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Physiological Responses of Tomato (*Solanum lycopersicum* L.) to Salt Stress Mediated by Native Halotolerant *Bacillus* spp. Strains using LC-MS/MS

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Abstract

Background and aims

Climate change is increasing global land salinity, causing plant deterioration, stunted growth, nutrient imbalances, ion toxicity, and physiological disruption. This study explores the potential of halotolerant PGPB (Plant Growth-Promoting Bacteria) particularly *Bacillus* species, to alleviate salinity stress in tomato plant physiology.

Material and methods

Two halotolerant strains, *Bacillus albus* strain FA26 and *Bacillus anthracis* LOS6, were isolated from dominant plant species in saline regions. Greenhouse experiments were conducted on tomato plants exposed to 200 mM NaCl with and without bacterial inoculation. The key physiological parameters assessed included K^+ , Na^+ , and Ca^{2+} concentrations (determined by flame photometry and atomic absorption spectroscopy), chlorophyll content (measured by spectrophotometry), and proline levels (determined by a ninhydrin colorimetric assay). Metabolic profiling of phenolic compounds was performed using Fourier-Transform Infrared spectroscopy (FT-IR) and Liquid Chromatography–Mass Spectrometry (LC-MS/MS).

Key results

Inoculated plants, improved K^+/Na^+ homeostasis under saline conditions, and reduced Na^+ accumulation (approximately 66.70% with *Bacillus anthracis* LOS6). Treatment with *Bacillus* spp. improved chlorophyll content, while *Bacillus albus* strain FA26 showed the highest total chlorophyll (42.12 mg L^{-1}), chlorophyll A (23.21 mg L^{-1}), and chlorophyll B (16.34 mg L^{-1}) contents, indicating improved photosynthetic efficiency, also supported proline osmotic adjustment under salt-stressed conditions. proline accumulation increased, with the *Bacillus anthracis* LOS6 strain showing the highest concentration ($5052\text{ }\mu\text{g g}^{-1}$), representing an increase of approximately 10,43% compared to non-stress conditions, reflecting its strong role in osmotic adjustment and stress tolerance. Secondary metabolites facilitate defense gene activation through salicylic acid biosynthesis.

Conclusion

These findings highlight the importance of using halotolerant PGPB as a strategy to address salt stress challenges in agriculture, demonstrating their potential to alleviate the adverse effects of salinity stress on tomato plant physiology.

Keywords

Climate change, ion homeostasis, bio-inoculants, PGPB, salinity tolerance.

Statements and Declarations

- **Ethics approval and consent to participate:** Not applicable.
- **Consent for publication:** Not applicable.
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- **Author contributions:** All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by SK, YK, YOD, and DAS. FD and RD contributed to supervision. AEA handled visualization. The first draft of the manuscript was written by SK. All authors read and approved the final manuscript.

1 Introduction

Climate change exacerbates environmental issues, particularly in agriculture. For instance, during hot seasons, rising temperatures accelerate water evaporation and soil desiccation, which in turn leads to increased soil salinity a critical problem that significantly reduces crop yields globally (Kramer et al. 2025). Soil is considered saline when the electrical conductivity of its saturation extract in the root zone exceeds 4 ds/m at 25 °C, approximately equivalent to 40 mM NaCl (Pierzynski et al. 2005). This condition severely restricts plant growth and crop production in affected areas. Many crops, including tomato varieties, are susceptible to moderate salinity levels (Singh et al. 2012). Furthermore, salinity stress disrupts crucial physiological functions and metabolic processes, such as ion toxicity, photosynthesis, and nutrient uptake (Parihar et al. 2015). These challenges pose a significant threat to global food security, particularly in regions prone to soil salinization.

To address this issue, researchers have explored various strategies to enhance crop resilience to salinity stress. One promising approach involves the use of plant growth-promoting rhizobacteria (PGPR), particularly halotolerant PGPR strains. *Bacillus* species, for instance, have shown potential in enhancing plant growth under saline conditions (Ayaz et al. 2022). They support plant growth through various mechanisms, including the production of phytohormones like indole-3-acetic acid (IAA), phosphate solubilization, nitrogen fixation, ammonia production, and antifungal properties (Egamberdieva et al. 2019). Recent studies have demonstrated the efficacy of *Bacillus* strains in improving plant resilience to abiotic stress. Rojas-Solis et al. (2020) reported that inoculating tomato plants with *Bacillus* strains resulted in notable physiological improvement under salt stress, including higher chlorophyll content, increased plant biomass, and enhanced root and shoot development. These findings suggest that PGPR inoculation could be a viable strategy for mitigating the adverse effects of salt stress on crop plants.

Despite these advancements, the specific physiological and biochemical mechanisms by which halotolerant *Bacillus* strains confer salt tolerance remain unclear, particularly under local environmental conditions. Therefore, this study aims to address this gap by evaluating the potential of two *Bacillus* strains, *Bacillus albus* strain FA26 and *Bacillus anthracis* LOS6, to enhance the growth and yield of tomatoes under salt stress. We focus on the *Solanum lycopersicum* L. tomato variety, which is moderately tolerant to salinity and widely cultivated in regions prone to soil salinization (Rashed et al. 2016; Wang et al. 2024). Following the approach of Wang et al. (2024), and integrating physiological measurements and LC-MS/MS analyses, this study seeks to elucidate the mechanisms underlying *Bacillus*-mediated stress mitigation, contributing to sustainable agriculture and improved crop resilience in saline environments.

2. Materials and methods

2.1 Soil sample collection

Soil samples were collected from four distinct salt lakes (Table 1), and their chemical and physical properties were thoroughly analyzed. Bacterial strains from endophytic and rhizospheric environments were isolated from the predominant plant species. The potential of these bacterial isolates as PGPR was assessed in vitro, leading to the identification of two *Bacillus* species: *Bacillus albus* strain FA26 and *Bacillus anthracis* LOS6. The investigation was conducted at the University of Mostaganem, Algeria, in the Faculty of Life and Natural Sciences during spring 2024. Tomato plants (*Solanum lycopersicum* L.) were cultivated, and each plot was harvested 45 days after planting to ensure that the plants remained viable while exhibiting clear physiological and biochemical responses to salinity (Xiong et al. 2019).

