with enhanced yield contributing traits were selected in var. VL 44 (Am1, Am2, Am3, Am4 and Am5) and var. VL 110 (AM1, AM2, AM3, AM4, AM5, and AM6) in lower and medium treatments of $Cd(NO_3)_2$ and $Pb(NO_3)_2$. Several valuable mutations in morphology, physiology and yield contributing traits were recorded in selected mutant lines induced at lower and medium doses of the mutagens. High yielding superior dwarf mutant line (Am5) with multiple branches was observed in var. VL 44 under the treatment of 20ppm $Cd(NO_3)_2$, while in var. VL 110 superior dwarf mutant line (AM6) with useful mutations was observed under the treatment of 30ppm $Pb(NO_3)_2$ (Fig. S1). The agronomic traits with enhanced genetic diversity in selected mutant lines are represented in Fig. 6 and Tables 4 & 5.

Genetic component analysis

In M_3 generation, genetic components were analyzed for the assessment of genetic variability of selected mutant lines in quantitative traits (Table 5). Thousand seed weight exhibited the highest genetic coefficient of variation (GCV) in variety VL 44, recording 25.24% and 23.70% under $Cd(NO_3)_2$ and $Pb(NO_3)_2$ treatments, respectively, whereas in variety VL 110, the corresponding values were lower at 14.70% and 13.82%, respectively (Table 5). The highest heritability (h^2bs) in variety VL 44 was observed for plant height, with values of 97.96% and 98.59% under $Cd(NO_3)_2$ and $Pb(NO_3)_2$ treatments, respectively. Similarly, in variety VL 110, the highest heritability was recorded for grain yield, with values of 98.11% and 97.52% under $Cd(NO_3)_2$ and $Pb(NO_3)_2$ treatments, respectively (Table 5). The highest genetic advance as percent of mean (GAM) was also observed for plant height, recording 57.40% and 62.20% in variety VL 44 and 57.70% and 54.60% in variety VL 110 under $Cd(NO_3)_2$ and $Pb(NO_3)_2$ treatments, respectively.

Discussion

Morphological variations

The mutant lines selected from effective doses in the M_3 generation showed significant variations in inflorescence traits, including spike length, density, color, panicle architecture, panicle morphology, and plant height, compared with the untreated control (Fig. 3 and S1). High heritability estimates indicated minimal environmental influence on trait expression. Several mutants exhibited reduced plant height, an advantageous trait in grain amaranth due to improved lodging resistance and reduced yield loss under strong winds. The semi-dwarf phenotype at lower and moderate mutagen doses may be linked to changes in genes regulating cell division and phytohormone pathways. The selected M_3 mutant lines also showed an increased number of spikelets per plant and enhanced grain yield, particularly at lower and moderate mutagen doses. These traits highlight the potential of the mutants as valuable genetic resources for grain yield improvement in grain amaranth.

Biological parameters

A declining trend in seed germination with increasing concentrations of the mutagens was recorded. Roy et al. (2024) reported a decreasing trend of seed germination in grain amaranth with enhanced concentration of Cd^{2+}, and Cd^{2+} ions due to their penetrance into seed coat and interfere with nucleic acids and production of reactive oxygen species (Talanova, Titov, and Boeva 2001). Baruah et al. (2019) were also reported a reduction in seed germination at variable concentrations of heavy metals in wheat, pea and tomato crops.

The highest reduction in pollen fertility was recorded at higher concentration of both the mutagens and var. VL 44 showed more reduction in seed germination than var. VL 110. (Nayab, PLANT, and 2024 2024) reported the sensitivity of pollen grain towards the heavy metals and changes in pollen morphology, reduced fertility and viability, and enhanced allergenic potential of pollen grains of various crops including grain amaranth. Reduce pollen fertility and viability was also reported in peas under the treatment of cadmium (Cd) and cobalt (Co) (Nessem et al. 2023).

Quantitative trais

Plant height increased at lower concentrations of both $Cd(NO_3)_2$ and $Pb(NO_3)_2$ in treated populations of var. VL 44 and var. VL 110. (Roy et al. 2024) reported a decrease in plant height and root length of grain amaranth with increased concentrations of cadmium. The plants grown under the treatment of a substrate with 20 mg Cd kg^{-1}dw do not have expected effect on plant height (Haller et al. 2023).

The variations in morphology of spikelet and panicle were observed and a slight increase was also recorded at lower concentrations of $Cd(NO_3)_2$ and $Pb(NO_3)_2$. Mutations in panicle shape and size, and number of spikelet per panicle were reported in sorghum (Wanga et al. 2023) under the treatment of EMS. Prabhakaran et al. (2026) reported panicle variations in early maturing amaranth crop under the treatment of variable concentrations of heavy metals. In the present study, flowering and maturity reflected positive effects of $Cd(NO_3)_2$ and $Pb(NO_3)_2$ at lower concentrations (10ppm) and delayed at medium and higher concentrations. Early flowering was reported in amaranth growing on sludge based composed containing heavy metals (Baniasadi et al. 2022). Late flowering was reported in some millets species growing on heavy metals contaminated soil (Vasilachi, Stoleru, and Gavrilescu 2023).

