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The synergy of halotolerant PGPB and mauran mitigates salt stress in tomato (*Solanum lycopersicum*) via osmoprotectants accumulation

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Abstract

Salinity stress is one of the major abiotic factors limiting sustainable agriculture. Halotolerant plant growth-promoting bacteria (PGPB) increased salt stress tolerance in plants, but the mechanisms underlying the tolerance are poorly understood. This study investigated the PGP activity of four halotolerant bacteria under salinity stress and the tomato salt-tolerance mechanisms induced by the synergy of these bacteria with the exopolysaccharide (EPS) mauran. All PGPB tested in this study were able to offer a significant improvement of tomato plant biomass under salinity stress; *Peribacillus castrilensis* N3 being the most efficient one. Tomato plants treated with N3 and the EPS mauran showed greater tolerance to NaCl than the treatment in the absence of EPS and PGPB. The synergy of N3 with mauran confers salt stress tolerance in tomato plants by increasing sodium transporter genes' expression and osmoprotectant content, including soluble sugars, polyols, proline, GABA, phenols and the polyamine putrescine. These osmolytes together with the induction of sodium transporter genes increase the osmotic adjustment capacity to resist water loss and maintain ionic homeostasis. These findings suggest that the synergy of the halotolerant bacterium N3 and the EPS mauran could enhance tomato plant growth by mitigating salt stress and could have great potential as an inductor of salinity tolerance in the agriculture sector.

1 | INTRODUCTION

Agriculture can be affected by abiotic and biotic stresses (Ali et al., 2022), salinity being one of the major abiotic factors limiting global agricultural productivity. Soil salinity is spreading globally in over 100 countries; currently, no continent is completely free from salt stress. To face that situation, it has been proposed to promote agriculture on saline soils, but most crops have a low salt tolerance (Etesami and Glick, 2020). Salinity causes an osmotic, ionic and oxidative stress affecting normal plant development. The first leads to deficient water uptake by plants due to their osmotic potential, the second causes sodium toxicity effects and disturbs plants' nutritional balance, and the third leads to production of reactive oxygen species (ROS) damaging different cellular components (Gupta et al., 2021). To counteract all those stresses, morphological, physiological and biochemical changes occur in plants, such as a decrease in transpiration rate, stomatal closure and photosynthesis alteration, leading to plant growth and yield reduction (Chourasia et al., 2022).

Under salinity conditions, plants have developed different salt tolerance mechanisms, such as compartmentalization or exclusion of

toxic ions, activation of ROS-scavenging enzymes and the production of different compounds protecting against oxidative stress, osmotic adjustment and hormonal regulation. The synthesis and accumulation of organic compounds maintain tissue turgidity, allowing water and nutrient uptake, which is critical for plant survival in salt stress. These compounds, collectively osmoprotectans, can accumulate to high levels without disturbing intracellular biochemistry and are either carbohydrates, amino acids, glycine betaine, polyamines or polyphenols (Verslues et al., 2006). Carbohydrates are a significant category of compatible solutes that include polyols (myo-inositol, inositol, mannitol), hexoses (fructose and glucose) and disaccharides (sucrose). Among the amino acids, proline is considered to be the most efficient and abundant compatible solute, whose synthesis can be dramatically enhanced by salt stress (Peña Calzada et al., 2023). The γ-aminobutyric acid (GABA) is a non-protein amino acid that also accumulates in many plant species in response to environmental stress, performing a dual function during salinity, acting as both an energy-providing metabolite and a carbon source to feed an alternative route for the Krebs cycle (Dabravolski and Isayenkov, 2023). Further, polyamine metabolism is closely linked to amino acids such as GABA and proline, all known to be involved in stress tolerance (Verslues et al., 2006).