2.2 Plant material and growth conditions

Tomato plants (*Solanum lycopersicum* L.) from an Italian cultivar were used in this study. Seeds were sourced from Italy and germinated under controlled conditions. Seeds were surface-sterilized by immersing them in 70% (v/v) ethanol for 2 min, followed by 2% (v/v) sodium hypochlorite for 3 min, and then rinsed five times with sterile distilled water. Seeds were germinated on sterilized Petri dish (3:1 v/v) substrate within 7.5 cm plastic pots containing 65 g of sterilized soil, maintained in a controlled-environment greenhouse at 25 ± 2 °C. For inoculation, *Bacillus albus* strain FA26 and *Bacillus*

anthracis LOS6 or both strains were cultured in Luria–Bertani (LB) medium for 24 h, harvested by centrifugation at 4,500 rpm for 10 min, washed once with sterile distilled water, and re-suspended in sterile distilled water to achieve a final optical density of $OD_{600} = 0.8$ (10^8 CFU mL⁻¹). Tomato seedlings were inoculated by root drenching with 35 mL of bacterial suspension every two days for three applications. Control plants received an equal volume of sterile distilled water. Five days after the initial inoculation, NaCl was added to the irrigation water to reach a final concentration of 200 mM (2% w/v). Plants were watered every two days with 35 mL of NaCl solution for a total of five irrigations. After seven days of exposure to salt stress, the seedlings were analyzed (Xiong et al. 2019).

2.3 Inoculant growth conditions

Bacteria were isolated from halophytic plants by collecting root segments with adhering soil from depths of 1 to 15 cm. Sampling was performed in triplicate. The samples were suspended in sterile 0.85% NaCl solution and serially diluted from 10^{-1} to 10^{-6} . Aliquots of 100 µL from appropriate dilutions were spread onto LB plates and incubated at 30 °C for 24–48 h. Distinct colonies were selected based on morphology and streaked onto fresh LB agar plates to obtain pure cultures; this purification step was repeated at least three times. Pure isolates were maintained on LB slants at 4°C. Molecular identification was performed using 16S rRNA gene amplification and sequencing. The draft genome sequences of both strains are available in GenBank, showing 95% and 98% similarity to *Bacillus anthracis* strain LOS6 (S1) and *Bacillus albus* strain FA26 (S2), respectively. A bacterial suspension was prepared at a concentration of 10^8 CFU/mL and adjusted to an optical density of approximately 0.8 after 24 h of incubation. The suspension was washed thrice with sterile physiological water, and centrifugation (4000 rpm for 10 min) was used to collect a pellet for each species that was inoculated individually (S1 or S2) or in combination (ST).

2.4 Determination of K⁺, Na⁺ and Ca⁺ contents

The mineral content (K⁺, Na⁺ and Ca⁺) of the tomato leaves was analyzed by first drying the leaf samples in an oven at 75 °C for 48 h. A 100 mg sample of the resulting dry powder was placed in crucibles and ashed in a muffle oven at 600 °C for 6 h. The ash was then dissolved in 5.1% HNO₃ at 110 °C. After cooling, the solution was filtered into a 100 mL volumetric flask and diluted to volume with distilled water. The potassium, sodium and calcium concentrations in the digested extracts were quantified using a flame photometer (model 410) and expressed in mg g⁻¹ dry weight (Astaneh et al. 2018).

2.5 Proline content

The proline content in tomato plant leaves was determined by homogenizing 500 mg of fresh leaf tissue with 10 mL of 3% sulfosalicylic acid and filtering through Whatman No. 2 filter paper. Two milliliters of ninhydrin reagent and 2 mL of glacial acetic acid were added to 2 mL of the filtered extract, following the method described by Bates et al. (1973). The mixture was incubated in a water bath at 100°C for 1 h, then placed on ice and extracted with 4 mL toluene. Absorbance was measured at 528 nm. Proline content was expressed as mg/g of fresh weight and quantified using a calibration curve prepared with standard proline.

2.6 Photosynthetic pigments content

A 0.5 g sample of fresh material was cut and crushed in an 80% acetone solution. The content of photosynthetic pigments (chlorophyll and carotenoids) was determined after filtration using optical density readings at 663, 645, and 470 nm. The contents of chlorophyll A, B, and total were determined according to the method described by Arnon (1949). Total carotenoid content was calculated from absorbance measurements using the equations of Lichtenthaler (1987) as follows:

$$\text{Chl A (mg. L}^{-1}\text{)} = 12.25 \times A_{663} - 2.79 \times A_{645}$$

$$\text{Chl B (mg. L}^{-1}\text{)} = 21.50 \times A_{645} - 5.10 \times A_{663}$$

$$\text{Chl T (mg. L}^{-1}\text{)} = \text{Chl A} + \text{Chl B}$$

$$\text{Carotenoids (mg. L}^{-1}\text{)} = \frac{1000 \times A_{470} - 1.90 \times \text{Chl A} - 63.14 \times \text{Chl B}}{214}$$

2.7 Fourier Transform Infrared (FT-IR) spectroscopy characterization of tomato plant biomolecules under stress

FT-IR spectroscopy is a crucial analytical tool for identifying a range of phytochemicals, including phenolics and flavonoids. The spectral fingerprint region (1800–900 cm^{-1}), as noted by Kainat et al. (2022), is critical for evaluating the biochemical composition of tomato plants, particularly for quantifying the total phenolic and flavonoid content. This study extends the application of FT-IR spectroscopy to a broader spectral range of 4000–400 cm^{-1} .

