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Synergistic effects of salicylic acid and GR24 in enhancing salinity tolerance in common bean (*Phaseolus vulgaris* L.): an integrated physiological, biochemical, and molecular approach

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Abstract

Soil salinity severely limits crop productivity worldwide, particularly in legumes such as common bean. This study investigated the independent and combined effects of salicylic acid (SA; 1 mM) and the strigolactone analogue GR24 (10 μ M) on two bean cultivars exposed to 200 mM NaCl. Salt stress markedly decreased relative water content (RWC), photosynthetic pigments, and total protein, while increasing electrolyte leakage (EL), malondialdehyde (MDA), proline, and hydrogen peroxide (H_2O_2). Application of SA and GR24 significantly alleviated these effects by improving RWC, restoring pigments, reducing EL, MDA, and H_2O_2 , and modulating proline levels. All measurements were based on three biological $\times$ three technical replicates. SA + GR24 also enhanced antioxidant enzyme activities (CAT, APX, SOD, GPX) and up-regulated the corresponding genes. These results provide an integrated hormonal approach for improving crop resilience under saline environments.

Keywords *Phaseolus vulgaris* L., Salt, Abiotic stress, Salicylic acid, Strigolactone, Antioxidant system

1 Introduction

Salinity is one of the most serious abiotic stresses, affecting nearly 20% of irrigated agricultural land and significantly reducing global food security. In legumes such as common bean (*Phaseolus vulgaris* L.), salt stress disrupts osmotic balance, induces ionic toxicity, and generates reactive oxygen species (ROS), thereby damaging photosynthesis and reducing yield [1].

Recent research emphasizes integrated mitigation strategies that combine physiological and molecular approaches with phytohormone regulation [2, 3].

Salicylic acid (SA) functions as a signaling molecule that boosts antioxidant defenses, preserves membrane stability, and influences stomatal behavior. Strigolactones (SLs),



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including their synthetic analogue GR24, control plant structure and stress response by maintaining ROS balance and enhancing resource distribution [4].

Added a missing background sentence explaining the global importance of salinity research in legumes. Although SA and SLs have been studied separately, their synergistic interaction under salinity has not yet been thoroughly investigated in common beans.

Furthermore, recent research highlights modern strategies for stress mitigation: Touzout et al. [5] demonstrated that cyanobacterial extracts enhance wheat growth under salt stress; Manzoor et al. [2] reported the role of zinc-oxide nanoparticles in increasing tolerance in fruit crops; Ahmad et al. [6] and Ahmad et al. [7] described biochemical pathways that improve stress resilience in trees and *Ziziphus*. These examples underscore the importance of integrating multiple signaling molecules to enhance yield stability.

Therefore, the present work aims to (1) evaluate the independent and combined effects of SA and GR24 on physiological parameters of common bean under 200 mM NaCl; (2) analyze biochemical and oxidative stress responses; (3) assess the transcriptional regulation of key antioxidant genes; and (4) incorporate the latest 2023–2025 references to strengthen scientific relevance. This comprehensive study offers new insights into the hormonal crosstalk between SA and GR24 and its role in improving salinity tolerance in common bean.

2 Materials and methods

2.1 Plant material and growth conditions

Two *Phaseolus vulgaris* L. cultivars, Jules (salt-tolerant) and Naz (salt-sensitive), were obtained from the Seed Bank at the University of Tehran. Seeds were surface-sterilized with 70% ethanol for 3 min, rinsed with sterile distilled water, and planted in 5 kg plastic pots filled with sandy-loam soil (84% sand, 13% silt, 3% clay). Seven seeds were sown per pot, then thinned to five uniform plants. Growth conditions were maintained at 25 ± 1 °C, 60% RH, and a 16-hour light/8-hour dark cycle with a light intensity of $250 \mu\text{mol m}^{-2} \text{s}^{-1}$.

2.2 Salinity stress and hormone treatments

At the eight-leaf stage, plants were exposed to 200 mM NaCl for three weeks. This stage corresponds to floral-bud differentiation and ensures physiological uniformity. The salinity solution was applied through irrigation every three days. To maintain a stable NaCl concentration, the electrical conductivity (EC) of drainage water was measured weekly with a portable EC meter.

Four foliar treatments were imposed:

1. Control (0 mM SA + 0 μM GR24).
2. SA (1 mM salicylic acid).
3. GR24 (10 μM GR24, a synthetic strigolactone analogue).
4. SA + GR24 (1 mM SA + 10 μM GR24 combined).

Solutions were sprayed until the leaves were fully covered. Treatments were applied during the second week of salinity, not at stress onset. Concentrations were selected according to published protocols [8] and confirmed through preliminary tests.

2.3 Experimental design and sampling

The experiment used a factorial completely randomized design (CRD) with three biological replicates for each treatment. Samples were collected 48 h after hormone spraying, when stress-response gene expression is usually stable.

2.4 Physiological measurements

Relative water content (RWC) was calculated as $RWC (\%) = (FW - DW)/(TW - DW) \times 100$ [9].

Electrolyte leakage (EL) was measured from 0.3 g of leaf discs immersed in 10 mL deionized water; electrical conductivities before (EC_1) and after autoclaving (EC_2) were recorded, and $EL (\%) = (EC_1/EC_2) \times 100$.

2.5 Biochemical assays

Malondialdehyde (MDA) content was measured using the thiobarbituric-acid (TBA) method [10].

Hydrogen peroxide (H_2O_2) was quantified according to Velikova et al. [11] at 390 nm.

Proline was determined using the acid-ninhydrin method [12].

Total soluble protein was measured using the Bradford [13] assay with bovine serum albumin standards.

2.6 Photosynthetic pigments

Pigments were extracted with 80% acetone and quantified spectrophotometrically [14].