The higher concentrations of both mutagens have negative effects and reduced the 1000-seed weight and grain yield. Detrimental effects of heavy metals on grain yield in amaranth were also reported by (Berseneva et al. 2023).

Physiological parameters

Physiological parameters were shown to improve at lower concentrations of mutagens in both varieties, with Pb(NO3)2 exhibiting more favorable effects than Cd(NO3)2. The impacts of cadmium (Cd) and lead (Pb) on physiological parameters such as stomata conductance, transpiration rate, and photosynthetic rate have been documented in amaranth (Huang et al. 2019) and *Capsicum annuum* L. (Hasan, Choudhary, Jahan, et al. 2022). An elevation in physiological parameters was observed with reduced concentrations of heavy metals in amaranth (Huang et al. 2019). Algethami et al. (2023) reported reduced stomata conductance, transpiration rate, and photosynthetic rate with increased concentrations of Cd and Pb in wheat. Consequently, the current findings corroborate prior studies regarding physiological parameters under treatments including varied concentrations of heavy metals.

Mutagenic effectiveness and efficiency

Mutagenic effectiveness and efficiency based on chlorophyll mutants showed a decreasing trend with increasing concentrations of $Cd(NO_3)_2$ and $Pb(NO_3)_2$ in the M_2 generation. Lead nitrate was found to be more effective and efficient than cadmium nitrate, and variety VL 110 was more sensitive to the mutagens than variety VL 44. Similar findings regarding the mutagenic effectiveness and efficiency of $Cd(NO_3)_2$ and $Pb(NO_3)_2$ have been reported in various crops, including *Capsicum annuum* L. (Hasan, Choudhary, Laskar, et al. 2022), supporting the present study. Effective doses of mutagens such as gamma rays, electron beam, and EMS have also been determined in cowpea, and the lower doses of mutagens were more effective in inducing desirable mutations (Eswaramoorthy et al. 2021). Reductions in mutagenic efficiency observed at higher concentrations of cadmium nitrate and lead nitrate are primarily attributable to increased biological damage such as lethality, sterility, and physiological injury (Shahwar et al. 2018). Although elevated doses may initially enhance DNA damage events, the associated cytotoxic effects markedly impair seed germination, plant survival, and reproductive fitness (Jarin et al. 2025). Consequently, the apparent decline in mutagenic efficiency at higher concentrations should be interpreted mainly as a result of lethal and sublethal toxicity rather than diminished mutagenic capacity (Yousuf et al. 2023).

Chlorophyll mutations

In the present study, significant variations in chlorophyll mutants (chlorina, aurea, xantha, viridis, and albino types—including one-type, two-type, and multiple-type mutants) were observed at moderate doses of $Cd(NO_3)_2$ and $Pb(NO_3)_2$ in both varieties of grain amaranth. Among the two mutagens, $Pb(NO_3)_2$ exhibited more pronounced effects on chlorophyll mutant frequency and their types than $Cd(NO_3)_2$. Furthermore, var. VL 110 showed a higher frequency of chlorophyll mutations and greater diversity of mutant types at moderate doses of both mutagens compared to var. VL 44 (Fig. 6; Tables 1 and 2). Genotype-dependent variation in response to mutagenic treatments has also been reported in groundnut (Mondal et al. 2017) and *Lupinus* (Kozak et al. 2019). Heavy metals are known to reduce chlorophyll content by interfering with enzymes involved in chlorophyll biosynthesis (Pietrini et al. 2024). Chlorophyll-deficient mutants, including one-type, two-type, and multiple-type forms, have also been documented in cowpea following exposure to physical and chemical mutagens (Eswaramoorthy et al. 2021).These chlorophyll-deficient mutants can serve as valuable genetic, physiological, and biochemical markers in grain amaranth breeding programs, facilitating gene mapping, functional validation, and the elucidation of metabolic pathways associated with chloroplast development and photosynthetic regulation.

Genetic components

Enhanced genetic components revealed the variability of quantitative traits and selected mutant lines of M_3 generation. Higher values of genetic components were reported in mutant lines of mungbean (Raturi et al. 2015). High genetic advance in association with heritability is important for effective selection of mutant lines (Akinwale et al. 2011). Heritability estimates should be regarded as preliminary indicators rather than conclusive evidence of reduced environmental influence on the traits. Therefore, future studies should include multi-environment evaluation of the selected mutant lines, along with their assessment in multilocation varietal trials.

The heavy metal induced mutagenesis broaden genetic diversity, and desirable mutants identified in advanced generations can be incorporated into breeding pipelines through hybridization, backcrossing, and pedigree selection to stabilize target traits while maintaining agronomic performance. When combined with molecular tools such as marker-assisted selection (MAS), QTL mapping, and genome wide association studies, induced mutations can be precisely identified, tracked, and deployed in breeding populations.

