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OPTIMIZATION OF 24-EPIBRASSINOLIDE DOSE FOR GROWTH ENHANCEMENT AND OXIDATIVE STRESS AMELIORATION IN RAGI (*Eleusine coracana* L.) SEEDLINGS UNDER INDIVIDUAL AND COMBINED STRESSES

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ABSTRACT

The application of plant hormones is an environmentally sustainable approach to mitigating the adverse effects of abiotic stresses on seedling establishment. Finger millet (*Eleusine coracana* L.), also known as Ragi, is highly sensitive to abiotic stress during early seedling growth. Damage caused by abiotic stresses can be mitigated by applying brassinolides. In this study, we evaluated the effects of 24-epibrassinolides (24-epiBL) by providing them in irrigation water solutions twice at two-day intervals, with a 3rd application by spraying on the 8th day, on Ragi seedlings at 0.0 μ M, 1.0 μ M, and 2.0 μ M under drought (DS), temperature (TS), and combined stress (CS). Germination, seedling growth, and pigment content decreased, while lipid peroxidation increased under DS, TS, and CS; however, CS caused the most severe damage. The 24-epiBL treatments reduced the adverse effects of abiotic stress on physio-biochemical processes at both concentrations. The treatments at 1.0 μ M and 2.0 μ M significantly improved germination (5.47–8.04%; 10.38–11.78%), seedling growth (10.53–15.60%; 18.95–34.04%), and seedling vigor index (17.22–21.86%; 38.94–43.69%), and promoted chlorophyll a (11.11–20.79%; 30.56–41.58%), chlorophyll b (26.67–39.47%; 51.11–55.26%), total carotenoids (2.86–23.26%; 8.57–32.56%), and peroxidase activity (11.98–17.30%; 18.36–21.74%), while reducing lipid peroxidation (11.18–15.11%; 21.05–23.24%) in 10-day-old seedlings. Most notably, the application of 24-epiBL at 2.0 μ M showed a better response than at 1.0 μ M under all stresses. The study results suggest that exogenous application of 24-epiBL, primarily at 2.0 μ M, can improve seedling growth and survival under individual and combined stress, with DS showing among the greatest 24-epiBL-mediated improvements across genotypes in several growth- and antioxidant-related traits.

Key words: Finger millet, 24-epibrassinolide, drought stress, temperature stress, pigments

Introduction

Finger millet (*Eleusine coracana* L., Family Poaceae) is an important food crop with considerable cultural, historical, and nutritional significance, particularly in the dryland farming systems (Sundaresan *et al.*, 2026). It is a self-pollinating, allotetraploid annual herbaceous C_4 crop ($2n = 4x = 36$) predominantly cultivated in the arid and semi-arid regions of India and Central Africa

(Goron & Raizada, 2015). Finger millet ranks third in importance among all millets, usually behind pearl millet and foxtail millet (Gebreyohannes *et al.*, 2021). Globally, it is cultivated over an estimated 4–4.5 million hectares, producing approximately 5 million tons of grain annually, with India contributing nearly 2.2 million tons (ICRISAT, 2026). Several studies have evaluated finger millet germplasm and cultivars, demonstrating substantial

genotypic variation in stress tolerance, providing opportunities for the development and selection of stress-resilient genotypes (Talabi *et al.*, 2026; Mude *et al.*, 2022).

Despite its inherent adaptation to harsh environments, finger millet is vulnerable to stresses, especially during critical growth stages. Abiotic stress at germination and early seedling stages can limit plant growth, tiller formation, and leaf area by increasing ROS and lipid peroxidation, leading to smaller ear heads (Alves *et al.*, 2026; Liu *et al.*, 2026). Similarly, deviations from the optimum temperature range can adversely affect the initiation and duration of phenological stages and crop yield by altering physiological and biochemical processes (Meena *et al.*, 2025). Drought-induced reductions in seedling length, seedling dry matter, germination percentage, seedling vigor index, and increased oxidative damage have been reported in black and brown finger millet landraces (Khatoom & Singh, 2017). Moreover, drought stress during seedling growth significantly impacts seedling vigor and physio-biochemical attributes (Nivethitha *et al.*, 2024). High temperatures also impair seedling growth, starch biosynthesis, and limit biomass accumulation, thereby reducing grain and fodder yields (Chakraborty *et al.*, 2026). Both drought and temperature stress impact more adversely in plant growth and hormonal biosynthesis, thereby reducing plant productivity (Kumari *et al.*, 2026).

In this context, brassinolides (BLs), a group of ubiquitous polyhydroxy steroidal phytohormones, have emerged as important regulators of plant growth, development, and stress responses. Brassinolides promote cell elongation, cell division, and differentiation and contribute to plant adaptation to diverse abiotic stresses (Kakei *et al.*, 2026; Unterholzner *et al.*, 2026). Exogenous BLs application can enhance stress tolerance by reducing oxidative stress and stimulating antioxidant defense systems (Avalbaev *et al.*, 2024). In proso millet and mung bean seedlings, BLs application increases chlorophyll, MSI, protein content, photosynthesis, and antioxidant enzymes, boosting germination and vigor under stress (Qiao *et al.*, 2026; Madaan *et al.*, 2026). Similar effects are seen in wheat and rice (Suresh *et al.*, 2025; Thomas *et al.*, 2025). BLs are eco-friendly and safe, making them promising for improving crop resilience without harming ecosystems.

Numerous studies have assessed finger millet germplasm and cultivars, revealing significant variation in stress tolerance among genotypes. It is hypothesized that the effects of combined drought and high-temperature stresses on morpho-physiological parameters differ significantly from those when these stresses are imposed

separately, and that 24-epibrassinolide (24-epiBL) modulates these responses in a stress-specific, dose-dependent manner, with an optimal concentration enhancing stress tolerance. Therefore, the present study was undertaken to address two key research questions:

- (i) How does exogenous 24-epiBL supplementation affect physiological and biochemical mechanisms during finger millet germination under drought, temperature, and combined stress?
- (ii) What is the optimal 24-epiBL dose for stress mitigation in finger millet seedlings? Exploring these questions could reveal 24-epiBL's role in enhancing early-stage stress resilience and help determine an effective concentration for use under combined stress conditions.

Materials and Methods

Plant Materials

For the experiment, ten finger millet (*Eleusine coracana* L.) genotypes (CMFV 1, GPU-67, GPU 48, Indaf-9, KMR-630, KMR 316, KMR 340, ML-365, MR-1, VL-352) were collected from ICAR-IIMR, Hyderabad, Telangana, India. Seeds were sorted by handpicking healthy ones, which were then used in the following experiments. Selected seeds were washed with distilled water to remove dust and other particles and then sterilized with 0.1% sodium hypochlorite (NaOCl).