2.8 LC-MS/MS analysis

Fresh plant tissue (50 mg) was placed in a 2 mL Eppendorf tube and extracted with 1 mL of a solvent mixture (acetonitrile: methanol: water, 1:1:1, v/v/v). The tube was vortex-mixed thoroughly to ensure complete dissolution, with ultrasonication applied if any residue remained. Subsequently, 0.8 mL of hexane was added for extraction, and the sample was centrifuged at 7000 rpm for 5 min at 4 °C. The lower (aqueous) phase was collected and diluted 1:4 (v/v) with the extraction solvent. For higher purity, the extracts were further purified using C18 solid-phase extraction cartridges (preconditioned with methanol and water). The cartridges were washed with 5% methanol and eluted with an 80% methanol solution. The eluate was evaporated under reduced pressure to near dryness and reconstituted in 500 μL of mobile phase A before analysis. Finally, the reconstituted samples were filtered through 0.25 μm PTFE syringe filters into LC vials and stored at –20 °C until instrumental analysis. Chromatographic separation was conducted on a Poroshell 120 EC-C18 column (50 mm $\times$ 4.6 mm, 2.7 μm) using an Agilent 6460 Triple Quadrupole LC–MS system. The injection volume was 4 μL , and the flow rate was 0.4 $\text{mL} \cdot \text{min}^{-1}$. Mobile phase A consisted of water containing 0.1% formic acid and 5 mM ammonium formate, while mobile phase B was acetonitrile with 0.1% formic acid. The total runtime was 40 min, and the column temperature was maintained at 30 °C.

Mass spectrometric detection employed electrospray ionization in both positive and negative ion modes, depending on the analyte class. The ion source temperature was set at 350 °C. The drying gas (nitrogen) flow rate was 10 $\text{L} \cdot \text{min}^{-1}$, the nebulizer pressure was 35 psi, and the capillary voltage was 3,500 V. Fragmentor voltages and collision energies were individually optimized for each compound during method development. Data acquisition and quantification were performed using the Agilent MassHunter software, with compound identification based on specific retention times and multiple reaction monitoring transitions of authentic standards.

2.9 Statistical analysis

The experiments aimed to identify statistically significant effects using analysis of variance (ANOVA) with two factors: NaCl stress and PGPB (*Bacillus* sp.) inoculation, incorporating randomization and control treatments. This was followed by

a Newman-Keuls test at $P < 0.05$ to determine significant effects, using StatBox v6.4 statistical software for pairwise mean comparisons.

3. Results

3.1 Effect of PGP bacteria on tomato plants under saline conditions

This research investigated the effects of PGPB on the physiological characteristics of tomato plants grown in both saline and non-saline environments. All PGPB strains tested exhibited significant effects when compared to the control, with both *Bacillus* strains displaying particularly noteworthy results. The increase in external salt concentration is known to elevate osmotic potential by inducing hyperosmotic pressure, which is likely the primary factor contributing to reduced growth observed in both plants and PGPB. This study assessed the selected strains for their potential to enhance plant growth traits, such as ion homeostasis, proline response and chlorophyll content.

3.2 Ion hemostasis

A significant decrease in potassium (K^+) concentration was observed under salt stress, particularly in control plants (0.007) compared to non-stressed plants (1.999). Conversely, bacterial treatments (S1, S2, and ST) significantly increased K^+ accumulation, with the ST treatment showing the highest levels under both conditions.

Sodium (Na^+) content rose sharply in control plants exposed to salt stress (2.261), while bacterial inoculation significantly reduced Na^+ accumulation under non-stressed conditions (0.603), particularly in S2-treated plants. A highly significant finding was observed for Ca^{2+} under salt stress, with a marked reduction in the control (9.678) compared to non-stressed conditions (22.744). S1-treated plants maintained higher Ca^{2+} concentrations, indicating an improved ionic balance under both stressed and non-stressed conditions. Detailed numerical values are presented in Table 2.

Table 1 Chemical and physical properties of the soil

Parameter	Type of soil				
	Norm	Macta	Arzew	Morsli	Elmelh
pH	ISO 10390	8.64	8.64	8.47	8.47
CEC (mS/cm)	ISO 11265	94.18	70.29	30.21	104.95
M.O (%)	/	3.22	1.58	1.10	0.41
Total carb (%)	/	0.21	9.1	16.13	4.59
N2	ISO 11261	0.191	0.064	0.052	0.085
P (mg/l)	/	0.24	0.55	0.92	0.19
Ca (mg/l)	/	97.4	56.71	41.1	134
Mg (mg/l)	/	591	77.06	61.35	68.47
Na (mg/l)	/	22	425.6	164	244.8
K (mg/l)	/	98.4	29.37	52.59	43.7
Sand (%)	/	64	56	64	44
Clay (%)	/	12	24	20	36
Silt (%)	/	24	20	16	20

Table 2 Effects of salt stress and *Bacillus* sp. inoculant treatment on mineral ion hemostasis of tomato plants compared to the control

		F1XF2 interaction		Stress F1		Strains F2
		Without NaCl	with NaCl	Without NaCl	with NaCl	
K⁺	Control	1.999±0.035 ^c	0.007±0.002 ^h	1.703±0.132 ^a	0.382±0.005 ^b	1.003±0.023 ^c
	S1	2.016±0.058 ^c	0.411±0.007 ^g			1.214±0.039 ^b
	S2	2.212±0.208 ^b	0.009±0 ^h			1.11±0.138 ^c
	ST	2.542±0.304 ^a	1.409±0 ^c			1.975±0.203 ^a
P Value		0		0		0
Na⁺	Control	0.603±0.024	2.261±0.015	2.741±0.077	1.608±0.063	1.432±0.019
	S1	1.931±0.029	2.379±0.072			2.155±0.052
	S2	4.02±0.071	0.753±0.015			2.387±0.048
	ST	2.384±0.184	4.263±0.015			3.323±0.123
P Value		0		0		0
Ca⁺	Control	22.744±0.156 ^g	9.678±0.044 ^m	34.873±0.198 ^a	14.025±0.122 ^b	16.211±0.108 ^g
	S1	48.348±0.043 ^b	29.327±0.232 ^e			38.838±0.157 ^a
	S2	23.682±0.237 ^f	10.061±0.132 ^h			16.871±0.18 ^f
	ST	23.907±0.119 ^f	13.124±0.073 ^l			18.516±0.093 ^c
P Value		0		0		0

Data: S1(*Bacillus anthracis* LOS6), S2 (*Bacillus albus* strain FA26), ST (combination of S1 and S2), F1 (factor of salt stress), and F2 (factor of strains). Values are expressed as mean ± standard deviation. Values followed by different letters indicate statistically significant differences among treatments at P < 0.05, as determined by ANOVA followed by a post hoc test. Treatments sharing the same letters are not significantly different from each other.