Conclusion

The current research illustrate the favorable response of agro-morphological traits to low and moderate concentrations of heavy metals in grain amaranth, hence enabling the selection of promising genotypes in subsequent generations. High estimates of genetic parameters indicated the efficacy of heavy metal mutagens in grain amaranth. Lower concentrations of both the heavy metals demonstrated high mutagenic effectiveness and efficiency. The current investigation shown that var. VL 110 exhibited more sensitivity to mutagens than Var. VL-44, and lower concentrations (10ppm, 20ppm, and 30ppm) of $Pb(NO3)_2$ were more successful in inducing favorable mutations in both varieties of grain amaranth compared to $Cd(NO3)_2$. Eleven promising mutant lines (Am1 – Am5 in variety VL 44 and AM1 – AM6 in variety VL 110) were selected in M_3 generation. Selection of mutant lines with desitabe agronomic traits represents the success of heavy metal induce mutagenesis in grain amaranth. The stable and promising mutants identified in the M_3 generation can undergo multilocation trials alongside standard checks for their release as new and superior cultivars of grain amaranth. Besides, these agronomically desirable mutant lines can serve as parents in recombination breeding programs to broaden the genetic base of grain amaranth. Finally, it will provide a working mutagenesis protocol for an underutilized crop like grain amaranth. Molecular approaches enable direct detection of mutagen-induced genomic alterations, confirmation of mutation events at the molecular level, and differentiation between true genetic changes and environmentally induced phenotypic variation. Genetic and transcriptomic studies are advised between the mutant lines and control cultivars to assess the differential gene expression profiling. The large mutagenized populations (M_2) of the both the heavy metals can serve as an ideal TILLING population for facilitating functional genomics studies in grain amaranth.

Declarations

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Conflict of interest

The author declares no conflict of interest. All authors have approved the manuscript and consented to publish.

Ethics approval

Not applicable

Ethics and Consent to participate

Grain amaranth (*Amaranthus hypochondriacus* L.) varieties VL 44 and VL 110 with wide adaptability to rainfed agro-ecology of North Western Himalayan region, high yield potential and creamish colour grains and small grain size and susceptibility to lodging were developed by Indian Council of Agricultural Research - Vivekananda Institute of Hill Agriculture, Almora, Uttarakhand, India. Both varieties were used in this study to test the mutagenic effectiveness and efficiency of heavy metals as mutagens.

Plant Guidelines Statement

Plant materials used in this investigation were obtained from cultivated populations and maintained at the affiliated institutional research farm. No samples were collected from protected areas or from wild populations. All experiments were performed in compliance with institutional regulations and applicable national legislation governing plant research.

Consent of publish

Not applicable

Data availability

The data used to support the findings of this study are included in the article.

Author contributions

DCJ: Conceptualization, Funding acquisition, Investigation, wrote the first draft and editing; MSB: Execution of field experiments and Data collection; RSP: Co-Investigation, assisted in standardizing chemical mutagen experiments, writing; NH: Bench work, planting and harvesting of field experiments, Writing – review & editing; NKH and LK: Writing – review & editing.

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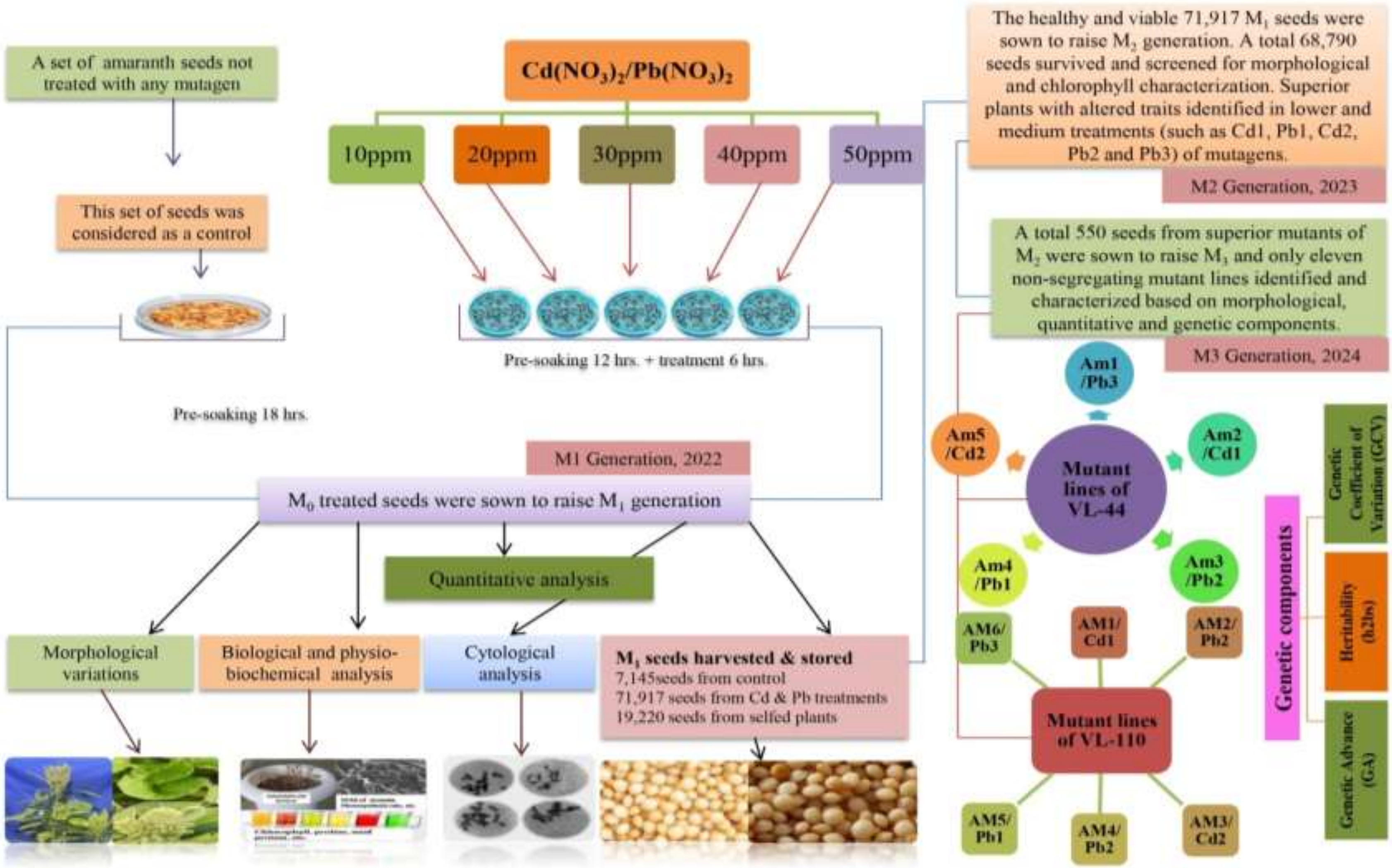

