



Single and Multi-Strain PGPR Enhance Salinity Tolerance in Canola (*Brassica napus* L.) Seedlings

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Abstract

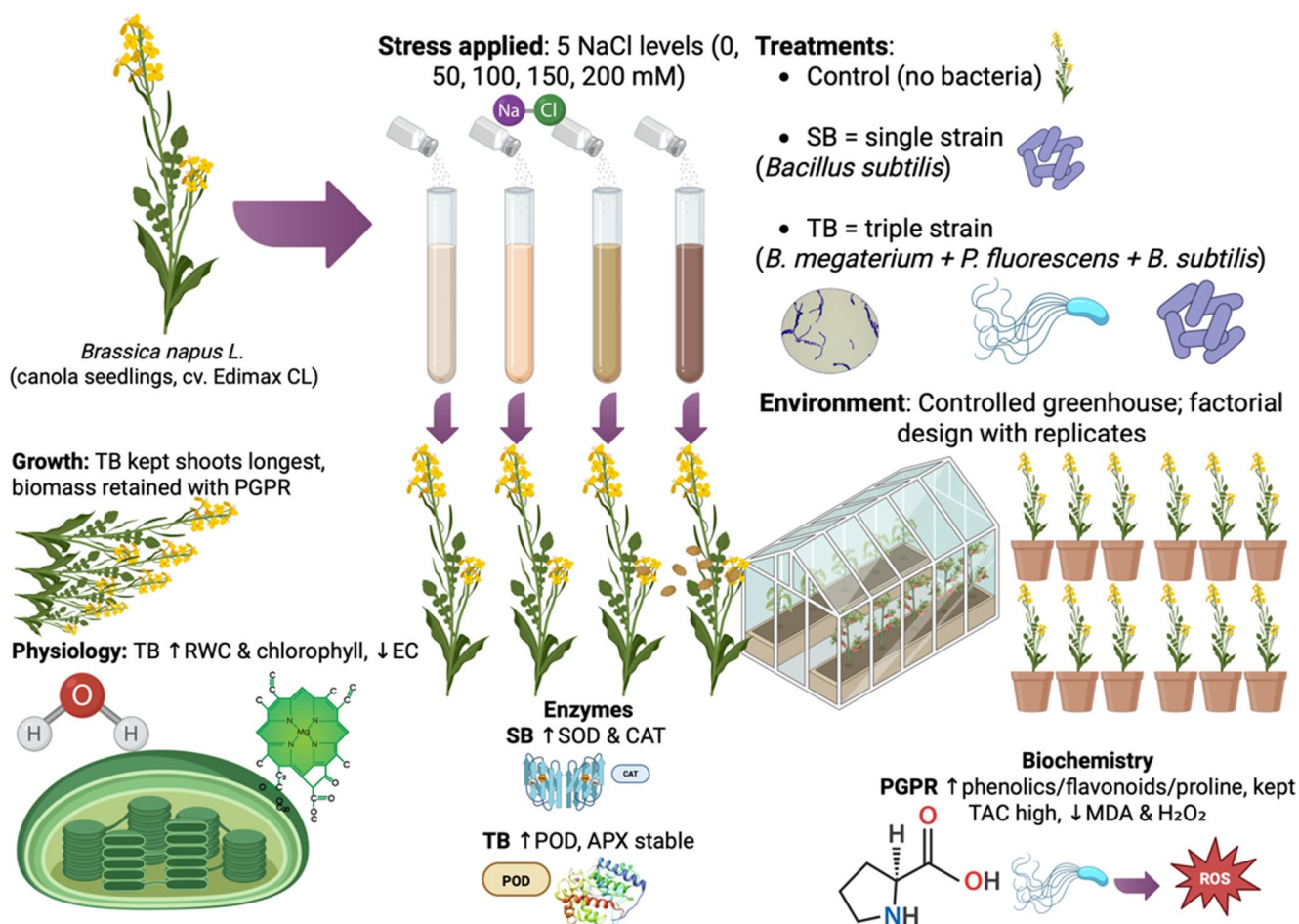
Salinity is a major abiotic stress limiting canola (*Brassica napus* L.) productivity, especially in arid regions. This study aimed to evaluate the efficiency of plant growth-promoting rhizobacteria (PGPR) in enhancing early seedling tolerance to graded sodium chloride (NaCl) stress and to compare single- and multi-strain inoculations. A greenhouse experiment was conducted under NaCl concentrations ranging from 0 to 200 mM using three treatments: uninoculated control, single strain (*Bacillus subtilis*, SB), and a triple-strain consortium (*Bacillus megaterium* + *Pseudomonas fluorescens* + *B. subtilis*, TB). Growth, physiological, and biochemical traits were analysed, including relative water content (RWC), electrolyte leakage (EC), photosynthetic pigments, phenolic compounds, oxidative stress markers, and antioxidant enzyme activities such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX). Salinity significantly reduced relative water content (RWC) from 60.10% to 48.63% and increased electrolyte leakage (EC) from 62.79% to 77.95%. TB inoculation mitigated these effects by maintaining higher RWC (53.57%) and lower EC (66.40%) than the control (71.93%). TB-treated plants showed higher chlorophyll (0.84 mg g⁻¹ FW), phenolics (10.34 mg GAE g⁻¹ DW), flavonoids (4.10 mg QE g⁻¹ DW), antioxidant capacity (85.98%), and proline (122.36 mg g⁻¹ FW) compared to controls. Oxidative damage decreased, with hydrogen peroxide (H₂O₂) and malondialdehyde (MDA) reduced to 14.01 μmol g⁻¹ FW and 60.22 nmol g⁻¹ FW, respectively. Enzymatic responses differed: SB enhanced SOD and CAT under moderate salinity, while TB increased POD and APX activities under mild stress. PGPR inoculation, particularly multi-strain consortia, alleviates salt-induced oxidative stress and improves osmotic adjustment and antioxidant defense in canola seedlings, offering an eco-friendly strategy for sustainable production in saline soils.

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Graphical Abstract



Highlights

- Salt stress reduced growth, biomass, and photosynthetic pigments in canola seedlings.
- PGPR inoculation improved shoot and root biomass under saline conditions.
- Bacterial priming enhanced water status, membrane stability, and pigment retention.
- Phenolics, flavonoids, proline, and antioxidant capacity were elevated by PGPR.
- Multi-strain PGPR consortia provided stronger stress tolerance than single strains.

Keywords *Brassica napus* L. · Plant growth-promoting rhizobacteria (PGPR) · Salinity stress · Antioxidant defense · Multistrain consortium; oxidative stress markers

1 Introduction

Brassica napus L. (canola) is recognized as the third most important oilseed crop worldwide after soybean and palm oil, accounting for approximately 13–16% of global vegetable oil production (Moreno, 2025). With its high economic value in both human nutrition and biodiesel production, canola has become increasingly important due to its favorable oil profile—rich in unsaturated fatty acids and low in saturated fats (Raboanatahiry et al., 2021). In recent years,

canola cultivation has expanded into wider geographic regions, including arid and semi-arid areas, owing to its adaptability and economic significance (Rathore et al., 2022). However, this expansion is increasingly constrained by widespread soil salinity, which affects nearly 20% of the world's arable lands (Singh 2021).

Soil salinity is one of the most critical abiotic stress factors threatening agricultural productivity, particularly in arid and semi-arid regions. It induces osmotic stress and ion toxicity, thereby disrupting cellular homeostasis, reducing

photosynthetic efficiency, limiting nutrient uptake, and triggering oxidative stress in plants (Khoza et al., 2025; Kumari et al., 2024). Consequently, there is a growing need for effective and environmentally friendly biological strategies to mitigate the adverse effects of salinity, especially during the seedling stage. In Türkiye, salinity has become an increasingly serious problem in the Central Anatolian Region, where canola has high production potential, primarily due to natural salinization processes and inadequate irrigation management. Therefore, minimizing the detrimental effects of salinity at early growth stages is crucial to maintaining yield stability and ensuring sustainable production under saline conditions (İrik and Bıkmaz, 2024).

Plants have developed several adaptive mechanisms to cope with salinity stress, including osmotic adjustment, ion compartmentalization, enhanced antioxidant defense, and modulation of growth regulators (Zaid et al. 2022). However, these mechanisms are often insufficient under prolonged or severe salinity, particularly in salt-sensitive species such as canola (Singhal et al., 2021). In this regard, Plant Growth-Promoting Rhizobacteria (PGPR) have emerged as a sustainable and eco-friendly biotechnological approach to enhance plant tolerance to abiotic stresses, as they employ beneficial microbial strains as biological tools that modulate plant physiological, biochemical, and molecular responses. PGPR colonization in the rhizosphere supports plant growth through various mechanisms, such as the production of indole-3-acetic acid (IAA), gibberellins, and cytokinins, nutrient solubilization, nitrogen fixation, and pathogen suppression. Under saline conditions, PGPR can further improve root architecture, enhance water use efficiency, and stimulate proline accumulation, thus maintaining metabolic balance in plants (Sharma et al., 2025).

