

Screening gamma-induced *Leucaena tarramba* mutants for drought tolerance under PEG-simulated osmotic stress in hydroponics

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Abstract

Drought tolerance remains a major challenge in improving *Leucaena tarramba*, a legume widely cultivated in arid and semi-arid tropics. Effective screening for drought tolerance is critical for identifying *Leucaena tarramba* putative mutants with improved adaptation to water-limited environments. A hydroponic system combined with polyethylene glycol (PEG)-induced osmotic stress provides a controlled and reproducible platform for evaluating drought-related responses. This study aimed to screen M_2 mutants and identify promising drought-tolerant putative mutants based on estimated estimates of genetic variability and key morpho-physiological responses under drought-simulating osmotic stress induced by polyethylene glycol (PEG) 6000 in a hydroponic deep-water culture system. Seeds from the Livestock Breeding and Forage Crops Center, West Nusa Tenggara, Indonesia were irradiated with Co-60 gamma rays at Center for Isotope and Radiation Application under the National Nuclear Energy Agency of Indonesia (PAIR-BATAN Indonesia) at doses ranging from 50 to 1000 Gy, with an estimated LD_{50} of 820 Gy based on survival at 21 days after planting. Following phenotypic evaluation of the M_1 generation, materials derived from 0, 250, 500, and 750 Gy were advanced to the M_2 generation. A split-plot design was arranged with PEG levels consisted of 0, 10, 20% as main plot and irradiation doses included 0, 250, 500, and 750 Gy as subplots. The putative mutants of M_2 were screened under hydroponic PEG stress until 42 days after planting (DAP), followed by post-hydroponic greenhouse evaluations at 60 and 90 DAP. Interaction between PEG and irradiation dose significantly affected several morpho-physiological traits. Under 10% PEG stress, the 250- and 500-Gy-derived M_2 putative mutants showed relatively better maintenance of several growth-related traits together with adaptive physiological responses, whereas 20% PEG caused more pronounced reductions in growth and physiological performance. Drought-tolerance screening based on individual dry-weight values revealed considerable variation among M_2 putative mutants derived from 250, 500, and 750 Gy. Stress selection indices varied among the M_2 irradiation-dose groups under PEG-induced osmotic stress. Stress intensity increased from 0.68 under 10% PEG to 0.85 under 20% PEG. Under 10% PEG, STI increased from 0.23 in the wild type to 0.41 in the 750 Gy group, while SSI decreased from 1.10 to 0.90. The same trend was observed under 20% PEG, although STI values were lower overall. The 500 and 750 Gy groups

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had SSI values below 1 under both stress conditions, indicating lower relative susceptibility than the wild type and 250 Gy groups. PCA based on individual morpho-physiological traits explained 89.5% of the total variation and separated entries with maintained and reduced performance. Several M₂ putative mutants under 10% PEG, including 250Gy_2P1, 250Gy_3P1, 250Gy_4P1, and 500Gy_1P1–500Gy_4P1, showed maintained performance and were identified as promising candidates. Preliminary broad-sense heritability estimates ranged from 0.24 to 0.60 during the hydroponic phase and from 0.11 to 0.80 during the post-hydroponic evaluations at 60 and 90 DAP. Hydroponic PEG screening provides a useful preliminary framework for identifying promising gamma-induced M₂ putative mutants with maintained morpho-physiological performance under stress, which can be prioritized for further progeny testing and molecular validation.

Keywords Drought tolerance · Estimates of genetic variability · Hydroponic screening · *Leucaena tarramba* · Polyethylene glycol (PEG) · Putative mutants

Abbreviations

DAP	Day after planting
DWC	Deep water culture
EC	Electrical conductivity
GCV	Genetic coefficient variability
h ²	Heritability
LD ₅₀	Lethal dose 50
PCA	Principal component analysis
PCV	Phenotypic coefficient variability
PEG	Polyethylene glycol
SI	Stress intensity
SSI	Stress susceptibility index
STI	Stress tolerance index
TDS	Total dissolved solids

Introduction

Plants are continually exposed to changing environmental conditions and must cope with various abiotic stresses, including drought, salinity, UV-B radiation, extreme temperatures, and nutrient imbalances [1–4]. Drought is among the most pervasive abiotic stresses affecting agricultural production, as it disrupts plant water relations, impairs physiological and metabolic processes, and progressively reduces crop growth and productivity. When prolonged or recurrent, these effects can diminish soil–plant system resilience, destabilize production, and ultimately threaten the long-term sustainability of agricultural systems [5–7]. In addition, the intensification of drought driven by climate change has resulted in decreased biomass production, accelerated land degradation, and growing risks to global food and feed security [8–12]. These circumstances emphasize the urgent need for strategic efforts to develop crop varieties capable of surviving in water-limited environments through integrated approach in physiological adaptations, agronomic practices, and genetic enhancements [13–15].

While research on genetic improvement has advanced substantially in food legumes, studies focusing on forage legumes [16, 17], particularly those adapted to marginal environments, remain comparatively limited. This research gap is critical, as forage legumes such as *Leucaena tarramba* (*Leucaena leucocephala* cv. Tarramba) provide multifaceted benefits beyond feed quality, encompassing soil fertility restoration and ecosystem resilience. *Leucaena tarramba*, a perennial tree legume extensively cultivated as forage in tropical regions, plays a pivotal role in sustainable livestock production systems by providing a protein-rich feed source from 23.7% to 34% [18],

contributing to biological nitrogen fixation [19] and supporting the rehabilitation of degraded or marginal soils [20, 21]. Although observable phenotypic variation exists among putative mutants in terms of drought tolerance performance, the underlying natural genetic diversity within the available *Leucaena tarramba* germplasm remains relatively narrow [22, 23]. This limited genetic study challenges for conventional breeding, which is further constrained by the species' long generation cycles [24, 25]. Harnessing and expanding this diversity through alternative approaches, such as mutation breeding is therefore crucial to enhance drought adaptability and ensure long-term forage sustainability in arid environments.

Mutations have been widely applied in plant breeding to generate novel genetic variations [26–28]. Induced mutagenesis has emerged as a powerful tool for expanding estimates of genetic variability in forages, enabling the exploration of stress tolerance genetics [29]. While mutation have revolutionized with precision genome-editing techniques such as CRISPR-Cas9 targeted gene modification in many crops [30, 31], their application in non-model forage legumes remains because of limited genomic resources, inefficient transformation/regeneration protocols, and associated logistical challenges [32, 33]. Traditional mutagenesis approaches do not require detailed genome annotations or elaborate transformation systems, and they can induce genome-wide variation in a cost-effective manner, making them a valuable strategy for species with narrow genetic bases such as some perennial forage legumes and limited breeding resources [34–36]. This approach remains highly relevant for preliminary genetic improvement and the discovery of novel alleles associated with drought tolerance and other adaptive traits. In addition, in mutation breeding studies, genetic potential can be inferred from the extent of phenotypic variability expressed among mutant populations [37, 38]. In this context, the present study estimated the genetic potential of *Leucaena tarramba* mutants based on observable differences in morphological and physiological traits within the putative mutants of M_2 . Because all mutants were derived from a single parent line and cultivated under identical environmental conditions, the observed variation in plant height, leaf morphology, and other quantitative traits likely reflects newly induced genetic changes rather than environmental effects. Thus, phenotypic divergence serves as a practical indicator of underlying genetic diversity among the mutants [39, 40].

In some legume species such as *Leucaena tarramba*, the exploration of potential mutants developed under controlled environments represents a critical step toward identifying adaptive putative mutants with long-term breeding potential [41, 42]. By combining controlled environmental screening systems such as hydroponic simulation, mutagenesis enables the systematic identification of potential mutants that present superior performance under controlled drought conditions [38, 43]. Compared to soil-based systems, hydroponics eliminates substrate heterogeneity and provides uniform PEG-induced osmotic stress, enabling faster and more practical screening of drought tolerance traits [44, 45]. Although several studies have examined the morpho-physiological responses of *leucaena* to abiotic stress [46, 47], studies on the estimates of genetic variability of induced mutants under artificially controlled environments remains limited. This gap highlights the need to identify putative mutants capable of maintaining growth and physiological performance under drought stress and to determine whether the resulting phenotypic variation can support forage-breeding programs. To broaden the phenotypic variation available in *Leucaena tarramba*, gamma irradiation was used to generate putative mutant populations, which were subsequently evaluated under a controlled hydroponic system using PEG 6000-induced drought-simulating osmotic stress. The novelty of this study lies in the integrated application of gamma-induced mutagenesis, PEG-based hydroponic screening, morpho-physiological characterization, stress-tolerance indices, multivariate analyses, and preliminary genetic-parameter estimation within a single screening framework. This study aimed to identify phenotypically promising M_2 putative mutants and determine the traits most informative for early-stage drought-tolerance selection. We hypothesized that gamma irradiation would generate sufficient phenotypic variation to produce differential responses to PEG-induced drought-simulating osmotic stress, enabling the identification of putative mutants with a greater ability to maintain growth-related traits and exhibit adaptive physiological responses under water-deficit conditions. The findings provide a practical basis for prioritizing promising putative mutants for subsequent field evaluation, multi-environment testing, progeny-stability assessment, and molecular validation, with potential application in the development of drought-resilient forage germplasm.

Materials and methods

Genetic source preparation (M_1 - M_2 generation)

The seeds of *Leucaena tarramba* (*Leucaena leucocephala* cv. Tarramba) used as genetic source were obtained from Livestock Breeding and Forage Crops Center of Mataram Livestock and Animal Health Service, West Nusa Tenggara, Indonesia. Seeds with a moisture content of 12–14% (measured using an MC-7821 moisture meter), blackish-brown coloration weighing 0.06–0.07 g per seed were selected for gamma irradiation. Irradiation was conducted at the Center for Isotope and Radiation Application under the National Nuclear Energy Agency of Indonesia (PAIR-BATAN Indonesia) using a 3-kCi Gamma Cell equipped with a Co-60 source, with doses ranging from 50 to 1000 Gy, while 200 non-irradiated seeds served as the wild-type control. To break dormancy, the seeds were soaked in hot water at 60–70 °C for 3 h before being sown in separate trays according to irradiation dose and maintained in a greenhouse for 21 days. Following germination, the seedlings were cultivated during the early growth phase under controlled greenhouse conditions (26–27 °C and 75–80% relative humidity) in polybags (40 × 60 cm) and subsequently transplanted to the experimental field at a spacing of 2.0 × 2.5 m for comprehensive phenotypic evaluation. The irradiation doses used for LD₅₀ determination were derived from the same initial irradiation experiment applied to the entire seed population, covering doses from the non-irradiated control (0 Gy) to 1000 Gy. The response data obtained across this dose range were used to estimate the LD₅₀ and determine the irradiation treatments subsequently evaluated in the M_1 and M_2 generations. In the M_1 generation, all surviving putative mutants derived from irradiation doses ranging from 50 to 750 Gy were evaluated for morphological and physiological traits from germination to the flowering stage. Based on the M_1 evaluation, four irradiation-doses such as control (no irradiation), 250, 500, and 750 Gy, were selected for subsequent drought-tolerance screening in the M_2 generation. Within these dose groups, the putative mutant of M_1 showing favorable morphological and physiological performance were selected and advanced for M_2 seed production. Thus, the M_2 population was established from seeds produced by selected M_1 putative mutants rather than from the entire surviving M_1 population. The selected M_1 putative mutants were maintained through the reproductive phase, including flowering, pod development, and seed maturation, and seeds were harvested at approximately 80% physiological maturity, 7–8 months after planting.

The selected putative mutant of M_1 were grown to reproductive maturity, allowed to flower and develop pods, and maintained until seed maturation. Mature seeds were subsequently harvested from these selected M_1 and germinated to produce the M_2 generation used in the present experiment. For the current screening, M_2 population was established from seeds produced by selected M_1 putative mutants. Within each irradiation-dose were evaluated collectively according to their irradiation-dose origin and were not treated as separate maternal families in the statistical design. Accordingly, maternal parent identity was not included as an independent experimental factor.

The putative mutant of M_2 were screened for drought response using a hydroponic deep-water culture (DWC) system under greenhouse conditions at 26–27 °C and approximately 80% relative humidity, with different levels of polyethylene glycol (PEG 6000)-induced osmotic stress. Genetic-related parameters derived from the M_2 population were interpreted as preliminary estimates based on observed phenotypic variation rather than as definitive evidence of genetically fixed differences. Further progeny evaluation and biomolecular validation are therefore required to confirm the genetic basis and stability of the observed responses.

Experimental design

A split-plot experimental design was used to evaluate the effects of PEG-induced drought-simulating osmotic stress, irradiation dose, and their interaction on plant responses. PEG treatment was assigned as the main-plot factor because each hydroponic tray received a single PEG concentration, whereas irradiation dose was assigned as the subplot factor within each tray.

The main-plot factor consisted of three PEG 6000 treatments: P0 (control, no PEG), P1 (moderate stress, 10% PEG; approximately -0.6 MPa), and P2 (severe stress, 20% PEG; approximately -1.2 MPa). Each PEG treatment was represented by four independent hydroponic trays corresponding to four experimental replications, resulting in a total of 12 trays. Within each tray, four irradiation-dose treatments were arranged as subplots: wild type (0 Gy), 250, 500, and 750 Gy. Thus, the experiment comprised 48 subplot experimental units (3 PEG levels $\times$ 4 replications $\times$ 4 irradiation treatments).

Each subplot contained five of putative mutants (M_2), resulting in 20 plants per tray and 240 plants overall. For the split-plot analysis of treatment effects, the five plants within each subplot were treated as subsamples rather than independent experimental replicates. Because plant mortality occurred in some PEG-induced osmotic stress subplots, particularly under severe stress, measurements from the surviving plants available at the time of assessment were averaged to obtain one subplot-level mean for each combination of replication, PEG treatment, and irradiation dose. These subplot means were used for the inferential analysis of PEG, irradiation dose, and PEG $\times$ irradiation-dose interaction effects. Thus, the subplot remained the experimental unit, and individual plants were not treated as additional independent replicates.

For the full split-plot analysis of morphological traits, the statistical model included replication, PEG treatment, irradiation dose, and the PEG $\times$ irradiation-dose interaction. The replication $\times$ PEG interaction was used as the main-plot error term for testing the PEG effect, whereas the subplot residual was used to test irradiation dose and the PEG $\times$ irradiation-dose interaction. The corresponding degrees of freedom were 3 for replication, 2 for PEG, 6 for replication $\times$ PEG, 3 for irradiation dose, 6 for PEG $\times$ irradiation dose, and 27 for the subplot residual. The split-plot model was expressed as follows [48, 49]:

$$Y_{ijk} = \mu + R_i + P_j + (RP)_{ij} + I_k + (PI)_{jk} + \varepsilon_{ijk}$$

Where;

Y_{ijk} = the observed mean response for replication i , PEG level j , and irradiation dose k .

μ = the overall mean.

R_i = the effect of replication i ($i = 1, 2, 3, 4$).

P_j = the effect of PEG level j (P0, P1, and P2) as the main-plot factor.

$(RP)_{ij}$ = the replication $\times$ PEG interaction

I_k = the effect of irradiation dose k (0, 250, 500, and 750 Gy) as the subplot factor.

$(PI)_{jk}$ = the PEG $\times$ irradiation interaction effect.

ε_{ijk} = the subplot residual error used to test the irradiation effect and the PEG $\times$ irradiation interaction.

Chlorophyll content, stomatal traits, and proline concentration were evaluated using three biological replicates for each PEG $\times$ irradiation-dose treatment combination. These physiological measurements therefore followed a reduced biological sampling design rather than the full four-tray replication structure used for the morphological traits. The three biological replicates represented plant-level biological observations within each treatment combination and were not considered additional tray-level experimental replications. Physiological traits were therefore analyzed separately according to this reduced sampling structure.

Identification of individual M_2 putative mutants of *Leucaena tarramba*

In addition to the treatment-level analysis, individual M_2 *Leucaena tarramba* were retained as identifiable entries for phenotypic screening and multivariate analyses. Irradiated M_2 individuals were referred to as individual M_2 putative mutant entries, whereas non-irradiated individuals were referred to as wild-type entries. Individual trait values were retained for these analyses and were not replaced by subplot means. Each entry code consisted of three components: irradiation-dose origin, experimental replication, and PEG treatment. The irradiation-dose component was designated as Wildtype, 250 Gy, 500 Gy, or 750 Gy; the numerical component (1–4) indicated the experimental replication in which the individual plant was evaluated; and P0, P1, or P2 indicated exposure to 0%,

10%, or 20% PEG, respectively. Therefore, each complete code denotes the phenotypic observation of a single M_2 *Leucaena tarramba* and is distinct from a subplot mean, replication mean, maternal family, or established mutant line. The entries sharing the same irradiation-dose origin and replication number but evaluated under different PEG treatments correspond to different M_2 individuals.