Experiment Site and Conditions

The study was conducted in the Department of Plant Physiology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh (25.2964°N, 83.0011°E). The germination assay was performed in Petri dishes (9-cm-diameter glass Petri dishes) in two Plant Growth Chambers under controlled conditions (32/28°C day/night temperatures; 80–90% humidity) and high-temperature conditions (42/32°C day/night temperatures; 80–90% humidity). The 16 seeds of each genotype were randomly selected and placed on filter paper in each Petri plate, with 8 replicates. 1 mL of distilled water (in control and temperature-treated) was applied daily; 24-epiBL solutions were applied thrice (in BL-treated), and 15% PEG solutions (in drought-treated) were applied twice on the 1st and 3rd day to each Petri dish. Finger millet seedlings were grown for 10 days.

Experimental Design

High temperature stress was applied at 42 °C/32 °C (TS), and drought stress was applied using 15% PEG (DS). The three concentrations of 24-epibrassinolide (24-epiBL), 0.0 µM (BL0), 1.0 µM (BL1), and 2.0 µM (BL2), were applied in irrigation water solutions twice at two-

day intervals (1st day and 3rd day), with a 3rd application by spraying on the 8th day in control, drought, temperature, and combined drought and temperature stress (CS) treated seedlings. The experiment was designed as a three-factor completely randomized design.

Determination of Seedling Growth Components

Primary data on seed germination were collected daily for up to 7 days. The germination percentage (%) was calculated as the total number of seeds germinated (Scott *et al.*, 1984). Plumule and radicle lengths of randomly selected 10-day-old fresh seedlings were recorded in centimeters. Seedlings were oven-dried at 60 °C until a constant weight was reached, recorded as dry weight (DW) in grams (g). Seedling vigor index was calculated using the formula suggested by Abdul-Baki and Anderson (1973).

(i) Germination percentage:

$$\text{Germination \%} = \frac{\text{Number of germinated Seeds}}{\text{Total No. of Seeds in Petri Plates}} \times 100$$

(ii) Seedling Vigor Index:

$$\text{Seedling Vigor Index} = \text{Germination percentage} \times \text{Seedling Dry weight (mg)}$$

Determination of Biochemical Traits

(i) Chlorophyll and Carotenoid contents:

Chlorophyll and carotenoid contents were estimated using the method described by Hiscox & Israelstam (1979). 25 mg of 10-day-old fresh seedlings were added to test tubes containing 3 mL of DMSO. Tubes were incubated in the dark at 55 °C for 4 h. Samples were then cooled to room temperature in the dark, and absorbance was recorded at 663, 645, 510, and 480 nm, with DMSO as the blank. The results were expressed as mg g⁻¹ fresh weight.

Chlorophyll a (mg g⁻¹ FW)

$$\text{Chlorophyll a} = (12.7 \times A_{663} - 2.69 \times A_{645}) \times \left(\frac{\text{Volume of DMSO (mL)}}{\text{Weight of Seedlings (mg)}} \right) \times 1000$$

Chlorophyll b (mg g⁻¹ FW)

$$\text{Chlorophyll b} = (22.9 \times A_{645} - 4.68 \times A_{663}) \times \left(\frac{\text{Volume of DMSO (mL)}}{\text{Weight of Seedlings (mg)}} \right) \times 1000$$

Total Carotenoids (mg g⁻¹ FW)

$$\text{Total Carotenoids} = (7.6 \times A_{480} - 1.49 \times A_{510}) \times \left(\frac{\text{Volume of DMSO (mL)}}{\text{Weight of Seedlings (mg)}} \right) \times 1000$$

(ii) **Lipid Peroxidation:** Damage to the membrane was estimated in terms of malondialdehyde (MDA) content or lipid peroxidation (Heath & Packer, 1968). 10-day-old fresh seedlings (500 mg) were ground in 10 mL of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged

at 15,000 x g for 15 minutes. An aliquot of 1.0 mL of the supernatant was mixed with 4.0 mL of 0.5% thiobarbituric acid (TBA) in 20 % TCA. This mixture was heated in a water bath at 95 °C for 30 minutes, then cooled to room temperature. After a second centrifugation at 10,000 x g for 10 minutes, the absorbance of the supernatant was measured at 532 nm. The TBARS concentration was calculated using an extinction coefficient of 155 mM⁻¹ cm⁻¹. Non-specific absorbance at 600 nm was subtracted.

(iii) Ascorbate Peroxidase (APX) activity:

Ascorbate peroxidase (APX) enzyme (EC 1.11.1.11) activity was determined according to Nakano and Asada (1981). The reaction mixture (3 mL) contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM EDTA, and 0.1 mM H₂O₂. The reaction was initiated by adding 0.1 mL of crude enzyme extract. Oxidation of ascorbate was observed for 30 seconds by measuring the decrease in absorbance at 290 nm every 15 s (e = 2.8 mM⁻¹ cm⁻¹). Enzyme activity is quantified as the amount of ascorbic acid oxidized (initial reading - final reading) per minute per milligram of protein.

Statistical Analysis

Data were collated and initially organized in Microsoft Excel 2019. Statistical analyses were conducted in RStudio 2026.08.2+200 ("Yellow Yarrow" Release; Posit Software, PBC, Boston, MA, USA) using R. A three-way analysis of variance (ANOVA) assessed the effects of genotypes, stress, 24-epibrassinolide treatment, and their interactions on the measured parameters. Significant differences among treatment means were evaluated with Tukey's multiple-comparison test at $P \leq 0.05$. Principal component analysis (PCA) assessed multivariate relationships among the measured traits and identified patterns of variation across experimental treatments. Heatmap analysis used standardized Z-scores of the 24-epiBL response to visualize relative response patterns across genotypes and stress conditions. Figures and graphical representations were generated using R packages, primarily ggplot2, ComplexHeatmap, and circlize.

Results

Effects of 24-epiBL on Germination Percentage and Seedling Growth of Ragi genotypes under Individual and Combined Stress

The ameliorative effects of 24-epiBL applications on

Table 1: Ameliorative impacts of 24-epiBL supplementation on germination percentage and seedling growth of Ragi seedlings under drought, temperature, and combined stress.

Traits	A. Germination Percentage (%)			B. Radicle Length (cm)			C. Plumule Length (cm)		
Stress	Mean Value			Mean Value			Mean Value		
	0.0 μ M	1.0 μ M	2.0 μ M	0.0 μ M	1.0 μ M	2.0 μ M	0.0 μ M	1.0 μ M	2.0 μ M
C	89.81 $\pm$ 3.54 e	91.95 $\pm$ 3.16 de	94.91 $\pm$ 2.85 d	2.50 $\pm$ 0.34 d	2.67 $\pm$ 0.36 cd	2.86 $\pm$ 0.31 c	1.37 $\pm$ 0.18 d	1.40 $\pm$ 0.17 d	1.47 $\pm$ 0.18 d
DS	79.32 $\pm$ 4.60 ab	85.70 $\pm$ 5.83 f	88.66 $\pm$ 5.77 ef	1.46 $\pm$ 0.40 ab	1.67 $\pm$ 0.40 be	1.87 $\pm$ 0.41 e	0.95 $\pm$ 0.13 ab	1.05 $\pm$ 0.16 aef	1.13 $\pm$ 0.14 ef
TS	80.19 $\pm$ 3.97 ab	85.43 $\pm$ 4.42 f	88.51 $\pm$ 5.15 ef	1.41 $\pm$ 0.40 ab	1.63 $\pm$ 0.38 be	1.89 $\pm$ 0.37 e	0.94 $\pm$ 0.11 ab	1.04 $\pm$ 0.11 ae	1.17 $\pm$ 0.09 f
CS	72.56 $\pm$ 5.07 c	76.53 $\pm$ 4.86 a	80.74 $\pm$ 5.23 b	1.14 $\pm$ 0.33 a	1.27 $\pm$ 0.37 a	1.44 $\pm$ 0.40 ab	0.81 $\pm$ 0.15 c	0.92 $\pm$ 0.15 bc	1.01 $\pm$ 0.17 abe