2.7 Antioxidant enzyme activities

Leaf extracts were analyzed for enzyme activities:

- Superoxide dismutase (SOD) – Dhindsa et al [15].
- Catalase (CAT) – Aebi [16].
- Ascorbate peroxidase (APX) – Amako et al [17].
- Glutathione peroxidase (GPX) – Resende et al. [18]

2.8 Gene-expression analysis

Each assay was performed with three biological and three technical replicates. Total RNA was extracted using the GeneAll Ribospin kit; cDNA was synthesized with the RevertAid First Strand cDNA Synthesis Kit. QRT-PCR was carried out using SYBR Green on a StepOnePlus system (Applied Biosystems). Gene-specific primers (Table 1) were designed with PrimerQuest software based on known cDNA sequences. Relative expression was calculated using the $\Delta\Delta Ct$ method on the $2^{(-\Delta\Delta Ct)}$ scale after confirming primer efficiency (90–110%).

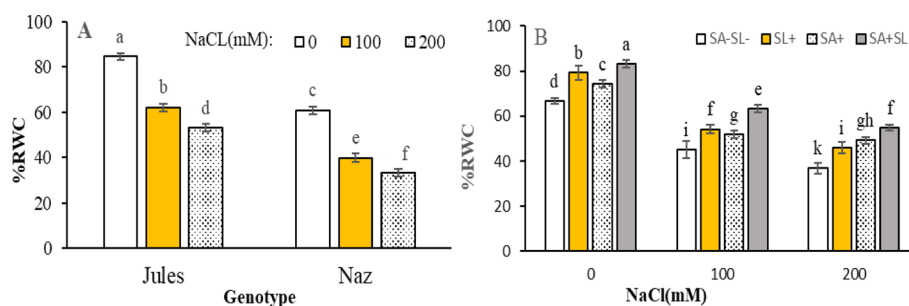
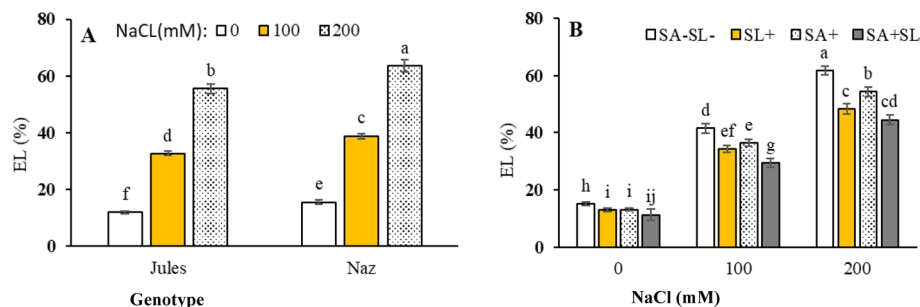
3 Results

3.1 Plant growth and relative water content

Salinity (200 mM NaCl) caused visible growth inhibition in both bean cultivars, with leaf wilting and chlorosis more pronounced in the salt-sensitive 'Naz' than in the tolerant 'Jules'. As shown in Fig. 1A, relative water content (RWC) decreased by 41% in Naz and

Table 1 Primer sequences used for antioxidant gene expression analysis in common beans under salt stress

Gene IDs	Gene name	Product length	Annealing temp	Sequence
Pv08g123450	<i>SOD-F</i>	140	62	F: GGGTGACCTGGGAAACATAG
	<i>SOD-R</i>		62	R: TCTTCCACCAGCATTTCAG
Pv05g067890	<i>CAT-F</i>	135	61	F: ACCAAACTATCTGCAACTTCC
	<i>CAT-R</i>		61	R: TTCCAGAGCAGATAGCAGG
Pv07g045330	<i>GPX-F</i>	150	60	F: TCGCTTTAAATCTGAATTCCC
	<i>GPX-R</i>		60	R: TAATAGCGATCTACCACTTGC
Pv02g102114	<i>APX-F</i>	150	61	F: TTGGAGCGGCACACAAGG
	<i>APX-R</i>		62	R: GGGCGGAATACAGGGTCAG
Pv01g017620	<i>Actin-F</i>	163	61	F: TGGCCGTACAACCTGGTATTG
	<i>Actin-R</i>		61	R: GCTCTGCAGATGTGGTGAAA

**Fig. 1** Effects of salinity and genotype interactions (A), and salinity and exogenous SA and GR24 application interactions (B), on relative water content (RWC) in common beans. Values with different letters differ at $p < 0.05$ (LSD test)**Fig. 2** Effects of salinity and genotype interactions (A), salinity and exogenous SA and SL application interactions (B), on Electrolyte leakage in common beans. Values with different letters differ at $p < 0.05$ (LSD test)

25% in Jules. Exogenous application of SA and GR24, alone or combined, significantly improved growth attributes and relative water content (RWC) compared with salt-stressed controls. The combined SA+GR24 treatment produced the highest RWC in both cultivars, restoring approximately 86% of the control level in Naz and 95% in Jules (Fig. 1B). RWC is the first sign of osmotic pressure disorder, and hormonal treatments modulated this disorder.

3.2 Electrolyte leakage and membrane injury

Salt stress increased EL by $\approx 120\%$ in Naz and 75% in Jules (Fig. 2A). All hormonal treatments reduced EL significantly; the lowest value was recorded in SA + GR24-treated

‘Jules’. This indicates enhanced membrane integrity, as evidenced by reduced oxidative lipid peroxidation. (Fig. 2B).

3.3 Oxidative stress indicators

Under salinity, both MDA and H_2O_2 contents increased sharply, especially in Naz.

Application of SA and GR24 decreased MDA and H_2O_2 by 35–50% relative to salt-only plants.

The combined treatment resulted in the most significant decline in oxidative markers, confirming synergistic mitigation (Fig. 3A–D).

3.4 Photosynthetic pigments

Chlorophyll a, b, total chlorophyll, and carotenoids declined significantly under salt stress.

SA and GR24 treatments restored pigment content; the combination recovered up to 90% of control values, but complete restoration was not achieved (Fig. 4A–D).