3.3 Chlorophyll

Chlorophyll (Chl) A levels were generally low under normal conditions (control). However, under stress, the Chl A content increased significantly, particularly in the F2 strain, which showed the highest value (23.21), and S2 exhibited substantial accumulation (22.04), potentially enhancing photosynthetic efficiency and reducing oxidative damage under stress. Similarly, for chlorophyll B, the F2 control group had the highest content (16.34), followed by S2 (20.09), showing a significant increase relative to the control, which may have improved the chlorophyll stability and photosynthetic capacity under stress. S1 and ST showed lower levels for these parameters. For total chlorophyll, S2 showed the highest value (42.12) under stress, indicating a significant increase relative to the control, playing a protective role in maintaining photosynthetic pigments and potentially enhancing photosynthetic efficiency by reducing the chlorophyll degradation under stress.

Although carotenoid levels increased under stress, variability among replicates resulted in non-significant P values. Under stress, S1 and S2 exhibited a significant increase in carotenoid levels relative to the control, indicating their potential to mitigate oxidative stress. Under non-saline conditions, carotenoid levels were relatively moderate in all treatments. However, upon exposure to salt stress, a pronounced increase in carotenoid accumulation was observed, especially in the control plants, which exhibited a substantial increase from 126.94 to 26681.24. Interestingly, the bacterial treatments (S1, S2, and ST) led to moderate carotenoid accumulation under salinity, with values ranging from 1246.80 to 1672.06, suggesting that the inoculated plants might have experienced less oxidative pressure. This observation suggests that the microbial inoculants

helped mitigate the severity of stress, reducing the need for the plants to produce high levels of carotenoids as a defensive response.

Table 3 Effects of salt stress and *Bacillus* sp. inoculant treatment on chlorophyll and carotenoid content in tomato plants relative to the control

		F1XF2 interaction		Stress F1		Strains F2
		Without NaCl	with NaCl	Without NaCl	with NaCl	
Chlo A	Control	0.67±0.29 ^g	10.75±1.01 ^f	0.43±0.11 ^b	23.21±1.46 ^a	5.71±0.70 ^f
	S1	0.50±0.08 ^g	16.94±0.40 ^c			8.72±0.27 ^e
	S2	0.43±0.04 ^g	22.04±3.46 ^d			11.24±2.31 ^d
	ST	0.23±0.04 ^g	10.31±0.41 ^f			5.27±0.27 ^f
P Value		0		0		0
Chlo B	Control	1.019±0.31 ^c	15.348±6.49 ^b	0.72±0.15 ^b	16.34±6.88 ^a	8.18±4.33 ^b ^c
	S1	0.83±0.13 ^c	5.517±6.03 ^c			3.17±4.02 ^c ^d
	S2	0.68±0.10 ^c	20.091±4.59 ^b			10.39±3.06 ^b
	ST	21.01±0.08 ^c	21.013±3.54 ^b			10.71±2.36 ^b
P Value		0		0		0
Chlo T	Control	1.68±0.59 ^f	26.09 ±5.8 ^{de}	1.15±0.24 ^b	39.54±6.24 ^a	13.89±3.89 ^{de}
	S1	1.33±0.18 ^f	22.46±5.68 ^c			11.89±3.79 ^e
	S2	1.11±0.14 ^f	42.12±5.01 ^b			21.62±3.34 ^{bc}
	ST	0.64±0.06 ^f	31.32±3.43 ^{cd}			15.98±2.29 ^{de}
P Value		0		0		0
Carotenoid	Control	126.94±54.11	26681.24±59418.858	121.96±24.75	5039.29±20381.5	13404.09±39612.59
	S1	157.94±29.24	1246.80±347.46			702.37±232.46
	S2	111.93±2.47	1672.06±58.785			891.99±39.23
	ST	97.00±8.902	1521.81±242.57			809.41±161.82
P Value		0.5006		0.19763		0.50007

Data: S1(*Bacillus anthracis* LOS6), S2 (*Bacillus albus* strain FA26), ST (combination of S1 and S2), F1 (factor of salt stress), and F2 (factor of strains). Values are expressed as mean ± standard deviation. Values followed by different letters indicate statistically significant differences among treatments at P < 0.05, as determined by ANOVA followed by a post hoc test. Treatments sharing the same letters are not significantly different from each other.

3.4 Proline

Proline concentration exhibited notable variations between non-inoculated and inoculated tomato plants, with all samples demonstrating low levels under normal conditions. Control plants exhibited the lowest proline concentration (48 µmol g⁻¹). However, when subjected to salt stress, there was a marked increase in proline content across all strains, underscoring its significance as a stress-responsive metabolite. In stressed control plants, proline levels rose markedly (4332 µmol g⁻¹), likely serving as a protective compensatory mechanism. Among the inoculated strains, S1 displayed the highest proline accumulation (5052 µmol g⁻¹), suggesting its significant role in osmotic regulation. Conversely, S2 and ST accumulated

less proline under stress (696 and 1012 $\mu\text{mol g}^{-1}$, respectively), particularly S2, indicating that these strains may employ alternative protective strategies to cope with salt stress.