The application of multiple PGPR strains—either singly or in microbial consortia—to improve stress tolerance can produce additive or synergistic effects through the integration of complementary genetic and metabolic traits. Recent studies have highlighted that PGPR consortia enhance stress tolerance synergistically via diverse functions such as IAA production, ACC deaminase activity, and siderophore biosynthesis (Choudhury et al. 2021; Khan et al. 2022). Although single-strain inoculations offer benefits, multi-strain combinations generally result in greater improvements in plant growth, antioxidant defense, water balance, and metabolite accumulation (Krishna et al. 2022; Saleem et al. 2021). Moreover, consortia can stabilize the root microbiota and regulate hormonal balance, thereby optimizing plant stress responses (Saleem et al. 2021).

Several studies have investigated the use of PGPR to enhance salt tolerance in canola. However, most previous works have focused on single or dual inoculations, mainly

involving *Enterobacter cloacae*, *Azotobacter chroococcum*, *Pseudomonas putida*, *Bacillus subtilis*, or *Pseudomonas fluorescens*, and have primarily evaluated responses in mature plants or based on general growth parameters (Abdel Latef et al. 2021; Lally et al. 2017; Li et al. 2017; Saghafi et al. 2018). This indicates a significant knowledge gap regarding the synergistic interactions of diverse PGPR species and their impact on physiological and biochemical adaptations during early seedling development.

The bacterial strains used in this study were selected based on their proven ability to alleviate salt stress and their complementary physiological traits. *Bacillus subtilis* is known to protect plants from osmotic and salt stress by activating antioxidant defense systems and regulating osmoregulation (Hasanuzzaman et al. 2022). *B. megaterium* enhances physiological tolerance by promoting proline accumulation, increasing antioxidant enzyme activities, and improving root development, photosynthesis, and water balance through hormone-regulatory mechanisms (Nascimento et al. 2020). *Pseudomonas fluorescens*, through ACC deaminase activity and nitrogen fixation, mitigates oxidative damage and modulates stress-related hormones, thereby enhancing root and shoot growth under saline conditions (Sofy et al. 2021). Owing to their complementary mechanisms, multi-strain inoculation is expected to elicit a more robust physiological and biochemical response in canola under salt stress.

This study was therefore designed to evaluate the role of single and multi-strain PGPR inoculations—comprising *B. megaterium*, *Pseudomonas fluorescens*, and *B. subtilis*—in enhancing the salt stress tolerance of *Brassica napus* seedlings. The experiment compared their effects on morphological, physiological, and biochemical traits under graded NaCl conditions in a controlled greenhouse environment.

It was hypothesized that PGPR inoculation, particularly in multi-strain consortia, would effectively mitigate salinity-induced stress by improving water relations, maintaining membrane integrity, stabilizing photosynthetic pigments, and stimulating antioxidant formation. Accordingly, the study aimed to: (i) assess the influence of PGPR on seedling growth under varying salinity levels; (ii) determine physiological responses associated with water homeostasis, photochemistry, and membrane stability; and (iii) evaluate the effects of PGPR on secondary metabolite production, antioxidant activity, and membrane lipid peroxidation.

Overall, the findings are expected to provide new insights into the mechanisms of PGPR-mediated salt tolerance and contribute to the development of microbial-based sustainable production systems for saline agricultural environments.

2 Materials and methods

2.1 Plant Material and Growth Substrate

The winter canola (*Brassica napus* L.) cultivar “Edimax CL” was selected due to its early maturity, high adaptability to Central Anatolian agroecological conditions, and known sensitivity to salt stress, making it a suitable model species for evaluating the mitigating effects of PGPR under salinity. Certified seeds were obtained from a commercial supplier and stored at 4 °C until sowing. The growth substrate consisted of a sterilized mixture of soil, peat, and perlite (1:1:1, v/v/v). In this study, the term ‘substrate’ specifically refers to this sterilized soil–peat–perlite mixture used as the growth medium for seedling establishment. Pots (5 kg capacity) were filled with the substrate and randomly placed on greenhouse benches to ensure uniform light and ventilation. Plants were maintained under controlled greenhouse conditions at 25 ± 3 °C temperature, $60 \pm 10\%$ relative humidity, and a natural photoperiod of approximately 12–14 h ($250\text{--}350 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR). Seedlings were irrigated with deionized water to maintain field capacity until salt stress treatments were initiated. Deionized water was specifically used to prevent any interference from mineral ions present in tap water, and no fertilizer or additional nutrient solution was applied to isolate the effects of bacterial inoculation and salinity treatments. Physiological and biochemical measurements were conducted after a four-week salinity exposure period, corresponding to approximately six weeks after sowing.

2.2 Bacterial Strains and Inoculum Preparation

Two inoculation treatments were used: a single-strain treatment (SB, *Bacillus subtilis*) and a triple-strain consortium (TB, *Bacillus megaterium* + *Pseudomonas fluorescens* + *Bacillus subtilis*). All isolates were obtained from the GÜBRETAS (Gübre Fabrikaları Türk A.Ş., Türkiye) culture collection and had been previously characterized for Gram reaction, phosphate solubilization, growth on nitrogen-free medium, and oxidase/catalase activities. *B. subtilis* is recognized for its ability to fix atmospheric nitrogen, whereas *B. megaterium* is notable for its phosphate-solubilizing capacity and its role in enhancing root development (Elkoca et al. 2010). In contrast, *P. fluorescens* is characterized by its rapid rhizosphere colonization and its ability to induce indole-3-acetic acid (IAA) production (Sivasakthi et al. 2014). For the single-strain treatment, *B. subtilis* was selected because it is one of the best-characterized PGPR species, exhibiting strong salt tolerance, efficient root colonization, and the ability to produce growth-promoting metabolites such as IAA, ACC deaminase, and exopolysaccharides, making

it a reliable model strain for salinity studies. Each strain was first revived from stock cultures on Plate Count Agar (PCA) and incubated at 27 ± 1 °C for 24 h. A single colony was transferred into 50 mL nutrient broth (NB) and shaken at 150 rpm for 48 h until mid-logarithmic phase ($\text{OD}_{600} \approx 0.8\text{--}1.0$). The resulting bacterial cultures were adjusted to 5×10^8 CFU (colony-forming units) mL^{-1} in sterile 10% sucrose solution, based on OD_{600} readings and confirmed by viable plate counts, following the method of Çakmakçı et al. (2023). For the consortium inoculum (TB), equal volumes of each suspension were mixed at a 1:1:1 ratio immediately before use to maintain viable cell balance.

Surface-sterilized seeds were immersed in the respective bacterial suspensions at a ratio of 2 mL inoculum per 100 seeds and incubated on a rotary shaker (100 rpm, 24 h, 25 °C). For the uninoculated control, seeds were soaked in sterile 10% sucrose solution—the same carrier medium used for inoculated treatments—to ensure experimental consistency. After sowing, 10 mL of the same bacterial suspension (or sterile sucrose solution for control) was applied evenly to the substrate surface of each pot. A second drench (10 mL pot^{-1}) was supplied at the two-leaf stage to reinforce rhizosphere colonization. The inoculation density and procedure were standardized across all treatments to ensure reproducibility. No additional carrier materials or surfactants were used.

2.3 Seed Sterilization, Inoculation, and Salt Stress Application

Seeds were surface-sterilized in 5% NaOCl (10 min), rinsed twice with sterile water, and air-dried under laminar flow (Tanur and Yorgancılar 2020). Salt stress was applied after the three-leaf stage. To avoid osmotic shock, NaCl was supplied gradually (starting at 25 mM, increased every three days) until the target concentrations were reached after 14 days. Thereafter, pots were irrigated twice weekly with the respective NaCl solutions, with periodic leaching to prevent salt buildup. Stress exposure lasted four weeks, after which plants showing clear stress symptoms (particularly at higher salinity levels) were harvested.

2.4 Experimental Site, Design, and Treatments

The experiment was conducted in the greenhouse of Selçuk University, Faculty of Agriculture (Konya, Türkiye), under controlled environmental conditions of 25 ± 3 °C temperature, $60 \pm 10\%$ relative humidity, and natural daylight (approximately 12–14 h photoperiod). Plants were irrigated with deionized water until salinity treatments began. The experiment followed a two-factor factorial arrangement in a split-plot design with four replicates (blocks) to minimize

random error. Main plots consisted of five NaCl concentrations: 0 mM (S_0 , control), 50 mM (S_1), 100 mM (S_2), 150 mM (S_3), and 200 mM (S_4). Subplots included three bacterial inoculation levels: uninoculated control (C), SB (*Bacillus subtilis*, single strain), and TB (*Bacillus megaterium* + *Pseudomonas fluorescens* + *Bacillus subtilis*, triple consortium). Each factorial combination corresponded to one pot (5 kg substrate capacity) containing six seeds, which were later thinned to five uniform seedlings per treatment. Each combination was replicated four times, resulting in a total of 60 pots [5 salinity levels $\times$ 3 inoculation treatments $\times$ 4 replicates]. Pots were randomly distributed within each block and rotated weekly on greenhouse benches to ensure homogeneous light exposure and ventilation. Salinity stress was applied when seedlings reached the three-leaf stage by gradually increasing NaCl concentration in the irrigation water (increments of 25 mM every three days) until the target levels were reached after 14 days. Thereafter, pots were irrigated twice weekly ($\sim$ 200 mL per irrigation) with the respective NaCl solutions, and leachate electrical conductivity (EC, %) was monitored weekly to ensure uniform salinity levels and to prevent salt accumulation. Stress exposure lasted four weeks, after which plants were harvested for morphological, physiological, and biochemical analyses.