3.5 Compatible solutes and protein content

Proline content increased ≈ 3 -fold under salt stress, particularly in Naz.

Hormone treatments decreased proline accumulation, suggesting lower osmotic stress.

Total soluble protein content, which declined under salinity, was significantly restored by SA and GR24 application, with maximum recovery in SA + GR24 (Fig. 5A–D).

3.6 Proline and hydrogen peroxide

Proline content increased substantially under salt stress, particularly in the ‘Naz’ cultivar. Proline increased about threefold, acting as an osmoprotectant. (Fig. 4A). SA and GR24 reduced proline levels under salinity (Fig. 4B). The decrease in proline content in treated plants indicates reduced stress intensity and a lower need for osmotic adjustment. Salt

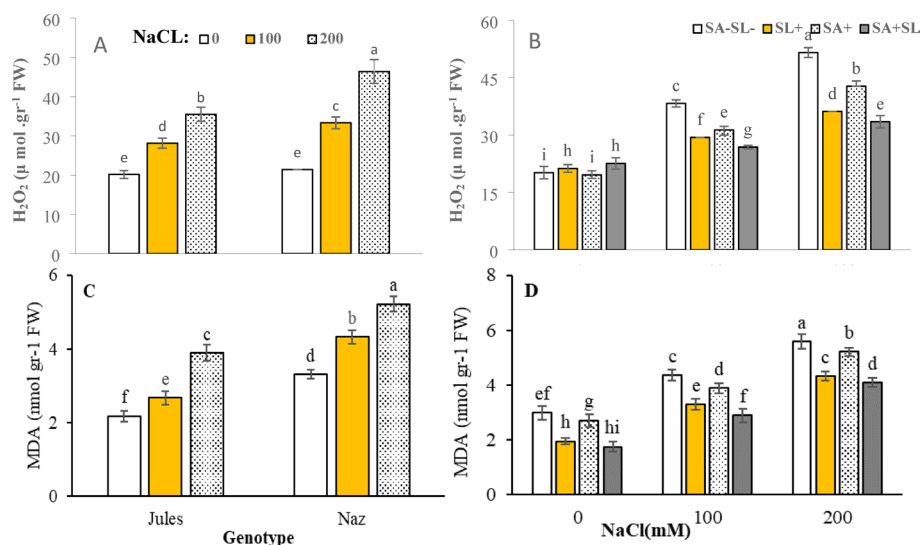


Fig. 3 Effects of salinity and genotype interactions (A, C), salinity and exogenous SA and SL application interactions (B, D) on H_2O_2 contents and lipid peroxidation (MDA) in common beans. A, B: H_2O_2 . C, D: MDA. Values with different letters differ at $p < 0.05$ (LSD test)

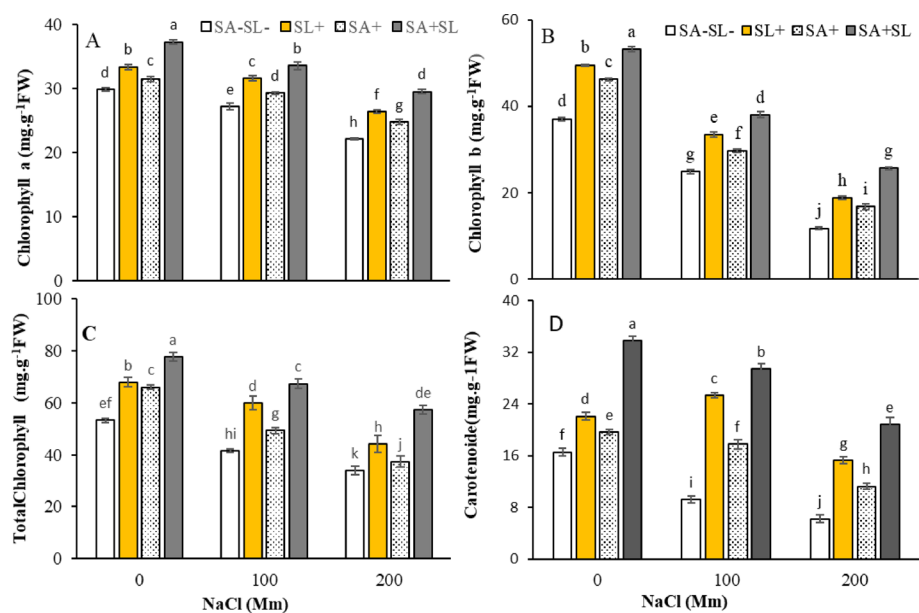


Fig. 4 Effects of salinity and exogenous SA and SL application interactions on photosynthetic pigments in common beans. **A:** chlorophyll a, **B:** chlorophyll b, **C:** total chlorophyll, **D:** carotenoids. Values with different letters differ at $p < 0.05$ (LSD test)