Figure 1: Pictorial representation of induce mutagenesis and selection of high yielding mutant lines in grain amaranth.

Effective Doses: Cd1/Pb1: 10ppm, **Cd2/Pb2:** 20ppm and **Cd3/Pb3:** 30ppm

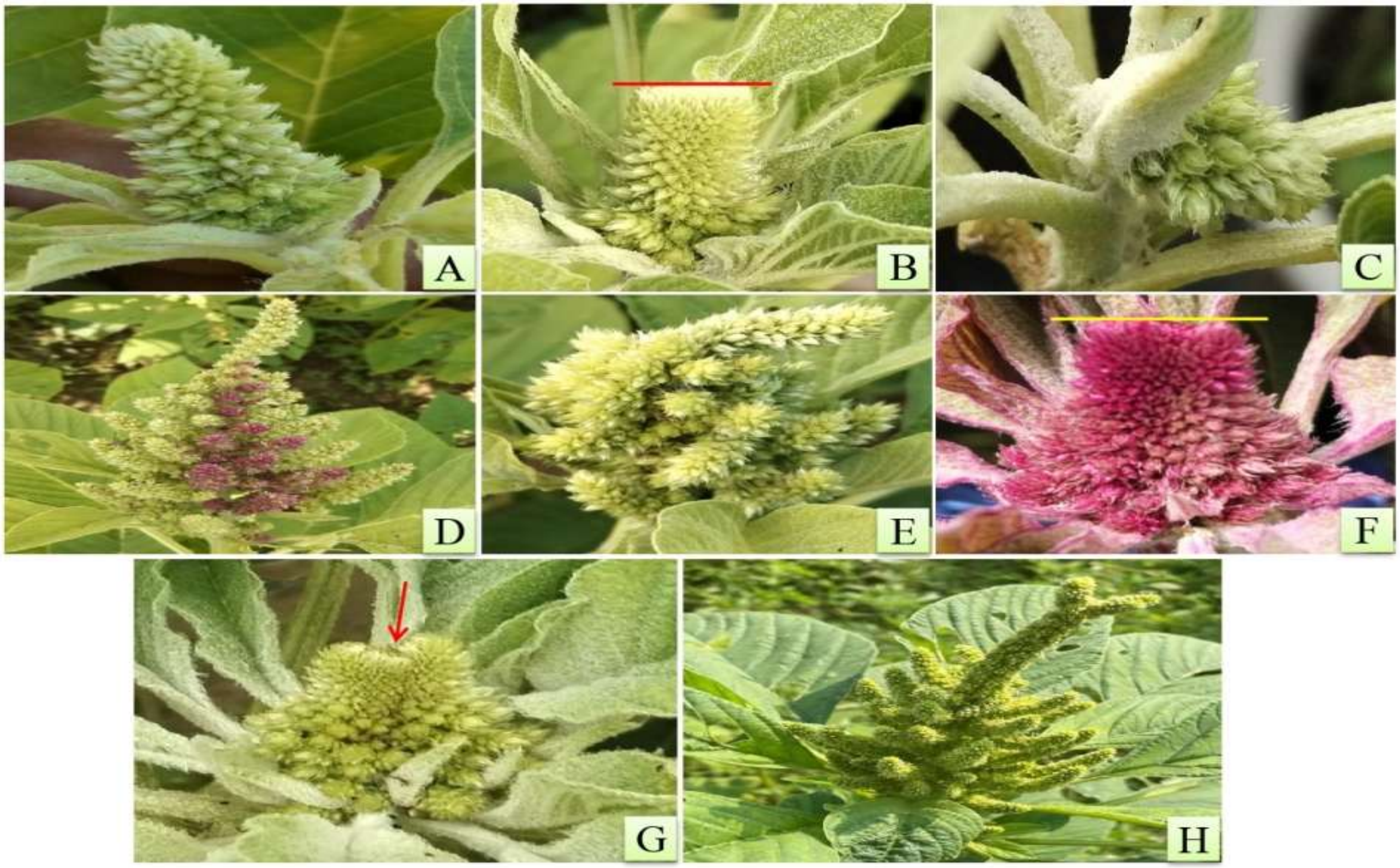

Figure 2: Penical variations in M_2 populations induced by $Cd(NO_3)_2$ and $Pb(NO_3)_2$ in var. VL 44 and var. VL 110 of *Amaranthus spp.*

A-Control, **B-**Blunt end panicle (VL 110)/10ppm $Cd(NO_3)_2$, **C-**Bend panicle (VL 44)/20ppm $Pb(NO_3)_2$, **D-**Blended panicle (VL 10)/30ppm $Cd(NO_3)_2$, **E-**Gigas spikelet mutant (VL 440)/20ppm $Cd(NO_3)_2$, **F-**Red blunt end panicle (VL 110)/30ppm $Pb(NO_3)_2$, **G-**Broad notched panicle (VL 44)/20ppm $Cd(NO_3)_2$ and **H-**Bifurcate panicle (VL 110)/10ppm $Pb(NO_3)_2$.