2.5 Measurement of Growth Parameters

Clear and unified seedlings per container were sampled and analyzed inclusively at the beginning of the harvest phase. The root length (RL) was measured by tracing the major root tip-to-the base of the hypocotyl with the help of a calibrated ruler, whereas the shoot length (SL) was measured by extending the measure axis to the soil surface by the apical meristem. The roots fresh weight (RFW) and shoots fresh weight (SFW) were instantaneously weighed in the precision balance, and the obtained specimens were further oven-dried at 70 °C (constant mass) at 48 h, which allowed calculating the root dry weight (RDW) and shoot dry weight (SDW).

2.6 Physiological Measurements

In situ measurement of the chlorophyll content (CC, SPAD) was carried out using Minolta SPAD-502 chlorophyll meter (Konica Minolta, Japan). The youngest fully expanded leaf was used in the determination and three readings per plant taken, then the mean value of each pot used as the final reading. The relative water content (RWC, %) was calculated using the procedure by Arora et al. (2002); discs excised from each plant were weighed for fresh weight (FW), incubated in distilled water overnight, weighed again for turgid weight (TW), dried at 65 °C during 48 h to measure dry

weight (DW). The RWC was determined using the following formula:

$$\text{RWC (\%)} = (\text{FW} - \text{DW}) / (\text{TW} - \text{DW}) \times 100 \quad (1)$$

Leakage of electrolytes provided characterization of membrane permeability. Twenty leaf discs (diameter 1 cm) were placed in 10 mL deionized water and incubated at 24 °C overnight after which the initial conductivity (EC_1) was determined. Samples were then autoclaved at 120 °C under 20 min to release total electrolytes and final conductivity (EC_2) measured. Membrane permeability was expressed as $EC_1/EC_2 \times 100$ (Lutts et al. 1996).

Fresh leaf tissue was homogenized in 80% acetone and 0.1 g used to extract pigments. Centrifuging (12 000 g, 15 min) of the homogenate allowed the measurement of supernatant absorbance at wavelengths 663 nm and 645 nm to obtain the values of chlorophyll a (Abs 663), b (Abs 645) and total chlorophyll (Abs 663 + Abs 645), and 480 nm to determine carotenoids (Abs 480). Standard equations were used to compute the concentrations (Lichtenthaler, 1987).

2.7 Biochemical Assays

The Folin -Ciocalteu reagent was used as the method analyzing total phenolic content (TPC), reported in mg gallic acid equivalents (GAE) per g dry weight with 760 nm absorbance as endpoint Sarker and Oba (2019). The total flavonoid content (TFC) was determined by the aluminum chloride colorimetric assay and represented as mg quercetin equivalents (QE) per g of dry weight at 415 nm Singleton et al. (1999). The total antioxidant capacity (TAC) was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging technique, where absorbance was set on 517 nm and reported as percent inhibition comparing to the control (Blois 1958). The membrane damage was assessed by lipid peroxidation-malondialdehyde (MDA) content as a measure of thiobarbituric acid (TBA) reaction 0.5% TBA was added to plant homogenates in 20% trichloroacetic acid, boiled 20 min, 5 min centrifugation at 15,000 g, and the absorbance was measured at 532 and 600 nm. An extinction coefficient of 155 mM⁻¹ cm⁻¹ was employed in calculating the MDA concentration (Sreenivasulu et al. 1999). Proline content was determined according to the method described by Praveen Kumar et al. (2012). Fresh tissue samples were homogenized in the appropriate solution to prepare the reaction mixture. Toluene was used as a blank, and absorbance values were recorded at 520 nm. The obtained results were calculated using a standard curve and expressed as mg g⁻¹ fresh weight (FW). The hydrogen peroxide (H₂O₂) content was measured following the procedure reported by Velikova et al. (2000). Fresh leaf and

root samples were homogenized in 0.1% trichloroacetic acid (TCA) and centrifuged. The resulting supernatant was reacted with 1 M potassium iodide (KI) and 10 mM potassium phosphate buffer (pH 7.0). Absorbance was measured at 390 nm, and H_2O_2 concentration was calculated from a standard curve. Results were expressed as $\mu\text{mol g}^{-1}$ FW.

2.8 Antioxidant Enzyme Assays

The activities of key antioxidant enzymes, including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX), were determined using established spectrophotometric methods with slight modifications. SOD activity was assayed according to Beauchamp and Fridovich (1971) based on its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). Absorbance was recorded at 560 nm, and one unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT reduction. Results were expressed as units per gram of fresh weight (U mg^{-1} FW). POD activity was measured following the method of Chance and Maehly (1955). Enzyme activity was determined by monitoring the increase in absorbance at 420 nm due to guaiacol oxidation in the presence of hydrogen peroxide. The activity was expressed as units per gram of fresh weight (U mg^{-1} protein). CAT activity was determined according to Aebi (1974) by monitoring the decomposition of hydrogen peroxide. Absorbance was recorded at 240 nm for one minute, and enzyme activity was calculated using the extinction coefficient of H_2O_2 . The results were expressed as units per gram of fresh weight ($\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein). APX activity was assayed using the method of Nakano and Asada (1987). The reaction mixture contained ascorbate as the electron donor, and the decrease in absorbance at 290 nm was monitored for 30 s. APX activity was expressed as units per minute per gram of fresh weight ($\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein).

2.9 Statistical Analysis

Data were analyzed according to a two-factor factorial split-plot design, where salinity levels represented the main-plot factor and PGPR inoculation treatments were the subplot factor. Each pot served as one experimental unit, and the mean of five seedlings per pot was used in statistical evaluation. The effects of salinity, inoculation, and their interaction were tested using two-way ANOVA. Prior to analysis, the assumptions of normality (Shapiro–Wilk test) and homogeneity of variances (Levene's test) were examined to confirm model adequacy. Mean comparisons were performed using Tukey's multiple range test ($P \leq 0.05$) in GraphPad Prism

v10, and results were expressed as mean $\pm$ standard error (SE). To ensure consistency, Tukey's test was also applied to the same dataset in JMP Pro 17 (SAS Institute, USA). Multivariate analyses, including heatmap visualization, principal component analysis (PCA), and correlation matrix analysis, were conducted in RStudio (version 2024.12.1) using the *heatmaply*, *ggbiplot*, and *corrplot* packages. All figures were generated in GraphPad Prism to produce publication-quality visual outputs.

3 Results

3.1 Effects on Growth Parameters

Salt stress markedly restricted the morphological development of canola seedlings, and this response was significantly influenced by bacterial inoculation (Supplementary Table 1). Shoot length decreased progressively with increasing NaCl concentrations ($P \leq 0.01$), whereas bacterial inoculation alleviated this reduction. Under non-saline conditions (S0), the highest shoot length was recorded in the triple-strain inoculation (TB, 38.0 cm), followed by the single-strain treatment (SB, 30.25 cm) and the uninoculated control (31.0 cm). Although the salt $\times$ bacteria interaction for root length was not significant, TB-inoculated plants consistently developed longer roots than the control across all salinity levels.

Both shoot and root fresh and dry weights declined with increasing salinity ($P \leq 0.01$), but these reductions were substantially mitigated by bacterial inoculation. A significant salt $\times$ bacteria interaction for shoot dry weight ($P \leq 0.05$) indicated that bacterial treatments contributed to improved biomass retention under stress. TB-treated plants exhibited higher shoot dry weights ($15.95 \text{ g plant}^{-1}$) compared with SB ($15.58 \text{ g plant}^{-1}$) and the control ($17.82 \text{ g plant}^{-1}$) at lower salinity levels, while under higher salinity (S3–S4), all treatments showed reduced biomass, though TB maintained relatively higher values.

Similar trends were observed for root biomass. Root fresh weight was highest at low-to-moderate salinity, with TB showing the greatest mean value ($17.14 \text{ g plant}^{-1}$) compared to SB ($15.93 \text{ g plant}^{-1}$) and the control ($16.68 \text{ g plant}^{-1}$). Root dry weight followed a comparable pattern, with TB ($4.27 \text{ g plant}^{-1}$) slightly exceeding SB ($4.06 \text{ g plant}^{-1}$) and the control ($4.14 \text{ g plant}^{-1}$). Overall, the triple bacterial consortium (TB) was more effective than the single-strain (SB) and control treatments in maintaining shoot and root biomass, demonstrating that multi-strain inoculation exerts a synergistic effect in mitigating salt-induced growth reduction in canola seedlings.

3.2 Effects on Physiological Parameters

Salt stress exerted significant effects on water relations, membrane stability, and photosynthetic pigment contents in canola seedlings (Table 1). Relative water content (RWC, %) progressively declined with increasing NaCl concentrations, decreasing from 60.10% at S0 to 48.63% at S4. Among bacterial treatments, SB recorded the highest mean RWC (54.92%), indicating a modest improvement in water retention compared with the control. Electrolyte leakage (EC, %) increased markedly under salinity, confirming membrane destabilization. The lowest EC was observed at S0 (62.79%) and the highest under S3 (77.95%). TB inoculation maintained relatively greater membrane stability (66.40%) than the control (71.93%), suggesting that bacterial inoculation alleviated salt-induced membrane damage. Photosynthetic pigments, including SPAD values, chlorophyll a, b, carotenoids, and total chlorophyll, were all significantly affected by salinity. TB-inoculated plants maintained higher SPAD

values (47.96) than the control (45.26) and preserved higher chlorophyll and carotenoid contents, particularly under moderate salinity (S2–S3). Overall, PGPR inoculation mitigated the negative effects of salinity mainly by improving membrane stability and maintaining photosynthetic pigments, while its influence on RWC was relatively limited.