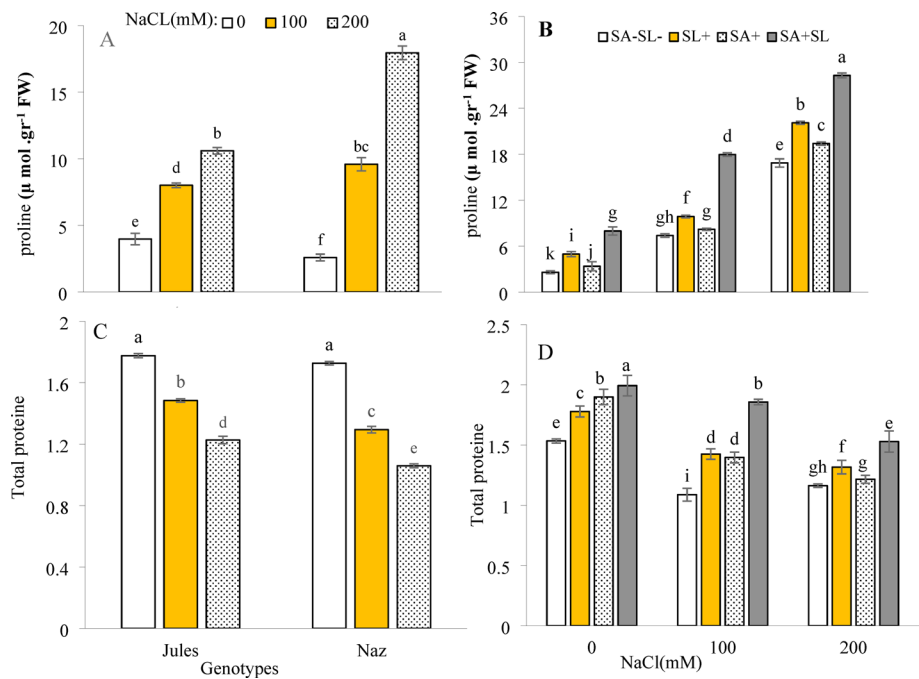


Fig. 5 Effects of salinity and genotype interactions (**A-C**), salinity and exogenous SA and SL application interactions (**B-D**) on Proline content (**A, B**) and total protein (**C, D**) in common beans. Values with different letters differ at $p < 0.05$ (LSD test)

stress increased H_2O_2 accumulation in both cultivars, with the highest levels in 'Naz' (Fig. 4C). SA and GR24—especially SA+GR24—markedly reduced H_2O_2 (Fig. 4D).

3.7 Antioxidant enzyme activities

Salinity increased antioxidant enzyme activities (SOD, CAT, APX, GPX), reflecting stress induction.

SA and GR24 further enhanced these enzymes beyond salt-induced levels, and the combination produced the highest activities across both cultivars. The increase in CAT and APX activity under SA+GR24 was ≈ 1.7 -fold compared with salt-stressed plants. The increase in SOD and CAT activities represents the first line of defense for the elimination of O_2 and H_2O_2 Fig. 6.

3.8 Gene expression profiles of antioxidant enzymes

The relative expression of SOD, CAT, and GPX genes is shown in Fig. 7.

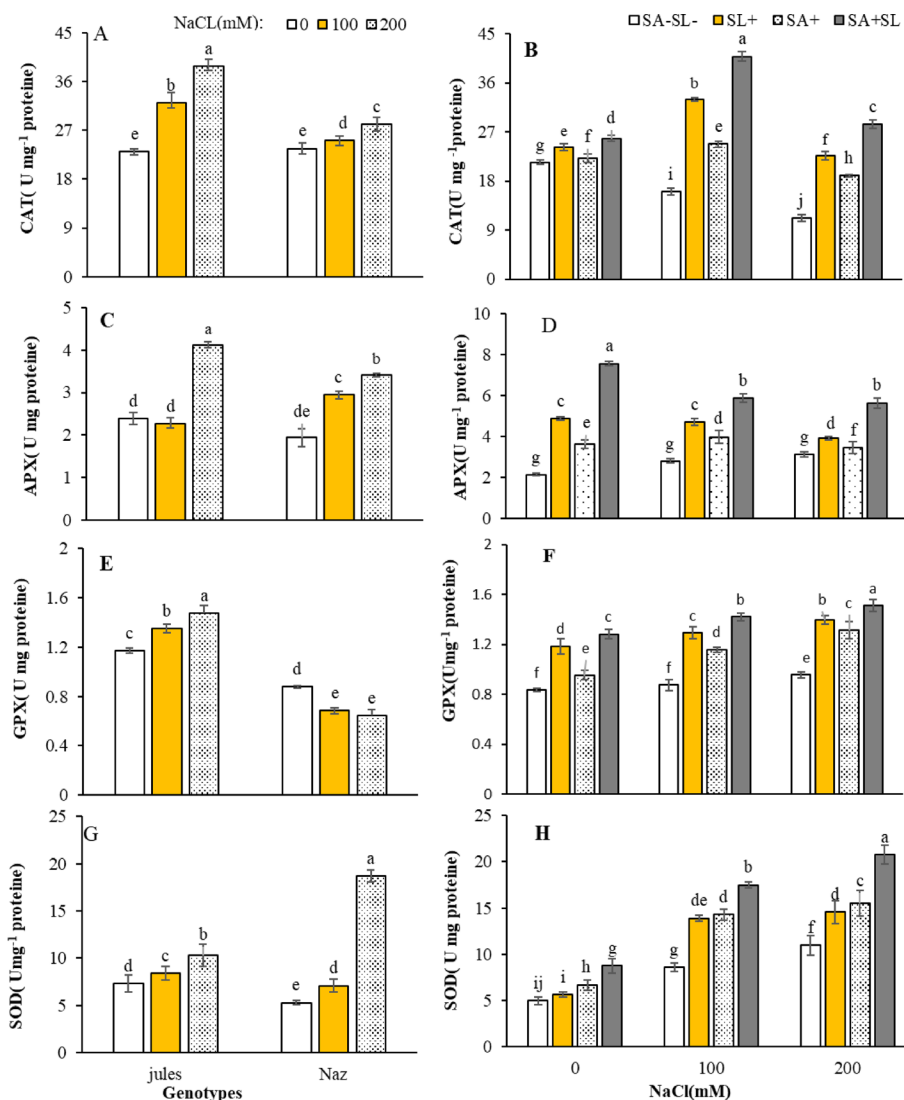


Fig. 6 Effects of salinity and genotype interactions (A, C, E, G), as well as salinity and exogenous SA and SL application interactions (B, D, F, H) on CAT, APX, GPX, and SOD activity in common beans. Panels A and B illustrate common beans. A, B: CAT. C, D: APX. E, F: GPX. G, H: SOD. Values with different letters differ at $p < 0.05$ (LSD test)