Note: Values in the table are means $\pm$ SE of three replicates (n = 3) across 10 Ragi genotypes. Different lowercase letters indicate significant differences among Stress $\times$ 24-epiBL treatments according to Tukey's multiple-range test at $P \leq 0.05$. C, control; DS, Drought stress; TS, Temperature stress; CS, Combined stress.

Ragi seedlings from 10 genotypes were observed under abiotic stress conditions. The 24-epiBL enhanced germination under both individual and combined drought and temperature stress. Both individual and combined stress imposition markedly reduced germination percentage, plumule length, and radicle length of Ragi seedlings compared with the unstressed control. At BL0 (0.0 μ M 24-epiBL), germination, radicle length, and plumule length decreased by 11.68%, 41.60%, and 30.66%, respectively, under DS and 10.71%, 43.60%, and 31.39%, respectively, under TS, relative to the control. However, the highest inhibitory effect was observed under CS, decreasing these parameters by 19.21%, 54.40%, and 40.88%, respectively, relative to the control (Table 1A, B & C). Thus, across all stresses, CS showed the strongest suppression of germination and seedling growth,

followed by temperature and drought stress. Tukey's multiple-range test ($P \leq 0.05$) also showed significant differences among Stress $\times$ 24-epiBL combinations, indicating that the magnitude of stress-induced inhibition and the response to 24-epiBL concentration varied significantly across treatments.

The BL mean response curve showed that the application of 24-epiBL progressively alleviated the adverse effects of stress, with 2.0 μ M generally providing the highest response in both germination and seedling growth. Relative to the respective stressed seedlings with no brassinolide application, 2.0 μ M enhanced germination by 11.78%, 10.38%, and 11.27% under DS, TS, and CS, respectively (Fig. 1A). Radicle length showed a stronger ameliorative response at 2.0 μ M, with increases of 28.08%, 34.04%, and 26.32% under DS, TS, and CS,

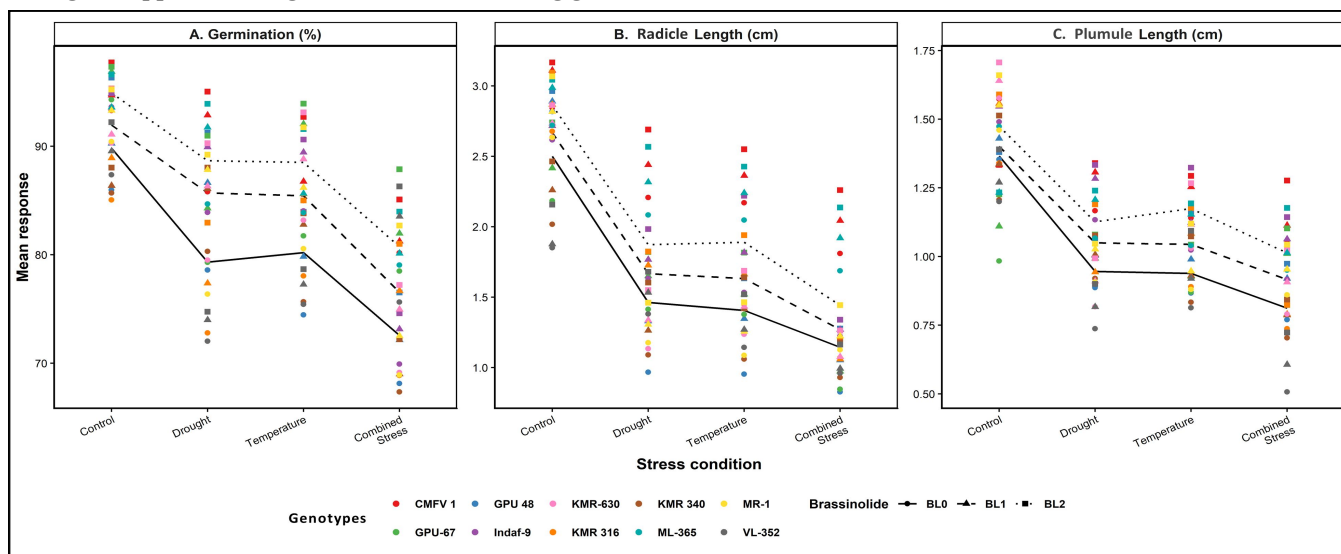
**Fig. 1:** Response of Ragi genotypes to 24-epibrassinolide (24-epiBL) supplementation under control, drought, temperature, and combined stress conditions: (A) germination percentage, (B) radicle length, and (C) plumule length. BL₀, BL₁, and BL₂ represent 0.0, 1.0, and 2.0 μ M 24-epiBL, respectively. Each point represents the mean response of an individual genotype; lines indicate the treatment means across genotypes.

Table 2: Ameliorative impacts of 24-epiBL supplementation on pigments and Seedling Vigor Index of Ragi seedlings under drought, temperature, and combined stress.

Traits	A. Chl a (mg g ⁻¹ FW)			B. Chl b (mg g ⁻¹ FW)			C. Seedling Vigor Index		
Stress	Mean Value			Mean Value			Mean Value		
	0.0 μ M	1.0 μ M	2.0 μ M	0.0 μ M	1.0 μ M	2.0 μ M	0.0 μ M	1.0 μ M	2.0 μ M
C	1.90 $\pm$ 0.56 d	2.04 $\pm$ 0.53 d	2.21 $\pm$ 0.56 d	1.09 $\pm$ 0.23 f	1.17 $\pm$ 0.25 ef	1.31 $\pm$ 0.28 e	347.47 $\pm$ 48.71 e	374.30 $\pm$ 44.90 de	412.01 $\pm$ 42.61 d
DS	1.08 $\pm$ 0.44 abc	1.28 $\pm$ 0.47 bc	1.46 $\pm$ 0.56 c	0.38 $\pm$ 0.10 ab	0.53 $\pm$ 0.14 cd	0.59 $\pm$ 0.15 cg	192.45 $\pm$ 49.91 ab	234.51 $\pm$ 57.64 fgh	267.39 $\pm$ 58.97 gh
TS	1.01 $\pm$ 0.44 ab	1.22 $\pm$ 0.51 bc	1.43 $\pm$ 0.53 c	0.45 $\pm$ 0.14 abcd	0.57 $\pm$ 0.19 cg	0.68 $\pm$ 0.20 g	189.44 $\pm$ 47.51 ab	229.39 $\pm$ 45.92 afg	272.21 $\pm$ 47.96 h
CS	0.72 $\pm$ 0.36 a	0.80 $\pm$ 0.35 a	0.94 $\pm$ 0.39 ab	0.31 $\pm$ 0.07 a	0.42 $\pm$ 0.08 abd	0.47 $\pm$ 0.09 bcd	143.13 $\pm$ 40.78 c	167.77 $\pm$ 41.89 bc	199.00 $\pm$ 49.37 abf