3.3 Pearson Correlation Analysis of Growth Parameters

Pearson correlation analysis revealed strong associations among growth, physiological, and pigment-related traits of canola seedlings under salinity (Fig. 1). Root length (RL) was positively correlated with root fresh (RFW) and dry weight (RDW), while shoot fresh (SFW) and dry weights (SDW) showed a strong positive relationship with relative water content (RWC). In contrast, all growth-related parameters (RL, RFW, RDW, SFW, SDW) exhibited significant negative correlations

Table 1 Responses of water relations, electrolyte leakage, and photosynthetic pigment parameters of Canola (*Brassica Napus* L.) seedlings under salinity and plant growth-promoting rhizobacteria (PGPR) inoculation

NaCl (mM)	Treatment	RWC (%)	EC (%)	CC (SPAD)	Chl a (mg g ⁻¹ FW)	Chl b (mg g ⁻¹ FW)	Car. (mg g ⁻¹ FW)	Total Chl (a + b) (mg g ⁻¹ FW)
S0 (Control)	C	59.38±1.15	44.56±2.12 ^b	42.51±0.96 ^c	0.35±0.03	0.13±0.01	1.42±0.12	0.43±0.04
	TB	60.25±0.57	75.21±0.77 ^{ab}	44.23±0.2 ^{ce}	0.31±0.03	0.14±0.02	1.47±0.17	0.41±0.04
	SB	60.68±2.01	68.60±2.47 ^{ab}	42.59±0.45 ^c	0.26±0.04	0.11±0.02	1.26±0.18	0.34±0.05
	S0 Mean	60.10^A	62.79	43.11^B	0.31^C	0.13^B	1.38^B	0.39^B
S1	C	56.54±0.88	67.86±3.89 ^{ab}	43.83±0.61 ^{de}	0.56±0.03	0.22±0.01	2.17±0.09	0.71±0.04
	TB	56.78±0.45	67.63±1.23 ^{ab}	48.21±0.33 ^{ac}	0.47±0.05	0.19±0.02	1.82±0.17	0.6±0.07
	SB	52.12±0.36	72.60±2.56 ^{ab}	48.19±0.61 ^{ac}	0.54±0.06	0.17±0.02	1.79±0.19	0.65±0.08
	S1 Mean	55.15^{AB}	69.36	46.74^{AB}	0.52^B	0.19^{AB}	1.93^{AB}	0.65^A
S2	C	51.11±0.95	74.75±4.31 ^{ab}	47.58±0.21 ^{bd}	0.53±0.03	0.17±0.01	1.77±0.11	0.64±0.03
	TB	49.66±0.07	64.24±1.66 ^{ab}	47.16±0.45 ^{bd}	0.48±0.02	0.17±0.01	1.72±0.1	0.58±0.03
	SB	50.0±0.47	70.65±4.4 ^{ab}	46.87±0.75 ^{bd}	0.67±0.02	0.21±0.01	2.03±0.07	0.8±0.03
	S2 Mean	50.26^B	69.88	47.20^{AB}	0.56^{AB}	0.18^{AB}	1.84^{AB}	0.67^A
S3	C	49.12±0.46	82.74±1.84 ^a	46.63±0.86 ^{bc}	0.78±0.05	0.23±0.01	2.18±0.12	0.91±0.05
	TB	53.47±0.51	70.65±1.95 ^{ab}	51.92±0.84 ^a	0.66±0.01	0.22±0.01	2.31±0.08	0.75±0.02
	SB	63.2±6.14	80.46±3.44 ^a	50.65±0.67 ^{ab}	0.72±0.03	0.23±0.01	2.39±0.13	0.87±0.04
	S3 Mean	55.26^{AB}	77.95	49.73^A	0.72^A	0.23^A	2.29^A	0.84^A
S4	C	49.61±0.37	89.79±1.59 ^a	45.76±0.4 ^{ce}	0.61±0.03	0.19±0.01	1.89±0.09	0.73±0.03
	TB	47.68±0.87	54.28±5.82 ^{ab}	48.30±0.41 ^{ac}	0.57±0.02	0.21±0.01	2.12±0.11	0.74±0.03
	SB	48.61±0.99	56.50±6.59 ^{ab}	45.38±0.13 ^{ce}	0.61±0.03	0.19±0.0	2.01±0.08	0.72±0.03
	S4 Mean	48.63^B	66.86	46.48^{AB}	0.60^{AB}	0.20^{AB}	2.01^{AB}	0.73^A
TrtMean	C	53.15	71.93	45.26 ^B	0.57	0.19	1.89	0.68
	TB	53.57	66.4	47.96 ^A	0.49	0.19	1.89	0.61
	SB	54.92	69.76	46.74 ^{AB}	0.56	0.18	1.9	0.67
	S**	S: ns, Trt: ns;	S: ns, Trt: ns;	S: ** Trt: **;	S: ** Trt: ns;	S: ** Trt: ns;	S: ** Trt: ns;	S: ** Trt: ns;
	SxTrt:	SxTrt: **	SxTrt: **	SxTrt: **	SxTrt: ns	SxTrt: ns	SxTrt: ns	SxTrt: ns

RWC relative water content (%), EC electrolyte leakage (%), CC chlorophyll content (SPAD value), Chl a chlorophyll a (mg g⁻¹ FW), Chl b chlorophyll b (mg g⁻¹ FW), Car. carotenoids (mg g⁻¹ FW), Chl (a + b) total chlorophyll (mg g⁻¹ FW)

Treatments: S₀ control (0 mM NaCl), S₁ 50 mM NaCl, S₂ 100 mM NaCl, S₃ 150 mM NaCl, S₄ 200 mM NaCl. C uninoculated control, SB single-strain bacterial inoculation, TB triple-strain bacterial consortium

Data represent mean±standard error. Mean values in the same column followed by different letters indicate significant differences according to Tukey's HSD test ($p < 0.05^*$, $p < 0.01^{**}$, ns not significant)

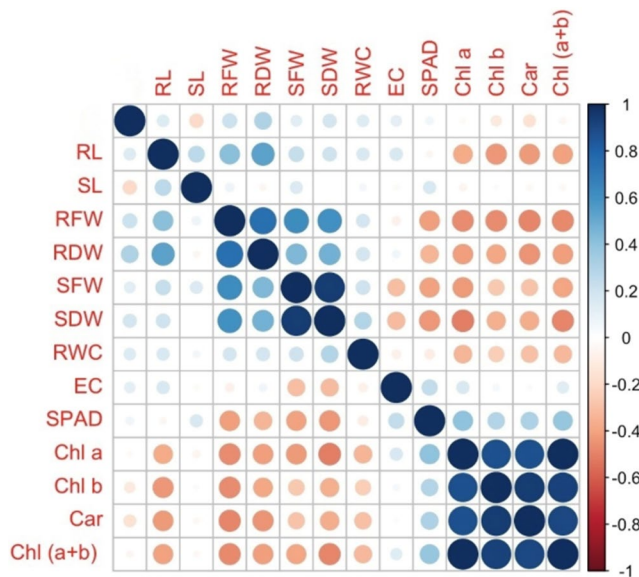


Fig. 1 Correlation matrix of growth, physiological, and photosynthetic pigment parameters in canola (*Brassica napus* L.) seedlings under salinity and plant growth-promoting rhizobacteria (PGPR) inoculation. The size and color intensity of the circles indicate the strength and direction of Pearson's correlation coefficients (r). Strong positive correlations (dark blue) were found among root and shoot biomass traits (RFW, RDW, SFW, SDW) and physiological parameters (RWC, SPAD, Chl a + b), while negative correlations (dark red) were mainly associated with electrolyte leakage (EC)

with electrolyte leakage (EC), indicating that membrane instability was inversely associated with biomass production. Pigment-related traits were strongly and positively interrelated, highlighting their coordinated role in photosynthetic efficiency. Particularly, chlorophyll a was closely associated with chlorophyll b, carotenoids, and total chlorophyll. These findings underline that PGPR-induced improvement in water balance and biomass production likely influences photosynthetic efficiency under salt stress.

4 Effects on Biochemical Parameters

4.1 Total Phenolic Content (TPC, Mg GAE g⁻¹ DW)

The phenolic response increased notably after salt exposure, peaking at moderate salinity (S2). The highest TPC values were obtained in TB (10.34 mg GAE g⁻¹ DW) and SB (10.59 mg GAE g⁻¹ DW), both significantly higher than the uninoculated control (8.93 mg GAE g⁻¹ DW) under the same salinity (Table 2). The lowest phenolic level was recorded under non-saline conditions (S0, 5.28 mg GAE g⁻¹ DW). In comparison to the uninoculated control, both bacterial inoculations promoted phenolic accumulation, with TB exerting a slightly stronger effect. Salinity, bacterial

treatment, and their interaction showed significant effects on TPC ($P < 0.01$).