Plant growth-promoting bacteria (PGPB) are non-pathogenic microorganisms present in the rhizosphere of plants, enhancing plant growth and productivity and promoting tolerance to various abiotic and biotic stresses. The use of PGPB is an alternative strategy to improve plant tolerance mechanisms. It is then of interest to isolate bacteria with salt-tolerance characteristics from a halophyte rhizosphere (Saghafi et al., 2019; Etesami and Glick, 2020). Several PGPB have been assessed with regard to salt stress amelioration and have been found to help plants overcome salt stress by different direct and indirect mechanisms (Velázquez-Becerra et al., 2011; Hakim et al., 2021), such as phytohormone production, nitrogen fixation, nutrient solubilization, production of lytic enzymes, antibiosis, siderophores production, biofilm formation or production of volatile organic compounds among others. All these mechanisms cause in plants an increased production of different compounds protecting against osmotic and oxidative stress, such as polyamines, amino acids, polyphenols or soluble sugars. The aim of the present study was to investigate the use of halotolerant PGPB in combination with the exopolysaccharide mauran in order to increase the salt tolerance of tomato plants and to elucidate the mechanisms implicated in this stress protection.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains

The four strains used in this study (*Pseudomonas brassicacearum* subsp. *neoaurantiaca* B22, *Kushneria endophytica* B23, *Pseudomonas segetis* P6 and *Peribacillus castrilensis* N3) belong to the bacterial collection isolated by the BIO-188 research group. *P. castrilensis* N3 was isolated from *Lutra lutra* feces in Castril, Granada (37°52′00″N 2°45′58″W), while the other ones were isolated from the rhizosphere of the halophyte plant *Salicornia europaea* (strain P6) and from the shoot and rhizosphere of the halophyte plant *Arthrocaulon* in the Saladar de El Margen, Cúllar, Granada (37° 38′50.6"N 2°37′22.2"W). These strains were selected in previous studies of the BIO-188 lab according to their PGP activity and quorum quenching activity (Rodríguez et al., 2020; Rodríguez et al., 2022).

2.2 | EPS isolation and purification

Mauran, the EPS produced by *Halomonas maura* S30, was isolated and purified as previously described (Quesada et al., 1994; Arias et al., 2003). Briefly, strain S30 was cultivated in MY medium supplemented with 7.5% w/v marine salts' solution (Rodríguez-Valera et al., 1981) for 5 days. Then, the culture was centrifuged and the supernatant precipitated with cold ethanol before being ultracentrifuged, dialyzed against distilled water and, finally, lyophilized. Strain S30 was able to produce 3.8 g of mauran per liter of medium.

2.3 | Salt tolerance of bacterial isolates

The optimum growth and growth range were determined by plate count on tryptone soya agar (TSA, Difco®) medium with different NaCl concentrations ranging from 0 to 2053 mM.

2.4 | Plant Growth Promoting assays in abiotic conditions

2.4.1 | Tomato plant assays

Tomato seeds were surface sterilized according to the protocol described by Molan et al. (2010). PGP activity under salinity conditions was determined in tomato plants grown for two weeks from sterile seeds sown in plastic pots containing a sterilized mix of perlite/vermiculite (1:3 v/v). The experimental design was as follows: (i) non-inoculated control plants, (ii) plants inoculated with one of the 4 PGPB strains, (iii) plants treated with 100 mM NaCl, (iv) plants primed with 100 mM NaCl and one of the 4 PGPB strains. For PGPB treatment, 250 µL of bacterial suspension was applied with three days interval for 15 days. Control plants were treated with the same volume of sterile water. Salt stress was applied at the end of the bacterial inoculation period (15 days) with three days interval over 28 days with Hoagland Solution at 0 and 100 mM NaCl. Each treatment was composed of 9 plants.

The study of the PGP activity of the strain N3 in combination with the EPS produced by the halophilic bacterium *H. maura* S30 under non-salinity and salinity conditions was performed as mentioned above with the inoculation of bacterial suspension and 250 µL of EPS at the same time. The concentration of the EPS inoculated was 3.8 g of mauran per liter of sterile water.