Table 4 Effects of salt stress and *Bacillus* sp. inoculant treatment on proline levels in tomato plants relative to the control

		F1XF2 interaction		Stress F1		Strains F2
		Without NaCl	with NaCl	Without NaCl	with NaCl	
Proline	Control	48±13.42	4332±167.33			2190±111.91
	S1	120±8.37	5052±356.37	310.57±16.56	261.29±145.49	2586±237.65
	S2	136±16.73	696±27.02			416±21.19
	ST	248±30.50	1012±55.227			630±42.06
P Value		0		0		0

3.5 Fourier Transform Infrared Spectroscopy FT-IR characterization of tomato plant biomolecules under different conditions

The FT-IR spectra of tomato plant tissues (leaves and stems) revealed characteristic absorption bands associated with major plant biomolecules, such as carbohydrates, proteins, lipids, and phenolic compounds (Fig 1). The broad band at 3343.90 cm^{-1} was attributed to O–H stretching vibrations of hydroxyl groups in cellulose, hemicellulose, and pectins. This band's intensity decreased significantly under salt stress, indicating reduced hydrogen bonding and moisture content. However, inoculated plants maintained higher intensity values, suggesting enhanced cell wall hydration and structural integrity. Bands at 2948.62 and 2838.78 cm^{-1} , linked to C–H stretching of aliphatic groups (CH_2 , CH_3) in waxes and lipids, were less intense in stressed plants, indicating potential membrane lipid degradation. In contrast, inoculated treatments showed stronger bands, suggesting protection of lipid components and improved membrane stability. The absorption band at 1648.57 cm^{-1} , assigned with C=O stretching (amide I) vibrations, increased under salinity, likely due to the accumulation of lignin and phenolic compounds related to stress adaptation. Inoculated plants exhibited moderate intensity, reflecting balanced biosynthesis of these metabolites. The bands at 1451.29 and 1407.88 cm^{-1} corresponded to C–H bending vibrations in carbohydrates and proteins. Their reduction under stress suggested partial degradation of cell wall polysaccharides, while the recovery of intensity in inoculated plants indicated cell wall stabilization facilitated by *Bacillus* strains.

Similarly, the absorption peaks at 1113.34 and 1014.37 cm^{-1} , corresponding to C–O–C and C–O stretching in cellulose, hemicellulose and pectins, showed a clear decline under salt stress, whereas inoculated plants exhibited relatively stronger signals, suggesting maintenance of polysaccharide structure. Minor peaks at 625.15 cm^{-1} and 583.44 cm^{-1} , associated with aromatic or mineral components, displayed slightly higher intensities under stress, indicating the accumulation of phenolic or inorganic constituents involved in defense responses. Overall, FT-IR spectral variations demonstrated that salinity stress induced biochemical changes in tomato tissues, particularly in cell wall polysaccharides, proteins, and lipids, while *Bacillus* inoculation mitigated these changes, preserving cellular structure and biochemical stability under saline conditions.

