



Brassinolide Mediated Modulation of Biochemical and Antioxidant Responses in Mungbean (*Vigna radiata* L.) under Salinity Stress

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ABSTRACT

Background: Salinity stress is a major abiotic constraint limiting crop productivity worldwide, particularly in legumes such as mungbean (*Vigna radiata* L.). Brassinosteroids (BRs), especially 24-epibrassinolide (EBL), are recognized for their role in regulating plant growth and enhancing tolerance to abiotic stresses by modulating physiological, biochemical and antioxidant defense mechanisms.

Methods: The present investigation evaluated the ameliorative effects of EBL on two mungbean genotypes, HUM23 and HUM16, under salinity stress conditions. HUM23 and HUM16 were selected based on their contrasting responses to abiotic stress (Tolerant vs. sensitive, respectively). Seeds were pretreated with EBL at concentrations of 0.005 mM and 0.01 mM (Selected based on literature-reported effective dose ranges for legumes), followed by exposure to 100 mM NaCl. Biochemical parameters including leaf greenness index (SPAD), protein content, nitrate reductase (NR) activity, proline and hydrogen peroxide levels were analyzed along with antioxidant enzyme activities (SOD, APX, CAT) at 5, 10, 15, 30 and 50 days after sowing (DAS). Experiments were conducted at the Department of Plant Physiology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, during 2023-2024.

Result: Salinity stress significantly reduced chlorophyll content, protein content and nitrate reductase activity, while markedly increasing hydrogen peroxide accumulation, indicating enhanced oxidative stress. Exogenous application of EBL mitigated the adverse effects of salinity by improving chlorophyll retention, protein synthesis and antioxidant enzyme activities, thereby reducing oxidative damage. Among the genotypes, HUM16 exhibited greater salinity tolerance when treated with 0.005 mM EBL, whereas HUM23 showed superior overall biochemical performance under both control and EBL-treated conditions.

Key words: Antioxidant enzymes, Brassinosteroids, Mungbean, Nitrate reductase, Proline, Salinity stress.

INTRODUCTION

Mungbean (*Vigna radiata* L.) is a shortduration pulse crop valued for its protein rich seeds (18-36%) that are highly digestible and widely consumed across Asia and Africa. Brassinosteroids (BRs) have emerged as pivotal regulators in plant responses to salinity stress, operating through multiple interconnected physiological, biochemical and molecular pathways. Under saline conditions, plants are challenged by osmotic imbalance, excessive uptake of Na⁺/Cl⁻ ions, disruption of K⁺/Na⁺ homeostasis, accumulation of reactive oxygen species (ROS) and membrane lipid peroxidation, which cumulatively impede photosynthesis, growth and yield (Soni *et al.*, 2021; Kumar *et al.*, 2021). BRs help mitigate these effects in two major ways: by reinforcing plant growth and development under adverse conditions and by enhancing specific stress-defence mechanisms. Exogenous application of BRs (such as 24-epibrassinolide) has been shown to restore many physiological functions under salinity: improvement in chlorophyll content, photosynthetic performance, stomatal conductance and root architecture and biomass accumulation (Singh *et al.*, 2026; Monisha *et al.*, 2025). In salt stressed maize, treatment with EBL reduced leaf Na⁺ accumulation, improved K⁺ uptake, enhanced superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) activities and ultimately