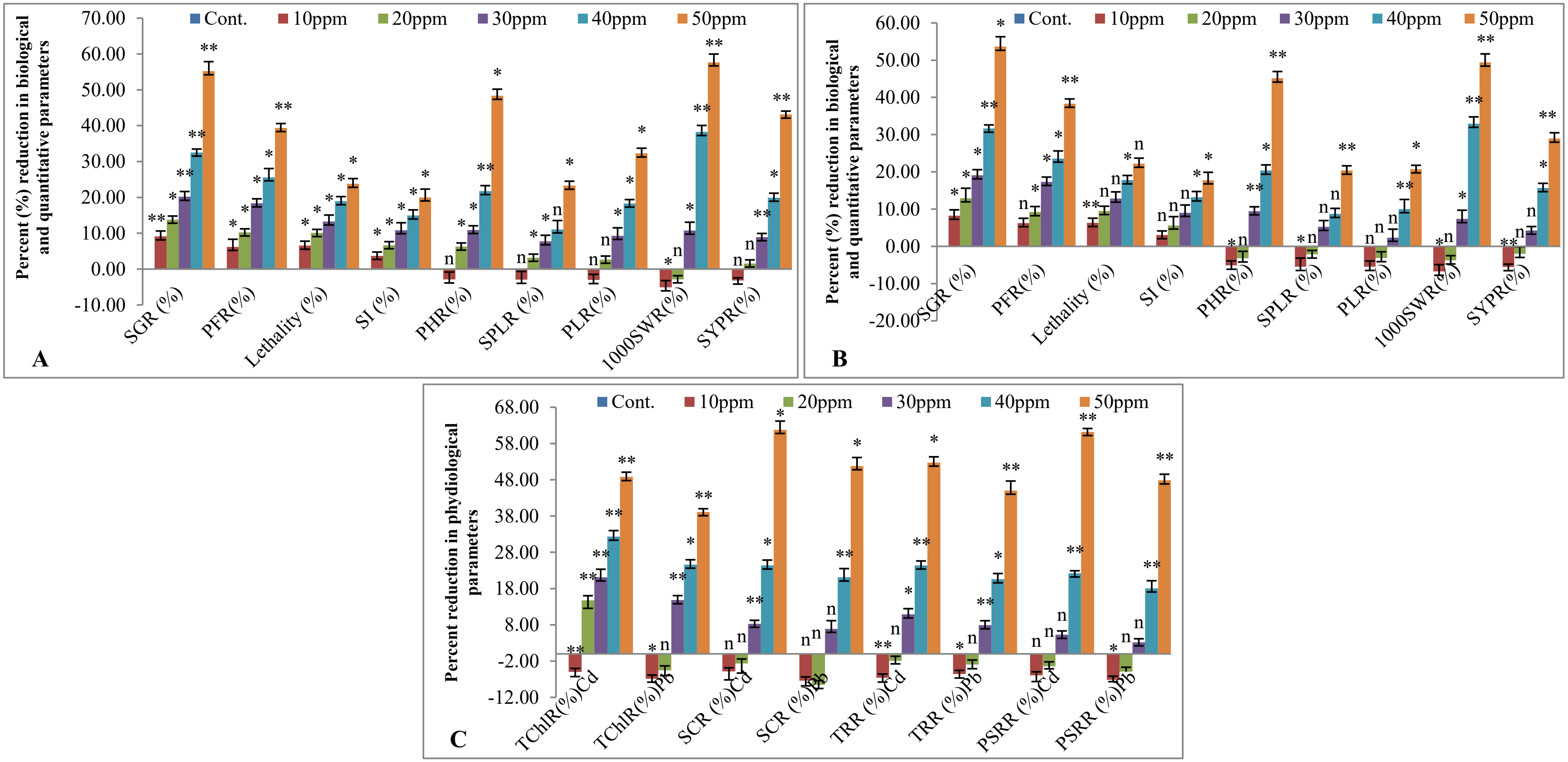

Figure 3: Percent reduction in biological, quantitative and physiological parameters in chemically **(A)** $Cd(NO_3)_2$, **(B)** $Pb(NO_3)_2$ and **(C)** $Cd(NO_3)_2$/ $Pb(NO_3)_2$ induced M_2 populations of var. VL 44 of *Amaranthus spp.* The significant level represents at 0.5% [LSD ($P \leq 0.05$)*] and 0.01% [LSD ($P \leq 0.01$)**], and non-significant level represents by 'n' letter.
SGR; Seed germination reduction, **SI;** Seedling injury, **PLR;** Panicle length reduction, **PFR;** Pollen fertility reduction, **PHR;** Plant height reduction, **1000SWR;** 1000-seed weight reduction, **SPLR;** Spikelet per panicle reduction, **SYPR;** Seed yield reduction, **TChlR**; Total chlorophyll reduction, **SCR**; Stomata conductance reduction, **TRR**; Transpiration rate reduction and **PSRR**; Photosynthetic rate reduction.