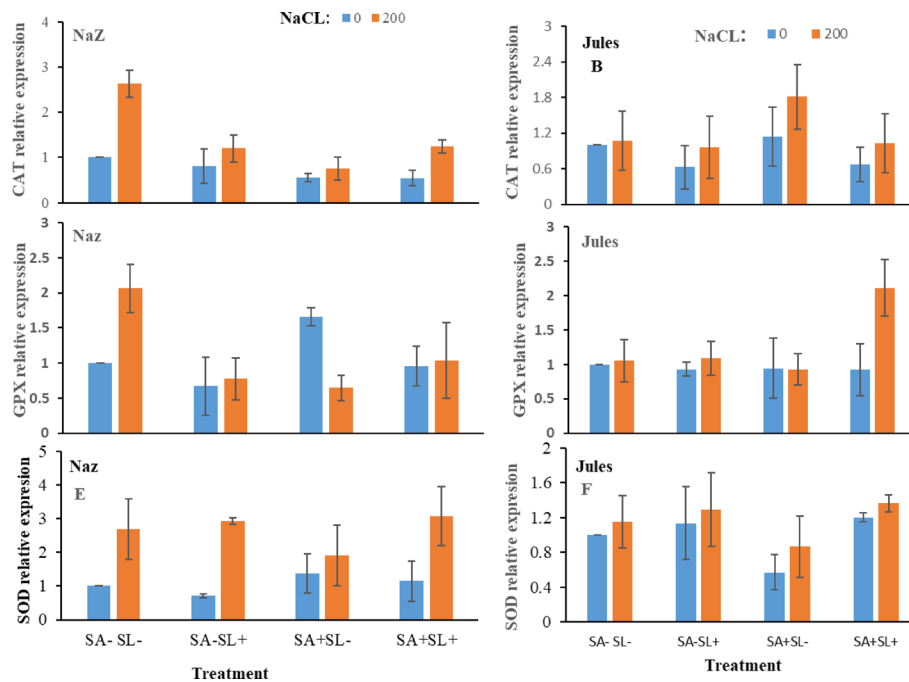


Fig. 7 Effects of salinity and exogenous SA and GR24 interactions on antioxidant gene expression levels in common bean leaves. Relative expression of antioxidant-related genes under salinity and hormone treatments. All figure subpanels (A–F) were corrected for mislabeling and aligned with genes CAT, GPX, and SOD

Salt stress up-regulated all four genes, while SA + GR24 further increased expression, particularly in Jules.

3.9 Pearson correlation analysis

Pearson correlation analysis showed positive relationships between proline, EL, MDA, SOD, H_2O_2 , and EL in leaves (Fig. 9). Notably, EL was strongly linked with H_2O_2 ($r=0.91$), proline ($r=0.81$), MDA ($r=0.81$), and SOD ($r=0.71$). In contrast, EL had strong negative correlations with RWC ($r=-0.89$), chlorophyll b ($r=-0.83$), carotenoids ($r=-0.66$), and protein ($r=-0.93$). Protein content was moderately negatively related to MDA ($r=-0.70$), proline ($r=-0.71$), and EL, but positively associated with RWC, chlorophyll b, and carotenoids. Additionally, a positive relationship was observed between enzyme activity and expression for CAT, APX, SOD, and GPX (Fig. 8).

3.10 Hierarchical clustering analysis

The similarities and differences among all treatments were evaluated using the group average and Euclidean distance index, then presented as two cluster dendrograms for the Naz and Jules cultivars (Fig. 9). Cluster analysis grouped treated and untreated plants based on physicochemical traits and gene expression, with a polar heat map illustrating the relationships between clusters (Fig. 10).

The Naz cultivar formed three distinct clusters (Fig. 9A). Cluster A included unstressed and untreated plants, as well as those treated with SA + SL, characterized by high relative water content (RWC) and moderate levels of chlorophyll b (Chl.b) and carotenoids. Cluster B consisted of stressed, untreated plants with high electrolyte leakage index (ELI) and moderate RWC, along with notable H_2O_2 levels. Cluster C included

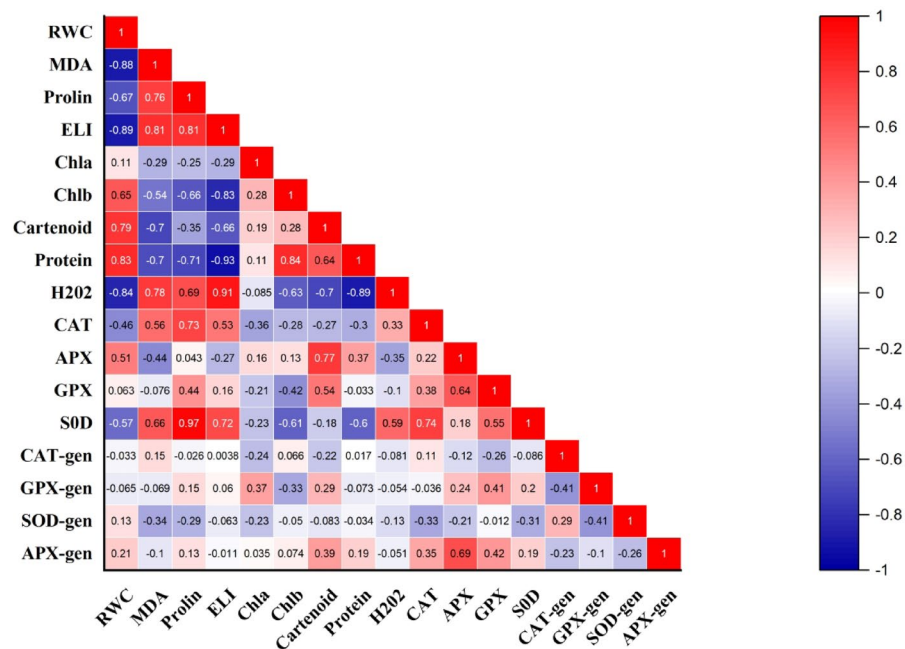


Fig. 8 Correlation matrix showing how different parameters of bean plants are affected by salt stress and foliar spraying of SA and the synthetic form of SL. These parameters include: Chla (Chlorophyll a), Chlb (Chlorophyll b), RWC (Relative water content), EL (Electrolyte leakage), APX (Ascorbate peroxidase), POX (Guaiacol peroxidase), CAT (Catalase), H₂O₂ (Hydrogen peroxide), and MDA (Malondialdehyde)

plants treated with SA + SL and those grown in 200 mM NaCl (T8), which displayed high RWC, moderate H₂O₂, and moderate ELI.