Artificial screening by hydroponic Deep-Water Culture (DWC)

The hydroponic Deep-Water Culture (DWC) system was established using containers with an 8 L capacity, serving as the reservoirs for the nutrient solution. An artificial growth substrate was employed to support the putative mutants of M_2 which were positioned on styrofoam boards perforated with planting holes at a spacing of 25×30 cm. Each tray was equipped with air pumps and air stones to maintain sufficient aeration and prevent hypoxic stress in the root zone. Prior to the application of drought stress treatments, M_2 *Leucaena tarramba* seeds were germinated for 14 days to ensure uniform seedling establishment.

The hydroponic system was established following a modified International Rice Research Institute [50] protocol using an AB mix nutrient solution. Seedlings were acclimatized for 14 days before stress induction. Drought stress was simulated using PEG 6000, with three designated treatments: P0 (0% PEG; non-stress control), P1 (10% PEG, w/v; approximately -0.6 MPa), and P2 (20% PEG, w/v; approximately -1.2 MPa) [47, 51–53]. For P1, PEG 6000 was added at 14 DAP to establish a final concentration of 10%, which was subsequently maintained throughout the hydroponic stress period. For P2, PEG 6000 was introduced stepwise, beginning at 14 DAP and reaching the designated final concentration of 20% at 21 DAP, to minimize abrupt osmotic shock. The hydroponic solutions were continuously aerated and mixed to ensure uniform solute distribution and prevent PEG stratification or precipitation.

Morpho-physiological responses of M_2 *Leucaena tarramba* were assessed at 42 days after planting (DAP), representing the completion of the hydroponic PEG-induced drought-simulating osmotic stress screening phase. After the hydroponic phase, the plants were transferred to greenhouse conditions for post-hydroponic evaluations at 60 and 90 DAP.

The system was controlled throughout the hydroponic screening phase, with temperature maintained at 26 – 27 °C and relative humidity at approximately 80%. The nutrient solution pH was kept within the range of 5.2 – 6.2 , monitored using a digital pH meter (PH-009(I) A, HWB, China) and adjusted with KOH or H_3PO_4 as required. The electrical conductivity (EC) and total dissolved solids (TDS) of the nutrient solution were measured using a TDS/EC meter (TDS & EC Hold, generic model, China). To sustain stable nutrient availability and solution volume during the stress period, the reservoirs were supplemented weekly with fresh nutrient solution containing the corresponding PEG concentration for each treatment (Fig. 1).

Observed parameter

The experiment comprised three sequential observations such as LD_{50} determination, evaluation of M_1 and M_2 putative mutants generation.

During the LD_{50} test, the observed parameter was seedling survival rate, assessed 21 days to estimate the irradiation dose causing 50% mortality through regression analysis.

In the M_1 generation, the observed morphological parameters included plant height (cm), plant length (cm), number of leaves (unit), number of branches (unit), number of twigs (unit), stem diameter (cm), number of pods (unit), and leaf area (cm^2). Biomass-related traits consisted of whole plant weight (g), leaf fresh weight (g), leaf dry weight (g), branch fresh weight (g), branch dry weight (g), twig fresh weight (g), twig dry weight (g), pod fresh weight (g), and pod dry weight (g). Yield-related parameters included number of pod samples (unit), average number of seeds per pod (unit), seed mass per 100 g (g), and pod sample mass (g). All parameters were recorded monthly until the reproductive stage (7–8 months) to obtain M_2 seeds.

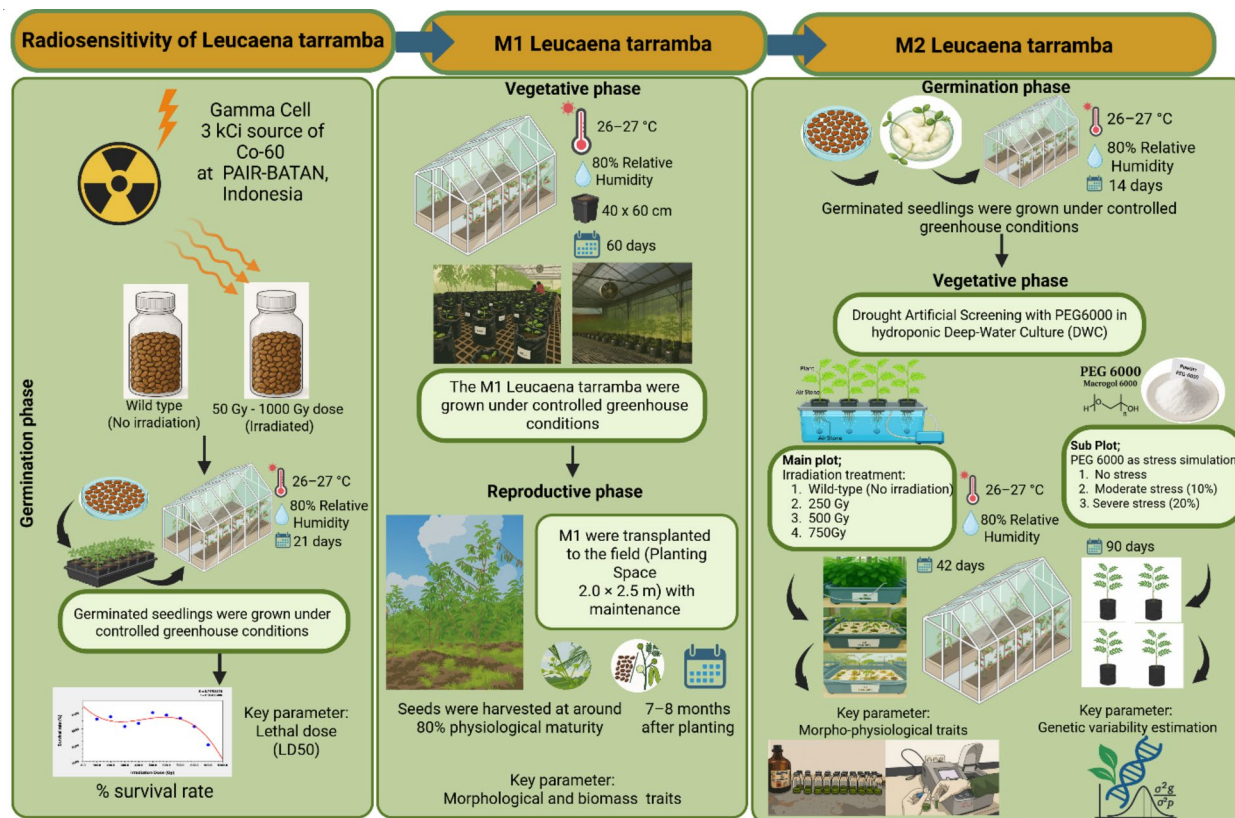


Fig. 1 Graphical abstract *M₂ Leucaena tarramba* under hydroponically induced drought stress

For the *M₂* generation, the observed morphological parameters included plant height (cm), number of leaves (unit), number of branches (unit), and leaf area (cm²), fresh weight (g), dry weight (g), root weight (g), and root length (cm). Physiological parameters comprised chlorophyll a, chlorophyll b, and total chlorophyll content (μmol m⁻²), which were quantified using a UV–Vis spectrophotometer (Shimadzu UV–Vis 1800) at 665 nm and 652 nm following the method of Porra et al. [54]. Stomatal characteristics were determined following Weyers and Johansen [55], including number of stomata (unit) and stomatal density (stomata mm⁻²). The number of stomata is expressed as the number of stomata per microscopic field, whereas stomatal density is expressed as stomata mm⁻² with images analyzed using ImageJ software (version 1.53). Proline content was measured spectrophotometrically using the ninhydrin method, with absorbance determined at 520 nm according to Bates et al. [56]. Chlorophyll content, stomatal traits, and proline concentration were measured using three biological replicates for each PEG × irradiation-dose treatment combination. Physiological measurements were performed on surviving plants with sufficient viable tissue for analysis. These three biological replicates constituted a reduced biological sampling design and were analyzed separately from the full four-replication split-plot dataset used for morphological traits.

Stress-selection indices were calculated using plant dry weight (DW) under non-stress and PEG-induced osmotic-stress conditions [57, 58]. Parameters related to the stress selection indices include stress tolerance index (STI), stress intensity (SI), and Stress susceptibility index (SSI) based on formula [59].

$$STI = Y_p \times \frac{Y_p \times Y_s}{(\bar{Y}_p)^2}$$

$$SI = 1 - \left(\frac{\bar{Y}_s}{\bar{Y}_p} \right)$$

$$SSI = \left[1 - \left(\frac{\bar{Y}_s}{\bar{Y}_p} \right) \right] / SI$$

Where;

STI=Stress tolerance index.

SI=Stress intensity.

SSI=Stress susceptibility index.

$\bar{Y}_p$ =Dry weight of a putative mutant under non-stress (control) conditions.

$\bar{Y}_s$ =Dry weight of the same putative mutant under stress conditions.

$\bar{Y}_p$ =Mean dry weight of all evaluated putative mutants under non-stress (control) conditions.

$\bar{Y}_s$ =Mean dry weight of all evaluated putative mutants under stress conditions.

Those stress selection indices parameters had categories. The category and interpretation of the stress selection indices according to Fernandez [59] as presented in Table 1.

Potential estimates of genetic variability was preliminarily estimated from phenotypic variation in the M_2 population. Phenotypic variance (σ^2M_2) was calculated from individual M_2 observations, while environmental variance (σ^2M_0) was estimated from the non-irradiated parental population evaluated under comparable conditions. Genetic variance was estimated as $\sigma^2g = \sigma^2M_2 - \sigma^2M_0$. The preliminary genetic-parameter estimates based on phenotypic variation have been mentioned below [60];

$$\sigma^2 = \frac{\sum x^2 - \left[\frac{(\sum x)^2}{n} \right]}{n - 1}$$

$$\begin{aligned} \sigma^2g &= \sigma^2p - \sigma^2e \\ &= \sigma^2M_2 - \sigma^2M_0 \end{aligned}$$

Where;

σ^2 =variance.

n=number of populations.

σ^2p =phenotypic variance.

σ^2g =genetic variance.

σ^2e =environmental variance.

σ^2M_2 = M_2 population variance.

σ^2M_0 =variance of parents of *Leucaena tarramba*.

Broad-sense Heritability values were calculated by equation below;

Table 1 The interpretation of the stress selection indices value

Parameter	Value	Category
Stress Tolerance Index (STI),	STI > 1.0	Tolerant
	0.5 < STI < 1.0	Moderate tolerant
	STI < 0.5	Sensitive
Stress Intensity (SI)	SI = 0.0	Very low stress
	SI > 0.0	Moderate stress
	SI = 1.0	Maximum stress
Stress Susceptibility Index (SSI)	SSI = 0	Very low susceptibility to stress
	SSI < 1	Relatively tolerant to stress
	SSI = 1	Susceptibility to stress
	SSI > 1	Sensitive to stress

$$h^2 = \frac{\sigma^2_g}{\sigma^2_p}$$

Where;

σ^2_p = phenotypic variance.

σ^2_g = genetic variance.

According to Standfield [61], Heritability was expressed as a proportion from 0 to 1 and classified as high ($h^2 > 0.5$), moderate ($0.2 < h^2 \leq 0.5$), and low ($h^2 < 0.2$). Genetic variation was determined based on the Phenotypic Coefficient Variability (PCV) and Genetic Coefficient Variability (GCV). The GCV was calculated in the following equation;

$$PCV(\%) = \frac{\sqrt{\sigma^2_p}}{\bar{x}} \times 100$$

$$GCV(\%) = \frac{\sqrt{\sigma^2_g}}{\bar{x}} \times 100$$

Where;

σ^2_p = phenotypic variance.

σ^2_g = genetic variance.

$\bar{x}$ = the average of observed parameter values.

PCV and GCV values were classified as low (<10%), moderate (10–20%), and high (>20%) [62–64]. Because phenotypic variance includes both genetic and environmental components, PCV is expected to be equal to or greater than GCV when $\sigma^2_p \geq \sigma^2_g$. A small difference between PCV and GCV indicates a relatively limited environmental influence on trait expression, whereas a larger difference suggests a greater environmental contribution to phenotypic variation [64, 65].

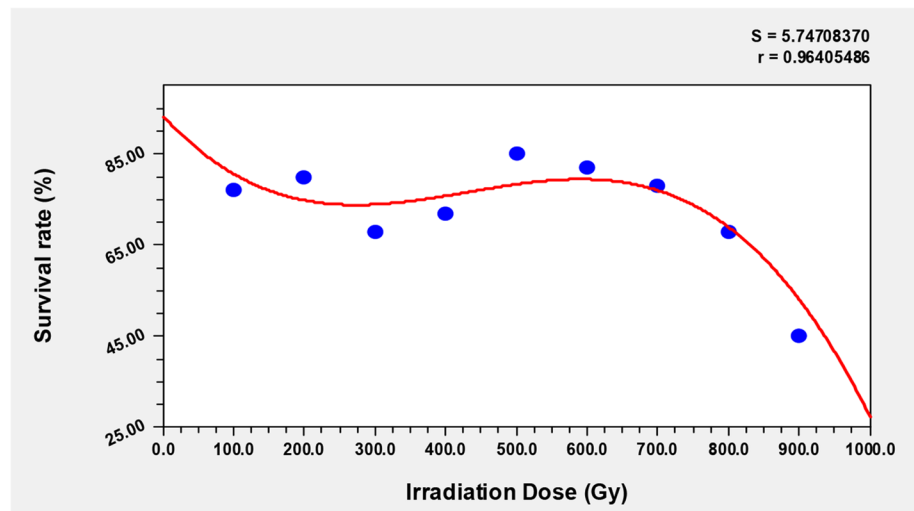
Data analysis

The LD₅₀ value was estimated through regression analysis using CurveExpert 1.3, based on the survival rate data of irradiated seeds. Data M₁ and M₂ mutants were analysed using both univariate and multivariate statistical approaches to test for significant differences among irradiation doses, PEG concentrations, and their interactions across the evaluated traits. Before analysis of variance (ANOVA), residual normality and homogeneity of variance were assessed using the Shapiro–Wilk, respectively. Residual plots were also visually inspected to identify potential deviations from ANOVA assumptions. When significant effects were detected, mean separation was conducted using Tukey’s Honest Significant Difference (HSD) test at $p \leq 0.01$. For multivariate analysis, the M₁ and M₂ datasets were subjected to cluster analysis, principal component analysis (PCA), polar heatmap analysis, and Pearson correlation analysis. The M₁ mutants were analysed using SAS 9.4 and RStudio version 4.2.3, with trait standardization, K-means clustering, and hierarchical clustering performed to identify morphological groupings using Ward’s method. The optimal number of clusters was determined using the elbow method. The M₂ mutants were further explored using RStudio version 4.2.3 for PCA, correlation matrix analysis, and heatmap construction. Final data visualization, including PCA biplots, cluster heatmaps, and Pearson correlation plots, was completed using OriginPro 2025b.

Table 2 Regression equation for LD₅₀ of *Leucaena tarramba* gamma irradiation at 21 Days after Planting (DAP)

Parameter	Model	Equation	R	LD ₅₀
Survival rate (%)	Degree of polynomial	$y = 49.9 + 0.27x - 0.00 \times 2 + 1.53 \times 3$	0.96	820 Gy

y predicted survival rate (%), x gamma irradiation dose (Gy), R correlation coefficient, LD₅₀ estimated irradiation dose resulting in 50% survival

Fig. 2 LD₅₀ of *Leucaena tarramba* gamma irradiation at 21 days after planting

Results

LD₅₀ *Leucaena tarramba* post gamma irradiation

The regression equation used to estimate the lethal dose (LD₅₀) is presented in Table 2, while the relationship between irradiation dose and survival rate is illustrated in Fig. 2.

Seedling survival showed a nonlinear response to increasing gamma-irradiation dose at 21 days after planting (DAP) (Fig. 2), with observed survival ranging from approximately 25% to 85% across the tested doses. Survival remained relatively high at intermediate irradiation levels, reaching its maximum at 500 Gy, before declining markedly at higher doses. A third-degree polynomial regression provided a good fit to the survival response ($r=0.964$; $R^2=0.929$) (Table 2). The LD₅₀ was estimated by setting the predicted survival rate to 50% ($y=50$) and solving the fitted equation for irradiation dose (x). The relevant root within the experimental dose range corresponded to an approximate LD₅₀ of 820 Gy.