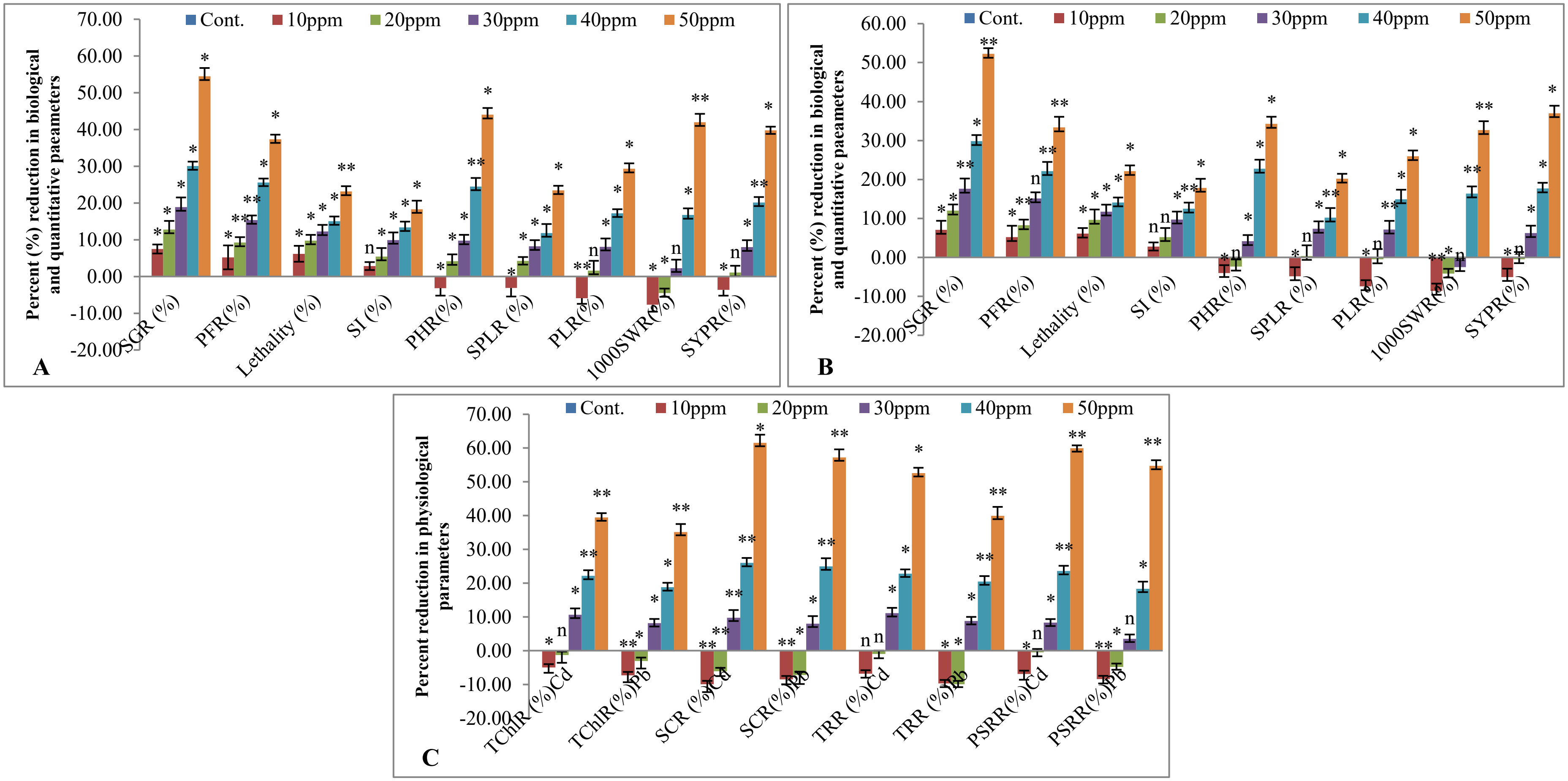

Figure 4: Percent reduction in biological, quantitative and physiological parameters in chemically **(A)** $Cd(NO_3)_2$, **(B)** $Pb(NO_3)_2$ and **(C)** $Cd(NO_3)_2$/ $Pb(NO_3)_2$ induced M_2 populations of var. VL 110 of *Amaranthus spp.* The significant level represents at 0.5% [LSD ($P \leq 0.05$)*] and 0.01% [LSD ($P \leq 0.01$)**], and non-significant level represents by 'n' letter.

SGR; Seed germination reduction, **SI;** Seedling injury, **PLR;** Panicle length