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improved grain yield (Sivakumar and Priya, 2021; Jin *et al.*, 2024). On the biochemical front, BRs enhance the antioxidant system (both enzymatic and non-enzymatic) to scavenge ROS, maintain redox homeostasis and preserve membrane integrity. They also stimulate osmolyte (such as glycine-betaine, sugars and proline) accumulation which contributes to osmotic adjustment under saline conditions (Yadav and Hemantaranjan, 2017; Zhang *et al.*, 2024). For example, exogenous BRs improved sugar and glycine-betaine levels in salt-stressed pepper plants, thus easing osmotic stress and improving growth (Zolkiewicz *et al.*, 2025). At the molecular level, BRs influence the expression of stress-responsive genes *via* signaling cascades involving BZR1/BES1 transcription factors, reinforcing both growth and defence responses (Soliman *et al.*, 2020). Moreover, BRs do not act in isolation but function within a complex hormonal network. Crosstalk between BRs and other phytohormones like abscisic acid (ABA), gibberellins (GA), ethylene, jasmonic acid (JA) and strigolactones helps fine-tune the plant's response to salinity. For instance, in rice under NaCl stress, combined exogenous BR and ABA treatments showed synergistic effects on growth and stress tolerance (Rehman *et al.*, 2022). Recent reviews have emphasized the intricate interactions between BR signalling and environmental stimuli including salinity and nutrient deficiencies (Farooq *et al.*, 2024). Finally, though much of this research has focussed on cereals and model plants, the potential benefits of BRs in legumes (Such as mungbean) are highly relevant but under-explored. Given that legumes face additional constraints under salinity (*e.g.*, symbiotic nitrogen fixation disruption), BR-mediated mechanisms may offer dual benefits by protecting both stress physiology and symbiotic functions. The present study investigates the role of EBL in mitigating salinity induced biochemical and oxidative stress in two mungbean genotypes (HUM23 and HUM16). By evaluating biochemical parameters and antioxidant enzyme activities at different growth stages, this research aims to clarify the mechanisms of BR induced tolerance and provide a basis for improving mungbean productivity under saline conditions.

MATERIALS AND METHODS

Experimental location and period

The experiment was conducted at the Department of Botany, Institute of Agricultural Sciences, Banaras Hindu University (BHU), Varanasi, India, during 2023-2024.

Plant material and genotype selection

Seeds of mungbean (*Vigna radiata* L.) genotypes HUM23 and HUM16 were procured from the Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, BHU, Varanasi, India. These two genotypes were deliberately selected based on their documented contrasting responses to abiotic stress: HUM23 is recognized as a relatively stress-tolerant genotype, while HUM16 is considered stress-sensitive, making them ideal

for comparative evaluation of EBL-mediated responses (Yadav and Hemantaranjan, 2017).

EBL concentrations and treatments

Seeds were surface sterilized with 0.2% HgCl₂ and soaked in different concentrations of 24-epibrassinolide (0.005 mM and 0.01 mM) prior to sowing. These concentrations were selected based on literature-reported effective dose ranges of EBL for legume crops under abiotic stress (Kumar *et al.*, 2023; Soliman *et al.*, 2020). Salinity stress was induced by applying 100 mM NaCl to the soil. Treatments included: T0 (control), T1 (100 mM NaCl), T2 (100 mM NaCl + 0.01 mM EBL), T3 (100 mM NaCl + 0.005 mM EBL) and T4 (0.01 mM EBL alone, no NaCl). Note: A T5 treatment of 0.005 mM EBL alone was not included in the current design due to experimental space constraints; this is acknowledged as a limitation for future investigation.

Experimental design

Experiments were carried out in a completely randomized design (CRD) with three replications. Biochemical parameters and antioxidant enzymes were measured at 5, 10, 15, 30 and 50 days after sowing (DAS).

Biochemical analyses

Standard methods were employed: leaf greenness index (SPAD meter-a non-destructive, indirect estimate of leaf chlorophyll status based on green colour; not a direct pigment measurement), protein (Assink *et al.*, 2023), proline (Sena *et al.*, 2024), nitrate reductase activity (Singh and Dhal, 2023), ascorbate peroxidase (Ramadan *et al.*, 2025), superoxide dismutase (Farooq *et al.*, 2024) and catalase (Uthra *et al.*, 2022).

Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) using SPSS software (version 21.0, IBM Corp.). Means were compared using Tukey's Honestly Significant Difference (HSD) test at $p \leq 0.05$. Results are expressed as mean $\pm$ SE of three independent replications (Mishra *et al.*, 2020).