Morphological traits of M₁ *Leucaena tarramba* post gamma irradiation

The analysis of morphological traits in the M₁ *Leucaena tarramba* population revealed clear phenotypic variation among the irradiated mutants. Cluster analysis, performed using Ward's method, was applied to identify patterns of similarity and divergence across putative mutants. The optimal number of clusters was estimated using the Elbow method, where a distinct inflection point at $K=4$ indicated the most suitable grouping. Four clusters were retained to capture intermediate putative mutants with overlapping characteristics (as illustrated in Fig. S1). Additionally, the resulting dendrogram and polar heatmap illustrating the distribution of standardized all traits of M₁ *Leucaena tarramba* is presented in Fig. S2. Therefore, the cluster group for those traits of M₁ *Leucaena tarramba* is presented in Table 3.

Table 3 showed that Group I represented the high-performing cluster, characterized by the plant height (397.00 ± 14.06 cm), the largest number of leaves (432.91 ± 31.59), and the highest biomass and pod yield (whole plant weight: $42,411.45 \pm 409.90$ g; pod dry weight: 58.90 ± 9.91 g). In contrast, Group IV consisted of

Table 3 Morphological and biomass traits of M₁ *Leucaena tarramba* post gamma irradiation in reproductive phase

Trait	Group I	Group II	Group III	Group IV
Plant Height (cm)	397.00±14.06 ^a	366.89±6.36 ^a	374.85±6.72 ^a	317.75±7.95 ^b
Plant Length (cm)	414.00±14.53 ^a	369.42±8.93 ^b	389.76±6.26 ^{ab}	335.16±8.15 ^c
Number of Leaves (unit)	432.91±31.59 ^a	269.47±18.55 ^{bc}	289.68±18.64 ^b	224.5±15.68 ^{bc}
Number of Branches (unit)	22.09±2.14 ^{ab}	20.21±1.32 ^{ab}	23.20±0.97 ^a	15.19±0.70 ^c
Number of Twig (unit)	55.64±12.82 ^a	13.42±1.58 ^b	15.32±1.41 ^a	13.22±1.61 ^b
Stem Diameter (cm)	34.50±1.11 ^a	30.43±1.22 ^b	31.07±0.70 ^{ab}	25.95±0.74 ^c
Number of Pod (unit)	236.36±45.22 ^a	108.26±21.71 ^{bc}	38.79±11.73 ^c	75.34±14.17 ^{bc}
Leaf Area Meter (cm ²)	207.93±15.94	213.63±6.76	222.16±6.93	184.06±6.16
Whole Weight (g)	4241.45±409.90 ^a	2790.47±193.14 ^{bc}	2936.12±198.92 ^b	2021.66±119.91 ^c
Leaf Fresh Weight (g)	121.18±10.83 ^a	142.63±10.14 ^a	60.70±4.14 ^c	77.34±5.84 ^{bc}
Leaf Dry Weight (g)	51.18±5.60 ^a	56.21±3.06 ^a	28.00±1.90 ^b	30.94±2.03 ^b
Branch Fresh Weight (g)	124.63±5.71	165.05±14.43 ^a	95.79±6.76 ^b	106.16±6.43 ^b
Branch Dry Weight (g)	59.09±7.66 ^b	114.11±31.54 ^a	52.64±3.76 ^b	52.19±2.93 ^b
Twig Fresh Weight (g)	35.45±7.59 ^b	53.53±6.29 ^a	35.38±3.54 ^b	30.09±2.73 ^b
Twig Dry Weight (g)	18.63±3.03 ^b	27.74±3.58 ^a	19.64±1.96 ^{ab}	16.69±1.30 ^b
Pod Fresh Weight (g)	107.90±17.02 ^a	54.68±9.68 ^b	66.61±5.41 ^b	35.25±6.37 ^b
Pod Dry Weight (g)	58.90±9.91 ^a	29.11±4.15 ^b	39.55±3.47 ^b	21.47±4.15 ^b
Number of Pod Samples (unit)	157.00±21.85 ^b	88.00±13.54 ^c	122.00±11.86 ^{bc}	158.10±12.47 ^b
Average Number of Seeds (unit)	18.48±0.26 ^{ab}	18.86±0.51 ^a	16.90±0.34 ^b	18.47±0.32 ^{ab}
Mass of Seed per 100 Gram (g)	5.09±0.21 ^b	5.71±0.21 ^b	5.33±0.11 ^b	5.66±0.14 ^{ab}
Mass of Pod Sample (g)	119.30±22.61 ^b	103.37±15.45 ^b	104.74±11.58 ^b	151.41±11.23 ^b

Clusters were generated using SAS. Values are presented as mean±SE; different superscript letters indicate significant differences according to Tukey's HSD test at $p \leq 0.01$

the lowest-performing individuals, with significantly reduced plant height (317.75 ± 7.95 cm), number of leaves (224.5 ± 15.68), and pod dry weight (21.47 ± 4.15 g). Groups II and III captured intermediate phenotypes.

A clear dose–response relationship was observed when irradiation treatments were mapped to these groups (attached as Fig. S2). The wild-type group was mostly classified into Groups II and III, indicating moderate growth performance. Its values were lower than Group I (high performance) but better than Group IV (low performance). Treatment with low to medium doses (50–250 Gy) often placed individuals in Group I, confirming this range as optimal for producing high morphological and biomass performance, highlighting high biomass and yield potential. At higher doses (300–500 Gy), most putative mutants were placed in Group IV, which was marked by reduced all traits. At very high doses (500–750 Gy), no high-performing putative mutants (Group I) were found, and nearly all mutants fell into Groups III and IV. To complement the data presented in Table 3, Fig. 3 shows Principal Component Analysis (PCA) that illustrates the multidimensional variation among *Leucaena tarramba* mutants.

Figure 3 illustrates the phenotypic distribution of M1 *Leucaena tarramba* individuals based on principal component analysis. PC1, PC2, and PC3 explained 20.6%, 15.9%, and 14.0% of the total variance, respectively, accounting for a cumulative 50.5% of the observed variation. Wildtype individuals (G2-Wildtype-2, G2-Wildtype-3, G3-Wildtype-4, G2-Wildtype-5, G3-Wildtype-6, and G3-Wildtype-7) occupied a relatively overlapping region, reflecting comparatively similar agronomic characteristics. The 250 Gy individuals (G1-250–1, G2-250–4, and G4-250–6) remained relatively close to the wildtype distribution, although some individuals shifted along

PCA-Mapping

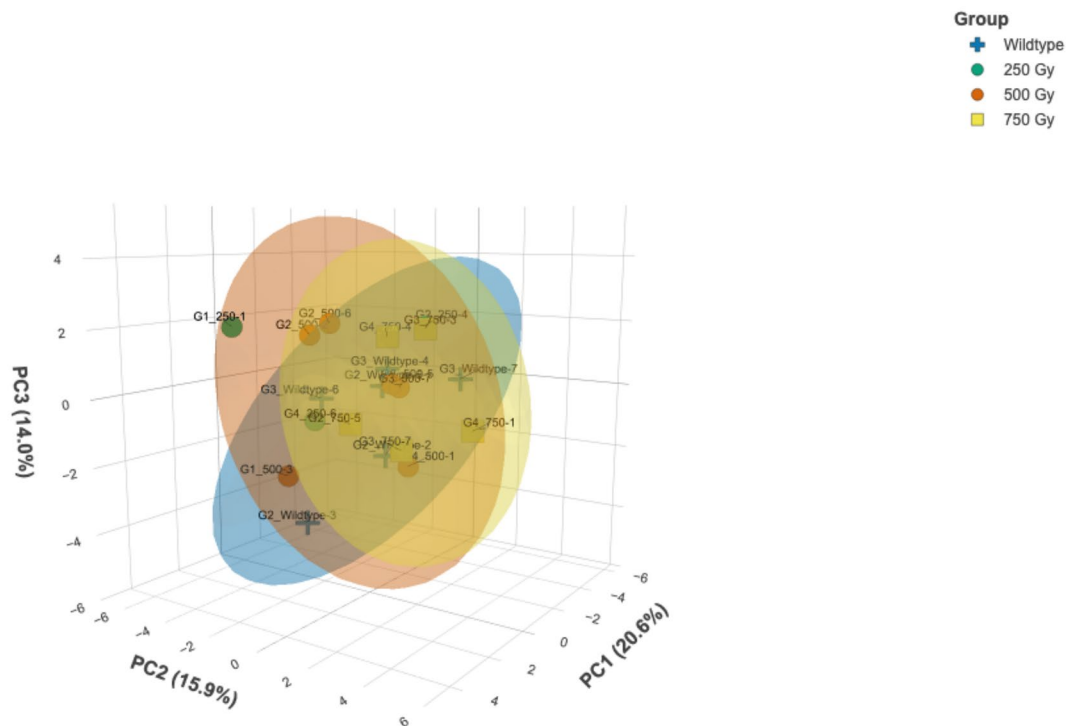


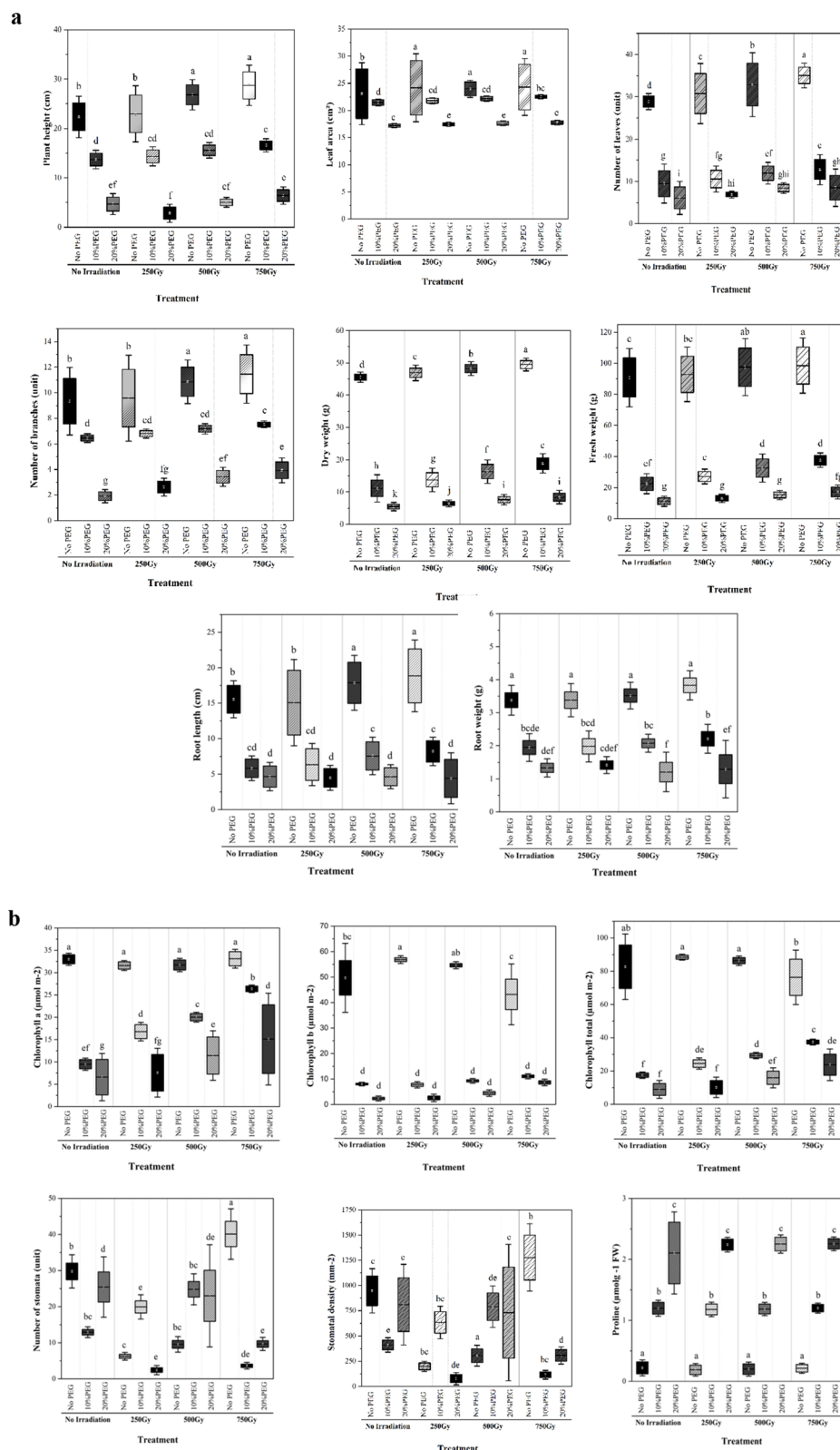
Fig. 3 Principal component analysis (PCA) of *M1* *Leucaena tarramba*

the principal component axes, indicating changes in particular agronomic traits. The 500 Gy individuals (G4-500-1, G2-500-2, G1-500-3, G3-500-5, G2-500-6, and G3-500-7) showed a broader distribution and considerable overlap with both the wildtype and irradiated individuals. Similarly, the 750 Gy individuals (G4-750-1, G3-750-3, G4-750-4, G2-750-5, and G3-750-7) were dispersed across the PCA space, indicating heterogeneous responses to the higher irradiation dose. The substantial overlap among treatment groups suggests that irradiation dose did not produce completely distinct phenotypic groups; instead, gamma irradiation generated considerable individual variation within and among doses. These trends suggest that gamma irradiation contributed to increased phenotypic variation in *Leucaena tarramba*, providing a useful basis for identifying putative mutants with potentially adaptive traits. This pattern highlights the heterogeneous response of *M1* generation to gamma irradiation and supports the selection of individual plants with distinct agronomic characteristics for further evaluation in subsequent generations.

Morpho-physiological traits of *M2* *Leucaena tarramba* under PEG-induced drought-simulating osmotic stress in hydroponics

The morpho-physiological assessment of the *M2* generation of *Leucaena tarramba* was conducted to evaluate the effects of gamma irradiation and the combined influence of gamma-induced mutations with drought stress at different levels. The results are presented into two parts. The first part reports the analysis of variance, which shows the differences among putative mutants under hydroponic phase (attached in Table S1). The second one describes the morpho-physiological traits of *M2* mutants grown under drought-simulating osmotic stress induced by polyethylene glycol (PEG), illustrated in Fig. 4.

Fig. 4 a Morphological traits of M₂ mutants grown under hydroponically induced PEG 6000 as drought stress; The data are presented as Mean $\pm$ Standard Deviation. Within the same row, values followed by different superscripts denote significant differences between the interactions of irradiation doses and PEG at different levels ($p < 0.01$). Tukey's test of significance was conducted at a 1% level of significance **. **b** Physiological traits of M₂ mutants grown under hydroponically induced PEG 6000 as drought stress; The data are presented as Mean $\pm$ Standard Deviation. Within the same row, values followed by different superscripts denote significant differences between the interactions of irradiation doses and PEG at different levels ($p < 0.01$). Tukey's test of significance was conducted at a 1% level of significance**



According to Table S1, irradiation doses significantly affected the morpho-physiological traits of the mutants. Plant height in the mutants ranged from 14.33 to 20.24 cm, which was higher than the wild-type group, with heights ranging from 15.07 to 17.15 cm. Leaf area also showed an increase, ranging from 21.65 to 22.40 cm² in mutants, and 20.86 to 21.37 cm² in the control. Similar significant increases ($p < 0.01$) were observed for the number of leaves, number of branches, dry weight, fresh weight, and root length, but root weight did not show a significant effect. Chlorophyll a content ranged from 20.88 to 27.46 $\mu\text{mol m}^{-2}$ higher than in the wild-type group 17.40 to 19.86. Similar significant increases were also observed for chlorophyll b and c. Stomatal traits showed a different pattern, with the 250 Gy-treated putative mutants having about half the control's values. Among these, putative mutant 250Gy_4 had the lowest values where 9.44 unit number of stomata and 318.309 mm⁻² stomatal density), while wild-type 4 had the highest (24.40 unit number of stomata and 776.67 mm⁻² stomatal density). Proline content was lowest in 250Gy_1 (0.78 $\mu\text{mol g}^{-1}$ FW) and highest in 500Gy_3 (1.52 $\mu\text{mol g}^{-1}$ FW). Mutants 500Gy_1, 500Gy_2, 750Gy_1, 750Gy_3, and 750Gy_4 were the best performers in both morphological and physiological traits, particularly in chlorophyll content. 250Gy_1 had the highest proline content, and wild-type 4 in stomatal traits. In accordance with Table S1, those mutants were treated with different concentrations of PEG 6000 to stimulate drought stress under hydroponic conditions. The interactions between gamma irradiation dose and PEG 6000 as drought stress grown under hydroponic system are presented in Fig. 4a and b.