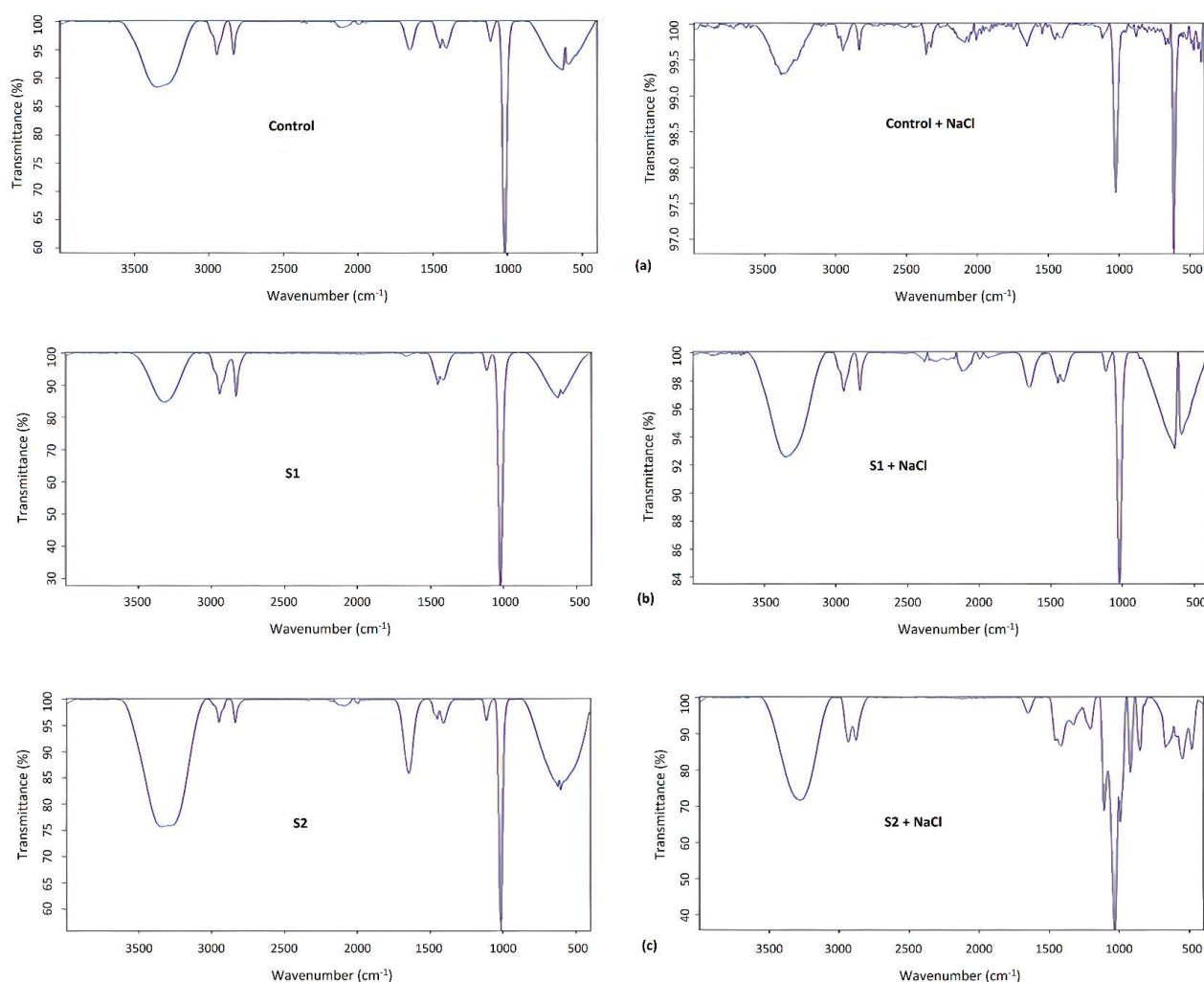


Fig 1 FT-IR analysis of the chemical constituents of tomato plants under different treatments of A) control and control + NaCl, B) plants with *Bacillus anthracis* and plants with *Bacillus anthracis* + NaCl, and C) plants with *Bacillus albus* strain FA26 and plants with *Bacillus albus* strain FA26 + NaCl

3.6 LC-MS/MS analysis

This research identified notable reductions in phenolic compounds, with comparisons across various conditions depicted in Fig 2.

Salt stress exerts a considerable influence on various metabolic processes. In this study, the effect of NaCl stress was evident when comparing the control (Fig 2a) with the control + NaCl (Fig 2d); chlorogenic acid (0.00 (Fig 2a) vs 31.17×10^3 mg/g (Fig 2d)), syringic acid (0.00 (Fig 2a) vs 58.61×10^3 mg/g (Fig 2d)), vanillin (11.29 mg/g (Fig 2a) vs 70.92×10^3 mg/g (Fig 2d)), and trans-ferulic acid (28.40 mg/g (Fig 2a) vs 87.72×10^3 mg/g (Fig 2d)) showed a substantial increase under NaCl, suggesting the activation of phenolic biosynthesis pathways in response to salinity. In non-stressed conditions, *Bacillus* inoculation led to significant metabolic changes: caffeic acid was absent in the control (Fig 2a) but present in the *Bacillus albus* strain FA26 (4.73×10^3 mg/g (Fig 2b)). Gentisic acid levels decreased in both *Bacillus anthracis* (26.57×10^3 mg/g (Fig 2b)) and *Bacillus albus* strain FA26 (17.98×10^3 mg/g (Fig 2c)) compared to the control (47.13 μ g/g (Fig 2a)). Rutin levels remained relatively stable across all conditions but were slightly lower in *Bacillus albus* strain FA26 (38.90×10^3 mg/g (Fig 2c)). p-Coumaric acid was detected in *Bacillus anthracis* (4.13×10^3 mg/g (Fig 2b)), but was absent in the control and *Bacillus albus* strain FA26 (0.00 mg/g (Fig 2c)). Under salt stress, the combined effect of NaCl and bacterial inoculation resulted in

pronounced biochemical modulation. Chlorogenic acid levels increased significantly in *Bacillus anthracis* + NaCl (60.54×10^3 mg/g (Fig 2e)) compared to the control + NaCl (31.17×10^3 mg/g (Fig 2f)). Salicylic acid (52.86×10^3 mg/g) and hydroxybenzaldehyde (1.83×10^3 mg/g) were detected only in *Bacillus albus* strain FA26 + NaCl (Fig 2f). Syringic acid and rutin levels increased significantly in *Bacillus albus* strain FA26 + NaCl (111.86×10^3 mg/g and $160.98 \mu\text{g/g}$, respectively (Fig 2f)), indicating that *Bacillus* inoculation enhanced phenolic compound accumulation under salinity.