4.2 Total Flavonoid Content (TFC, Mg QE g⁻¹ DW)

The pattern of TFC closely mirrored that of TPC. Flavonoid content increased under moderate salinity and PGPR treatments ($P < 0.01$; Table 2). The highest TFC (4.10 mg QE g⁻¹ DW) was observed in TB-treated seedlings at 100 mM NaCl, whereas the lowest mean value (2.61 mg QE g⁻¹ DW) occurred in S0. Both SB and TB enhanced flavonoid levels relative to the control, with TB showing the greater improvement, indicating stronger stimulation of secondary metabolism.

4.3 Total Antioxidant Capacity (TAC, %)

In the DPPH radical-scavenging assay, TAC ranged from 77.38% to 85.98%. The maximum antioxidant capacity (85.98%) was detected at 50 mM NaCl (S1), whereas a gradual decline occurred at higher salinity, reaching a minimum of 77.38% under TB at 200 mM NaCl (S4) (Table 2). Despite this decrease, PGPR-inoculated seedlings maintained higher TAC values than the control, confirming that bacterial inoculation enhanced antioxidant potential under saline conditions. Salinity, inoculation, and their interaction had significant effects ($P < 0.01$).

4.4 Proline Content (mg g⁻¹ FW)

Proline accumulation varied strongly with salinity and inoculation ($P < 0.01$). The control plants showed the lowest value (6.36 mg g⁻¹ FW) at 0 mM NaCl, whereas TB and SB markedly increased proline to 60.23 and 73.33 mg g⁻¹ FW, respectively. The highest level (122.36 mg g⁻¹ FW) was recorded in TB at 150 mM NaCl (S3). Across all salt concentrations, bacterial treatments—especially TB—induced higher proline accumulation than the control, reflecting enhanced osmotic adjustment under salt stress (Table 2).

4.5 Malondialdehyde (MDA, Nmol g⁻¹ FW) and Hydrogen Peroxide (H₂O₂, μmol g⁻¹ FW) Content

MDA, an indicator of lipid peroxidation, increased with salinity but was partially mitigated by bacterial inoculation (Table 2). The highest MDA value (142.47 nmol g⁻¹ FW) occurred in S4-TB, whereas the lowest (41.94 nmol g⁻¹ FW) was found in the S3 control. Although TB stimulated phenolic and flavonoid accumulation, it did not completely suppress lipid peroxidation under severe salinity. Overall, salinity, bacterial inoculation, and their interaction showed no significant effect on MDA accumulation.

Table 2 Responses of phenolic compounds, antioxidant capacity, and oxidative stress markers of Canola (*Brassica Napus* L.) seedlings under salinity and plant growth-promoting rhizobacteria (PGPR) inoculation

NaCl (mM)	Treatment	TPC (mg GAE g ⁻¹ DW)	TFC (mg QE g ⁻¹ DW)	TAC (%)	MDA (nmol g ⁻¹)	Prolin (mg g ⁻¹ FW)	H ₂ O ₂ (μmol g ⁻¹ FW)
S0 (Control)	C	5.16±0.07 ^c	2.69±0.15 ^{ef}	82.61±0.52 ^{ab}	70.97±1.86	6.36±0.18 ^d	16.93±0.06 ^{ab}
	TB	5.59±0.16 ^{de}	2.85±0.05 ^{de}	82.85±0.34 ^{ab}	64.52±1.08	60.23±0.91 ^{bc}	14.01±0.02 ^e
	SB	5.10±0.06 ^e	2.31±0.02 ^f	83.79±0.25 ^{ab}	60.22±0.62	73.33±0.53 ^b	13.67±0.02 ^e
	S0 Mean	5.28^C	2.61^C	83.08^A	65.23	46.64^C	14.86^C
S1	C	6.93±0.06 ^c	3.30±0.08 ^{bd}	85.98±0.27 ^a	56.99±2.48	43.10±0.94 ^c	13.92±0.12 ^e
	TB	6.36±0.08 ^{cd}	3.02±0.07 ^{ce}	81.29±0.38 ^b	60.22±2.71	102.42±0.15 ^a	15.13±0.15 ^{ce}
	SB	5.73±0.10 ^{de}	2.73±0.03 ^{ef}	82.23±0.42 ^b	50.54±3.29	100.67±0.26 ^a	14.29±0.02 ^{de}
	S1 Mean	6.34^C	3.02^B	83.17^A	55.91	82.06^B	14.44^C
S2	C	8.93±0.21 ^b	3.57±0.06 ^b	82.94±0.23 ^{ab}	65.59±4.48	73.38±1.10 ^b	14.35±0.24 ^{de}
	TB	10.34±0.26 ^a	4.10±0.01 ^a	80.24±1.02 ^{bc}	56.99±3.78	103.75±0.28 ^a	14.91±0.05 ^{de}
	SB	10.59±0.42 ^a	3.42±0.02 ^{bc}	81.49±0.19 ^b	61.29±1.08	99.35±1.39 ^a	17.74±0.53 ^a
	S2 Mean	9.95^A	3.70^A	81.56^{AB}	61.29	92.16^B	15.66^{AB}
S3	C	9.05±0.14 ^b	3.32±0.02 ^{bd}	82.03±0.27 ^b	41.94±3.72	103.00±0.14 ^a	16.84±0.11 ^{ab}
	TB	9.14±0.15 ^b	3.53±0.03 ^b	82.70±0.38 ^{ab}	59.14±3.10	122.36±10.53 ^a	14.94±0.16 ^{de}
	SB	8.88±0.20 ^b	3.78±0.05 ^{ab}	81.62±0.17 ^{ab}	58.06±5.69	103.58±0.64 ^a	16.73±0.20 ^{ac}
	S3 Mean	9.02^{AB}	3.55^A	82.12^{AB}	53.04	109.64^A	16.17^A
S4	C	8.73±0.56 ^b	3.38±0.28 ^{bc}	81.88±0.34 ^b	64.52±2.15	104.15±0.49 ^a	14.49±0.07 ^{de}
	TB	8.59±1.63 ^b	3.51±0.65 ^{bc}	77.38±14.78 ^c	142.47±65.90	103.47±0.83 ^a	14.46±0.06 ^{de}
	SB	8.47±0.09 ^b	3.31±0.04 ^{bd}	82.94±0.40 ^{ab}	60.22±2.24	104.41±0.27 ^a	15.86±0.25 ^{bd}
	S4 Mean	8.60^B	3.40^A	80.73^B	104.66	104.01^A	14.93^{BC}
Trt.Mean	C	7.76	3.25 ^B	83.08 ^A	60.00	65.91 ^B	15.30 ^A
	TB	8.01	3.40 ^A	80.89 ^B	86.02	98.45 ^A	14.68 ^B
	SB	7.75	3.11 ^B	82.42 ^A	58.06	96.27 ^A	15.66 ^A
		S ^{**} ; Trt ^{ns} ; SxTrt: ^{**}	S ^{**} ; Trt ^{**} ; SxTrt: ^{**}	S [*] ; Trt ^{**} ; SxTrt: ^{**}	ns	S ^{**} ; Trt ^{**} ; SxTrt: ^{**}	S ^{**} ; Trt ^{**} ; SxTrt: ^{**}

TPC total phenolic content (mg GAE g⁻¹ DW), TFC total flavonoid content (mg QE g⁻¹ DW), TAC total antioxidant capacity (%), MDA malondialdehyde (nmol g⁻¹ FW), Proline (mg g⁻¹ FW), H₂O₂ hydrogen peroxide (μmol g⁻¹ FW)

Treatments: S₀ control (0 mM NaCl), S₁ 50 mM NaCl, S₂ 100 mM NaCl, S₃ 150 mM NaCl, S₄ 200 mM NaCl, C uninoculated control, SB single-strain bacterial inoculation, TB triple-strain bacterial consortium

Data represent mean±standard error. Mean values in the same column followed by different letters indicate significant differences according to Tukey's HSD test ($p<0.05^*$, $p<0.01^{**}$, ns not significant)

Hydrogen peroxide (H₂O₂) content was significantly influenced by salinity, inoculation, and their interaction ($P\leq0.01$). At 0 mM NaCl, the control exhibited the highest level (16.93 μmol g⁻¹ FW), whereas TB and SB markedly reduced it. At 50–100 mM NaCl, TB maintained moderate values (14.91–15.13 μmol g⁻¹ FW), while SB increased to 17.74 μmol g⁻¹ FW at 100 mM. Under 150 mM NaCl, TB reduced H₂O₂ to 14.94 μmol g⁻¹ FW, compared with 16.84–16.73 μmol g⁻¹ FW in control and SB. At 200 mM NaCl, all treatments showed similar levels, with TB remaining the lowest (14.46 μmol g⁻¹ FW). These results indicate that TB inoculation was more effective in limiting oxidative stress across salinity levels.

4.6 Effects on Antioxidant Enzyme Activities

Superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) activities in canola seedlings showed distinct responses to salinity and bacterial

inoculation (Supplementary Table 2; Fig. 2). SOD activity was highest in the control under non-saline conditions (4.79 U mg⁻¹ FW) but declined following TB and SB inoculation. Under moderate salinity (100–150 mM NaCl), SB maintained relatively higher SOD activity than TB, indicating a strain-specific modulation of superoxide detoxification. POD activity peaked sharply at 50 mM NaCl (48.70 U mg⁻¹ protein in control), and TB further enhanced it to 69.12 U mg⁻¹ protein. However, at higher salinity, TB-induced POD activity declined, while SB maintained a moderate increase, particularly at 150 mM NaCl. CAT activity was low in the control under non-saline conditions (1.01 μmol min⁻¹ mg⁻¹ protein) but increased markedly with bacterial inoculation, reaching 4.78 and 4.04 μmol min⁻¹ mg⁻¹ protein in TB and SB, respectively. At 150 mM NaCl, SB maintained higher CAT activity (3.68 μmol min⁻¹ mg⁻¹ protein) compared to the control and TB, suggesting better enzymatic adaptation under moderate stress. APX activity remained relatively stable (62–65 μmol min⁻¹ mg⁻¹ protein) across most treatments.