RESULTS AND DISCUSSION

Salinity stress (100 mM NaCl, T1) exerted a pronounced and consistent negative influence on all biochemical and physiological parameters measured in both mungbean genotypes HUM23 and HUM16 across the three growth stages (5, 10, 15, 30 and 50 DAS). Exogenous application of 24-epibrassinolide (EBL) at both concentrations (0.005 mM, T3; 0.01 mM, T2) significantly ameliorated these adverse effects, with differential responses between the two genotypes. All results are presented genotype-wise (Fig 1-6).

Effect of EBL on leaf greenness index (SPAD values)

Salinity stress (T1) caused a significant reduction in SPAD values a non-destructive, indirect index of relative leaf

chlorophyll status “SPAD readings reflect relative leaf greenness and are correlated with, but are not a direct measure of, chlorophyll pigment content” (Parida *et al.*, 2024) in both genotypes at all growth stages, with the sharpest decline recorded at 10 DAS (Fig 1). It must be noted that SPAD meter readings reflect leaf transmittance at 650 nm and 940 nm and represent a relative index of greenness correlated with, but not equivalent to, absolute chlorophyll pigment content (Parida *et al.*, 2024). In HUM23,

SPAD values under T1 declined by approximately 22-28% compared to T0 (control), while HUM16 exhibited a comparatively steeper decline of 28-34% under the same stress, indicating greater susceptibility of HUM16 to salinity-induced chloroplast impairment (Fig 1). EBL application significantly reversed this decline. In HUM23, treatments T2 (0.01 mM EBL + NaCl) and T4 (0.01 mM EBL alone) maintained SPAD values 18-24% higher than T1 at 30 and 50 DAS, effectively approaching control levels. In HUM16,

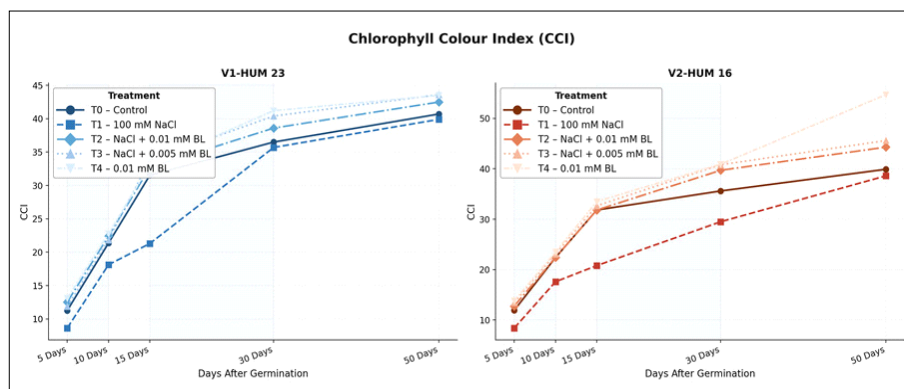


Fig 1: Effect of EBL on chlorophyll content (SPAD units) of mungbean genotypes under induced salinity stress.

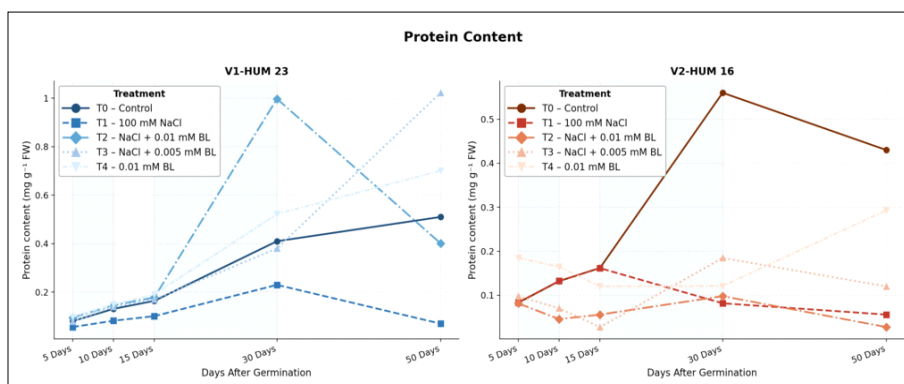


Fig 2: Effect of EBL on protein content (mg g⁻¹ FW) of mungbean genotypes under induced salinity stress.