In the Jules cultivar, three distinct clusters emerged (Fig. 9B). Group A consisted of untreated plants and those treated with SA + SL (T2-T4), both exhibiting high relative water content (RWC) and moderate levels of carotenoids and chlorophyll b (Chl. b). Group B included untreated plants grown with 200 mM NaCl, showing moderate RWC and a high electrolyte leakage index (ELI). Group C comprised plants treated with SA + SL in 200 mM NaCl, which displayed moderate RWC and ELI (Fig. 9B).

Hierarchical clustering confirmed genotype-specific responses:

Jules grouped closely with SA + GR24 treatments, while Naz remained distant under salt stress. These results demonstrate that combined SA and GR24 treatments synergistically improve physiological, biochemical, and molecular resilience against salt stress in common bean.

3.11 Principal component analysis

Principal component analysis (Fig. 10) explained 86.4% of the total variance (PC1 = 65.1%, PC2 = 21.3%). Control and SA + GR24 treatments clustered together and were clearly separated from salt-only plants, confirming a strong restorative effect of the combined hormones. The analysis grouped carotenoids, chlorophyll a, APX activity and gene expression, relative water content, and proteins, indicating strong similarities among these traits. In contrast, proline, SOD activity, electrolyte leakage, CAT activity, and GPX gene expression formed a distinct cluster (Fig. 10).

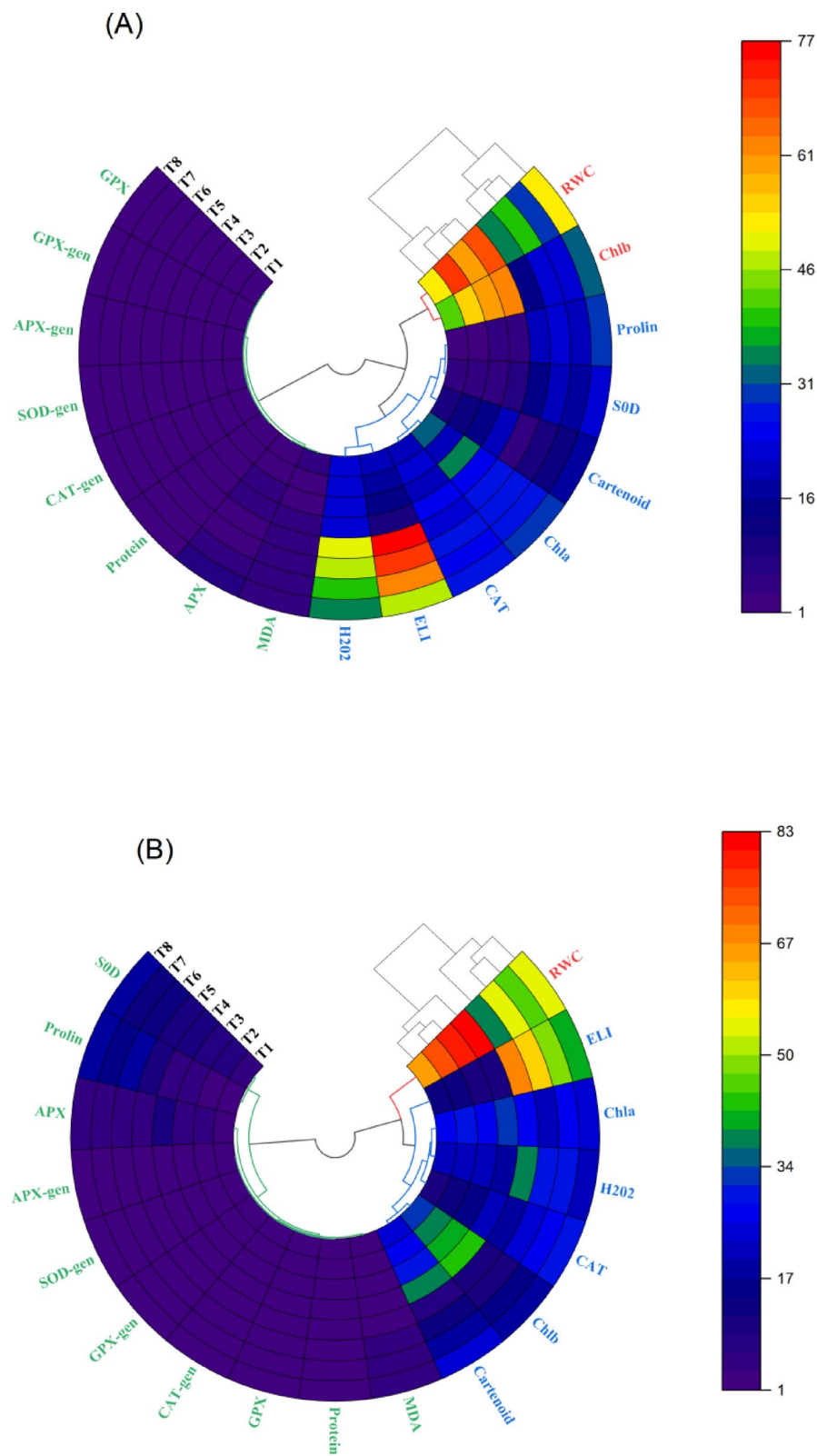


Fig. 9 (See legend on next page.)