The analysis in Fig. 4a and b indicated that the interaction between irradiation doses and PEG levels significantly affected ($p < 0.01$) the morpho-physiological traits of the *M₂ Leucaena tarramba*. Figure 4a revealed a clear trend regarding the effect of irradiation on the traits. Mutants exposed to 250 Gy and 500 Gy showed a significant increase in morphological traits compared to the wild-type group, with the best results observed under moderate PEG concentrations (10%). Mutants under the 250 Gy dose, those without PEG stress, showed moderate growth, plant height (22.95 cm), leaf area (24.18 cm²), and root weight (3.38 g). However, when exposed to 10% PEG, there was reduction in growth, plant height (14.34 cm), leaf area (21.80 cm²), and root weight (1.98 g). The addition of 20% PEG caused a more severe decline, with plant height (2.82 cm), leaf area (17.45 cm²), and root weight (1.42 g).

Conversely, putative mutants irradiated at 500 Gy showed better growth. Without PEG, putative mutants reached plant height (26.82 cm), leaf area (23.97 cm²), and root weight (3.52 g). Under 10% PEG, the reductions in growth were less severe than with 250 Gy, as the mutants maintained plant height (15.58 cm), leaf area (22.18 cm²), and root weight (2.07 g). However, under 20% PEG, growth still declined, with plant height (5.03 cm), leaf area (17.63 cm²), and root weight (1.21 g).

Interestingly, at 750 Gy without PEG, putative mutants showed optimal growth, with plant height (28.72 cm), leaf area (24.30 cm²), and root weight (3.83 g). Even under 10% PEG, the putative mutants showed moderate reductions, with plant height (16.60 cm), leaf area (22.53 cm²), and root weight (2.21 g). Under 20% PEG, the putative mutants still maintained better growth than those under 250 Gy and 500 Gy, with plant height (6.40 cm), leaf area (17.76 cm²), and root weight (1.29 g). For the non-irradiated (control) treatment, the putative mutants without PEG showed moderate growth, with plant height (22.34 cm), leaf area (23.07 cm²), and root weight (3.38 g). However, with the addition of 10% PEG, the putative mutants experienced a reduction in growth, with plant height (13.71 cm), leaf area (21.45 cm²), and root weight (1.95 g). Under 20% PEG, performance dropped significantly, plant height at 4.69 cm, leaf area at 17.21 cm², and root weight at 1.33 g, demonstrating the severe effects of high drought stress in the absence of irradiation. This trend is observed across other traits, including the number of leaves, branches, and dry weight (DW) and fresh weight (FW).

Figure 4b illustrates the physiological responses of *Leucaena tarramba* mutants under varying irradiation doses and PEG-induced drought-simulating osmotic stress. Overall, increasing PEG concentration caused a clear decline in chlorophyll pigments, stomatal traits, and proline accumulation across all irradiation levels. In the 250 Gy treatment, total chlorophyll content dropped from 88.4 $\mu\text{mol m}^{-2}$ at 0% PEG to 10.2 $\mu\text{mol m}^{-2}$ at 20% PEG, while stomatal density decreased from 634 mm⁻² to 77 mm⁻². A similar trend occurred at 500 Gy, where chlorophyll fell from 86.3 $\mu\text{mol m}^{-2}$ to 15.9 $\mu\text{mol m}^{-2}$, and stomatal density from 789 mm⁻² to 304 mm⁻². The 750 Gy mutants resulted in the sharpest decline. Interestingly, proline levels, expected to rise under stress,

remained stable under moderate PEG (10%) but declined at severe stress (20%), particularly in the 500 Gy and 750 Gy mutants. Plants without irradiation or PEG showed the highest chlorophyll ($82.7 \mu\text{mol m}^{-2}$) and stomatal density (946 mm^{-2}), confirming that both gamma exposure and osmotic stress substantially suppressed physiological performance.

Stress selection indices on *M₂* *Leucaena tarramba* under PEG-induced drought-simulating osmotic stress in hydroponics

Stress selection indices provide a comprehensive analysis of the Stress Tolerance Index (STI), Stress Intensity (SI), and Stress Susceptibility Index (SSI) for various mutants. The categories for each index offer the drought tolerance characteristics of these mutants as presented in Table 4.

Stress indices based on dry weight (DW) showed variation among the *M₂* irradiation-dose groups under PEG-induced osmotic stress (Table 4). Stress intensity (SI) was 0.684 under 10% PEG and increased to 0.854 under 20% PEG, reflecting the stronger effect of the higher PEG concentration on dry-weight reduction. Under 10% PEG, STI increased from 0.226 in the wild type to 0.285, 0.347, and 0.413 in the 250, 500, and 750 Gy groups, respectively. The same trend was observed under 20% PEG, although STI values were lower overall, ranging from 0.110 in the wild type to 0.183 in the 750 Gy group. According to the applied STI classification, all irradiation-dose groups were categorized as sensitive under both stress conditions. SSI showed an opposite trend, with lower values in the higher irradiation-dose groups. Under 10% PEG, SSI decreased from 1.102 in the wild type and 1.034 in the 250 Gy group to 0.968 and 0.905 in the 500 and 750 Gy groups, respectively. Under 20% PEG, SSI values were 1.031, 1.010, 0.987, and 0.973 for the wild type, 250, 500, and 750 Gy groups, respectively. Based on the applied SSI classification, values below 1 in the 500 and 750 Gy groups indicate lower relative susceptibility to PEG-induced drought-simulating osmotic stress than the wild type and 250 Gy groups. However, the differences among irradiation-dose groups were smaller under 20% PEG, suggesting that the relatively better dry-weight maintenance observed in the higher irradiation-dose groups became less pronounced under severe stress.

Polar heatmap and principal component analysis of *M₂* *Leucaena tarramba* under PEG-induced drought-simulating osmotic stress in hydroponics

To further examine the phenotypic patterns underlying these group-level stress responses, individual *M₂* entries were subsequently explored using a polar heatmap and PCA based on the combined morpho-physiological traits. These visualizations were used to provide a larger overview of trait variation among individual entries across irradiation-dose groups and PEG treatments.

The polar heatmap analysis comprehensively visualizes morpho-physiological traits of *M₂* *Leucaena tarramba* mutants treated between irradiation doses and PEG hydroponically simulated drought stress. The polar heatmap clustering among putative mutants is presented in Fig. 5.

Table 4 Stress selection indices of *M₂* irradiation-dose groups under PEG-induced drought-simulating osmotic stress

Irradiation-dose group	Stress treatment	STI	Category	SI	Category	SSI	Category
Wild type	10% PEG	0.23	Sensitive	0.68	Moderate stress	1.10	Sensitive to stress
250 Gy	10% PEG	0.28	Sensitive	0.68	Moderate stress	1.03	Sensitive to stress
500 Gy	10% PEG	0.35	Sensitive	0.68	Moderate stress	0.97	Relatively tolerant to stress
750 Gy	10% PEG	0.41	Sensitive	0.68	Moderate stress	0.90	Relatively tolerant to stress
Wild type	20% PEG	0.11	Sensitive	0.85	Moderate stress	1.03	Sensitive to stress
250 Gy	20% PEG	0.13	Sensitive	0.85	Moderate stress	1.01	Sensitive to stress
500 Gy	20% PEG	0.16	Sensitive	0.85	Moderate stress	0.99	Relatively tolerant to stress
750 Gy	20% PEG	0.18	Sensitive	0.85	Moderate stress	0.97	Relatively tolerant to stress

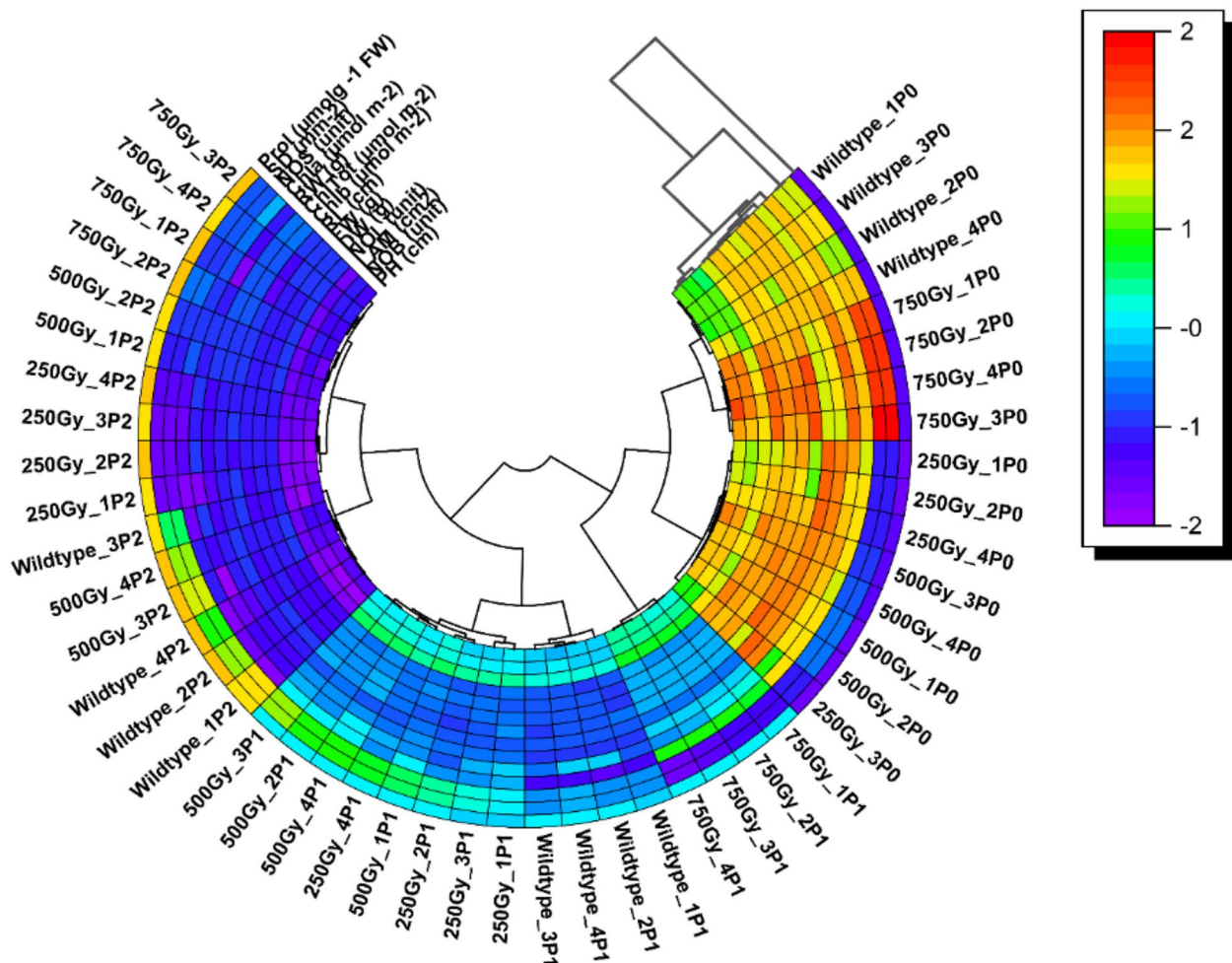


Fig. 5 Polar heatmap analysis of *M₂ Leucaena tarramba* under hydroponically induced drought stress based on standardized trait values (z-scores). Colors represent relative values from low (purple/blue) to high (yellow/red), and the dendrogram indicates mutant similarity. Plant height (PH), number of leaves (NOL), number of branches (NOB), and leaf area (LAM), fresh weight (FW), dry weight (DW), root weight (RW), and root length (RL), chlorophyll a, b and Total (Chla, Chlb, Chltot), Number of stomata (NOS), Stomatal density (SD), and Proline (Prol)

Figure 5 presents a polar heatmap of wildtype and putative mutants across all PEG concentrations (P0, P1, and P2), illustrating the overall variation in response from non-stress to severe stress conditions. The visualization highlights a clear dose–response trend, showing how trait performance gradually shifts as PEG concentration increases. The color gradient from red to blue delineates three major clusters: Cluster I, dominated by red–orange hues, comprises all P0 putative mutants such as Wildtype_1P0 to Wildtype_4P0, 250Gy_1P0 to 50Gy_4P0, 500Gy_1P0 to 500Gy_4P0, and 750Gy_1P0 to 750Gy_4P0), which maintained high morphological-physiological traits such as PH, NOB, NOL, DW, FW, chla, chlb, and chlTot indicating optimal growth under non-stress conditions. Cluster II, with intermediate green–yellow tones, includes putative mutants Wildtype_1P1 to Wildtype_14P1, 250Gy_1P1 to 250Gy_4P1, 500Gy_1P1 to 500Gy_4P1, and 750Gy_1P1 to 750Gy_4P1), representing reduced morpho-physiological traits, yet the putative mutants remain capable of surviving and maintaining minimal physiological activity under prolonged stress conditions. Cluster III, positioned opposite to Cluster I and shaded in blue–purple, groups all P2 putative mutants such as Wildtype_1P2 to Wildtype_4P2, 250Gy_1P2 to 250Gy_4P2, 500Gy_1P2 to 500Gy_4P2, and 750Gy_1P2 to 750Gy_4P2), which showed severe damage for the traits at 20% PEG. Interestingly, the parameters NOS and SD showed the opposite trend, with notable exceptions in Wildtype_3P2, 500Gy_3P2, and 750Gy_3P2, as well as in Wildtype_1P2–Wildtype_4P2,

which displayed higher NOS and SD values despite the high-stress environment. In contrast, the Prol parameter followed a reversed pattern compared to the general clustering trend, where higher stress levels corresponded to greater proline accumulation.

PCA was performed using the combined morpho-physiological traits under PEG-induced drought-simulating osmotic stress. This analysis was used to visualize the multivariate relationships among wild-type plants and M₂ putative mutants and to identify entries showing maintained or reduced performance profiles.

The Principal Component Analysis (PCA) biplot (Fig. 6) showed distinct grouping of individual screening entries based on their morpho-physiological traits. The first principal component (Dim1) explained 74.4% of the total variation, while the second principal component (Dim2) accounted for 15.1%, together explaining 89.5% of the variation in the dataset. Entries categorized as Maintained performance (blue circles) were predominantly distributed on the positive side of Dim1. These included several M₂ putative mutants, such as 250Gy_2P1, 250Gy_3P1, 250Gy_4P1, 500Gy_1P1, 500Gy_2P1, 500Gy_3P1, and 500Gy_4P1, which were associated with relatively higher values of plant height (PH), leaf area (LAM), dry weight (DW), total chlorophyll (ChlTot), number of branches (NOB), and fresh weight (FW).

In contrast, entries categorized as Reduced performance (yellow triangles) were predominantly positioned on the negative side of Dim1. These included several wild-type entries and M₂ putative mutants, such as Wildtype_1P1, Wildtype_2P2, Wildtype_3P2, 250Gy_1P2, 250Gy_2P2, and 250Gy_4P2. Their positions were generally associated with lower values of several growth- and physiological-performance traits, indicating a less favorable overall morpho-physiological profile under PEG-induced drought-simulating osmotic stress. The PCA also showed differences in the contribution of other physiological traits. Number of stomata (NOS) and stomatal density (SD) contributed mainly to variation along Dim2, whereas proline (Prol) was oriented toward the negative side of Dim1 and was more closely associated with entries showing reduced performance.