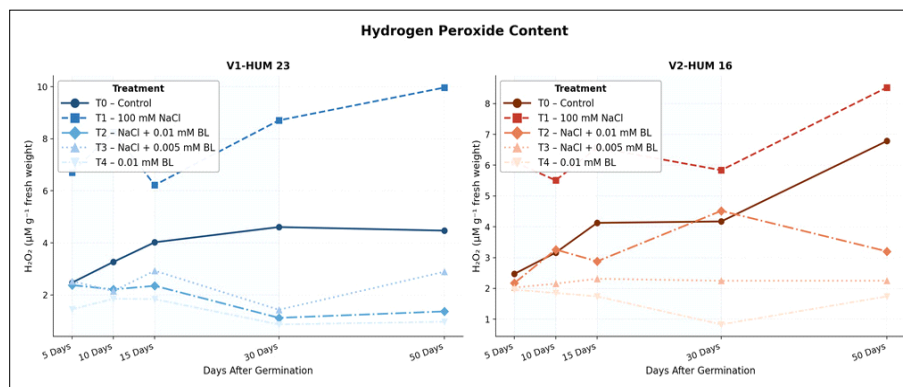


Fig 3: Effect of EBL on hydrogen peroxide (μM g⁻¹ FW) of mungbean genotypes under induced salinity stress.

T3 (0.005 mM EBL + NaCl) produced the most consistent SPAD recovery, restoring values to within 90-95% of the control at 50 DAS. HUM23 consistently showed higher absolute SPAD readings than HUM16 across all treatments, confirming its greater inherent photosynthetic stability. Similar EBL-mediated preservation of chlorophyll status under salinity has been reported in *Vigna* species (Kutay and Venkatesan, 2022; Yadav and Hemantaranjan, 2017). Furthermore, the differential SPAD recovery patterns between genotypes align with recent reports on BR-mediated chloroplast protection in salt-stressed legumes through upregulation of D1 protein stability and photosystem II repair pathways (Kausar and Komatsu, 2022; Seleiman *et al.*, 2023). The ability of EBL to maintain photosynthetic apparatus integrity under sustained ionic toxicity is particularly relevant for sustaining biomass accumulation in mungbean during the critical reproductive phase.

Effect of EBL on protein content

A significant and progressive decline in soluble protein content was recorded under NaCl stress (T1) in both genotypes at all sampling intervals, with the most severe reduction observed at 50 DAS. In HUM23, T1 reduced protein content by approximately 30-35% relative to T0 by 50 DAS, while in HUM16, the reduction under T1 was more pronounced at 35-40%, reflecting genotypic differences in proteolytic activity

and nitrogen utilization under osmotic stress (Fig 2). EBL application produced a marked improvement in protein content in both genotypes. In HUM23, T2 and T4 elevated protein content by 25-30% over T1 at 30 and 50 DAS, with T2 recording the highest values. In HUM16, T3 was particularly effective, restoring protein levels by 20-25% above T1 at 50 DAS, consistent with this genotype's greater sensitivity to lower EBL doses. The EBL-mediated improvement in protein synthesis is attributable to brassinosteroid-regulated enhancement of ribosomal protein activity and nitrogen assimilation-related enzyme stability (Hassan *et al.*, 2025). Comparable trends were observed by Soliman *et al.* (2020) in legumes under combined BR and nitrogen supplementation. The preservation of soluble protein pools under EBL treatment likely reflects brassinosteroid-mediated attenuation of salinity-induced proteolysis, possibly through modulation of the ubiquitin-26S proteasome pathway and stabilization of molecular chaperones (Chakraborty *et al.*, 2025). Similar protective protein responses to exogenous BR have been documented in *Vigna mungo* and soybean under ionic stress (Sivakumar and Priya, 2021; Sadura and Janeczko, 2024).