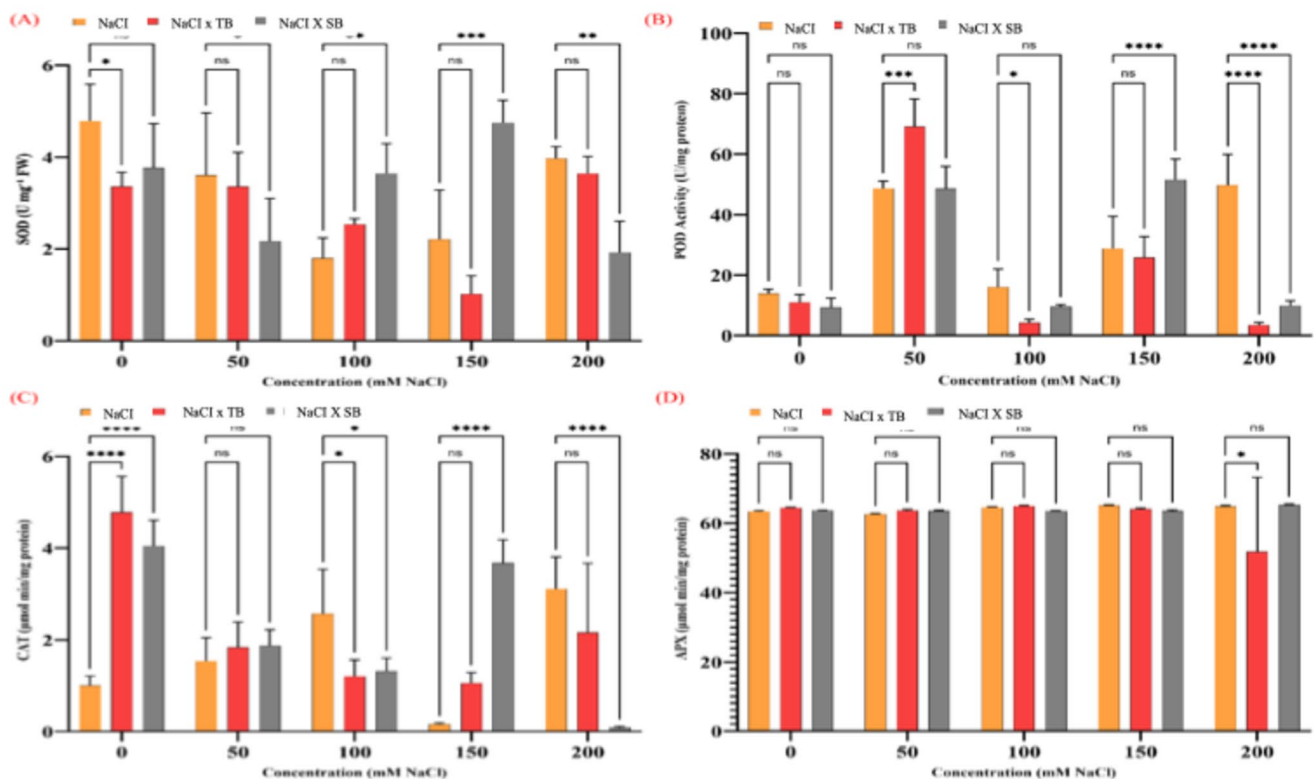


Fig. 2 Responses of antioxidant enzyme activities in canola (*Brassica napus* L.) seedlings under salinity and plant growth-promoting rhizobacteria (PGPR) inoculation (A) Superoxide dismutase (SOD) activity (U mg⁻¹ FW), (B) Peroxidase (POD) activity (U mg⁻¹ protein), (C) Catalase (CAT) activity (μmol min⁻¹ mg⁻¹ protein), and (D) Ascorbate peroxidase (APX) activity (μmol min⁻¹ mg⁻¹ protein), measured under different salinity levels. Treatments: NaCl=salinity treatment without bacterial inoculation; NaCl × TB=salinity combined with

triple-strain PGPR consortium (Bacillus megaterium + Pseudomonas fluorescens + Bacillus subtilis); NaCl × SB=salinity combined with single-strain PGPR inoculation (Bacillus subtilis). Data represent mean ± standard error (SE). Statistical significance was determined by two-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test. ns=not significant; **p*<0.05; ***p*<0.01; ****p*<0.001; *****p*<0.0001

A pronounced decline occurred only in the control under severe salinity (16.73 μmol min⁻¹ mg⁻¹ protein), while TB and SB maintained higher values. Overall, bacterial inoculation modulated antioxidant enzyme activities in a treatment- and concentration-dependent manner: SB enhanced SOD and CAT under moderate salinity, TB promoted POD under mild stress, and APX remained largely stable except in the uninoculated control under high NaCl.

4.7 Multivariate Analysis of Stress Responses

The multivariate analyses, including heatmap clustering and principal component analysis (PCA), provided an integrated view of biochemical and antioxidant responses in canola seedlings under different NaCl concentrations and bacterial inoculation treatments (Figs. 3 and 4).

Heatmap clustering clearly separated the treatments into two main groups. Uninoculated controls under high salinity clustered with elevated levels of MDA and H₂O₂, reflecting severe oxidative stress and disrupted redox balance. In contrast, PGPR-inoculated seedlings, particularly those treated

with the triple-strain consortium (TB), grouped together with higher levels of proline, TPC, TFC, and TAC, indicating enhanced osmolyte accumulation, secondary metabolism, and total antioxidant activity. The single-strain (SB) treatments formed an intermediate cluster, showing partial improvement compared to controls but less pronounced than TB. Antioxidant enzymes (SOD, CAT, POD, APX) clustered closely, illustrating their coordinated function in detoxifying reactive oxygen species (ROS). The opposite distribution of MDA and TAC across the heatmap emphasized the antagonistic relationship between oxidative damage and antioxidant defense. Principal component analysis (PCA) supported these clustering trends by explaining 57.7% of the total variance (PC1: 31.9%, PC2: 25.8%). Parameters with the highest contributions on PC1 included MDA, TPC, TFC, and proline, signifying that oxidative damage and secondary metabolite accumulation were the main factors driving the separation among treatments. Antioxidant enzymes (SOD, CAT, POD) and APX contributed strongly to PC2, representing the enzymatic component of stress tolerance. The orientation of TAC opposite to MDA

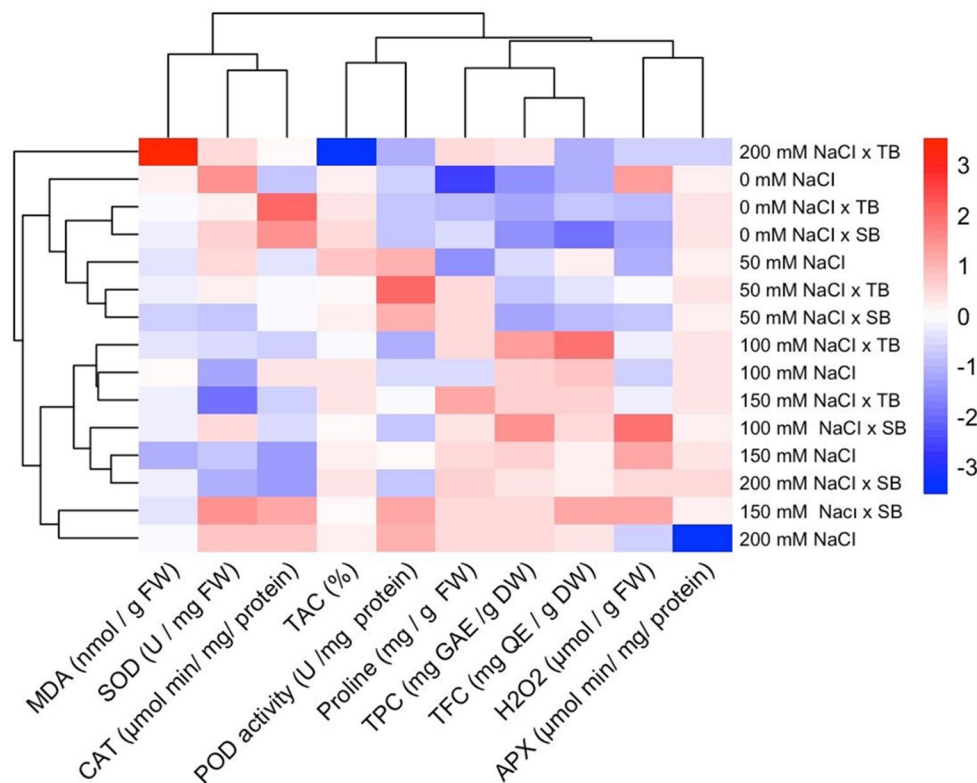


Fig. 3 Heatmap with hierarchical clustering of biochemical and antioxidant traits in canola (*Brassica napus* L.) seedlings under salinity and plant growth-promoting rhizobacteria (PGPR) inoculation. Seedlings were grown under different salinity levels (0–200 mM sodium chloride, NaCl) with or without bacterial inoculation. SB indicates single-strain PGPR inoculation with *Bacillus subtilis*, and TB indicates a triple-strain PGPR consortium consisting of *Bacillus megaterium* + *Pseudomonas fluorescens* + *Bacillus subtilis*. Color intensity

represents relative standardized values, with red indicating higher and blue indicating lower values. Hierarchical clustering reveals treatment-dependent patterns in oxidative stress markers, osmolyte accumulation, and antioxidant defense responses. Abbreviations: MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; POD, peroxidase; TAC, total antioxidant capacity; TPC, total phenolic content; TFC, total flavonoid content; H₂O₂, hydrogen peroxide; APX, ascorbate peroxidase

further highlighted the negative association between total antioxidant capacity and oxidative injury.