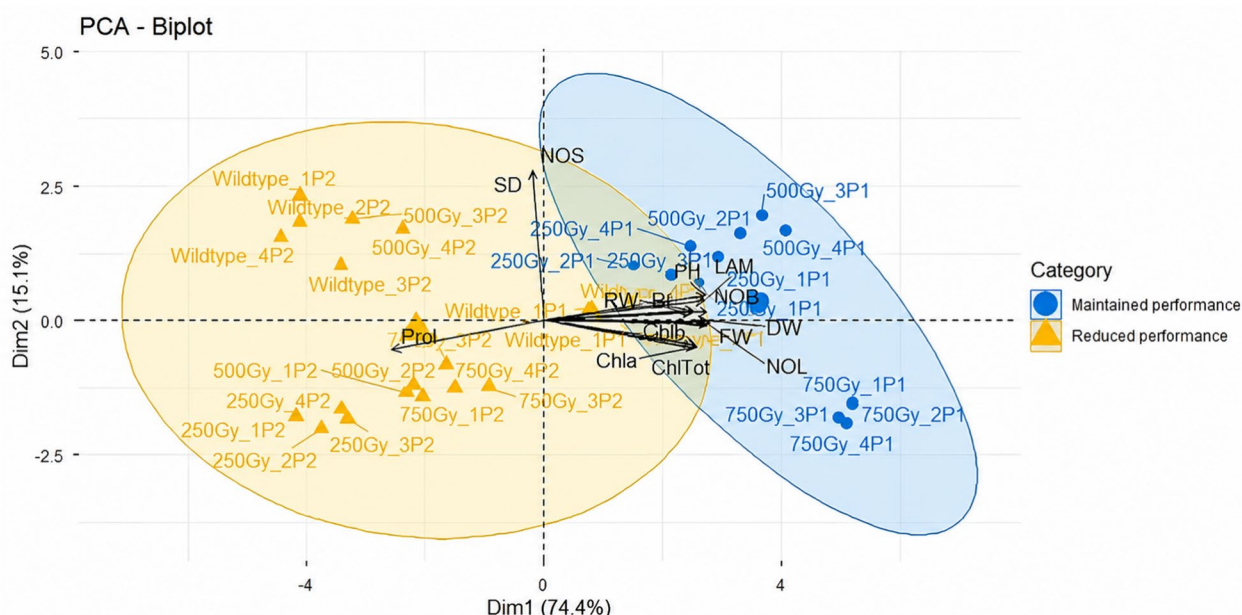


Fig. 6 Visualization of PCA based on morpho-physiological traits among wild-type plants and M₂ putative mutants of *Leucaena tarramba* under hydroponically induced drought stress. Plant height (PH), number of leaves (NOL), number of branches (NOB), and leaf area (LAM), fresh weight (FW), dry weight (DW), root weight (RW), and root length (RL), chlorophyll a and Total (Chla, Chlb, Chltot) Number of stomata (NOS), Stomatal density (SD), and Proline (Prol)

Correlation among morpho-physiological traits on putative mutants of *M₂ Leucaena tarramba* under PEG-induced drought-simulating osmotic stress in hydroponics

Further analysis after PCA, including correlation, provides a comprehensive study of morpho-physiological traits under hydroponically induced drought stress. Figure 7 illustrates the correlation among traits on *M₂ Leucaena tarramba* under hydroponically induced drought stress.

The correlation matrix revealed clear associations among morpho-physiological traits (Fig. 7). Plant height (PH) showed a highly significant and positive correlation ($p \leq 0.01$) with most traits, particularly number of branches (NOB, $r=0.99$), dry weight (DW, $r=0.91$), and root length (RL, $r=0.90$). Likewise, leaf area (LAM) and number of leaves (NOL) were strongly correlated to chlorophyll b (Chlb, $r=0.93$) and total chlorophyll content (ChlTot, $r=0.95$). A consistent pattern of positive correlations was observed among Fresh weight (FW) and dry weight (DW), and root weight (RW) demonstrated strong correlations ($r=0.87$ – 0.99). Chlorophyll pigments also correlated closely with growth attributes, with Chlb showing a strong association with DW ($r=0.94$) and ChlTot presenting the strongest correlation with RL ($r=0.97$). In contrast, stomatal traits, including number of stomata (NOS) and stomatal density (SD), showed weak and non-significant correlations with other variables ($r \leq 0.25$), indicating their limited contribution to agronomic performance under hydroponic stress conditions. Furthermore, proline (Prol), an osmoprotectant commonly accumulated under abiotic stress, displayed a significant negative correlation with all traits. The strongest inverse relationships were observed with PH ($r=-0.93$),

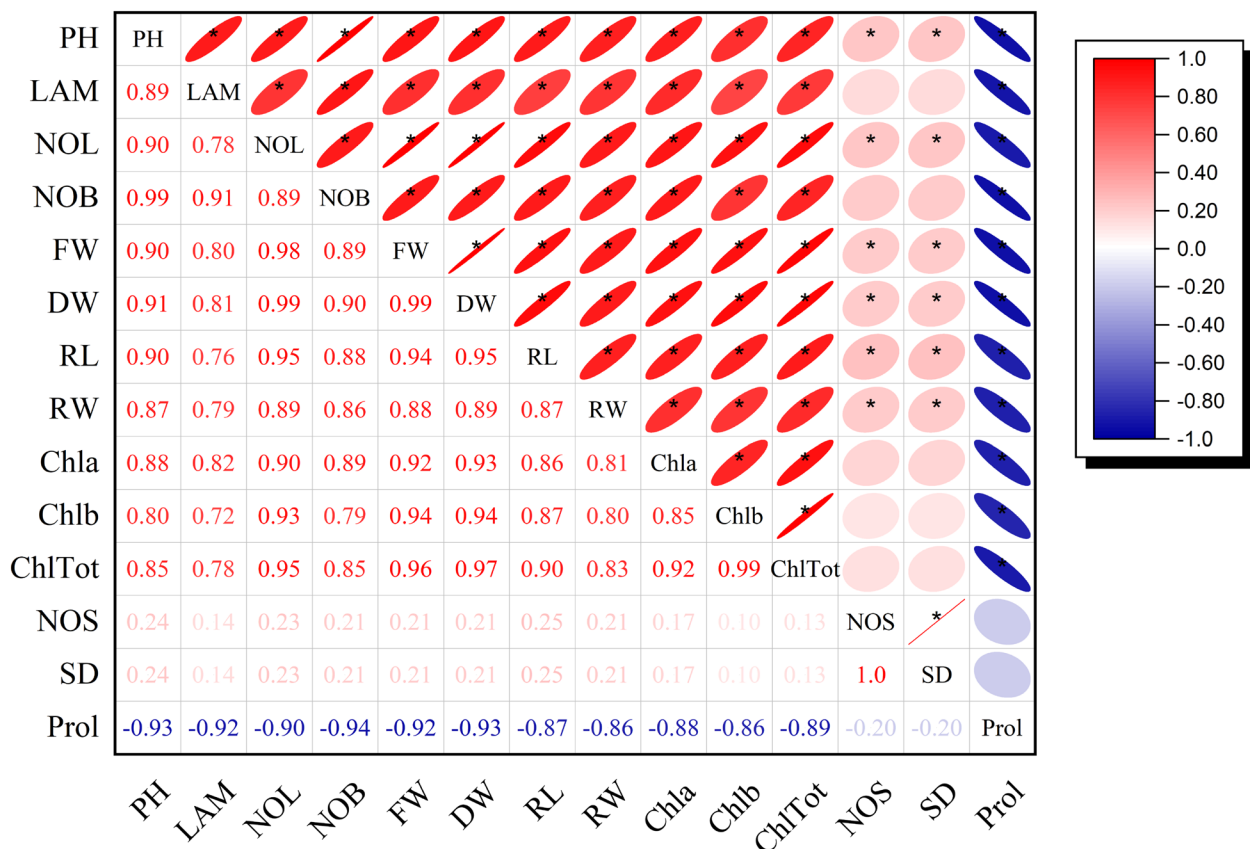


Fig. 7 Correlation among traits on putative mutants of *M₂ Leucaena tarramba* under hydroponically induced drought stress. Plant height (PH), number of leaves (NOL), number of branches (NOB), and leaf area (LAM), fresh weight (FW), dry weight (DW), root weight (RW), and root length (RL), chlorophyll a and Total (Chla, Chlb, Chltot) Number of stomata (NOS), Stomatal density (SD), and Proline (Prol). * p -value < 0.01 , * p -value

DW ($r=-0.92$), and ChlTot ($r=-0.89$), indicating that when proline levels increased, morpho-physiological traits declined.

Preliminary genetic-parameter estimates of *M₂* *Leucaena tarramba* following gamma irradiation

Gamma irradiation can induce phenotypic variation that may reflect underlying genetic changes in subsequent generations. Tables 5 and 6 present the morpho-physiological variation observed in *M₂* *Leucaena tarramba* during the hydroponic phase at 42 DAP and the post-hydroponic greenhouse phase at 60 and 90 DAP. Because irradiation dose represents an experimental treatment, the observed variation is interpreted as phenotypic evidence and as a basis for preliminary genetic-parameter estimates, pending further progeny and molecular validation.

Phenotypic variance (σ^2_p) was higher than genetic variance (σ^2_g) for all evaluated traits, indicating varying contributions of environmental effects to phenotypic expression. Table 5 showed that broad-sense heritability (h^2) ranged from 0.24 for chlorophyll b to 0.60 for root weight. High heritability was observed for plant height (0.56), number of leaves (0.52), number of branches (0.54), root weight (0.60), number of stomata (0.59), and stomatal density (0.59), whereas the remaining traits showed moderate heritability. The phenotypic coefficient of variation (PCV) ranged from 5.52% for stomatal density to 47.50% for root weight, while the genotypic coefficient of variation (GCV) ranged from 4.26% to 36.79% for the same traits. PCV was consistently higher than GCV across all traits. Root weight exhibited the highest PCV and GCV values (47.50% and 36.79%, respectively), followed by number of stomata (31.12% and 24.00%), indicating relatively high phenotypic and estimates of genetic variability for these traits. In contrast, stomatal density showed the lowest PCV and GCV values (5.52% and 4.26%, respectively). The differences between PCV and GCV across traits indicate that both genetic and environmental components contributed to the observed phenotypic variability.

Phenotypic variance (σ^2_p) was higher than genetic variance (σ^2_g) for all evaluated traits at both 60 and 90 DAP. Table 6 showed that, at 60 DAP, broad-sense heritability (h^2) ranged from 0.07 for stem diameter (DS) to 0.74 for plant height (PH). High heritability was observed for PH (0.74), number of leaves (NOL; 0.58), and number of branches (NOB; 0.54), whereas DS (0.07) and LAM (0.11) showed low heritability. PCV ranged from 13.02% for DS to 54.56% for LAM, while GCV ranged from 3.48% for DS to 21.24% for NOB. PCV was consistently higher than GCV, with the largest difference observed for LAM (54.56% vs. 17.81%), indicating a relatively strong environmental contribution to its phenotypic variation.

At 90 DAP, broad-sense heritability ranged from 0.35 for PH to 0.80 for DS. High heritability was observed for NOL (0.64), DS (0.80), and leaf area (LA; 0.70), whereas PH (0.35) and NOB (0.46) showed moderate heritability. PCV ranged from 14.60% for PH to 64.77% for LA, while GCV ranged from 8.59% to 54.15% for the same traits. LA exhibited the highest PCV and GCV values (64.77% and 54.15%, respectively), together with high heritability ($h^2=0.70$), indicating substantial phenotypic and estimates of genetic variability. Overall, PCV remained higher than GCV across all traits at both observation periods, reflecting varying degrees of environmental influence on trait expression.

Discussion

The optimal irradiation dose for mutation breeding typically lies near the lethal dose for 50% mortality (LD_{50}) [66, 67]. LD_{50} for *Leucaena tarramba* was determined to be 820 Gy. This relatively high value is attributed to the hard seed coat, which requires greater energy for radiation penetration and subsequent cellular damage [68]. Generally, seeds exhibit higher radiation tolerance than vegetative tissues due to their dormant state and protective seed coverings [69, 70]. Comparing to other legume species, the LD_{50} value for *Leucaena tarramba* is relatively higher, demonstrating its greater tolerance to ionizing radiation. This substantial difference indicates that *Leucaena tarramba* possesses stronger seed coat hardness and higher cellular repair capacity compared to other legumes such as *Phaseolus vulgaris* (318 Gy), *Indigofera zollingeriana* (330 Gy), and *Vigna subterranea*

Table 5 Genetic estimates of putative mutant (M_2) *Leucaena tarramba* in hydroponic phase

Doses	PH	LAM	NOL	NOB	FW	DW	RL	RW	Chla	Chlb	ChlTot	NOS	SD
σ^2p	137.66	113.33	210.31	22.24	1792.45	442.74	61.47	2.65	195.12	589.86	1453.63	254.52	257,886.36
σ^2e	59.89	63.91	100.50	10.31	1125.56	246.66	33.52	1.06	124.14	445.36	1039.77	103.16	104,524.98
σ^2g	77.77	49.42	109.81	11.93	666.89	196.08	27.95	1.59	70.98	144.49	413.86	151.36	153,361.38
h^2	0.56 ^H	0.44 ^M	0.52 ^H	0.54 ^H	0.37 ^M	0.44 M	0.45 ^M	0.60 ^H	0.36 ^M	0.24 M	0.28 M	0.59 ^H	0.59 ^H
PCV (%)	17.43	17.93	15.47	27.21	9.06	12.73	21.81	47.50	15.62	11.95	9.48	31.12	5.52
GCV (%)	13.10	11.84	11.18	19.93	5.53	8.47	14.71	36.79	9.42	5.91	5.06	24.00	4.26

σ^2p Phenotypic Variance, σ^2g Genetic Variance, σ^2e Environmental Variance, h^2 Broad-sense heritability expressed as a proportion (0–1), H High heritability ($h^2 > 0.5$), M Moderate heritability ($0.2 < h^2 \leq 0.5$), L Low heritability ($h^2 < 0.2$), PCV Phenotypic Coefficient Variability, GCV Genetic Coefficient Variability, H High Heritability, M Moderate Heritability, L Low Heritability; The abbreviations traits are: PH Plant height, NOL Number of leaves, NOB Number of branches, LAM Leaf area, FW Fresh weight, DW Dry weight, RW Root weight, RL Root length, $Chla$ Chlorophyll a, $Chlb$ Chlorophyll b, $ChlTot$ Total chlorophyll, NOS Number of stomata, and SD Stomatal density

Table 6 Genetic estimates of putative mutant (M_2) *Leucaena tarramba* in post-hydroponic phase

Parameter	60 DAP					90 DAP				
	PH	NOL	NOB	DS	LAM	PH	NOL	NOB	DS	LA
σ^2_p	347.82	88.11	4.95	0.1	1341.34	551.84	898.92	7.92	12.03	5734.69
σ^2_e	91.38	36.7	2.3	0.09	1198.48	360.7	324.7	4.3	2.38	1725.49
σ^2_g	256.44	51.41	2.65	0.01	142.86	191.14	574.22	3.62	9.65	4009.2
h^2	0.74 ^H	0.58 ^H	0.54 ^H	0.07 ^L	0.11 ^L	0.35 ^M	0.64 ^H	0.46 ^M	0.8 ^H	0.70 ^H
PCV (%)	18.51	23.12	29.03	13.02	54.56	14.6	27.44	27.42	28.14	64.77
GCV (%)	15.9	17.66	21.24	4.12	17.81	8.59	21.93	18.54	25.2	54.15

σ^2_p Phenotypic Variance, σ^2_g Genetic Variance, σ^2_e Environmental Variance, h^2 Broad-sense heritability expressed as a proportion (0–1), *H* High heritability ($h^2 > 0.5$), *M* Moderate heritability ($0.2 < h^2 \leq 0.5$), *L* Low heritability ($h^2 < 0.2$), *PCV* Phenotypic Coefficient Variability, *GCV* Genetic Coefficient Variability, *H* High Heritability, *M* Moderate Heritability, *L* Low Heritability, *PH* Plant Height, *NOL* Number of Leaves, *NOB* Number of Branches, *DS* Stem Diameter, *LAM* Leaf Area

(160 Gy) [71–73]. Similarly, *Vicia faba* putative mutants resulted genotypic differences in LD₅₀, varying from 140 to 669 Gy, depending on genotype and sowing time [74]. Such a high LD₅₀ value suggests that higher irradiation doses are required to induce effective mutagenesis while maintaining sufficient viability for subsequent generations, highlighting its potential genetic resource for next generations (M_2 and beyond) [73, 75–77].

Gamma irradiation has long been recognized as an effective mutagenic agent capable of inducing a wide range of genetic variations in plants. In the M_1 generation of *Leucaena tarramba*, noticeable alterations in morphological traits were observed, reflecting the immediate physiological and genomic disturbances caused by ionizing radiation. The morphological responses of M_1 mutants are often heterogeneous, as most induced mutations at this stage are not yet genetically stabilized or homozygous [8, 78]. Consequently, phenotypic variation within and among individual plants of the same treatment group tends to be high. Because these mutations remain unstable and heterogeneous, they segregate and are expressed differently within different parts of the same plant or among individual plants [79, 80]. It is important to emphasize that increasing irradiation doses in M_1 *Leucaena tarramba* led to a marked reduction in agronomic performance, particularly in traits related to vegetative growth. Generally, increasing radiation doses corresponded with a gradual decline in morphological and biomass traits. High doses often resulted in reduced germination and stunted seedling growth [81], likely due to damage to chromosomal integrity and the accumulation of reactive oxygen species (ROS) leading to oxidative stress [82]. In contrast, moderate doses occasionally stimulated slight increases in certain growth parameters, a phenomenon commonly referred to as “hormetic effects,” where low-level radiation induces adaptive metabolic responses that temporarily enhance growth or repair mechanisms [83]. Despite the phenotypic variability, certain putative mutant of M_1 showed unique or potentially beneficial morphological traits. However, since the M_1 generation primarily represents heterozygous and unstable mutants, such traits must be validated and stabilized in subsequent generations (M_2 and beyond) through selfing or clonal propagation. The identification and propagation of promising variants from the M_1 population are therefore critical for advancing to the selection phase of mutation breeding [26].