Note: Values in the table are means $\pm$ SE of three replicates (n = 3) across 10 Ragi genotypes. Different lowercase letters indicate significant differences among Stress $\times$ 24-epiBL treatments according to Tukey's multiple-range test at $P \leq 0.05$. C, control; DS, Drought stress; TS, Temperature stress; CS, Combined stress.

respectively (Fig. 1B). Similarly, plumule length increased by 18.95%, 24.47%, and 24.69% under DS, TS, and CS, respectively, at 2.0 μ M (Fig. 1C). The highest absolute responses at 2.0 μ M were recorded for germination under DS (88.66%), radicle length under TS (1.89 cm), and plumule length under TS (1.17 cm), while the greatest relative improvement in radicle growth occurred under CS (Fig. 1 & Table 1). Overall, these results indicate that 2.0 μ M 24-epiBL was the most effective concentration for mitigating stress-induced reductions in germination and seedling growth, with particularly pronounced effects on plumule and radicle development.

Effects of 24-epiBL on Pigments and Seedling Vigor Index of Ragi Seedlings under Individual and Combined Stress

Table 2 presents the ameliorative effects of 24-epiBL applications on pigments in Ragi seedlings of 10 genotypes under individual and combined drought and high temperature stress. The 24-epiBL enhanced pigment contents under both individual and combined stress. At 0.0 μ M 24-epiBL, a significant reduction in Chl a content was observed by 43.16% under DS, 46.84% under TS, and 62.11% under CS relative to the control (Table 2A). Chl b content decreased by 65.14%, 58.72%, and 71.56% under DS, TS, and CS, respectively (Table 2B). Similarly, Seedling Vigor Index declined significantly by 44.62% under drought, 45.48% under temperature, and 58.80% under combined stress (Table 2C). The results indicate that combined stress caused the greatest reductions in Chl a, Chl b, and Seedling Vigor Index, demonstrating a

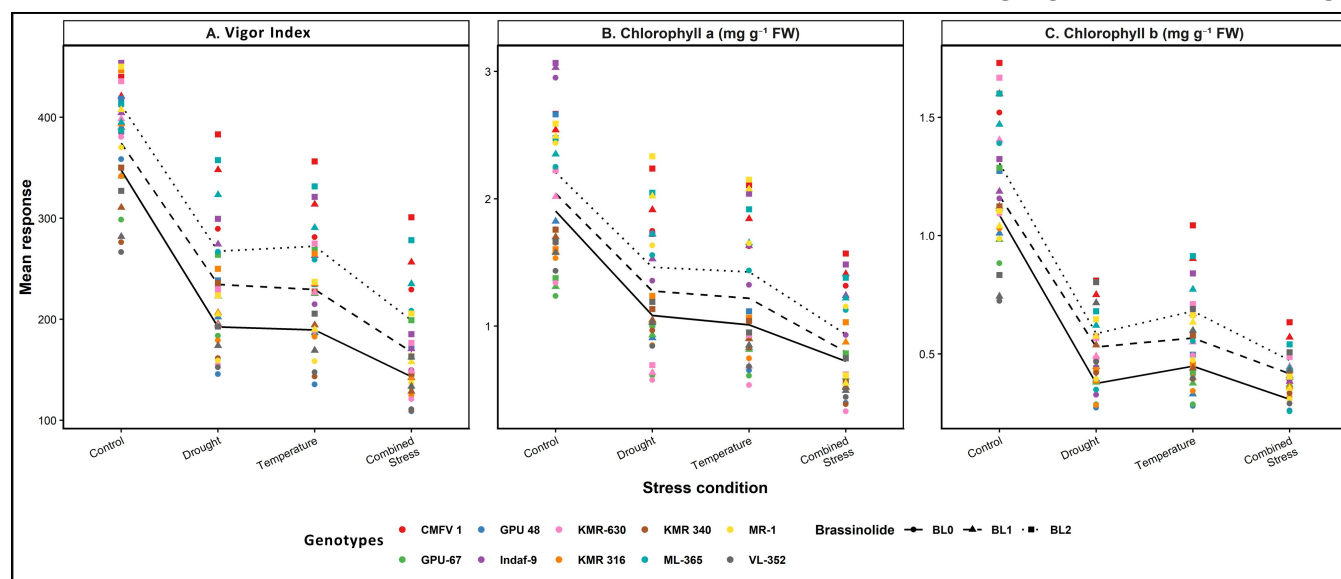
**Fig. 2:** Response of Ragi genotypes to 24-epibrassinolide (24-epiBL) supplementation under control, drought, temperature, and combined stress conditions: (A) Seedling Vigor Index, (B) chlorophyll a, and (C) chlorophyll b. BL₀, BL₁, and BL₂ represent 0.0, 1.0, and 2.0 μ M 24-epiBL, respectively. Each point represents the mean response of an individual genotype; lines indicate the treatment means across genotypes.

Table 3: Ameliorative impacts of 24-epiBL supplementation on Tcar, POX activity, and lipid peroxidation of Ragi seedlings under drought, temperature, and combined stress.

Traits	A. Tcar (mg g ⁻¹ FW)			B. POX activity ($\mu\text{mol mg}^{-1}$ protein min ⁻¹)			C. Lipid peroxidation ($\mu\text{mol g}^{-1}$ FW)		
	Stress	Mean Value		Mean Value			Mean Value		
	0.0 μM	1.0 μM	2.0 μM	0.0 μM	1.0 μM	2.0 μM	0.0 μM	1.0 μM	2.0 μM
C	0.41 $\pm$ 0.12 acd	0.46 $\pm$ 0.13 abcd	0.49 $\pm$ 0.16 bcd	422.57 $\pm$ 76.93 ah	498.62 $\pm$ 85.37 bce	516.32 $\pm$ 90.18 bcef	5.31 $\pm$ 0.95 eg	4.14 $\pm$ 1.32 de	3.86 $\pm$ 1.24 d
DS	0.45 $\pm$ 0.12 abcd	0.53 $\pm$ 0.13 bc	0.55 $\pm$ 0.12 b	471.20 $\pm$ 74.80 abc	527.63 $\pm$ 71.04 bcef	557.71 $\pm$ 70.71 ef	7.08 $\pm$ 1.59 abc	6.01 $\pm$ 1.58 cfg	5.59 $\pm$ 1.17 fg
TS	0.43 $\pm$ 0.14 abcd	0.53 $\pm$ 0.19 bc	0.57 $\pm$ 0.20 b	466.65 $\pm$ 63.82 ab	529.08 $\pm$ 66.13 cef	564.75 $\pm$ 71.90 f	7.40 $\pm$ 1.56 ab	6.54 $\pm$ 1.19 acf	5.68 $\pm$ 1.28 fg
CS	0.35 $\pm$ 0.29 a	0.36 $\pm$ 0.16 ad	0.38 $\pm$ 0.16 ad	299.82 $\pm$ 62.25 d	351.69 $\pm$ 63.38 dg	365.01 $\pm$ 67.55 gh	8.05 $\pm$ 1.81 b	7.15 $\pm$ 1.53 abc	6.24 $\pm$ 1.12 acfg