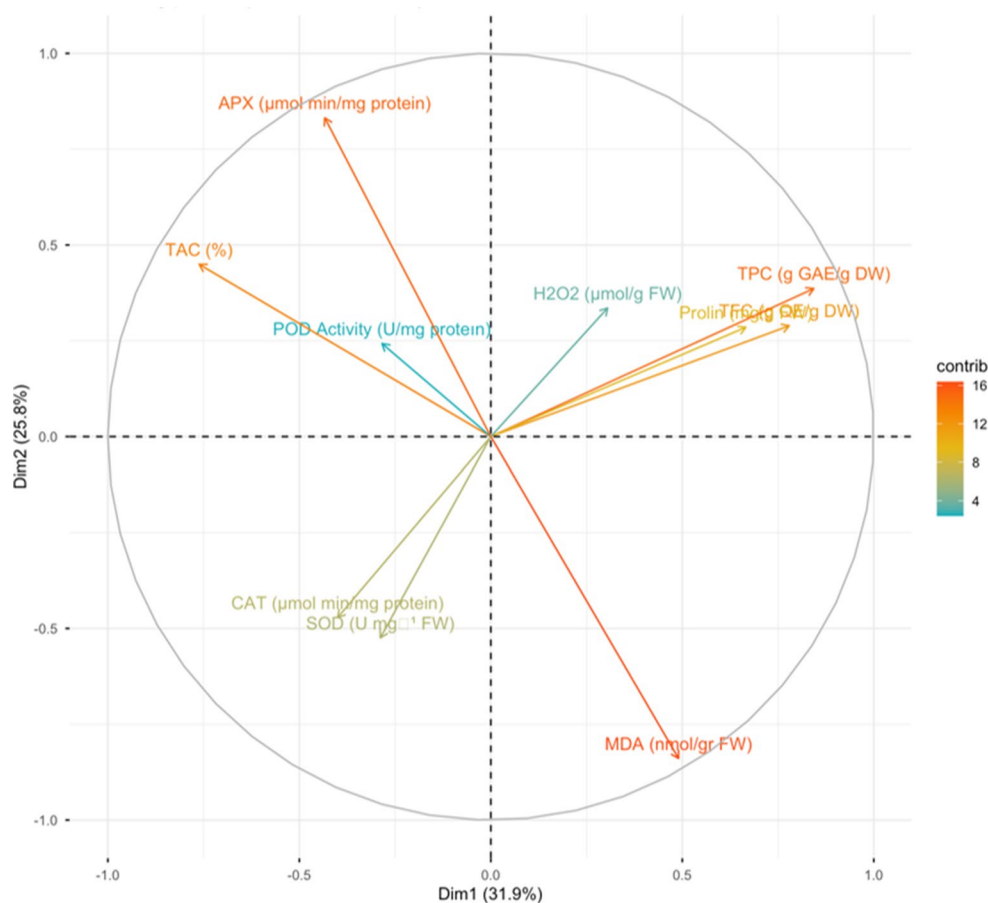
TB-treated plants were positioned near TPC, TFC, proline, and TAC vectors, reflecting their enhanced metabolic and antioxidant potential, whereas uninoculated controls under high salinity were associated with MDA, H₂O₂, and NaCl concentration vectors. SB treatments occupied an intermediate space, confirming partial mitigation of stress. Collectively, the PCA and heatmap results demonstrate that multi-strain PGPR inoculation reprograms the biochemical response of canola seedlings, reinforcing redox homeostasis, osmotic balance, and phenolic metabolism to counteract salinity-induced oxidative stress.

5 Discussion

Salinity is one of the most detrimental abiotic stresses, impairing plant growth through osmotic stress, ion toxicity, and oxidative damage, which collectively disrupt nutrient

balance, water uptake, and cellular metabolism (Munns and Tester 2008; Parihar et al. 2015). In the present study, canola seedlings exhibited marked reductions in shoot length, root length, and biomass under increasing NaCl concentrations, while bacterial inoculation, particularly with the triple-strain consortium, alleviated these growth reductions by maintaining greater shoot and root development. These findings are consistent with previous studies showing that plant growth-promoting rhizobacteria (PGPR) mitigate the adverse effects of salinity by improving nutrient acquisition, modulating phytohormones, and enhancing root architecture (Bharti et al. 2014; Egamberdieva et al. 2017). The underlying mechanisms behind this ameliorative effect are closely related to the multifaceted physiological and biochemical regulation capacity of PGPR. These bacteria can synthesize growth regulators such as indole-3-acetic acid (IAA), gibberellins, and cytokinins, while producing ACC-deaminase that reduces stress-induced ethylene accumulation—thereby stimulating root elongation, water uptake, and overall plant growth (Bouremani et al. 2023; Sofy et

Fig. 4 Principal component analysis (PCA) biplot showing the multivariate distribution of biochemical and antioxidant parameters in canola (*Brassica napus* L.) seedlings under salinity and plant growth-promoting rhizobacteria (PGPR) inoculation. Seedlings were grown under different salinity levels (0–200 mM NaCl) with or without bacterial inoculation. Treatments included an uninoculated control, single-strain PGPR inoculation with *Bacillus subtilis* (SB), and a triple-strain PGPR consortium consisting of *Bacillus megaterium* + *Pseudomonas fluorescens* + *Bacillus subtilis* (TB). Arrows represent the contribution and direction of biochemical variables, including total phenolic content (TPC), total flavonoid content (TFC), total antioxidant capacity (TAC), malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX)]. The first two principal components (PC1 and PC2) explained 35.2% and 23.6% of the total variance, respectively



al. 2021). In addition, bacterial exopolysaccharides form a protective film on the root surface, reducing ion leakage and strengthening membrane stability under saline conditions.

The improved performance of TB-treated plants in both shoot and root biomass compared to uninoculated controls aligns with reports that multi-strain PGPR consortia often provide synergistic benefits greater than single strains, owing to their complementary biological functions (Nadeem et al. 2006; Vimal and Singh 2019). This synergy can be attributed to the distinct yet complementary roles of individual strains: *Bacillus megaterium* enhances phosphate solubilization and proline accumulation, whereas *Pseudomonas fluorescens* contributes to ROS detoxification and membrane protection by activating antioxidant enzymes such as SOD, CAT, and POD (Khan et al. 2023; Moreno-Galván et al. 2020; Nawaz et al. 2020; Shyam and Narzary 2024). The combined action of these mechanisms maintains ion homeostasis, osmotic adjustment, and redox balance, thereby minimizing oxidative injury and improving cellular stability. Biomass retention under bacterial inoculation can thus be attributed to the enhanced production of phytohormones such as indole-3-acetic acid, improved accumulation of osmolytes (e.g., proline), and better regulation of ion homeostasis, all of which collectively support plant growth

under salt stress (Dodd and Pérez-Alfocea 2012; Zhang et al. 2022). These processes are also believed to strengthen the antioxidant defense system (SOD, CAT, POD, APX), which limits the accumulation of reactive oxygen species (ROS), protects membrane integrity, and sustains carbon assimilation (Khan et al. 2023; Kumari et al. 2024). Such biochemical reinforcement was particularly evident in the TB treatment, explaining its superior performance over the single-strain inoculation. Therefore, PGPR inoculation—especially the multi-strain consortium—appears to buffer the inhibitory effects of salinity on canola seedlings by maintaining growth and biomass allocation. Overall, these results indicate that bacterial inoculation acts simultaneously at both physiological (water relations, root development, ion regulation) and biochemical (phytohormone modulation, antioxidant activation, osmolyte accumulation) levels. In this way, the TB treatment establishes a multi-layered defense mechanism that collectively enhances salinity tolerance in canola seedlings.