reduction, **PFR;** Pollen fertility reduction, **PHR;** Plant height reduction, **1000SWR;** 1000-seed weight reduction, **SPLR;** Spikelet per panicle reduction, **SYPR;** Seed yield reduction, **TChlR**; Total chlorophyll reduction, **SCR**; Stomata conductance reduction, **TRR**; Transpiration rate reduction and **PSRR**; Photosynthetic rate reduction.

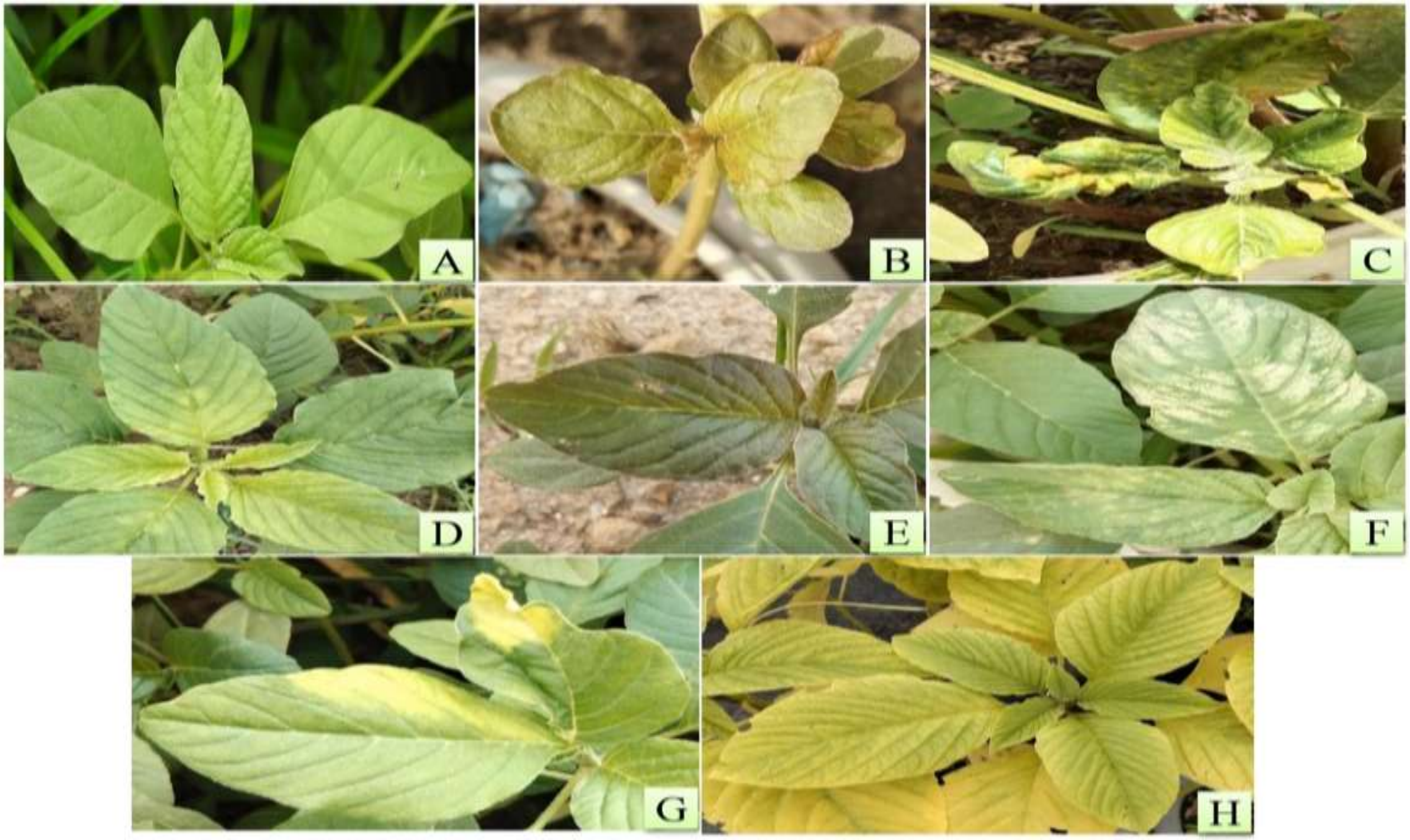

Figure 5: Chlorophyll mutant characterization in chemically induced M_2 populations in var. VL 44 and var. VL 110 of *Amaranthus spp.*