Polyethylene glycol (PEG) is widely used to simulate drought stress in stress screening [16, 84–86]. Higher concentrations of PEG (20% and above) severely inhibited growth and germination, while moderate levels (5–10%) allowed for differentiation among putative mutants, identifying those with higher drought tolerance [87, 88]. Similarly, in lentils, PEG concentrations of 5–10% did not significantly affect germinability, but higher concentrations (20–25%) completely inhibited growth, highlighting the importance of selecting appropriate PEG levels for stress studies [16]. Studies in maize and cucurbits have confirmed PEG’s effectiveness in differentiating tolerant from sensitive lines [89, 90]. Hydroponic deep water culture (DWC) combined with PEG-induced drought-simulating osmotic stress provides an efficient system for screening legumes under drought. This method facilitates the precise identification of tolerant putative mutants and strengthens the evaluation of morpho-physiological traits in mutant lines. Previous studies have shown that DWC optimizes nutrient uptake [38, 91, 92]. Combined with PEG-induced drought-simulating osmotic stress, offers an efficient method to screen legumes for drought tolerance. This approach enables precise identification of resilient putative mutants while reinforcing

the morpho-physiological potential, thereby supporting breeding strategies for improved resilience under water-limited conditions [93]. In this study, the application of 10% and 20% PEG effectively differentiated the drought response of *Leucaena tarramba* putative mutants, indicating its suitability as a screening medium. Interaction between PEG-induced drought-simulating osmotic stress and gamma irradiation, indicating PEG's effectiveness as a drought stress selection agent [94, 95]. This present study highlights the intricate interaction between induced drought stress and mutagenesis in plant performance. The results demonstrate that PEG-induced drought-simulating osmotic stress significantly reduces several morpho-physiological parameters of *Leucaena tarramba*. As Habtegebriel et al. [96] and Mahpara et al. [97] state, increasing PEG concentrations intensify drought-simulating osmotic stress, leading to substantial reductions in vegetative growth and overall plant vigor.

The hydroponic PEG system offers several advantages over soil-based screening. It enables uniform stress imposition, precise control of nutrient availability, and early detection of tolerant lines [38, 98]. In *Leucaena tarramba*, morphological indicators such as chlorophyll, stomata and proline emerged as reliable markers of drought tolerance. These responses suggest that certain putative mutants developed adaptive mechanisms to maintain osmotic balance, possibly through enhanced accumulation of osmolytes such as proline and soluble sugars. Such biochemical adjustments are known to stabilize cellular membranes and preserve enzymatic functions under osmotic stress [99, 100]. However, the hydroponic PEG-screening approach presents several methodological limitations. PEG induces osmotic stress uniformly in controlled environments, yet it cannot fully mimic complex field drought conditions involving fluctuating soil moisture and temperature [101]. In the present study, the absence of field validation limits the agronomic relevance of the findings, as putative mutant $\times$ environment interactions remain untested. Moreover, the lack of molecular and biochemical analyses constrains the understanding of stress adaptation mechanisms. Future studies should include field validation and molecular profiling, including antioxidant and epigenetic analyses, to strengthen drought-tolerance screening [100, 102–105].

Under 10% PEG level, several irradiated mutants maintained better growth and physiological performance than the wild type or control. This response may reflect a greater capacity to maintain cellular turgor, photosynthetic pigments, energy metabolism, and antioxidant protection under moderate osmotic stress. Gamma-induced wheat mutants with improved drought tolerance have similarly shown lower chlorophyll degradation and changes in proteins associated with chloroplasts, mitochondria, ATP production, and cellular homeostasis under PEG treatment [106–108]. However, because these mechanisms were not directly measured in the present study, the observed responses of the putative mutants should be interpreted as possible phenotypic adaptations, while the underlying biomolecular mechanisms responsible for these responses remain to be confirmed through further molecular and progeny-based analyses. In contrast, 20% PEG likely imposed a stronger osmotic constraint that severely restricted physiological activity. The simultaneous decline in multiple traits therefore indicates general stress injury rather than successful acclimation. Although proline commonly functions as an osmoprotectant, its reduction under severe stress may indicate that cellular damage exceeded the capacity to maintain proline biosynthesis and metabolic homeostasis or that the energy and substrates required for its synthesis became limited [109–111]. A progressive decline in proline content with increasing PEG-induced stress levels has been reported in *Moringa stenopetala*, while proline concentration in *Vicia sativa* was not correlated with field yield performance, indicating that proline may reflect the physiological response to water-deficit stress rather than serve as a reliable standalone predictor of drought tolerance [112–114].

The improvement physiological performance of M_2 mutants under hydroponic conditions suggests that gamma irradiation can expand adaptive plasticity to water-deficit environments, particularly under moderate stress. In legumes, water scarcity during vegetative and reproductive stages severely limits photosynthesis, assimilate allocation, and reproductive development [27, 28]. Nevertheless, legumes rely on complex defense networks integrating antioxidant enzymes, ascorbate peroxidase, catalase, and superoxide dismutase, with hormonal signaling to maintain redox homeostasis [115, 116]. Gamma irradiation may potentiate these mechanisms through transcriptional modulation of stress-related genes [117], accounting for the greater vigor of irradiated *Leucaena tarramba* under drought-simulating osmotic stress with PEG levels. However, the attenuation of growth at 20% PEG indicates that irradiation-derived tolerance remains effective only within species-specific physiological limits, as

also observed in gamma-irradiated chickpea [118]. Moderate irradiation (~500 Gy) stabilized chlorophyll pigments and enhanced proline accumulation, both essential components of drought adaptation. Proline acts as an osmoprotectant that sustains cell turgor, protects proteins and membranes, and regulates ROS detoxification [119, 120]. The heterogeneous responses among moderate-dose mutants under 10% PEG level stress reveal expanded estimates of genetic variability useful for mutation breeding.

Under PEG-induced osmotic stress simulating drought conditions, clear physiological differentiation arises between wild-type and putative mutants. The wild-type plants consistently exhibited superior vigor, greater leaf turgidity, and higher photosynthetic stability, reflecting their inherent physiological robustness under controlled osmotic stress [38, 121]. Stress indices showed that the 500 and 750 Gy groups had lower relative susceptibility than the wild type and 250 Gy groups. Under 10% PEG, the 750 Gy group had the highest STI (0.41) and the lowest SSI (0.90), while the 500 Gy group also showed an SSI below 1. A similar pattern was observed under 20% PEG, although STI values were lower overall and all irradiation-dose groups remained classified as sensitive based on STI. These results indicate that the higher irradiation-dose groups, particularly 500 and 750 Gy, maintained dry weight relatively better at the group level under PEG-induced drought-simulating osmotic stress. Mutants derived from high irradiation doses displayed significant growth inhibition, chlorosis, and reduced biomass accumulation, symptoms frequently associated with radiation-induced DNA damage and metabolic disruption [122, 123].

The maintenance of growth and physiological traits under 10% PEG level may reflect a greater capacity for osmotic adjustment and preservation of cellular function. However, these mechanisms were not directly measured in this study and should therefore be regarded as biological interpretations rather than confirmed responses. Independently of the stress-index analysis, individual morpho-physiological and multivariate screening identified several M₂ putative mutants with maintained performance under 10% PEG level. Entries from the 250, 500, and 750 Gy treatments showed relatively favorable combinations of growth, biomass, chlorophyll, and other physiological traits in the heatmap and PCA. Several individuals, including 250Gy_2P1, 250Gy_3P1, 250Gy_4P1, 500Gy_1P1–500Gy_4P1, and 750Gy_1P1–750Gy_4P1, were associated with the maintained-performance profile and can therefore be considered promising M₂ putative mutant candidates for further selection. Their identification was based on individual morpho-physiological and multivariate performance rather than on individual STI, SI, or SSI values. From a plant-breeding perspective, these individuals may provide useful preliminary material for further drought-response selection in *Leucaena*. However, their breeding value remains to be confirmed through progeny testing, field evaluation, multi-environment testing, forage-quality assessment, and molecular or genomic characterization.

The maintenance of growth and physiological traits under 10% PEG level may reflect a greater capacity to maintain cellular function under moderate osmotic stress. However, these mechanisms were not directly measured in the present study and should therefore be interpreted as possible biological explanations rather than confirmed mechanisms. Several M₂ putative mutants from the 250 and 500 Gy treatments, including 250Gy_2P1, 250Gy_3P1, 500Gy_1P1, and 500Gy_3P1, maintained several growth-related traits under 10% PEG level and showed comparatively favorable morpho-physiological profiles. Similar responses have been reported in *Medicago sativa* and alfalfa following gamma irradiation, where selected irradiated materials showed improved performance under water-deficit conditions associated with changes in osmolyte accumulation and antioxidant responses [32, 118, 124–126]. Nevertheless, such physiological mechanisms were not directly evaluated in the present study.

Preliminary genetic-parameter estimates further indicated that the relative contribution of genetic and environmental components varied among traits and developmental stages. During the post-hydroponic greenhouse phase at 60 and 90 DAP, estimated genotypic variance exceeded environmental variance for several traits, accompanied by moderate-to-high broad-sense heritability for selected traits. These results suggest that part of the observed phenotypic variation may have a genetic basis, although the estimates remain preliminary because they were obtained from early-generation M₂ materials. Progeny evaluation in subsequent generations is therefore required to determine whether the observed differences are stable and heritable. Similar studies in *Festuca pallescens* and

Medicago sativa have also reported genetic contributions to morpho-physiological variation under water-stress conditions [127]. The narrow PCV–GCV differences reflect strong genetic control, whereas wider gaps in traits indicate greater environmental influence [39, 63]. As emphasized by Yazdi et al. [128] and Ali [127] that heritability measured at later stages provides more reliable selection indices.

Conclusion

Under 10% PEG level, several irradiated M₂ putative mutants of *Leucaena tarramba* maintained growth and physiological performance better than under 20% PEG level, whereas the higher PEG concentration markedly reduced overall plant performance.

Stress selection indices, recalculated at the irradiation-dose-group level, provided complementary information on the relative response of each irradiation treatment. The 500 and 750 Gy groups had SSI values below 1 under both 10% and 20% PEG, indicating lower relative susceptibility than the wild type and 250 Gy groups. However, all irradiation-dose groups remained classified as sensitive based on STI, indicating that the relatively better response of some irradiation groups did not represent confirmed drought tolerance.

PCA explained 89.5% of the total variation and identified several M₂ putative mutants with maintained performance under PEG-induced osmotic stress, particularly under 10% PEG. Entries including 250Gy_2P1, 250Gy_3P1, 250Gy_4P1, 500Gy_1P1–500Gy_4P1, and 750Gy_1P1–750Gy_4P1 showed maintained multivariate performance and were therefore considered promising M₂ putative mutant candidates for further selection.

Preliminary broad-sense heritability estimates ranged from 0.24 to 0.60 during the hydroponic phase and from 0.11 to 0.80 during post-hydroponic evaluations at 60 and 90 DAP. These estimates indicate that the relative genetic contribution varied among traits and developmental stages.

This hydroponic screening PEG-induced osmotic drought stress was useful for identifying promising M₂ putative mutants at an early stage of mutation breeding. Group-level stress indices supported the comparison among irradiation treatments, while individual morpho-physiological and multivariate analyses helped select promising mutants for further progeny testing, field evaluation, and molecular validation.

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Authors' contributions Purnama Isti Khaerani: designed the experimental framework, collected and curated the data, analysis, and drafted the manuscript. Bernadetta Rina Hastilestari: methodological guidance, analysis and supervision. Yunus Musa: designed the experimental framework, collected and curated the data. Renny Fatmyah Utamy: provided methodological input and supported data validation. Giampiero Cai: conceptualization, supervision, and major revisions to the manuscript. Alan Álvarez-Holguín: assisted with data analysis and critical manuscript revisions. Wijaya Murti Indriatama: designed the experimental framework and experimental resources. Muhammad Fuad Anshori: conceptualization, designed the experimental framework, project coordination, and final approval of the manuscript for submission. Ali Husni, Fahrudin Wakano, Jabal Rahmat Ashar: validation, review, draft editing, and support for APC funds. All authors reviewed, edited, and approved the final version of this manuscript.

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Data availability The data and materials used in this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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References

1. Mareri L, Parrotta L, Cai G. Environmental stress and plants. *Int J Mol Sci.* 2022;23:5416. <https://doi.org/10.3390/ijms23105416>.
2. Nadir M, Anugrah MJ, Khaerani PI. Salt Salinity tolerance on nursery of *Indigofera zollingeriana*. *IOP Conf Ser Earth Environ Sci.* 2018;156:012027. <https://doi.org/10.1088/1755-1315/156/1/012027>.
3. Khaerani PI, Musa Y, Anichini S, Parri S, Faleri C, Cai G. Effect of UV-B stress on olive (*Olea europaea* L.) pollen tubes: a study of callose plug deposition and male germ unit integrity. *Protoplasma.* 2025;262:475–87. <https://doi.org/10.1007/s00709-024-02010-4>.
4. Rahman S, Iqbal M, Husen A. Medicinal plants and abiotic stress: An overview. *medicinal plants: their response to abiotic stress.* 2023;1-34. https://doi.org/10.1007/978-981-19-5611-9_1.
5. Khamis G, Alsherif EA, Korany SM, Aldailami DA, Aloufi AS, Khalaf MH, et al. Drought stress differentially influences growth, physiology, and metabolite accumulation in *Triticum Aestivum* (C3) and *Amaranthus Caudatus* (C4) Plants. *BMC Plant Biol.* 2025;25. <https://doi.org/10.1186/s12870-025-07022-7>.
6. Nardino M, Perin EC, Aranha BC, Carpes ST, Fontoura BH, Sousa DJPde, et al. Understanding drought response mechanisms in wheat and multi-trait selection. *PLoS One.* 2022;17:e0266368. <https://doi.org/10.1371/journal.pone.0266368>.
7. Fidiyawati E, Khaerani PI, Sulistiono W, Aji HB, Sobari I, Sakiroh S, et al. Improving sugarcane productivity and yield through single-bud planting in dryland smallholder systems for sustainable agriculture. *Discov Sustain.* 2026;7:452. <https://doi.org/10.1007/s43621-026-02774-0>.
8. Yuan X, Li S, Chen J, Yu H, Yang T, Wang C, et al. Impacts of global climate change on agricultural production: a comprehensive review. *Agronomy.* 2024;14:1360. <https://doi.org/10.3390/agronomy14071360>.
9. Hasan S, Mujnisa A, Khaerani PI, Sema, Natsir A. Potential of complete feed formulated from local raw materials on beef cattle performance. *Eurasian J Biosci.* 2020;14:1-6. <https://doi.org/10.20961/lar.v19i2.%2051971>.
10. Utamy RF, Ako A, Baba S, Zulkharnaim SG, Alhamd L, Indrawirawan AUN, et al. Effects of plant growth-promoting rhizobacteria and seedling density on morphologic characteristics and nutrient content of corn fodder. *Sarhad J Agric.* 2025;41:209–19. <https://doi.org/10.17582/journal.sja/2025/41.1.209.219>.
11. Khaerani PI, Musa Y, Utamy RF, Ishii Y. Botanical composition and yields of forages in natural pastures using principal component analysis and cluster dendrogram in South Sulawesi, Indonesia. *Online J Biol Sci.* 2024;24:613–23. <https://doi.org/10.3844/ojbsci.2024.613.623>.
12. Syaiful SA, Mantja K, Faisal F, Irmayanti I, Khaerani PI, Anshori MF. Synergistic effects of hormonal priming and *Trichoderma asperellum* on the morphological and physiological traits of true shallot seeds. *Sarhad J Agric.* 2025;41:796–806. <https://doi.org/10.17582/journal.sja/2025/41.2.796.806>.
13. Franco-Navarro JD, Padilla YG, Álvarez S, Calatayud Á, Colmenero-Flores JM, Gómez-Bellot MJ, et al. Advancements in water-saving strategies and crop adaptation to drought: A Comprehensive Review. *Physiol Plant.* 2025;177:e70332. <https://doi.org/10.1111/ppl.70332>.