Note: Values in the table are means $\pm$ SE of three replicates (n = 3) across 10 Ragi genotypes. Different lowercase letters indicate significant differences among Stress $\times$ 24-epiBL treatments according to Tukey's multiple-range test at $P \leq 0.05$. C, control; DS, Drought stress; TS, Temperature stress; CS, Combined stress.

stronger inhibitory effect on pigment accumulation and Seedling Vigor Index than either individual stress. Significance was determined by Tukey's multiple-range test at $P \leq 0.05$.

The mean BL response curve (Fig. 2) showed that 24-epiBL produced a concentration-dependent ameliorative response, with 2 μM consistently providing greater improvements across the pigments and Seedling Vigor Index under stress. Among the stress treatments, the maximum relative improvement with 24-epiBL at 2 μM was observed under TS (41.58%) in Chl a (Fig. 2A), under DS (55.26%) in Chl b (Fig. 2B), and under TS in Seedling Vigor Index (43.69%) (Fig. 2C), although substantial recovery was also evident under CS compared

with its respective untreated stress condition. Therefore, HS exhibited the strongest proportional responsiveness to 2 μM 24-epiBL, whereas CS remained the most severe condition despite substantial hormonal amelioration.

Effects of 24-epiBL on total carotenoids, POX activity and lipid peroxidation of Ragi genotypes under individual and combined stress

Table 3 presents the ameliorative effects of 24-epiBL applications on Ragi seedlings of 10 genotypes under individual and combined drought and high temperature stress. 24-epiBL enhanced total carotenoids and peroxidase (POX) activity while reducing lipid peroxidation under both individual and combined drought and temperature stress. The enhancement in Tcar content

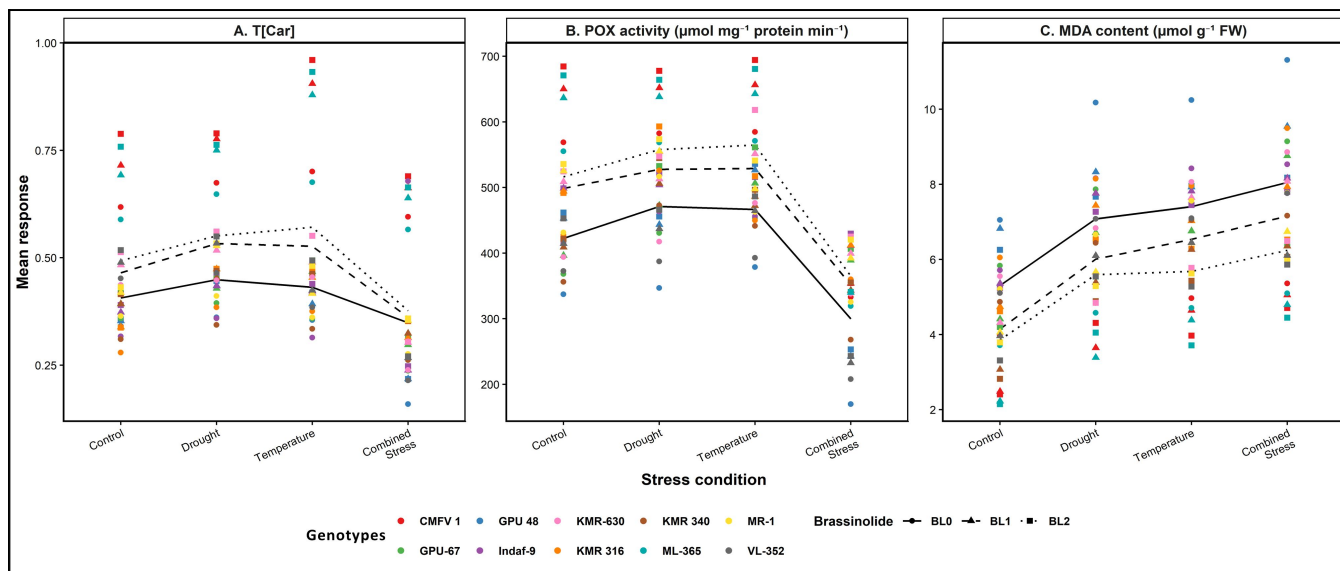


Fig. 3: Response of Ragi genotypes to 24-epibrassinolide (24-epiBL) supplementation under control, drought, temperature, and combined stress conditions: (A) total carotenoids (Tcar) (mg g⁻¹ FW), (B) peroxidase activity (POX), and (C) lipid peroxidation. BL₀, BL₁, and BL₂ represent 0.0, 1.0, and 2.0 μM 24-epiBL, respectively. Each point represents the mean response of an individual genotype; lines indicate the treatment means across genotypes.

was observed under DS and TS by 9.76% and 4.88%, respectively, whereas under CS it was reduced by 14.63% compared with the control; however, these changes were not significant (Table 3A). POX activity increased non-significantly by 11.50% and 10.42% under DS and TS, respectively, whereas CS caused a significant 29.13% reduction relative to the control (Table 3B). In contrast, lipid peroxidation increased significantly by 33.33%, 39.36%, and 51.60% under drought, temperature, and combined stress, respectively, indicating enhanced membrane lipid damage under all stress conditions, with the highest damage observed under combined stress (Table 3C). Overall, the results indicate that CS produced the strongest alteration in biochemical stress responses, particularly by increasing lipid peroxidation and reducing POX activity.

Whereas the BL mean response curve showed the highest per cent improvement in Tcar and POX activity and the highest per cent reduction in lipid peroxidation at 2.0 μM 24-epiBL application than at 1.0 μM 24-epiBL under all stress conditions, compared with the respective untreated stressed seedlings (Fig. 3 & Table 3). This result indicates that the strongest response for Tcar was

observed under TS (32.56%) at 2.0 μM 24-epiBL, whereas the response under CS was comparatively low, with the stress $\times$ treatment interaction nonsignificant ($p > 0.05$) (Fig. 3A). However, 2.0 μM 24-epiBL was the most effective for POX activity under CS (21.74%) among the stressed treatments and the greatest reduction in lipid peroxidation under TS (23.24%), indicating significant differential responses of 24-epiBL across stress conditions (Fig. 3B & 3C).