Hydrogen peroxide (H₂O₂) accumulation

Salinity stress (T1) caused a significant elevation in H₂O₂ accumulation in both genotypes across all growth stages,

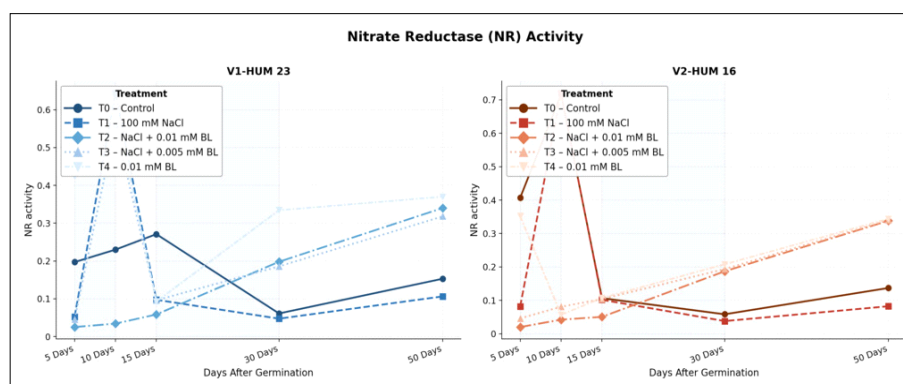


Fig 4: Effect of EBL on nitrate reductase (NR) activity of mungbean under salinity stress.

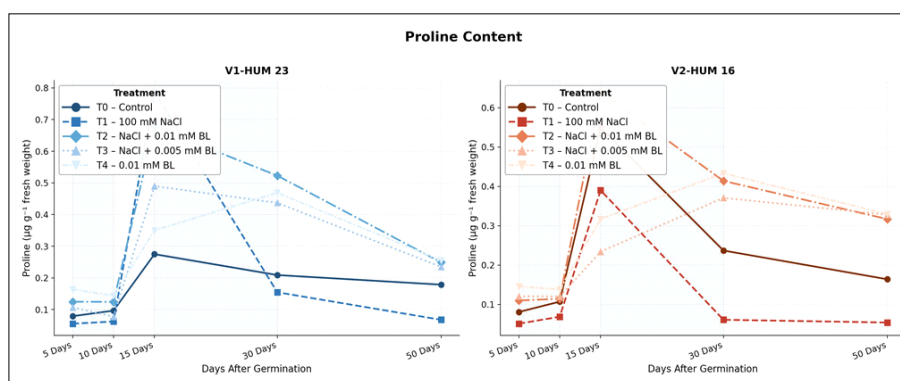


Fig 5: Effect of EBL on proline accumulation of mungbean under salinity stress.

confirming the onset of severe oxidative damage. In HUM16, H_2O_2 levels under T1 at 30 DAS were approximately 28-32% higher than in HUM23 under the same treatment, indicating that HUM16 generates more reactive oxygen species under ionic stress, consistent with its relatively lower antioxidant buffering capacity (Fig 3). EBL treatments (T2, T3 and T4) significantly reduced H_2O_2 accumulation in both genotypes. In HUM23, the lowest H_2O_2 was recorded under T4 at 30 DAS, which was 38-42% lower than T1. In HUM16, T2 showed the greatest suppression of H_2O_2 at 50 DAS, reducing levels by approximately 35% below T1. These results indicate effective EBL-mediated upregulation of ROS scavenging mechanisms, consistent with findings in legumes and cereals under brassinosteroid application (Jisha and Puthur, 2022; Sadura and Janeczko, 2024). The observed H_2O_2 suppression under EBL is mechanistically linked to brassinosteroid-triggered transcriptional

upregulation of NADPH oxidase regulatory subunits and glutathione reductase, which together sustain the ascorbate-glutathione cycle under ionic stress (Zolkiewicz, 2025; Jisha and Puthur, 2022). The greater basal H_2O_2 accumulation in HUM16 relative to HUM23 under T1 corroborates the genotypic differences in constitutive antioxidant buffering capacity and underscores the need for targeted hormonal interventions in stress-sensitive varieties. Limitation: The present study did not assess Na^+/K^+ ionic ratios or crop yield parameters. These represent important dimensions of salinity tolerance and will be incorporated in future field-scale studies correlating ionic homeostasis with oxidative stress markers (Seleiman *et al.*, 2023).