Biomass accumulation is typically reduced under salt stress due to impaired photosynthesis, disrupted carbon allocation, and restricted nutrient uptake, which together limit shoot and root growth (Munns and Tester 2008). In the current study, however, PGPR inoculation significantly

altered the biomass response of canola seedlings in a trait-specific manner, with TB and SB treatments maintaining higher shoot dry weight under intermediate salinity and TB in particular enhancing root biomass at low-to-moderate stress levels. Similar findings have been reported in wheat and maize, where PGPR inoculation promoted biomass retention under salinity by improving photosynthetic efficiency and nutrient-use efficiency (Pereira et al., 2020). These outcomes suggest that PGPR influence biomass partitioning through integrated hormonal and biochemical signaling. For instance, auxins and cytokinins produced by PGPR stimulate root branching and cell expansion, whereas ACC-deaminase activity reduces stress-induced ethylene accumulation, thereby maintaining shoot meristem activity and photosynthetic function (Gamalero et al. 2023). In addition to hormonal regulation, the maintenance of higher biomass in inoculated seedlings may result from enhanced antioxidative protection and osmotic adjustment, as PGPR can elevate antioxidant enzyme activities, increase proline accumulation, and stabilize chlorophyll pigments, collectively preventing oxidative damage and supporting energy metabolism (Al-Turki et al. 2023; Moreno-Galván et al. 2020). The TB treatment appears to coordinate these responses more efficiently by combining complementary bacterial traits such as phosphate solubilization, ROS scavenging, and osmolyte synthesis, resulting in greater photosynthetic efficiency and carbon allocation stability.

Salinity stress disrupts plant water relations by reducing osmotic potential and impairing membrane integrity, leading to decreased relative water content (RWC), increased electrolyte leakage (EC), and reduced pigment stability (Arif et al. 2020). In the current study, canola seedlings exhibited progressive declines in RWC and chlorophyll pigments, along with increased EC under elevated salinity, while bacterial inoculation—particularly with the TB consortium—ameliorated these effects by enhancing water retention, maintaining membrane stability, and improving chlorophyll content. At the biochemical level, PGPR likely improved osmotic regulation by inducing the accumulation of compatible solutes such as proline, soluble sugars, and glycine betaine, which stabilize cellular water potential and protect macromolecular structures (Al-Turki et al. 2023). In addition, bacterial exopolysaccharides may have contributed to improved soil aggregation and rhizosphere moisture retention, while the synthesis of cytokinins and abscisic acid-like compounds modulated stomatal behavior to reduce transpirational water loss (Arkhipova et al. 2020; Egamberdieva et al. 2017). In our experiment, TB-treated plants showed superior RWC and lower EC compared to SB and controls, suggesting that the multi-strain consortium provided broader functional coverage: while *Bacillus megaterium* improved osmolyte biosynthesis and membrane

integrity, *Pseudomonas fluorescens* enhanced ionic homeostasis and antioxidant protection. The coexistence of these traits likely resulted in synergistic improvement of water relations. The positive effects on chlorophyll and carotenoid concentrations align with findings that PGPR stimulate pigment biosynthesis and protect the photosynthetic apparatus from oxidative stress, thereby sustaining photosynthetic efficiency under saline environments (Heidari and Golpayegani 2012). Overall, the TB consortium's ability to stabilize RWC, EC, and chlorophyll levels under salinity suggests a cooperative action among strains that optimize water conservation, ionic balance, and pigment integrity through osmolyte synthesis, ion transport regulation, and ROS detoxification.

Excessive accumulation of reactive oxygen species (ROS) under salt stress triggers oxidative damage, which plants mitigate by producing secondary metabolites (phenolics, flavonoids) and osmolytes (e.g., proline) to enhance antioxidant defense and osmotic adjustment (Ikram et al. 2025). In the present study, canola seedlings exposed to moderate salinity accumulated the highest levels of total phenolics and flavonoids, particularly under PGPR inoculation, while extreme salinity suppressed these levels, highlighting the dual role of stress in inducing protective compounds and, at severe levels, overwhelming the antioxidant machinery. PGPR likely enhanced activation of the phenylpropanoid pathway, resulting in increased synthesis of phenolics and flavonoids with ROS-scavenging and membrane-stabilizing roles (Bharti et al. 2014; Giannelli et al. 2023). Elevated total antioxidant capacity (TAC) and proline accumulation under TB inoculation indicate that the consortium primed both enzymatic and non-enzymatic antioxidant systems, maintaining redox homeostasis under moderate salinity (Al-Turki et al. 2023; Singh et al., 2021). Proline functions not only as an osmoprotectant but also as a molecular chaperone and ROS scavenger, protecting proteins and membranes from oxidative damage. The higher proline and phenolic contents in TB-treated seedlings therefore reflect a coordinated activation of nitrogen metabolism, osmotic regulation, and antioxidant defense. Collectively, these findings demonstrate that PGPR inoculations—particularly multi-strain consortia—strengthen biochemical resilience by boosting antioxidant compounds and osmolyte accumulation, thereby mitigating oxidative and osmotic damage under saline conditions.

Salt stress is known to enhance oxidative stress in plants by overproducing ROS, leading to lipid peroxidation, membrane damage, and accumulation of oxidative markers such as MDA and H₂O₂ (Ahmad et al. 2019; Kesawat et al. 2023). In this study, MDA levels increased with salinity but were moderated by bacterial inoculation, with the lowest accumulation under *Bacillus subtilis* and a moderate rise

under TB at high salinity. This pattern indicates that while SB limits oxidative injury by reducing ROS accumulation, TB manages oxidative stress through both detoxification and controlled ROS signaling. Moderate ROS levels in TB-treated seedlings likely serve as signaling cues that activate systemic antioxidant defenses and redox regulatory genes (Su et al. 2024). Such “controlled oxidative signaling” is essential for acclimation and homeostatic adjustment. The reduction of H₂O₂ by TB under most conditions highlights strain-specific efficiency in ROS scavenging, potentially through activation of catalase and ascorbate peroxidase (Dang 2024). Furthermore, synergy between *Bacillus megaterium* and *Pseudomonas fluorescens* likely promoted both osmolyte-mediated membrane stabilization and the transcriptional activation of antioxidant enzymes, resulting in cumulative stress protection and improved redox balance.

Salt stress disrupts cellular redox homeostasis by enhancing superoxide, hydrogen peroxide, and hydroxyl radical production, and plants counter this imbalance with antioxidant enzymes such as SOD, CAT, POD, and APX, which act in concert to detoxify ROS (Anas et al. 2024; Pang and Wang 2008). In this study, canola seedlings displayed dynamic modulation of these enzymes under NaCl stress and PGPR inoculation, where SB mainly enhanced SOD and CAT activities under moderate salinity, TB promoted POD activity at mild salinity, and APX remained relatively stable except under severe conditions. The distinct patterns between SB and TB indicate different strategies of oxidative stress management: SB mainly supports primary ROS detoxification (SOD/CAT), whereas TB activates a broader antioxidant network including POD- and APX-mediated processes, maintaining redox stability and supporting lignification and cell wall strengthening (Grover et al. 2021; Kumari et al. 2024). TB-induced coordination of enzymatic antioxidants thus provides a sequential detoxification cascade—from radical dismutation (SOD) to H₂O₂ decomposition (CAT, POD, APX)—ensuring redox homeostasis and sustained stress signaling. This hierarchical activation suggests that TB does not merely alleviate oxidative stress but establishes an integrated regulatory framework connecting ROS detoxification, structural defense, and energy balance. Altogether, these findings indicate that multi-strain inoculation triggers a complex and coordinated antioxidant defense network that enhances stress tolerance in canola seedlings by integrating enzymatic, structural, and redox-buffering processes.

6 Conclusion

This study demonstrates that salinity stress severely disrupts the growth, physiology, and biochemical balance of canola (*Brassica napus* L.) seedlings, whereas inoculation

with plant growth-promoting rhizobacteria effectively alleviates these adverse effects. Inoculated canola seedlings exhibited enhanced shoot and root development, maintained water balance, stabilized cellular membranes, and preserved photosynthetic pigments under saline conditions. The single-strain bacterial inoculation showed notable effects under moderate salinity, mainly by reducing oxidative stress through the activation of antioxidant enzymes such as superoxide dismutase and catalase, thereby limiting the accumulation of reactive oxygen species. In contrast, the triple-strain bacterial consortium exerted a broader and more integrated impact, strengthening osmotic adjustment (proline accumulation and ion regulation) and biochemical defense (increased phenolic, flavonoid, and total antioxidant contents), thereby inducing a multi-layered tolerance response in canola. This demonstrates that the complementary physiological and biochemical functions of different bacterial strains act synergistically to enhance plant resilience under salt stress. Overall, the superior performance of the multi-strain consortium compared with the single strain indicates that microbial diversity is a key strategy to improve early-stage salt tolerance in canola. These findings provide a novel perspective for the use of mixed bacterial inoculants as sustainable biofertilizers and biostimulants in saline agriculture.

Future studies should investigate the responses of different canola genotypes to bacterial consortia, as well as their impacts on yield and quality parameters and long-term soil microbiome dynamics. Integrating multi-strain microbial inoculants with plant breeding and soil health management could provide a promising approach for developing salt-tolerant and climate-smart agricultural systems that ensure sustainable canola production under salinity stress.

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Author Contributions Münüre Tanur Erkoyuncu conceived and designed the study. Münüre Tanur Erkoyuncu performed the greenhouse experiments and physiological–biochemical analyses, while Muhammad Anas completed the antioxidant enzyme assays. Münüre Tanur Erkoyuncu and Muhammad Anas carried out the data analysis and wrote the draft manuscript. Erdogan Esref Hakkı contributed to resources, validation, and editorial revisions. All authors contributed to the final version of the manuscript.

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Data Availability The data supporting the findings of this study are available from the corresponding author.

Declarations

Ethics Approval Not applicable.

Conflict of Interest The authors declare that they have no conflict of interest.

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