Heat map analysis of concentration-dependent Brassinolide responses

The relative-response heat maps revealed clear dose- and stress-dependent variation in responses to 24-epiBL across all finger millet genotypes and traits (Fig. 4A, B). At 1.0 μM 24-epiBL, positive responses were predominantly observed for germination, plumule and radicle length, seedling vigor index, and POX activity, whereas MDA generally showed a negative relative response, particularly under DS and CS, indicating reduced lipid peroxidation (Fig. 4A). The responses varied among genotypes, with drought showing a particularly pronounced BL-mediated improvement in several growth- and antioxidant-related traits.

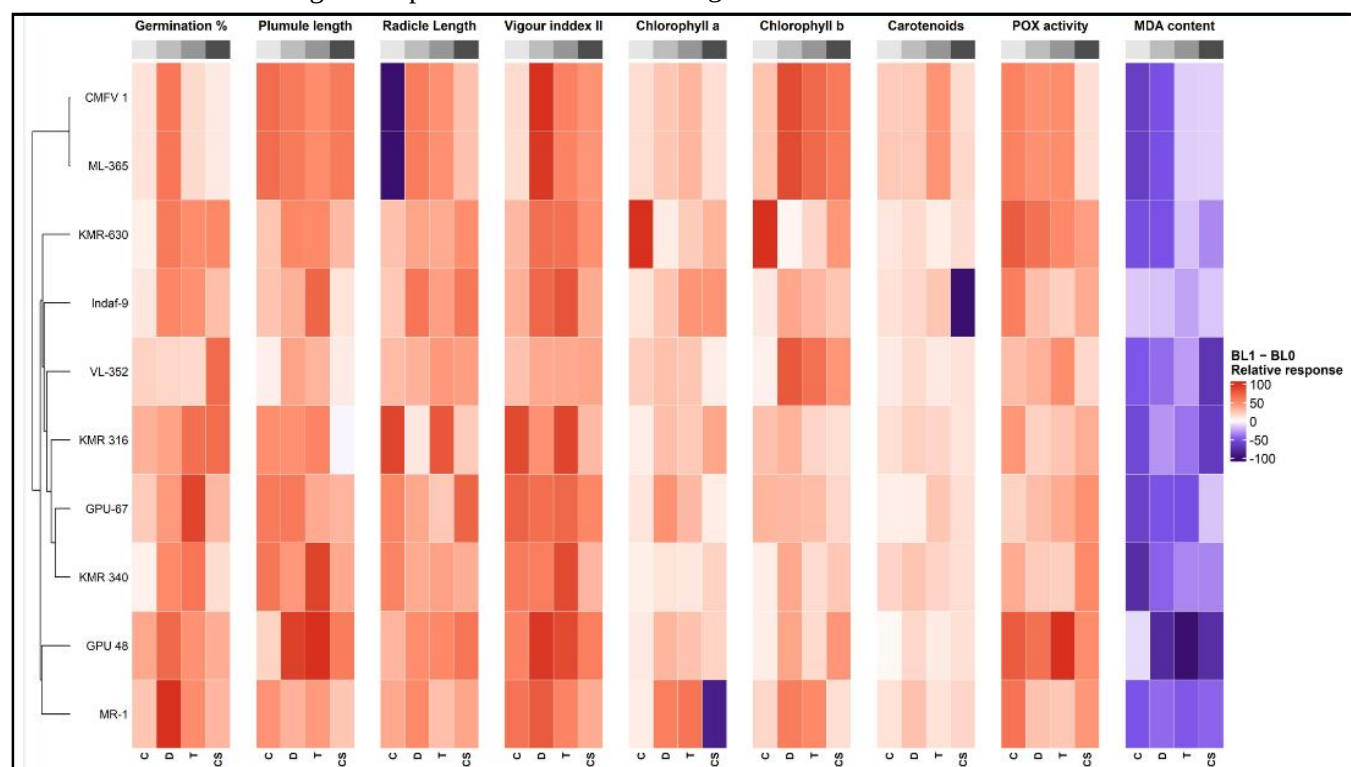


Fig. 4A: Hierarchical clustering heatmap illustrating the relative response (%) of 10 Ragi genotypes to 1.0 μM 24-epibrassinolide (BL_1) relative to the corresponding untreated control (BL_0) under control (C), drought stress (D), temperature stress (T), and combined stress (CS). The heatmap represents genotype-specific responses for growth and physio-biochemical traits. The color gradient indicates the magnitude and direction of the relative response. Red shades indicate positive responses, whereas blue/purple shades indicate negative responses relative to BL_0 ; color intensity reflects the magnitude of the relative response. The dendrogram on the left shows hierarchical clustering of genotypes based on similarities in their response patterns.

At 2.0 μM 24-epiBL, the heat map showed a generally stronger and more consistent positive response for several growth traits, particularly radicle length and seedling vigor index, together with enhanced POX activity across several genotype $\times$ stress combinations (Fig. 4B). The negative response of lipid peroxidation was also maintained across most stressed conditions, suggesting reduced membrane lipid peroxidation following 24-epiBL application. The comparison of both heat maps indicated that 2.0 μM BL generally produced a stronger overall response than 1.0 μM BL, particularly for growth and oxidative stress-related traits. However, the magnitude of the response differed among stresses and genotypes, indicating that BL efficacy was stress- and genotype-dependent rather than uniformly dose-dependent. Overall, the heat-map analysis identified 2.0 μM 24-epiBL as the most responsive concentration across multiple traits, while drought stress exhibited one of the greatest BL-mediated improvements among the genotypes (Fig. 4A, B).

Principal component analysis of 24-epiBL-mediated responses

Principal component analysis also showed a concentration-dependent change in the overall

physiological and biochemical responses of all finger millet genotypes (Fig. 5). The first two principal components explained 85.3% of the total variation under BL ϵ , with PC1 and PC2 accounting for 70.9% and 14.4%, respectively. At 1.0 μM 24-epiBL, PC1 and PC2 explained 69.2% and 12.9% of the variation, respectively, whereas at 2.0 μM 24-epiBL, they explained 66.3% and 14.1%, respectively (Fig. 5).