Effect of EBL on nitrate reductase (NR) activity

NR activity used here as an indicator of nitrate assimilation efficiency and not as a comprehensive index of total nitrogen

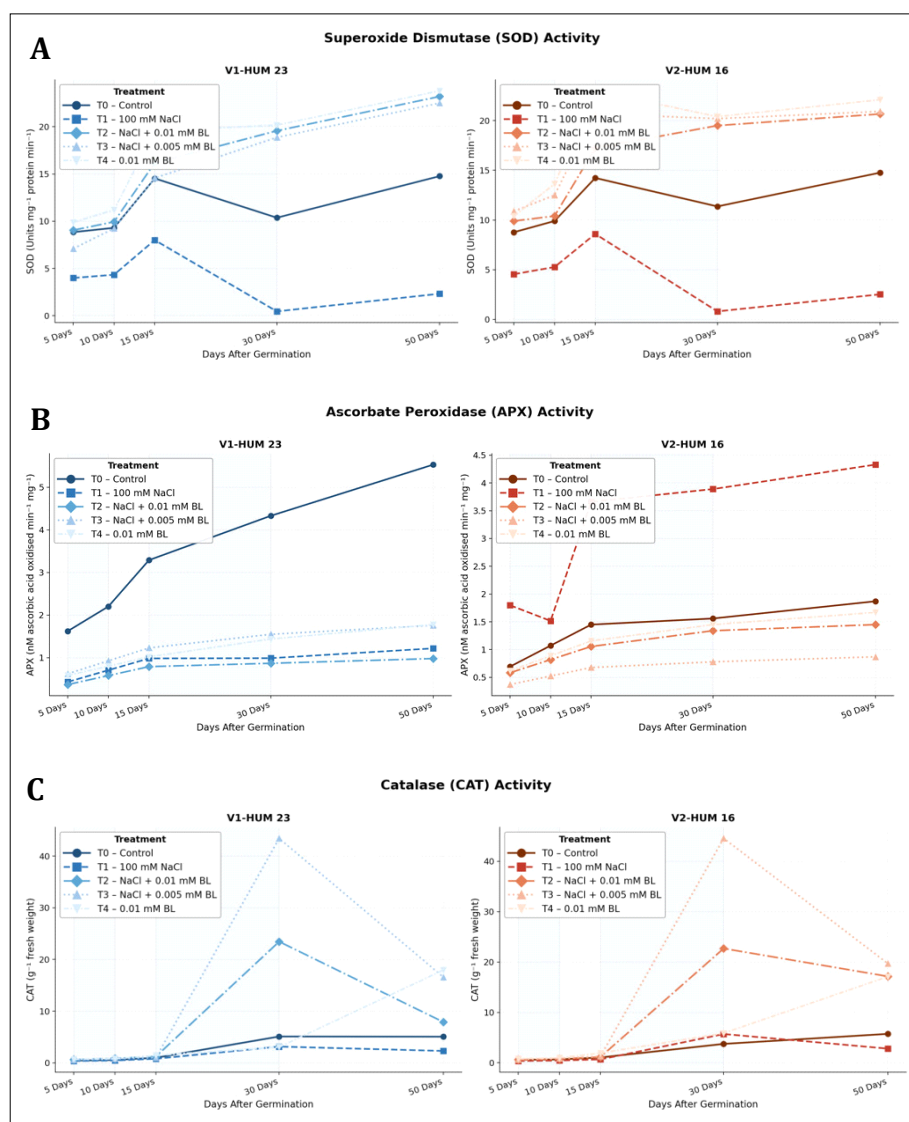


Fig 6: Effect of EBL on antioxidant enzyme activities (SOD (A), APX (B), CAT (C)) in mungbean under salinity stress.