A- Control, **B-**Aurea mutant (40ppm $Cd(NO_3)_2$), **C-**Xantha mutant (10ppm $Pd(NO_3)_2$), **D-**Viridis mutant (20ppm $Pd(NO_3)_2$), **E-**Striata mutant (30ppm $Cd(NO_3)_2$), **F-**Variegate mutant (10ppm $Cd(NO_3)_2$), **G-** Chlorina mutant (30ppm $Pd(NO_3)_2$) and **H-**Albino mutant (50ppm $Cd(NO_3)_2$)

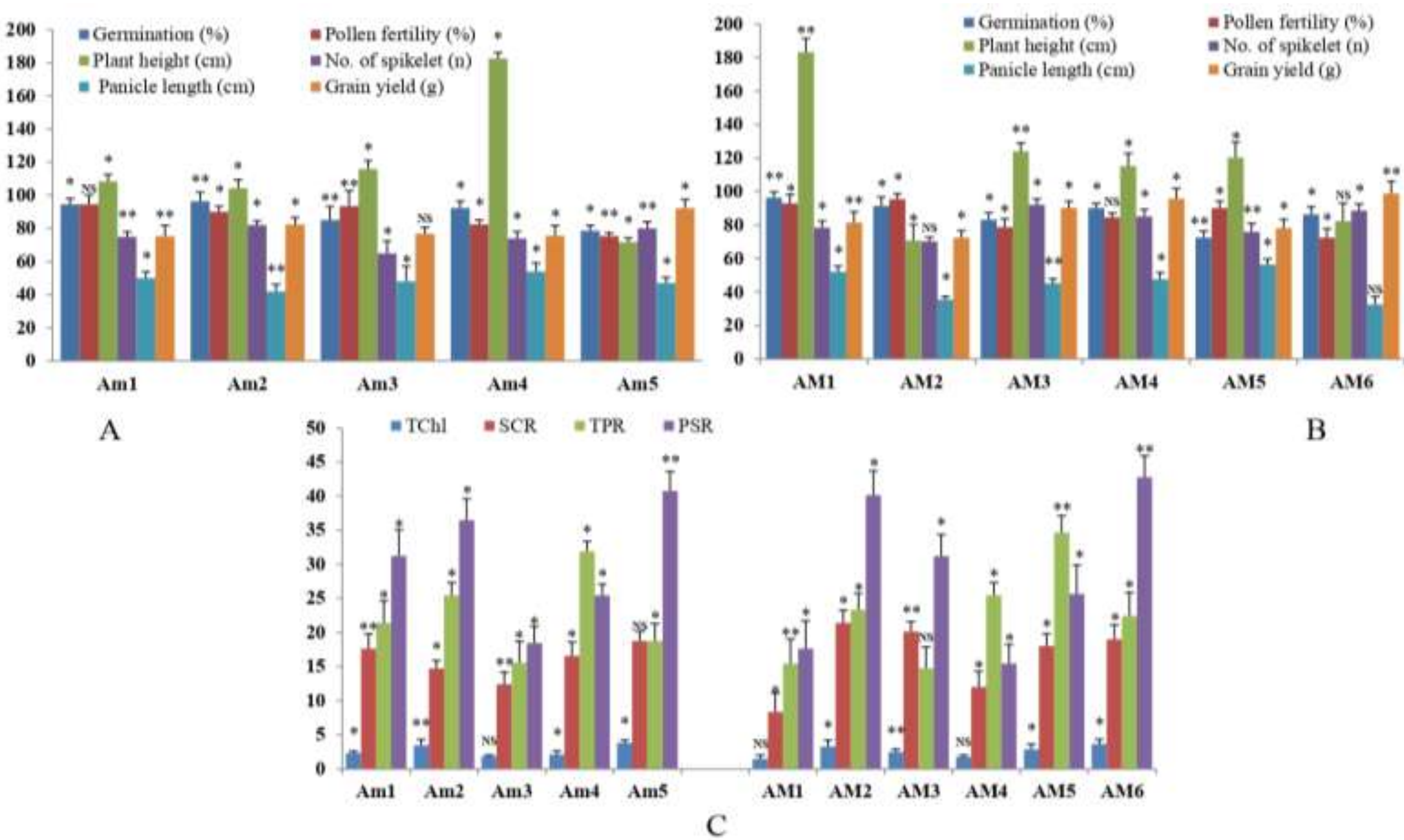

Figure 6: Biological, quantitative and physiological parameters in high yielding mutant lines of grain amaranth. ** represent significant at 1%, * significant at 5% and NS means non-significant, respectively.

A. Biological and quantitative parameters of mutant lines selected from var. VL 44

B. Biological and quantitative parameters of mutant lines selected from var. VL 110

C. Physiological parameters of mutant lines selected from both var. VL 44 & var. VL 110

Supplementary Files

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