14. Chand S, Indu, Singhal RK, Govindasamy P. Agronomical and breeding approaches to improve the nutritional status of forage crops for better livestock productivity. *Grass Forage Sci.* 2022;77:11–32. <https://doi.org/10.1111/gfs.12557>.
15. Widiayani N, Anshori MF, Nasaruddin N, Farid M, Ridwan I, Bahrun AH, et al. A new approach for evaluating maize transgressive segregants and their three-way cross potential in the S4 convergent breeding population. *BMC Plant Biol.* 2025;25:122. <https://doi.org/10.1186/s12870-025-06103-x>.
16. Çiğ F, Erman M, El SA, Ceritoglu M, Soysal S, Uçar Ö, et al. Screening of lentil genotypes during germination and early growth stages under PEG-induced drought stress. *Chil J Agric Res.* 2024;84:326–37. <https://doi.org/10.4067/S0718-58392024000300326>.
17. Mansour E, Desoky ESM, Ali MMA, Abdul-Hamid MI, Ullah H, Attia A, et al. Identifying drought-tolerant genotypes of faba bean and their agro-physiological responses to different water regimes in an arid Mediterranean environment. *Agric Water Manag.* 2021;247:106754. <https://doi.org/10.1016/j.agwat.2021.106754>.
18. Sutaryono YA, Dahlanuddin, Mardiansyah, Harjono, Sukarne, Sari NH. Introduction of the use of Tarramba leucaena (*Leucaena leucocephala* cv. tarramba) as a protein-rich feed for the Sambik Elen cattle farmers group in Bayan District, North Lombok Regency (in Indonesian). *Jurnal Pengabdian Magister Pendidikan IPA.* 2023;6(2):296–301. <https://doi.org/10.29303/jpmipi.v6i2.4358>.
19. Hasan S, Budiman, Natsir A, Sema, Khaerani PI. The investigation of biological nitrogen fixation in critical dryland pasture. *Online J Biol Sci.* 2019;19:152–8. <https://doi.org/10.3844/ojbsci.2019.152.158>.
20. Dagar JC, Gupta S. Agroforestry: potentials for rehabilitation of degraded lands, constraints and the way forward. In: *Agroforestry research developments*. New York: Nova Publishers; 2016. p. 47–98.
21. Fanindi A, Sutedi E, Herdiawan I. Productivity of two species of tree legume as sources forage on post-tin mining in Bangka Island. In: *IOP Conference Series: Earth and Environmental Science*. IOP Publishing; 2024. p. 12080. <https://doi.org/10.1088/1755-1315/1341/1/012080>.
22. Han Y, Abair A, van der Zanden J, Nageswara-Rao M, Vasani SP, Bhoite R, et al. Transcriptome-wide genetic variations in the legume genus leucaena for fingerprinting and breeding. *Agronomy.* 2024;14:1519. <https://doi.org/10.3390/agronomy14071519>.
23. Dalzell SA. Leucaena cultivars-current releases and future opportunities. *Trop Grassl-Forrajes Trop.* 2019;7:56–64. [https://doi.org/10.17138/tgft\(7\)56-64](https://doi.org/10.17138/tgft(7)56-64).
24. Khaerani PI, Musa Y, Utamy RF, Ishii Y, Idrus M, Indriatama WM, et al. Assessing Genetic Variability in M2 Mutants of Leucaena tarramba Through RAPD Molecular Markers. *Asian J Plant Sci.* 2024;23:272–80. <https://doi.org/10.3923/ajps.2024.272.280>.
25. Brewbaker JL. Breeding leucaena: Tropical multipurpose leguminous tree. *Plant Breeding Rev.* 2016;40(40):43–121. <https://doi.org/10.1002/9781119279723.ch2>.
26. Yali W, Mitiku T. Mutation breeding and its importance in modern plant breeding. *J Plant Sci.* 2022;10:64–70. <https://doi.org/10.11648/j.jps.20221002.13>.
27. Singer SD, Laurie JD, Bilichak A, Kumar S, Singh J. Genetic variation and unintended risk in the context of old and new breeding techniques. *Crit Rev Plant Sci.* 2021;40:68–108. <https://doi.org/10.1080/07352689.2021.1883826>.
28. Musa Y, Sjahril R, Nadir M, Khaerani PI, Sakinah AI. Early growth of post-gamma irradiated Indigofera zollingeriana. In: *IOP Conference Series: Earth and Environmental Science*. 2021. p. 807(3). <https://doi.org/10.1088/1755-1315/807/3/032026>.
29. Sustek-Sánchez F, Rognli OA, Rostoks N, Sömera M, Jaškūnė K, Kovi MR, et al. Improving abiotic stress tolerance of forage grasses—prospects of using genome editing. *Front Plant Sci.* 2023;14:1127532. <https://doi.org/10.3389/fpls.2023.1127532>.
30. Rasheed A, Gill RA, Hassan MU, Mahmood A, Qari S, Zaman QU, et al. A critical review: recent advancements in the use of CRISPR/Cas9 technology to enhance crops and alleviate global food crises. *Curr Issues Mol Biol.* 2021;43:1950–76. <https://doi.org/10.3390/cimb43030135>.
31. Guo Y, Zhao G, Gao X, Zhang L, Zhang Y, Cai X, et al. CRISPR/Cas9 gene editing technology: a precise and efficient tool for crop quality improvement. *Planta.* 2023;258:36. <https://doi.org/10.1007/s00425-023-04187-z>.
32. Kulkarni KP, Tayade R, Asekova S, Song JT, Shannon JG, Lee J-D. Harnessing the potential of forage legumes, alfalfa, soybean, and cowpea for sustainable agriculture and global food security. *Front Plant Sci.* 2018;9:1314. <https://doi.org/10.3389/fpls.2018.01314>.
33. Haq SIU, Zheng D, Feng N, Jiang X, Qiao F, He J-S, et al. Progresses of CRISPR/Cas9 genome editing in forage crops. *J Plant Physiol.* 2022;279:153860. <https://doi.org/10.1016/j.jplph.2022.153860>.
34. Nayagam JR. CRISPR and Beyond: The Future of Induced Mutagenesis in Plants. *Revista Latinoamericana de la Papa.* 2025;29:181–95.
35. Duensing N, Sprink T, Parrott WA, Fedorova M, Lema MA, Wolt JD, et al. Novel features and considerations for ERA and regulation of crops produced by genome editing. *Front Bioeng Biotechnol.* 2018;6:79. <https://doi.org/10.3389/fbioe.2018.00079>.
36. USDA. Induced Mutagenesis. *Induced Mutagen.* 1983;105:1–65. <https://doi.org/10.1007/978-1-4684-4382-0>.

37. Bado S, Forster BP, Nielen S, Ali AM, Lagoda PJJ, Till BJ, et al. Plant mutation breeding: current progress and future assessment. *Plant Breeding Rev.* 2015;39:23–88. <https://doi.org/10.1002/9781119107743.ch2>.
38. Anshori MF, Purwoko BS, Carsono N, Dewi IS, Suwarno WB, Ardie SW, et al. Optimizing hydroponic salinity screening: a novel approach using image-based phenotyping for double haploid rice selection. *Front Sustain Food Syst.* 2025;9:1659331. <https://doi.org/10.3389/fsufs.2025.1659331>.
39. Indriatama WM, Trikoesoemaningtyas SIA, Human S. Estimation of genetic variability and heritability of wheat agronomic traits resulted from some gamma rays irradiation techniques. *Jurnal Ilmiah Aplikasi Isotop dan Radiasi.* 2016;12:79–88. <https://doi.org/10.17146/jair.2016.12.2.2861>.
40. Ahloowalia BS, Maluszynski M. Induced mutations – A new paradigm in plant breeding. *Euphytica.* 2001;118:167–73. <https://doi.org/10.1023/A:1004162323428>.
41. Khaerani PI, Musa Y, Utamy RF, Indriatama WM, Kantong SA, Ishii Y. Germination growth of mutant (M1) *Leucaena leucocephala* cv. Tarramba induced by gamma ray irradiation. In: AIP Conference Proceedings. AIP Publishing; 2023. <https://doi.org/10.1063/5.0144831>.
42. Shelton M, Dalzell S, Tomkins N, Buck SR. *Leucaena*-The productive and sustainable forage legume. 2021.
43. Sarsu F, Penna S, Nikalje GC. Strategies for screening induced mutants for stress tolerance. In: *Mutation breeding for sustainable food production and climate resilience*. Springer; 2023. p. 151–76. https://doi.org/10.1007/978-981-16-9720-3_6.
44. Kylyshbayeva G, Bishimbayeva N, Jatayev S, Eliby S, Shavrukov Y. Polyethylene Glycol (PEG) application triggers plant dehydration but does not accurately simulate drought. *Plants.* 2024;14:92. <https://doi.org/10.3390/plants14010092>.
45. Shehzad M, Gul RS, Rauf S, Clarindo WR, Al-Khayri JM, Hussain MM, et al. Development of a robust hydroponic method for screening of sunflower (*Helianthus annuus* L.) accessions for tolerance to heat and osmotic stress. *Sci Rep.* 2021;11:1677. <https://doi.org/10.1038/s41598-021-81072-3>.
46. Yige C, Fangqing C, Lei L, Shunbo Z. Physiological responses of *Leucaena leucocephala* seedlings to drought stress. *Procedia Eng.* 2012;28:110–6. <https://doi.org/10.1016/j.proeng.2012.01.691>.
47. Honda MDH, Borthakur D. Mimosine is a stress-response molecule that serves as both an antioxidant and osmolyte in giant leucaena (*Leucaena leucocephala* subsp. *glabrata*) during environmental stress conditions. *Plant Stress.* 2021;2:100015. <https://doi.org/10.1016/j.stress.2021.100015>.
48. Gomez KA, Gomez AA. *Statistical procedures for agricultural research*. John Wiley & sons; 1984.
49. Montgomery DC. *Design and analysis of experiments*. John Wiley & sons; 2017.
50. IRRI. *Standard evaluation system for rice (SES)*. 4th Edition. Los Baños, Philippines: International Rice Research Institute; 1996.
51. Nayyar H, Walia DP. Water stress induced proline accumulation in contrasting wheat genotypes as affected by calcium and abscisic acid. *Biol Plant.* 2003;46:275–9. <https://doi.org/10.1023/A:1022867030790>.
52. Michel BE, Kaufmann MR. The osmotic potential of polyethylene glycol 6000. *Plant Physiol.* 1973;51:914–6. <https://doi.org/10.1104/pp.51.5.914>.
53. Verslues PE, Ober ES, Sharp RE. Root growth and oxygen relations at low water potentials. Impact of oxygen availability in polyethylene glycol solutions. *Plant Physiol.* 1998;116:1403–12. <https://doi.org/10.1104/pp.116.4.1403>.
54. Porra RJ, Thompson WA, Kriedemann PE. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochimica et Biophysica Acta (BBA)-Bioenergetics.* 1989;975:384–94. [https://doi.org/10.1016/S0005-2728\(89\)80347-0](https://doi.org/10.1016/S0005-2728(89)80347-0).
55. Weyers JDB, Johansen LG. Accurate estimation of stomatal aperture from silicone rubber impressions. *New Phytol.* 1985;101:109–15. <https://doi.org/10.1111/j.1469-8137.1985.tb02820.x>.
56. Bates LS, Waldren RP, Teare ID. Rapid determination of free proline for water-stress studies. *Plant Soil.* 1973;39:205–7. <https://doi.org/10.1007/BF00018060>.
57. Azman Halimi R, Raymond CA, Barkla BJ, Mayes S, King GJ. Development of selection indices for improvement of seed yield and lipid composition in Bambara groundnut (*Vigna subterranea* (L.) Verdc.). *Foods.* 2021;11:86. <https://doi.org/10.3390/foods11010086>.
58. Song R, Shi P, Xiang L, He Y, Dong Y, Miao Y, et al. Evaluation of barley genotypes for drought adaptability: based on stress indices and comprehensive evaluation as criteria. *Front Plant Sci.* 2024;15:1436872. <https://doi.org/10.3389/fpls.2024.1436872>.
59. Fernandez GCJ. Effective selection criteria for assessing plant stress tolerance. In: *Proceedings of International Symposium on Adaptation of Food Crops to Temperature and Water Stress*, Shanhua, Taiwan. AVRDS Publ. CABI Digital Library; 1992. p. 257–70. <https://doi.org/10.22001/wvc.72511>.
60. Singh RK, Chaudhary BD. *Biometrical methods in quantitative genetic analysis*. New Delhi, India: Kalyani; 1977.
61. Stansfield WD. *Theory and problems of genetics*. second Eds. New Delhi: McGraw-Hil; 1969.
62. Mahmoud AMA, Hassan AA, Abdel-Ati KEA, Osman NH, Mohamed HAA. Exploring Ty resistance genes and genetic diversity in improved tomato lines selected from commercial hybrids. *BMC Plant Biol.* 2025;25:1213. <https://doi.org/10.1186/s12870-025-07344-6>.