Across the 24-epiBL concentrations, MDA was strongly associated with the positive direction of PC1, whereas plumule length, radicle length, seedling vigor index, and chlorophyll-related traits were primarily associated with the negative direction of PC1. POX activity and carotenoids contributed strongly to PC2. The redistribution of genotype scores and trait associations following BL application indicated that exogenous BL modified the coordinated growth, photosynthetic, and oxidative-stress responses of the seedlings. The PCA therefore supported the heat-map analysis by demonstrating that BL concentration altered the overall multivariate response of finger millet genotypes, with the 2.0 μM treatment producing a distinct response pattern relative to 0.0 μM and 1 μM 24-epiBL (Fig. 5). Together,

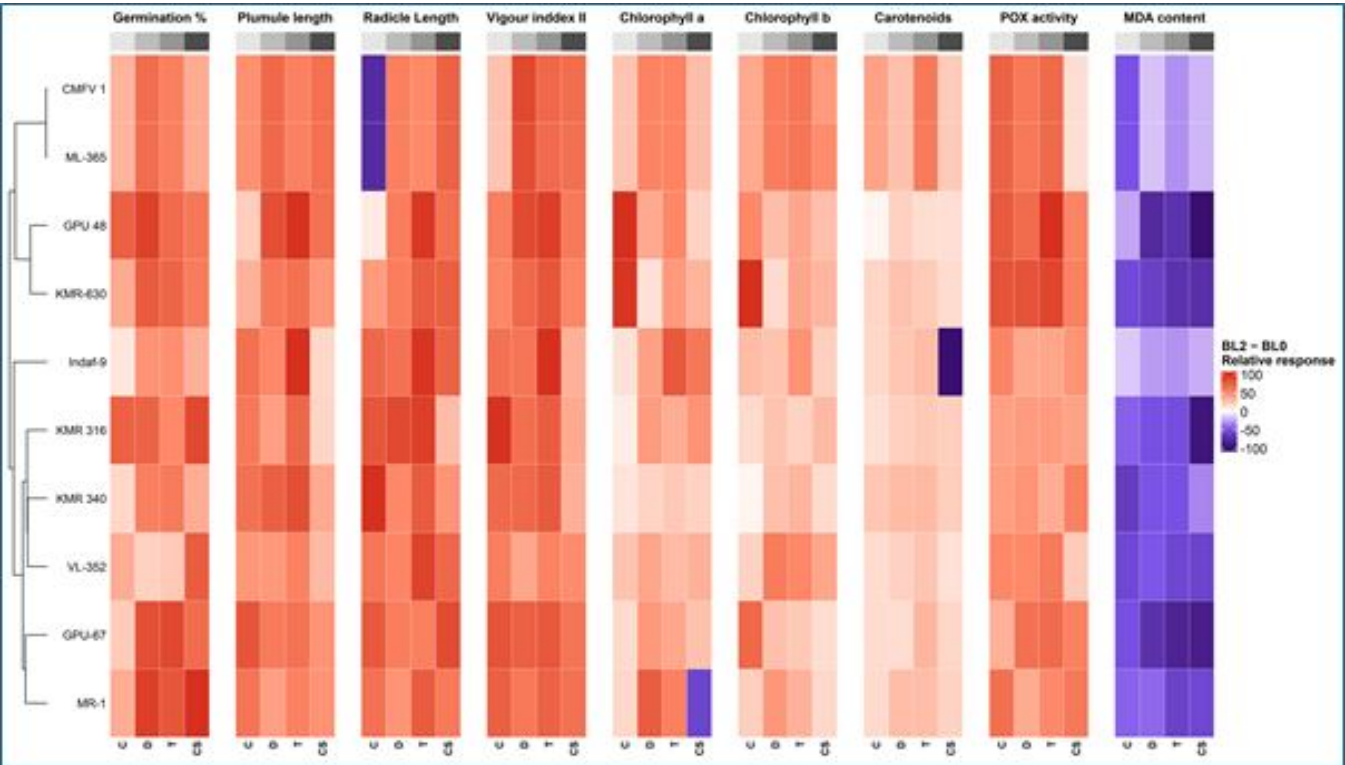


Fig. 4B: Hierarchical clustering heatmap illustrating the relative response (%) of 10 Ragi genotypes to 2.0 μM 24-epibrassinolide (BL $_2$) relative to the corresponding untreated control (BL $_0$) under control (C), drought stress (D), temperature stress (T), and combined stress (CS). The heatmap represents genotype-specific responses for growth and physio-biochemical traits. The color gradient indicates the magnitude and direction of the relative response. Red shades indicate positive responses, whereas blue/purple shades indicate negative responses relative to BL $_0$; color intensity reflects the magnitude of the relative response. The dendrogram on the left shows hierarchical clustering of genotypes based on similarities in their response patterns.

the multivariate analyses indicated that 2.0 μM 24-epiBL was generally more effective at improving stress-associated growth and antioxidant responses, with drought among the most responsive stress conditions.

Discussion

Drought and temperature stress are the most significant factors contributing to reduced agricultural yields, especially when they occur simultaneously. Seedlings are the most sensitive to these abiotic stresses, which reduce yield by restricting germination, seedling survival, tiller formation, and plant growth during the early growth period (Alves *et al.*, 2026; Mude *et al.*, 2022). Germination and seedling growth declined under all individual and combined stresses; however, the reduction was greater under combined stress than under individual stresses (Table 1). Thus, methods to increase germination and seedling survival rates in Ragi seedlings are needed. It was observed that 24-epiBL treatment of Ragi seeds at concentrations of 1.0 μM and 2.0 μM significantly increased the germination and seedling growth of Ragi genotypes under stress (Fig. 1). More specifically, the greatest improvements in germination and seedling growth were observed at 2.0 μM , with the most pronounced ameliorative effect under temperature stress. Qiao *et al.*,

(2026) reported that 0.01 $\mu\text{mol L}^{-1}$ 24-epiBL promoted germination and seedling vigor by improving water absorption and α -amylase activity in proso millet under PEG-induced DS. Thomas *et al.*, (2025) also reported that 2/ μM brassinosteroid promoted germination and seedling growth in rice seedlings by maintaining ion balance and osmotic regulation under salt stress. Shang *et al.*, (2023) reported that a 1.0 mg L^{-1} brassinosteroid treatment of rice seeds is optimal for enhancing rice seedling growth under salt stress. However, no reports describe the effects of exogenous 24-epiBL treatments on Ragi seed germination and seedling growth under combined and individual stresses.

The photosynthetic pigments are essential for the synthesis of numerous chemical compounds in plants (Krishnakripa & Thoppil, 2026). Abiotic stress reduces pigment production by affecting the electron transport pathway and protochlorophyllide reductase activity (Yang *et al.*, 2026). Both individual and combined stresses substantially reduced photosynthetic pigment content and the seedling vigor index, with combined stress producing the greatest reductions (Table 2). Across stresses and genotypes, the 24-epiBL treatments increased the seedling vigor index and photosynthetic pigment content, with