(See figure on previous page.)

Fig. 9 A polar heatmap illustrating the grouping of control and treated plants based on yield, growth, and physicochemical traits in the **(A)** salt-sensitive (Naz) cultivar and **(B)** salt-tolerant (Jules) cultivar. T1: NaCl0-SL-SA-, T2: NaCl0-SL-SA+, T3: NaCl0-SL + SA-, T4: NaCl0-SL + SA+, T5: NaCl100-SL-SA-, T6: NaCl100-SL-SA+, T7: NaCl100-SL + SA-, T8: NaCl100-SL + SA+, T9: NaCl200-SL-SA-, T10: NaCl200-SL-SA+, T11: NaCl200-SL + SA-, T12: NaCl200-SL + SA+. Here, Chla: chlorophyll a; Chlb: chlorophyll b; RWC: relative water content; EL: electrolyte leakage; APX: ascorbate peroxidase; GPX: guaiacol peroxidase; CAT: catalase; H₂O₂: hydrogen peroxide; MDA: malondialdehyde

3.12 Proposed mechanistic model

The conceptual model in Fig. 11 summarizes the integrated response of SA and GR24 under salinity.

SA enhances ROS scavenging and signal transduction through the MAPK and NPR1 pathways, while GR24 modulates antioxidant gene expression and stomatal regulation.

The combined signaling network results in balanced ROS levels, improved photosynthetic efficiency, and restored growth.

4 Discussion

Salinity creates complex physiological and biochemical challenges for plants, including osmotic imbalance, ion toxicity, oxidative stress, and impaired photosynthesis [1]. Our results confirmed that salt stress significantly affected both common bean cultivars, decreasing RWC, pigments, and protein content, and increasing EL, MDA, H₂O₂, and proline accumulation. However, exogenous application of SA and GR24, alone or together, effectively reduced these adverse effects.

Salt stress increased EL and MDA due to heightened lipid peroxidation caused by excessive ROS accumulation, a typical response observed under ionic and osmotic stress conditions [19]. The higher EL seen in the 'Naz' cultivar further indicates weak membrane stability and greater susceptibility to oxidative stress.

Proline accumulation under salinity is known to function as an osmoprotectant, stabilizing proteins and membranes while scavenging free radicals [12]. Therefore, higher proline levels in Naz reflect a stronger osmotic imbalance. Interestingly, SA and GR24 reduced proline accumulation under salinity, suggesting that hormonal treatment alleviated stress intensity and reduced the plant's need for osmotic adjustment.

The decline in chlorophyll pigments under salinity aligns with previous research indicating that salt stress hampers chlorophyll biosynthesis, increases chlorophyllase activity, and induces photo-oxidation [20]. Although SA and GR24 significantly boosted pigment content, complete restoration was not observed. This partial recovery may result from irreversible damage to pigment synthesis pathways and ongoing disruptions in thylakoid structure caused by prolonged salt stress [7].

Salt stress led to significant ROS accumulation, as evidenced by elevated H₂O₂ and MDA levels. Plants combat oxidative stress through antioxidant enzymes, including SOD, CAT, APX, and GPX [21] (Hasanuzzaman et al. 2021).

Hormonal treatments further increased these enzymes' activities and boosted their gene expression. SA is known to activate ROS-scavenging pathways via NPR1- and MAPK-mediated signaling [22]. GR24 also increases antioxidant activity, likely through SL-dependent transcriptional regulation and better stomatal function [4]. Therefore, the synergy of SA+GR24 probably results from the combined activation of transcriptional and enzymatic detoxification mechanisms.

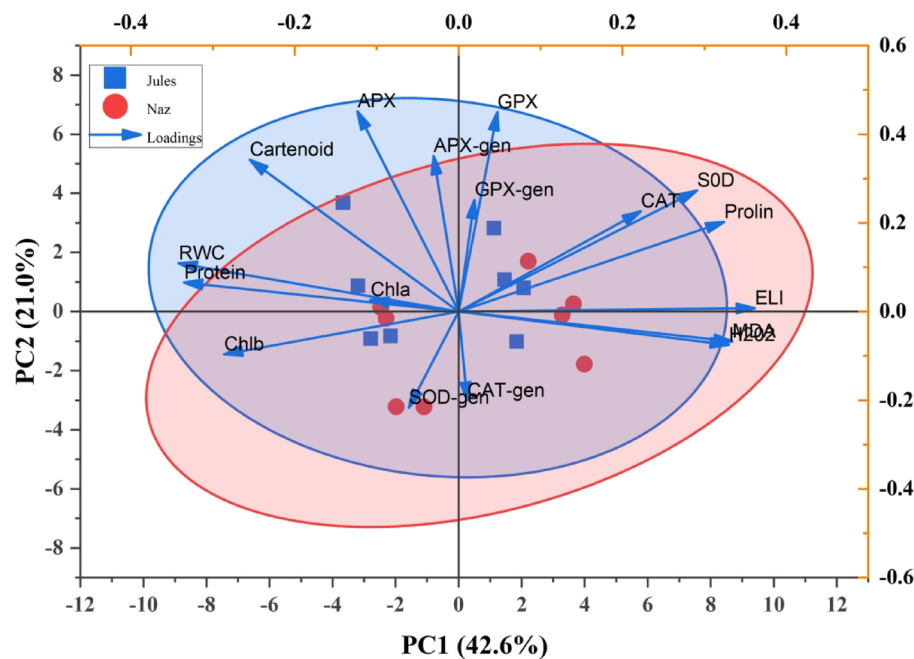


Fig. 10 shows the Principal Component Analysis (PCA) of various bean plant parameters affected by salt stress and foliar spraying of SA and synthetic SL: Chla (Chlorophyll a), Chlb (Chlorophyll b), RWC (Relative water content), EL (Electrolyte leakage), APX (Ascorbate peroxidase), GPX (Guaiacol peroxidase), CAT (Catalase), H_2O_2 (Hydrogen peroxide), and MDA (Malondialdehyde)