metabolism, declined significantly under NaCl stress in both genotypes, with reductions exceeding 40% compared to controls by 50 DAS. HUM16 exhibited a relatively greater NR suppression (~45% decline under T1 at 50 DAS) compared to HUM23 (~38% decline), suggesting that HUM16's nitrogen assimilation machinery is more vulnerable to ionic disruption under salinity (Fig 4). EBL treatments markedly restored NR activity in both genotypes. In HUM23, T2 recorded the highest NR values at 15 and 50 DAS, exceeding T1 levels by 35-42%. In HUM16, T3 (0.005 mM EBL) was most effective at 15 DAS, elevating NR activity by ~38% over T1. This EBL-mediated restoration may be attributed to brassinosteroid-regulated stabilization of the NR enzyme's molybdenum cofactor structure and improved cellular energy status through enhanced ATP availability (da Silva *et al.*, 2024). A complete characterization of nitrogen metabolism would require additional parameters including glutamine synthetase (GS), glutamate synthase (GOGAT) and total nitrogen content are recommended for future investigations. It is also noteworthy that under salinity, Na⁺ ions directly compete with molybdate (MoO₄²⁻) at the NR active site, suppressing enzyme kinetics; EBL-induced Na⁺ compartmentalization at the vacuolar level would thus indirectly protect NR functionality, an interaction warranting confirmation through ionic flux analysis in future studies (Soni *et al.*, 2021; Kumar *et al.*, 2021). The genotype-specific differential in NR recovery further highlights the agronomic importance of screening mungbean varieties for hormonal responsiveness in nitrogen assimilation pathways.

Effect of EBL on proline accumulation

Proline content increased in both genotypes under NaCl stress (T1) relative to the control (T0), confirming its role as an osmoprotective solute under ionic stress conditions. In HUM23, T1 increased proline by approximately 40-45% over T0 by 30 DAS. HUM16, accumulated comparatively higher proline under T1 (~50% above T0 at 30 DAS), which may partially account for its relatively better osmotic adjustment despite greater oxidative vulnerability. Notably, EBL-treated plants accumulated even higher proline than the saline control, with maximum levels recorded under T2 and T4 in HUM23 and under T3 in HUM16 at 30 and 50 DAS (Fig 5). This super-accumulation of proline under EBL + NaCl treatment suggests that EBL actively stimulates osmotic adjustment beyond the constitutive stress response, supporting cellular membrane protection and enzyme stability (Isayenkov and Maathuis, 2019). These findings are consistent with those of Monisha *et al.* (2025), who reported EBL-mediated proline enhancement in *Vigna radiata* under saline irrigation and with Singh *et al.* (2026), who reported similar trends in mungbean under phytohormone treatment. The EBL-mediated proline super-accumulation observed in this study is likely driven by brassinosteroid-induced upregulation of P5CS (Δ^1 -pyrroline-5-carboxylate synthetase) expression and

simultaneous suppression of proline oxidase activity, as reported in salt-stressed legumes (Jin *et al.*, 2024; Isayenkov and Maathuis, 2019). The higher constitutive proline in HUM16 under T1 may function as a compensatory buffer for its comparatively weaker antioxidant enzyme response, suggesting that osmotic adjustment through proline is a primary stress-coping mechanism in this genotype.