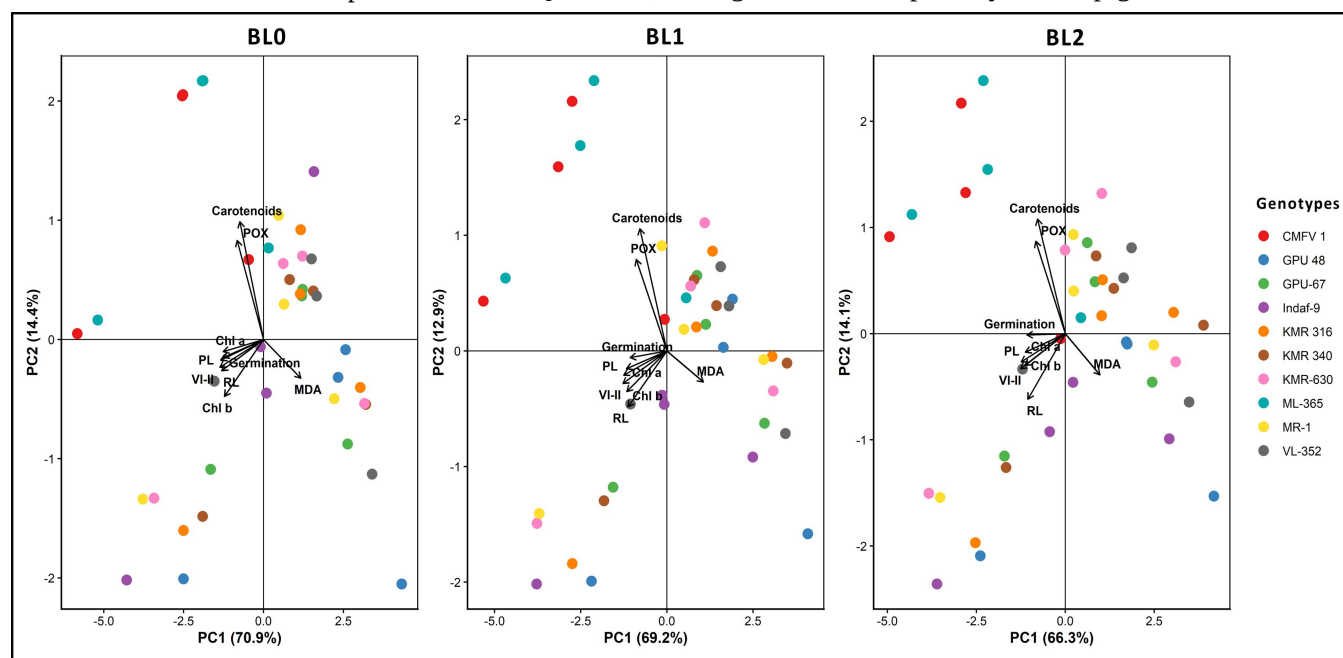


Fig. 5: Principal component analysis (PCA) biplots illustrate the effects of 24-epiBrassinolide (24-epiBL) on physiological and biochemical traits of ragi seedlings under stress conditions. The biplots show relationships among measured growth, pigments, and oxidative-stress-associated traits, as well as the distribution of the evaluated finger millet genotypes. PC1 and PC2 represent the first and second principal components, respectively. The percentage values in parentheses indicate the proportion of total variance explained by each principal component. Vector length and direction indicate the contribution and association of individual traits with the respective principal components, whereas the distribution of genotype observations reflects treatment-associated variation. The PCA demonstrates a progressive modification of the multivariate physiological response as 24-epiBL concentration increases, with 2.0 μM showing the strongest overall alteration in trait associations. 24-epiBL at 0.0 μM (BL0), 1.0 μM (BL1), and 2.0 μM (BL2)

greater response at 2.0 μM (Fig. 2). Consistent with these findings, earlier studies reported that 24-epiBL can increase photosynthetic pigments under alkaline salt stress in potato seedlings (Wang *et al.*, 2025) and under salt stress in rice seedlings (Mu *et al.*, 2022), suggesting that brassinosteroids improve photosynthetic capacity by stabilizing the thylakoid membrane and increasing pigment levels.

Heatmap analysis revealed a predominantly stronger and more consistent positive response across various growth traits, especially radicle length and seedling vigor index, along with increased POX activity in multiple genotype $\times$ stress combinations. However, lipid peroxidation remained largely suppressed under most stress conditions, indicating that BL application reduces membrane lipid damage (Fig. 4). Overall, the heat map analysis revealed that 2.0 μM 24-epiBL was the most responsive concentration across multiple traits. Antioxidants, such as carotenoids, are important for scavenging excess ROS and mitigating the adverse effects of abiotic stress (Matia *et al.*, 2026). In this study, lipid peroxidation increased significantly under both individual and combined stresses in Ragi seedlings, with more severe damage in seedlings under combined stress. However, applying 24-epiBL significantly increased Tcar and POX activity in Ragi seedlings, reducing lipid peroxidation and promoting seedling growth under stress. This is supported by studies in proso millet (Qiao *et al.*, 2026), rice, and maize under abiotic stress (Suresh *et al.*, 2025; Thomas *et al.*, 2025).

The protective benefits of 24-epiBL supplementation may result from increased antioxidant enzyme activity and higher antioxidant levels. Abiotic stress in seedlings raises ROS levels, causing lipid peroxidation and triggering antioxidant enzymes to mitigate oxidative stress, thereby allowing Ragi seedlings to continue growing (Mude *et al.*, 2020). Antioxidant enzymes, mainly POX, SOD, APX, CAT, and GR, and antioxidant compounds, such as carotenoids, protect seedlings from oxidative damage caused by abiotic stresses (Avalbaev *et al.*, 2024; Liu *et al.*, 2026). Although the POX enzyme activity decreased in combined-stressed seedlings of finger millet because combined stress caused more severe damage compared to the individual stresses, and total carotenoids decreased under all stresses, with more reduction under CS in Ragi seedlings (Table 3), the 24-epiBL treatment increased both the POX activity and Tcar contents and reduced oxidative stress, with the most significant effect at 2 μM compared to unstressed control seedlings (Fig. 3). Ragi seedlings showed the highest recovery in POX activity

in DS and Tcar content in TS with 24-epiBL treatment. But the 24-epiBL response under CS was comparatively low. This is consistent with earlier studies by Khan *et al.*, (2026) under combined drought and salt stress, Soares *et al.*, (2020) under Pb toxicity, and Khosravifar *et al.* (2025) under drought stress. The increase in Tcar and POX activity may be related to the restoration of 24-epiBL's effects on seedling vigor. However, the exact metabolic process behind this in the finger millet crop still needs to be identified and studied.

Conclusion

The combined drought and temperature stress severely damaged Ragi seedling growth. Exogenous application of 24-epiBL at appropriate concentrations (1.0 μM and 2.0 μM) to Ragi seedlings significantly reduced oxidative stress and increased germination, seedling growth, seedling vigor index, photosynthetic pigments, and POX enzyme activity. Furthermore, both concentrations are suitable for seed treatments and significantly increased seedling growth, but 2.0 μM 24-epiBL showed significant variations in germination, seedling growth, and physio-biochemical parameters under individual and combined stress.

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Authors' contributions

DRS: Writing – original manuscript and performed the experiments; **PP:** conceived, supervised, and designed the experiments; **PP** and **SKR:** Writing – review and editing of the manuscript; **DRS & IP:** analyzed the data; **KK & NPP:** prepared figures and tables. All authors have read and agreed to the published version of the manuscript.

Data Availability

The raw data supporting the conclusions of this manuscript will be made available by the authors without undue reservation.

Ethics declarations

Conflict of Interest

The authors declare no competing interests.

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