63. Chacko A, Jayalekshmy VG, Shahiba AM. Studies on PCV, GCV, heritability, and genetic advance in rice genotypes for yield and yield components. *Int J Plant Soil Sci.* 2023;35:324–30. <https://doi.org/10.9734/ijpss/2023/v35i163266>.
64. Zaki HEM, Radwan KSA. Estimates of genotypic and phenotypic variance, heritability, and genetic advance of horticultural traits in developed crosses of cowpea (*Vigna unguiculata* [L.] Walp). *Front Plant Sci.* 2022;13:987985. <https://doi.org/10.3389/fpls.2022.987985>.
65. Adedugba AA, Adeyemo OA, Adetumbi AJ, Amusa OD, Ogunkanmi LA. Evaluation of genetic variability for major agro-morphological and stalk sugar traits in African sorghum genotypes. *Heliyon.* 2023;9. <https://doi.org/10.1016/j.heliyon.2023.e14622>.
66. Ghasemi-Soloklui AA, Kordrostami M, Karimi R. Determination of optimum dose based on biological responses of lethal dose (LD25, 50, 75) and growth reduction (GR25, 50, 75) in ‘Yaghouti’ grape due to gamma radiation. *Sci Rep.* 2023;13:2713. <https://doi.org/10.1038/s41598-023-29896-z>.
67. Kumawat S, Rana N, Bansal R, Vishwakarma G, Mehrete ST, Das BK, et al. Expanding avenue of fast neutron mediated mutagenesis for crop improvement. *Plants.* 2019;8:164. <https://doi.org/10.3390/plants8060164>.
68. Wang J, Zhang Y, Zhou L, Yang F, Li J, Du Y, et al. Ionizing radiation: Effective physical agents for economic crop seed priming and the underlying physiological mechanisms. *Int J Mol Sci.* 2022;23:15212. <https://doi.org/10.3390/ijms232315212>.
69. Bera K, Dutta P, Sadhukhan S. Seed priming with non-ionizing physical agents: plant responses and underlying physiological mechanisms. *Plant Cell Rep.* 2022;41:53–73. <https://doi.org/10.1007/s00299-021-02798-y>.
70. Geras'kin S. Plant adaptation to ionizing radiation: mechanisms and patterns. *Sci Total Environ.* 2024;916:170201. <https://doi.org/10.1016/j.scitotenv.2024.170201>.
71. Ulukapi K, Ozmen SF. Study of the effect of irradiation (60Co) on M1 plants of common bean (*Phaseolus vulgaris* L.) cultivars and determined of proper doses for mutation breeding. *J Radiat Res Appl Sci.* 2018;11:157–61. <https://doi.org/10.1016/j.jrras.2017.12.004>.
72. Kang R, Seo E, Kim G, Park A, Kim WJ, Kang SY, et al. Radio sensitivity of cowpea plants after gamma-ray and proton-beam irradiation. *Plant Breed Biotechnol.* 2020;8:281–92. <https://doi.org/10.9787/PBB.2020.8.3.281>.
73. Khaerani PI, Musa Y, Sjahril R, Nadir M. Radiosensitivity of post-gamma irradiated *Indigofera zollingeriana*. *IOP Conf Ser Earth Environ Sci.* 2021;807:032025. <https://doi.org/10.1088/1755-1315/807/3/032025>.
74. Guha Mallick R, Pramanik S, Pandit MK, Gupta AK, Roy S, Jambhulkar S, et al. Radiosensitivity of seedling traits to varying gamma doses, optimum dose determination and variation in determined doses due to different time of sowings after irradiation and methods of irradiation in faba bean genotypes. *Int J Radiat Biol.* 2023;99:534–50. <https://doi.org/10.1080/09553002.2022.2107723>.
75. Khaerani PI, Musa Y, Utamy RF, Indriatama WM, Umpuch K, Holguin AA. Radiosensitivity of Super Napier Grass (*Pennisetum purpureum* × *Pennisetum glaucum*) induced by low- and high-activity rates of gamma irradiation. *Plant Breed Biotechnol.* 2024;12:30–42. <https://doi.org/10.9787/PBB.2024.12.30>.
76. Salgotra RK, Chauhan BS. Genetic diversity, conservation, and utilization of plant genetic resources. *Genes (Basel).* 2023;14:174. <https://doi.org/10.3390/genes14010174>.
77. Bharath RA, Prathmesh SP, Sarsu F, Suprasanna P. Induced mutagenesis using gamma rays: biological features and applications in crop improvement. *OBM Genet.* 2024;8(2):233. <https://doi.org/10.21926/obm.genet.2402233>.
78. Kitamura S, Satoh K, Oono Y. Detection and characterization of genome-wide mutations in M1 vegetative cells of gamma-irradiated *Arabidopsis*. *PLoS Genet.* 2022;18:e1009979. <https://doi.org/10.1371/journal.pgen.1009979>.
79. Nkurunziza R, Jankowicz-Cieslak J, Bocianowski J, Bhatnagar-Mathur P, Werbrouck SPO, Ingelbrecht ILW. Enhancing genetic diversity in *Coffea arabica* L. through induced mutagenesis. *Curr Plant Biol.* 2025;43:100514. <https://doi.org/10.1016/j.cpb.2025.100514>.
80. Habib SH, Akanda AL, Roy P, Kulsum U, Rohman M, Rahman MM. Mutant divergence in sunflower induced through gamma radiation. *J Crop Sci Biotechnol.* 2023;26:317–25. <https://doi.org/10.1007/s12892-022-00186-w>.
81. Kiani D, Borzouei A, Ramezanpour S, Soltanloo H, Saadati S. Application of gamma irradiation on morphological, biochemical, and molecular aspects of wheat (*Triticum aestivum* L.) under different seed moisture contents. *Sci Rep.* 2022;12:11082. <https://doi.org/10.1038/s41598-022-14949-6>.
82. Tan Y, Duan Y, Chi Q, Wang R, Yin Y, Cui D, et al. The role of reactive oxygen species in plant response to radiation. *Int J Mol Sci.* 2023;24:3346. <https://doi.org/10.3390/ijms24043346>.
83. Roy SD, Mukhopadhyay AK. Harnessing hormesis: exploring insecticide dose-response dynamics for sustainable pest management. *Uttar Pradesh J Zool.* 2025;46(4):12–28. <https://doi.org/10.56557/upjz/2025/v46i44800>.
84. Tsago Y, Andargie M, Takele A. In vitro selection of sorghum (*Sorghum bicolor* (L.) Moench) for polyethylene glycol (PEG) induced drought stress. *Plant Sci Today.* 2014;1:62–8. <https://doi.org/10.14719/pst.2014.1.2.14>.
85. Gopal J, Iwama K. In vitro screening of potato against water-stress mediated through sorbitol and polyethylene glycol. *Plant Cell Rep.* 2007;26:693–700. <https://doi.org/10.1007/s00299-006-0275-6>.
86. Nadir M, Ansary I, Khaerani PI, Syamsuddin. Effect of various polyethylene glycol concentrations on the growth of seedlings of *Indigofera zollingeriana*. *IOP Conf Ser Earth Environ Sci.* 2019;343:012040. <https://doi.org/10.1088/1755-1315/343/1/012040>.

87. Koskosidis A, Ebrahim K, Mavromatis A, Pavli O, Vlachostergios DN. Effect of PEG-induced drought stress on germination of ten chickpea (*Cicer arietinum* L.) genotypes. Not Bot Horti Agrobot Cluj Napoca. 2020;48:294–304. <https://doi.org/10.15835/nbha48111799>.
88. Vus N, Vasylenko A, Lutenko V, Kobyzeva L, Bezuhla O, Shevchenko L, et al. Concentration effect of polyethylene glycol in evaluation of grain legumes for drought tolerance. Žemės ūkio mokslai. 2020;27. <https://doi.org/10.6001/zemesukiomokslai.v27i2.4337>.
89. Mehmandar MN, Rasouli F, Giglou MT, Zahedi SM, Hassanpouraghdam MB, Aazami MA, et al. Polyethylene glycol and sorbitol-mediated *in vitro* screening for drought stress as an efficient and rapid tool to reach the tolerant *Cucumis melo* L. genotypes. Plants (Basel). 2023;12:870. <https://doi.org/10.3390/plants12040870>.
90. Islam NU, Rather MA, Sofi PA, Wani MA, Ali G, Wani FJ, et al. Elucidating drought tolerance potential of maize (*Zea mays* L.) inbred lines through polyethylene glycol induced drought stress. J Sci Res Rep. 2024;30:633–42. <https://doi.org/10.9734/jsrr/2024/v30i51982>.
91. Gillani SA, Abbasi R, Martinez P, Ahmad R. Comparison of energy-use efficiency for lettuce plantation under nutrient film technique and deep-water culture hydroponic systems. Procedia Comput Sci. 2023;217:11–9. <https://doi.org/10.1016/j.procs.2022.12.197>.
92. Nursyahid A, Setyawan TA, Sa'diyah K, Wardihani ED, Helmy H, Hasan A. Analysis of deep water culture (DWC) hydroponic nutrient solution level control systems. IOP Conf Ser Mater Sci Eng. 2021;1108:012032. <https://doi.org/10.1088/1757-899X/1108/1/012032>.
93. Korbee N, Bautista B, García-Sánchez M, Cobos P, Ferres-García JL, Figueroa FL, et al. Evaluating hydroponics and aquaponics: Comparative insights into sustainability and strawberry quality. Agric Water Manag. 2025;312:109412. <https://doi.org/10.1016/j.agwat.2025.109412>.
94. Pope EM, Opile W, Ngode L, Chepkoech E. Evaluation of upland rice response to water stress using Polyethylene Glycol (PEG-6000) at germination and early seedling stage. Asian J Res Crop Sci. 2024;9:37–49. <https://doi.org/10.9734/ajrcs/2024/v9i1244>.
95. Sarma H, Sen B, Basumatary T, Daimary D, Boro BB, Basumatary D. Impact of polyethylene glycol-induced osmotic stress on maize germination, growth, and physiological response. Towards Excell. 2023;15. <https://doi.org/10.37867/TE150318>.
96. Habtegebriel MH, Feyissa T, Setotaw TA, Melkie Y. Screening of barley (*Hordeum vulgare* L.) for early seedling growth traits for drought tolerance under polyethylene glycol 6000. Agrosyst Geosci Environ. 2025;8:e70203. <https://doi.org/10.1002/agg2.70203>.
97. Mahpara S, Zainab A, Ullah R, Kausar S, Bilal M, Latif MI, et al. The impact of PEG-induced drought stress on seed germination and seedling growth of different bread wheat (*Triticum aestivum* L.) genotypes. PLoS One. 2022;17:e0262937. <https://doi.org/10.1371/journal.pone.0262937>.
98. Das A, Munshi AD, Raju D, Kumar S, Singh D, Talukdar A, et al. Key physiological traits for drought tolerance identified through phenotyping a large set of slicing cucumber (*Cucumis sativus* L.) genotypes under field and water-stress conditions. Genet Resour Crop Evol. 2024;71:1855–68. <https://doi.org/10.1007/s10722-023-01737-y>.
99. Singh P, Choudhary KK, Chaudhary N, Gupta S, Sahu M, Tejaswini B, et al. Salt stress resilience in plants mediated through osmolyte accumulation and its crosstalk mechanism with phytohormones. Front Plant Sci. 2022;13:1006617. <https://doi.org/10.3389/fpls.2022.1006617>.
100. Kumar S, Seem K, Mohapatra T. Biochemical and epigenetic modulations under drought: remembering the stress tolerance mechanism in rice. Life. 2023;13:1156. <https://doi.org/10.3390/life13051156>.
101. Rajeswar S, Narasimhan S. Peg-induced drought stress in plants: A review. Res J Pharm Technol. 2021;14:6173–8. <https://doi.org/10.52711/0974-360X.2021.01069>.
102. Nguyễn CT, Gammatantrawet N, Susawaengsup C, Tandee K, Ramli ANM, Tongkoom K, et al. Drought-ready plant resilience: Harnessing nano-biotechnology techniques for swift screening and selection of organic crop varieties. S Afr J Bot. 2024;169:553–66. <https://doi.org/10.1016/j.sajb.2024.05.011>.
103. Saleem MH, Noreen S, Ishaq I, Saleem A, Khan KA, Ercisli S, et al. Omics technologies: unraveling abiotic stress tolerance mechanisms for sustainable crop improvement. J Plant Growth Regul. 2025;44:4165–87. <https://doi.org/10.1007/s00344-025-11674-y>.
104. Anshori MF, Purwoko BS, Dewi IS, Suwarno WB, Ardie SW, Carsono N, et al. Geometric image-based phenotyping and physiological analysis for validation of rice salinity tolerance screening under artificial pot conditions. BMC Plant Biol. 2026;26:947. <https://doi.org/10.1186/s12870-026-08810-5>.
105. Seem K, Kaur S, Kumar A, Krishnan GS, Viswanathan C, Mohapatra T, et al. Integrated histone modification and transcriptome analyses reveal the regulatory mechanism for sustained agronomic performance of rice under terminal drought stress. Plant Stress. 2026;20:101313. <https://doi.org/10.1016/j.stress.2026.101313>.
106. Ghatak A, Chaturvedi P, Weckwerth W. Cereal Crop Proteomics: Systemic analysis of crop drought stress responses towards marker-assisted selection breeding. Front Plant Sci. 2017;8:757. <https://doi.org/10.3389/fpls.2017.00757>.
107. Şen A, Gümüş T, Temel A, Öztürk İ, Çelik Ö. Biochemical and proteomic analyses in drought-tolerant wheat mutants obtained by gamma irradiation. Plants. 2024;13:2702. <https://doi.org/10.3390/plants13192702>.

108. Tian F, Gong J, Zhang J, Zhang M, Wang G, Li A-X, et al. Enhanced stability of thylakoid membrane proteins and antioxidant competence contribute to drought stress resistance in the *tasg1* wheat stay-green mutant. *J Exp Bot*. 2013;64:1509–20. <https://doi.org/10.1093/jxb/ert004>.
109. Fisher LHC, Han J, Corke F, Akinyemi A, Didion T, Nielsen K, et al. Linking dynamic phenotyping with metabolite analysis to study natural variation in drought responses of *Brachypodium distachyon*. *Front Plant Sci*. 2016;7:1751. <https://doi.org/10.3389/fpls.2016.01751>.
110. Raza A, Charagh S, Abbas S, Hassan MU, Saeed F, Haider S, et al. Assessment of proline function in higher plants under extreme temperatures. *Plant Biol (Stuttg)*. 2023;25:379–95. <https://doi.org/10.1111/plb.13510>.
111. Takahashi F, Kuromori T, Urano K, Yamaguchi-Shinozaki K, Shinozaki K. Drought stress responses and resistance in plants: From cellular responses to long-distance intercellular communication. *Front Plant Sci*. 2020;11:703149. <https://doi.org/10.3389/fpls.2020.556972>.
112. González JM, Loarce Y, Sánchez-Gordo N, la Rosa LD, Ramírez-Parra E. Combining in vitro and Field studies to predict drought tolerance in *Vicia sativa* L. *Genotypes Plants*. 2025;14:3376. <https://doi.org/10.3390/plants14213376>.
113. Hosseinifard M, Stefaniak S, Javid MG, Soltani E, Wojtyła Ł, Garnczarska M. Contribution of exogenous proline to abiotic stresses tolerance in plants: A Review. *Int J Mol Sci*. 2022;23:5186. <https://doi.org/10.3390/ijms23095186>.
114. Per TS, Khan NA, Reddy PS, Masood A, Hasanuzzaman M, Khan MIR, et al. Approaches in modulating proline metabolism in plants for salt and drought stress tolerance: Phytohormones, mineral nutrients and transgenics. *Plant Physiol Biochem*. 2017;115:126–40. <https://doi.org/10.1016/j.plaphy.2017.03.018>.
115. Matamoros MA, Becana M. Molecular responses of legumes to abiotic stress: Post-translational modifications of proteins and redox signaling. *J Exp Bot*. 2021;72:5876–92. <https://doi.org/10.1093/jxb/erab008>.
116. Sharma A, Sharma P, Kumar R, Sharma V, Bhardwaj R, Sharma I. Role of reactive oxygen species in the regulation of abiotic stress tolerance in legumes. In: *Abiotic stress and legumes: tolerance and management*. Elsevier; 2021. p. 217–243. <https://doi.org/10.1016/B978-0-12-815355-0.00012-6>.
117. Hong MJ, Kim DY, Jo YD, Choi HI, Ahn JW, Kwon SJ, et al. Biological effect of gamma rays according to exposure time on germination and plant growth in wheat. *Appl Sci*. 2022;12:3208. <https://doi.org/10.3390/app12063208>.
118. Rani A, Devi P, Jha UC, Sharma KD, Siddique KHM, Nayyar H. Developing climate-resilient chickpea involving physiological and molecular approaches with a focus on temperature and drought stresses. *Front Plant Sci*. 2020;10:1759. <https://doi.org/10.3389/fpls.2019.01759>.
119. Haghpanah M, Hashemipetroudi S, Arzani A, Araniti F. Drought tolerance in plants: physiological and molecular responses. *Plants (Basel)*. 2024;13:2962. <https://doi.org/10.3390/plants13212962>.
120. Renzetti M, Funck D, Trovato M. Proline and ROS: a unified mechanism in plant development and stress response? *Plants (Basel)*. 2025;14:2. <https://doi.org/10.3390/plants14010002>.
121. Peršić V, Ament A, Antunović Dunić J, Drezner G, Cesar V. PEG-induced physiological drought for screening winter wheat genotypes sensitivity-integrated biochemical and chlorophyll a fluorescence analysis. *Front Plant Sci*. 2022;13:987702. <https://doi.org/10.3389/fpls.2022.987702>.
122. Patade VY, Suprasanna P, Bapat VA, Kulkarni UG. Selection for abiotic (salinity and drought) stress tolerance and molecular characterization of tolerant lines in sugarcane. *BARC Newsletter*; 2006.
123. Álvarez-Holguín A, Morales-Nieto CR, Avendaño-Arrazate CH, Corrales-Lerma R, Villarreal-Guerrero F. Phenotypic and genetic variability induced in Lehmann's love grass (*Eragrostis lehmanniana*) through gamma irradiation. *Trop Grassl-Forrajes Trop*. 2022;10:63–8. [https://doi.org/10.17138/TGFT\(10\)63-68](https://doi.org/10.17138/TGFT(10)63-68).
124. Kakaei M, Mazahery-Laghab H. Study of genetic diversity, heritability and the correlation of different traits in alfalfa (*Medicago sativa* L.) related to alfalfa weevil (*Hypera postica* Gyll.) damage in alfalfa germplasm. *Plant Genet Resour*. 2025;2:63–76.
125. Sayed MRI, Alshallash KS, Safhi FA, Alatawi A, Alshamrani SM, Dessoky ES, et al. Genetic diversity, analysis of some agro-morphological and quality traits and utilization of plant resources of alfalfa. *Genes (Basel)*. 2022;13:1521. <https://doi.org/10.3390/genes13091521>.
126. Vozhehova R, Tyshchenko A, Tyshchenko O, Dymov O, Piliarska O, Lykhovyd P. Evaluation of breeding indices for drought tolerance in alfalfa (*Medicago*) genotypes. *Sci Papers Series A Agronomy*. 2021;64.
127. Ali A, Javed M, Ali M, Rahman SU, Kashif M, Khan SU. Genetic variability, heritability, and genetic gain in F3 populations of bread wheat (*Triticum aestivum* L.) for production traits. *SABRAO J Breed Genet*. 2024;56(2):505–18. <https://doi.org/10.54910/sabrao2024.56.2.5>.
128. Yazdi MH, Visscher PM, Ducrocq V, Thompson R. Heritability, reliability of genetic evaluations and response to selection in proportional hazard models. *J Dairy Sci*. 2002;85(6):1563–77. [https://doi.org/10.3168/jds.S0022-0302\(02\)74226-4](https://doi.org/10.3168/jds.S0022-0302(02)74226-4).

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