Effect of EBL on antioxidant enzyme activities

Activities of all three antioxidant enzymes including superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) declined significantly under NaCl stress (T1) in both genotypes, signifying salinity-induced impairment of the ROS detoxification system. In HUM23, SOD, APX and CAT activities under T1 were reduced by approximately 30-35%, 35-40% and 28-32% respectively compared to the control at 15 DAS. HUM16 showed a broadly similar pattern, though CAT suppression was comparatively more severe (~36-38% reduction at 30 DAS), indicating greater catalase sensitivity in this genotype under ionic toxicity (Fig 6C). EBL application significantly restored and in several cases exceeded control-level enzyme activities. In HUM23, the maximum SOD and APX activities were recorded under T2 at 15 DAS, exceeding T1 by 40-48% and 42-50% respectively (Fig 6A and B). The highest CAT activity in HUM23 was observed under T4 at 15 and 30 DAS, which was 35-40% above T1. In HUM16, T3 (0.005 mM EBL + NaCl) was most effective for SOD and APX recovery at 15 DAS, with values 38-45% above T1, while T4 produced the highest CAT activity at 30 DAS. These results collectively demonstrate that EBL up-regulates the enzymatic antioxidant defense system in a genotype- and dose-dependent manner, consistent with the reported BR-mediated activation of the ascorbate-glutathione cycle and enhancement of catalytic ROS scavenging under abiotic stress (Chakraborty *et al.*, 2025). The observed dose-dependent response with HUM16 responding preferentially to the lower EBL dose (T3) and HUM23 to the higher dose (T2) confirms differential hormonal sensitivity between the two genotypes (Yadav and Hemantaranjan, 2017). The BR-mediated antioxidant enzyme induction documented here is consistent with brassinosteroid-stimulated activation of the BES1/BZR1 transcription factor cascade, which directly enhances transcription of SOD, APX and CAT gene families under stress (Soliman *et al.*, 2020; Farooq *et al.*, 2024). The superior CAT recovery in HUM23 under T4 (EBL without NaCl) compared to its T2 response is notable and suggests that the absence of ionic interference allows EBL to maximally induce catalase synthesis, an observation with potential implications for preventive (pre-stress) brassinosteroid application strategies.

Genotypic variation

Comparative genotype-wise analysis across all six figures revealed consistent and interpretable physiological

differentiation between HUM23 and HUM16. HUM23 demonstrated superior inherent biochemical stability maintaining higher SPAD readings, protein content and NR activity under stress but required a higher EBL concentration (0.01 mM, T2) to achieve maximum antioxidant enzyme induction. In contrast, HUM16 showed greater sensitivity to lower EBL concentrations (0.005 mM, T3), achieving its best recovery in antioxidant enzymes, particularly SOD and APX, at this dose, while simultaneously exhibiting higher constitutive proline accumulation under salinity (Fig 1-6). This hormonal sensitivity differential is consistent with the differential stress response profiles of these BHU-developed mungbean varieties reported previously (Yadav and Hemantaranjan, 2017). Together, these findings confirm that EBL is a viable exogenous biostimulant for enhancing salinity tolerance in mungbean, with dose optimization depending on genotype-specific hormonal responsiveness. The genotypic contrast identified here is consistent with a broader body of literature documenting that salinity tolerance in mungbean is a quantitative trait governed by multiple loci regulating ionic compartmentalization, ROS scavenging capacity and hormone signal transduction efficiency (Kausar and Komatsu, 2022; Soni *et al.*, 2021). The fact that HUM16, despite being the more stress-sensitive genotype, exhibited superior recovery under the lower EBL dose (T3) for SOD and APX activities is particularly significant from an applied perspective: it implies that even varieties with lower inherent stress tolerance can be effectively rescued through optimized exogenous brassinosteroid regimes, thereby broadening the scope of EBL-based crop management strategies across diverse mungbean germplasm in salt-affected agroecosystems of South Asia (Monisha *et al.*, 2025; Singh *et al.*, 2026).

CONCLUSION

The present study demonstrates that exogenous application of 24-epibrassinolide (EBL) at 0.005 mM and 0.01 mM effectively alleviates salinity-induced (100 mM NaCl) biochemical stress in mungbean (*Vigna radiata* L.) by enhancing chlorophyll stability (SPAD index), soluble protein content, nitrate reductase (NR) activity and enzymatic antioxidant defense (SOD, APX, CAT), while significantly reducing H₂O₂ accumulation and stimulating proline super-accumulation for osmotic adjustment. Among the two genotypes tested, HUM16 exhibited superior biochemical recovery at the lower EBL dose (0.005 mM, T3), while HUM23 showed greater overall biochemical performance and responded maximally to the higher dose (0.01 mM, T2), confirming genotype-specific hormonal sensitivity thresholds. These findings establish EBL as an effective, chemical-free biostimulant for improving mungbean productivity in salt-affected soils; future field-scale studies incorporating Na⁺/K⁺ ionic ratios, yield parameters and transcriptomic profiling across a wider germplasm panel will be essential to translate these findings into actionable crop management recommendations.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the study design, data collection, analysis, interpretation, or the decision to publish the manuscript.

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