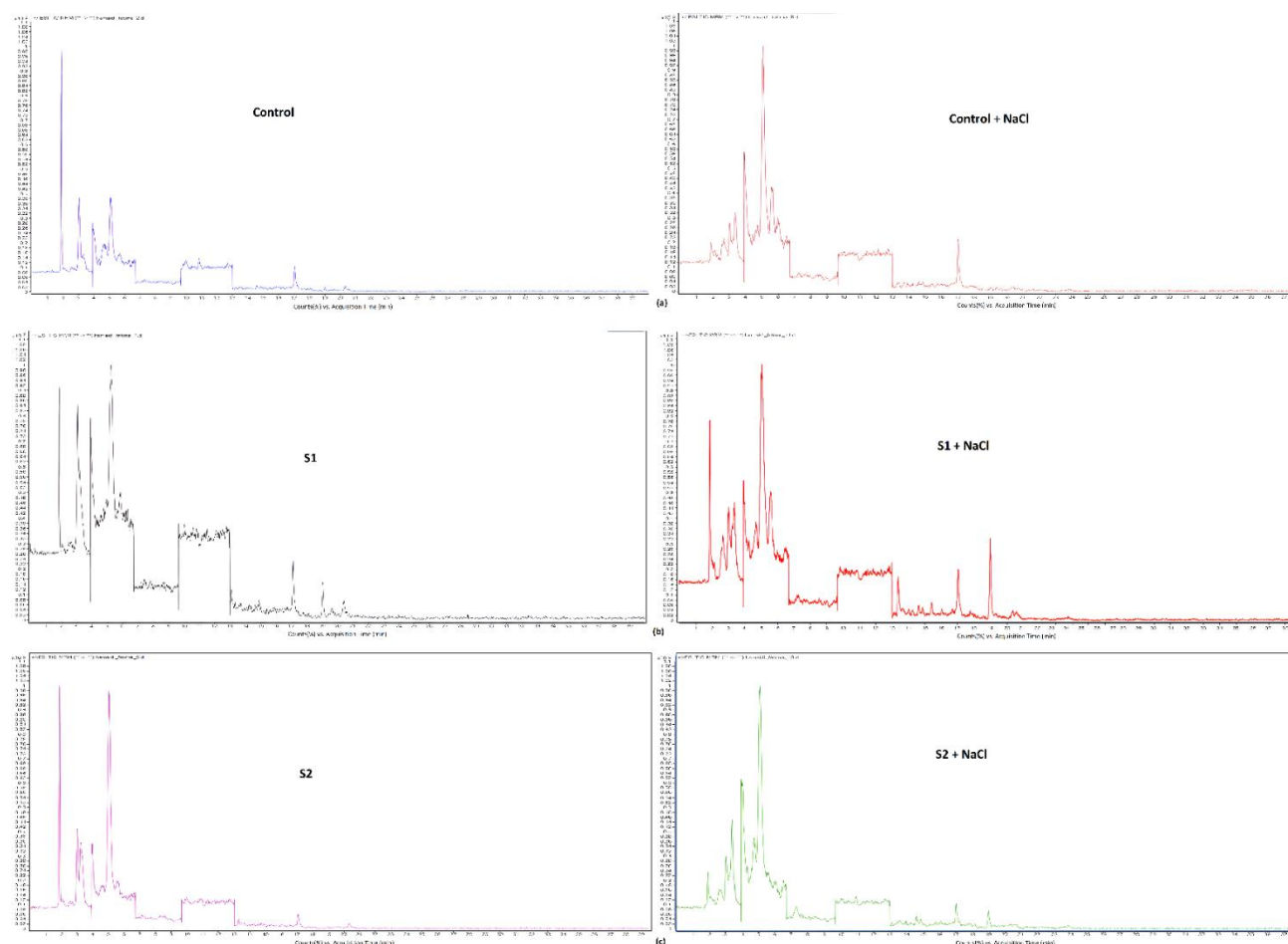


Fig 2 Representative chromatograms of LC MS/MS separation of chemical constituents extracted from tomato plants: a) control and control+NaCl, b) plants with *Bacillus anthracis* (S1) strain and S1+NaCl, c) plants with *Bacillus albus* strain FA26 (S2) and S2+NaCl

4. Discussion

Human-driven climate change and the overuse of chemical fertilizers are significant contributors to increasing soil salinity, particularly in semiarid regions. Salinity is a major abiotic stressor that severely disrupts plant growth and metabolism (Alghamdi et al. 2024). In such environments, the physiological and biochemical functions of both wild and cultivated plant species are greatly compromised, leading to considerable yield loss. Therefore, it is imperative to develop alternative and strategic solutions to enhance agricultural productivity to meet future challenges. Salt stress, in particular, adversely affects crop quality and yield, as observed in sensitive crops such as tomatoes. Tomatoes, being a vital food crop, suffer from osmotic stress, ion toxicity, and nutritional imbalances due to high soil salinity, all of which hinder plant growth (Atta et al. 2023; Gunes et al. 2023). A promising approach involves the use of halotolerant bacterial inoculants, such as *Bacillus* spp., which demonstrate salinity tolerance by thriving in high-salinity soils. The capacity of halotolerant *Bacillus* strains to prosper in saline environments, as isolated from salt-affected areas (Fig 1), supports their application in enhancing crop growth traits like IAA

production and phosphate solubilization. These traits can stimulate root elongation and branching, improve nutrient and water uptake, and enhance ammonia production and potential antifungal activity, thereby strengthening stress resistance. These findings align with those of Shahid et al. (2020). In this study, the evaluation of two *Bacillus* strains, *Bacillus albus* strain FA26 and *Bacillus anthracis* LOS6, highlighted their potential to enhance tomato growth. These results are consistent with those of Moustaine et al. (2017), who found that PGPB inoculation improved tomato growth by increasing root length, stem diameter, and chlorophyll content under field conditions. In our in vivo experiments under saline conditions, we assessed the growth performance of tomato plants that suffered morphological and physiological damage. Despite the adverse effects of salt stress on plant growth, the application of *Bacillus* sp. under these conditions yielded promising results, consistent with the findings of Egamberdieva et al. (2019). Our findings confirmed that *Bacillus* sp. (S1, ST, and to some extent S2) exhibited potential adaptability to stress and are the most promising strains for further research. They promote growth parameters under salt stress, comparable to non-stressed conditions and mitigate the adverse effects of salt stress. These results are consistent with those of previous studies demonstrating the ability of plant growth-promoting rhizobacteria to enhance plant tolerance to salinity by improving nutrient uptake, hormonal balance, and stress-responsive mechanisms (Egamberdieva et al. 2017; Bharti et al. 2021).

Salt stress in tomato plants leads to a disruption in ion homeostasis, characterized by a decrease in K^+ and Ca^{2+} levels and an increase in Na^+ levels, particularly in control plants. The decline in K^+ is attributed to the competitive inhibition by Na^+ during ion uptake. Güneş et al. (2025) reported that K^+/Na^+ values were lower in salt concentration applications compared to control groups and that differences in the K^+/Na^+ ratio between applications occurred as salt concentration increased. Conversely, *Bacillus* sp. was found to reduce Na^+ accumulation and enhance K^+ retention under salinity. Inoculation with S2 was shown to improve K^+ levels and limit Na^+ accumulation under moderate salinity by regulating the expression and activity of K^+ and Na^+ transporters and H^+ pumps, likely through the regulation of K^+/Na^+ transporters and plasma membrane H^+ -ATPase activity. Additionally, while ST (S1 + S2) plays a protective role by effectively mitigating K^+ loss under salt stress, thereby preserving ion homeostasis. The highest Ca^{2+} levels recorded in S1 suggest a role in stabilizing cell membranes and mediating signaling pathways crucial for stress adaptation. These findings underscore the role of halotolerant *Bacillus* spp. in maintaining ion homeostasis and reducing Na^+ toxicity, aligning with recent studies that have highlighted the ability of *Bacillus* inoculants to modulate ion transport and maintain optimal K^+/Na^+ ratios under saline conditions (Wu et al., 2024). These results are consistent with other research, such as Abdelaziz et al. (2019), which demonstrated that *Pseudomonas indica* colonization under salt stress led to a fourfold reduction in Na^+/K^+ ratios in tomato leaves and roots. Zhu (2003) emphasized the critical importance of maintaining high K^+ and reducing Na^+ concentrations in the cytosol of plants under salt stress.