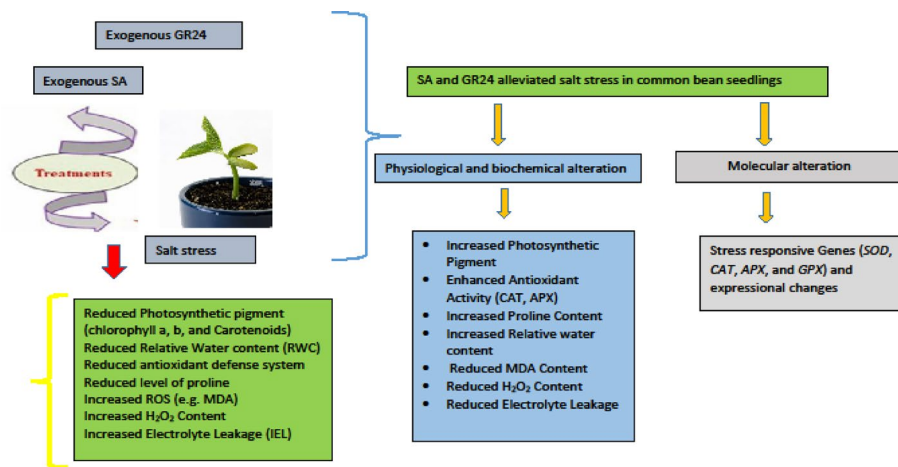


Fig. 11 Proposed mechanistic model illustrating how SA + GR24 interaction pathways mitigate salt stress in common bean

The correlated increases in antioxidant enzyme activity and gene expression levels align with earlier studies in rice, wheat, and *Ziziphus* germplasm, in which exogenous regulators enhanced salt tolerance through coordinated biochemical and molecular responses [5, 7].

Recent studies [2, 6] also indicate that maintaining a balanced ROS level is crucial for increasing crop yield under abiotic stress, consistent with our findings. Hierarchical clustering and PCA confirmed a clear separation among control, stressed, and hormone-treated plants. Salt stress led to significant ROS accumulation, as indicated by elevated H_2O_2 and MDA levels. Plants combat oxidative stress through antioxidant

enzymes, including SOD, CAT, APX, and GPX [23] (Hasanuzzaman et al. 2020). Hormonal treatments further enhanced antioxidant enzyme activities and increased their gene expression.

SA is known to activate ROS-scavenging pathways through NPR1- and MAPK-mediated signaling [22]. GR24 also enhances antioxidant activity, possibly through SL-dependent transcriptional regulation and improved stomatal conductance [24]. Therefore, the synergy of SA+GR24 likely results from complementary activation of transcriptional and enzymatic detoxification pathways. The observed increases in antioxidant enzyme activity and gene expression levels support earlier studies in rice, wheat, and *Ziziphus* germplasm, where exogenous regulators improved salt tolerance through coordinated regulation. Recent studies [2, 6] also suggest that maintaining a more balanced ROS profile is a key factor in improving crop yield under abiotic stress, findings that align with ours. Hierarchical clustering and PCA confirmed the clear separation between control, stressed, and hormone-treated plants.

Overall, the results clearly demonstrate that SA and GR24 mitigate salt stress through:

1. Improved water status (higher RWC).
2. Reduced membrane damage (lower EL, MDA).
3. Lower ROS accumulation (H_2O_2 decrease).
4. Stronger antioxidant defense (enzymatic + gene expression).
5. Improved photosynthetic performance (pigments).
6. Reduced osmotic stress (lower proline).

This work presents the first comprehensive report demonstrating how SA and GR24 interact to affect physiological, biochemical, and molecular parameters in common bean under salinity stress. These findings reveal a detailed, synergistic mitigation mechanism mediated by SA and GR24, offering a promising approach to improve salinity tolerance and increase yield stability in legumes. Such integrated hormonal treatments could serve as a practical method for boosting productivity in saline soils.

5 Conclusions

Salinity negatively affected common bean growth by reducing water content, photosynthetic pigments, and protein levels, while increasing markers of oxidative and osmotic stress (EL, MDA, H_2O_2 , proline). Application of SA and GR24, either alone or together, helped mitigate these harmful effects. Salt stress caused significant damage to the physiological, biochemical, and molecular functions of both bean cultivars, with the salt-sensitive 'Naz' being more affected. External application of salicylic acid (SA) and the strigolactone analogue GR24, either separately or in combination, lessened these impacts. The combined treatment (SA + GR24) was the most effective, enhancing water retention, restoring pigment levels, reducing electrolyte leakage and lipid peroxidation, and increasing antioxidant enzyme activity and gene expression. These findings support recent research highlighting integrated hormonal and biochemical strategies for salinity mitigation [2, 5, 6]. Overall, this study reveals, for the first time, that SA and GR24 work together to regulate antioxidant defenses, osmotic balance, photosynthetic stability, and gene expression in common beans under salt stress. This combined approach offers a promising and effective way to improve salinity tolerance and enhance legume resilience.

Author contributions

M.A.A. original draft writing, investigation, data evaluation, and validation. M.S. original draft writing, conceptualization, and methodology. G.P. Data analyses. B.D. Visualization, review, and final editing. All authors have read and agreed to the published final version of the manuscript.

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Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon request. All results obtained in this study are presented here. The datasets generated and analysed during the current study, including biochemical and enzymatic measurements and quantitative RT-PCR data, have been deposited in the Figshare repository. The files are currently under embargo; however, the data are accessible via the following persistent DOI: [<https://doi.org/10.6084/m9.figshare.31155238>]. The Data Availability section has been added to the revised manuscript, and the information has also been provided in the editorial system.

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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