Proline, a known osmoprotectant, accumulates in plants under salt stress conditions to counteract osmotic imbalance and oxidative damage. In our study, non-inoculated tomato plants exposed to salt stress showed a substantial proline accumulation ($4,332 \mu\text{mol g}^{-1}$), supporting the recognized role of proline as an osmoprotectant in saline environments (Forni et al. 2017). The inoculated treatment S1 further increased proline levels ($5,052 \mu\text{mol g}^{-1}$), aligning with previous studies indicating that certain PGPB can enhance osmolyte accumulation in tomatoes under salinity (El-Banna et al. 2024). In contrast, treatments S2 and ST exhibited significantly lower proline concentrations (696 and $1,012 \mu\text{mol g}^{-1}$, respectively), suggesting that these strains may alleviate salt-induced osmotic/ionic stress through mechanisms other than extensive proline accumulation. Literature indicates that PGPB may enhance salt tolerance through ion homeostasis, activation of antioxidant enzymes, and improved nutrient uptake, rather than solely through osmolytes (Karimi et al. 2025). Thus, the reduced proline content in S2 and ST may reflect a lower stress burden on the plant, rather than diminished tolerance, highlighting the importance of evaluating multiple physiological and biochemical parameters. This accumulation can damage chloroplast structures and

impair the photosynthetic machinery, thereby reducing the chlorophyll content (Wang et al. 2024). This may be attributed to improved antioxidant mechanisms or enhanced stability of photosynthetic pigment under stress. Among the strains tested, S1 retained the highest chlorophyll levels, particularly total chlorophyll and chlorophyll A, indicating superior salt stress tolerance. Conversely, strain ST exhibited lower chlorophyll levels under stress, suggesting greater susceptibility to salinity. While some bacterial strains may support pigment stability, further research is necessary to confirm their roles in stress tolerance mechanisms. The observed enhancement in root and shoot growth, as well as Chlorophyll stability, suggests that these bacteria may improve nutrient availability, hormonal regulation, and stress-related defense mechanisms. Similar trends have been observed in studies reporting that plant growth-promoting bacteria enhance plant resilience and promote growth under saline conditions. In this study, carotenoid content varied significantly among treatments and conditions, reflecting their functional roles in stress response mechanisms. These findings align with previous reports highlighting the antioxidative role of carotenoids during abiotic stress. The ability of *Bacillus* strains to support carotenoid homeostasis may significantly contribute to enhancing plant salt tolerance and maintaining photosynthetic efficiency. Furthermore, variability among strains suggests potential strain-specific mechanisms for modulating plant stress responses. These findings concur with those of Zafar et al. (2022), who reported increased carotenoid levels under salt stress conditions in *Gossypium hirsutum* (cotton) germplasm.

The dynamic nature of the stress response in tomato plants is effectively illustrated through FT-IR separation. This technique has shown the non-inoculated control plants under salt stress experienced significant increases in chlorogenic acid, syringic acid, and vanillin, suggesting the activation of phenolic metabolism as a defensive strategy. This rise in chlorogenic acid levels under NaCl stress highlights its protective function against oxidative damage, corroborating previous research that indicate phenolic accumulation enhances plant resilience in saline environments. In a manner akin to the response observed with *Bacillus anthracis*, chlorogenic acid levels increased significantly under NaCl stress, influencing phenolic biosynthesis, strengthening cell wall integrity and reducing oxidative stress. Salicylic acid was detected solely under NaCl stress, indicating a potential stress response in plants. Additionally, rutin and trans-ferulic acid were found to accumulate more in NaCl-stressed plants associated with the *Bacillus albus* strain FA26.

Research, such as that by Reimer et al. (2021), has combined FT-IR findings with LC-MS, and the results from LC-MS/MS validate the FT-IR data, providing quantitative confirmation of the identified compounds. FT-IR identified functional groups associated with phenolic compounds, while LC-MS/MS offered further quantification of these metabolites, enhancing the reliability of our findings. Flavonoids (rutin, chlorogenic acid) and phenolic acids (gentisic acid, syringic acid, trans-ferulic acid, and vanillin) are involved in oxidative stress protection, lignin biosynthesis, and defense mechanisms, particularly as salicylic acid, which was observed only under stress conditions. The major absorption peaks correspond to O–H (3340 cm^{-1}), C–H (2920 cm^{-1}), and C=O (1630 cm^{-1}) functional groups, indicating changes in biochemical composition under salt stress. These results align with those of a previous study by Al Hassan et al. (2015), which demonstrated that phenolic and flavonoid contents increased under high salinity stress (Hasanuzzaman and Fujita 2022). In summary, our findings suggest that salt stress induced a significant increase in several phenolic compounds, while inoculation with *B. albus* FA26 further enhances or modulates these responses, aiding in the biochemical defense and adaptation of tomato plants under saline conditions.

5. Conclusion

The integration of PGPB into agricultural systems offers a sustainable strategy to counteract the detrimental effects of salinity, thereby enhancing crop resilience and productivity. These findings highlight the pivotal role of ion homeostasis in salt stress

tolerance. *Bacillus albus* strain FA26 and *Bacillus anthracis* LOS6 proved to be effective in alleviating salt stress, with *Bacillus albus* strain FA26 excelling in Na⁺ exclusion and *Bacillus anthracis* LOS6 in Ca²⁺ regulation. However, *Bacillus anthracis* LOS6 combined with *Bacillus albus* strain FA26 was less effective in managing ion toxicity, suggesting its limited suitability for saline environments compared to other treatments. Further research into the molecular mechanisms underlying these differences will provide a deeper understanding of plant-microbe interactions in salt stress tolerance. The study also underscores the importance of proline as a stress biomarker in plants. While *Bacillus albus* strain FA26 relies significantly on proline accumulation for salt stress tolerance, *Bacillus anthracis* LOS6 appears to utilize alternative protective mechanisms, warranting further physiological and molecular exploration. This research demonstrates the potential of strain-specific microbial inoculants to enhance crop resilience against salinity stress.

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