Plants were grown in a phytotron growth cabinet with a long-day photoperiod (16:8 h light:dark) at 25°C with a relative humidity of 60%. Plant height, dry weight and stem diameter were measured for selected three plants in each treatment. The rest of the plant material (6 plants) was lyophilized for further analysis in this study.

2.4.2 | Determination of sodium

Digestion of the dried samples was performed in an acidic medium at 95°C (Bressy et al., 2013). Subsequently, the sodium content was determined by inductively-coupled plasma optical emission spectrometers (ICP-OES, Perkin Elmer, Optima 8300DV).

2.4.3 | Relative gene expression of *SlSOS1* and *NHX4* by quantitative RT-PCR

RNA was extracted from leaves by using the Direct-Zol RNA Miniprep Kit (Zymo Research) according to the manufacturer's instructions. The cDNA was synthesized from 1 µg RNA using Maxima Reverse Transcriptase (Thermo Fisher Scientific). For qRT-PCR, amplifications were run in a 96-well-plates iCycler iQ thermal cycler (Bio-Rad) using iQ SyBr Green Supermix (BioRad). Quantification was performed with the iCycler iQTM associated software (Real Time Detection System Software, version 2.0). Primers pairs used in this study are shown in Table S1. LeEF1α was used as the internal reference gene for normalizing the transcript profiles following the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.4.4 | Determination of polyamines

Polyamines were extracted from lyophilized material with 1% formic acid containing diaminoheptane as internal standard. Samples were then centrifuged at 3500 x *g* for 10 min. The supernatant was filtered with nylon filter (0.22 µm) and suitably diluted. Each sample was injected into a column (ACQUITY UPLC® HSS T3 12.1 mm × 100 mm, 1.8 µm). Solvent flow was 0.4 mL/min, and the elution gradient was prepared with eluent A (20 mM ammonium formiate) and eluent B (acetonitrile). 1 µL of each sample was injected and the gradient profile was applied as follows (t (min); %A): (0; 100%), (5; 100%), (6; 0%), (8; 0%), (8.1; 100%). Eluates were detected using a Xevo TQ-S triple quadrupole mass spectrometer (Waters) in the positive electrospray ionization (ESI) mode. The ion spray voltage was set at 2900 V, the source temperature at 150°C and desolvation temperature at 600°C. Putrescine, spermidine and spermine were detected in the multiple reaction monitoring mode of the tandem mass spectrometer with the following transitions: putrescine, m/z 89 → 30.05, m/z 89 → 44.05; diamineheptane, m/z 131.05 → 55.09, m/z 131.05 → 69.14; spermidine, m/z 146.06 → 72.06, m/z 146.06 → 84.09; spermine, m/z 203.18 → 72.11, m/z 203.18 → 84.07. The quantitative determination of each polyamine was performed by interpolation with a standard curve prepared for each polyamine, considering the value of the internal standard.

2.4.5 | Phenol content determination

Phenol extraction was determined following the protocol described by Singleton et al. (1999) with modifications. The lyophilized plant sample was extracted with acetone 80% in darkness for 30 min at 4°C at 1/100 proportion (lyophilized weight of sample/volume) and centrifuged at 8000 x *g* for 10 min at 4°C. The phenolic content of the supernatant (v/v) was determined by the Folin–Ciocalteu method according to [65]. The content of phenolic compounds was expressed as g of gallic acid per g of dry weight.

2.4.6 | Determination of glucose, fructose, sucrose and polyols

Carbohydrates were extracted from lyophilized material with a mix of ethanol/chloroform/water (12/5/1, v/v/v), adding lactose and ribitol as internal standards. The mixture was shaken for 30 min at 4°C and then centrifuged at 3500 x *g* for 10 min at 4°C. 1 mL of chloroform and 0.5 mL of water were added to the supernatant, strongly mixed and centrifuged at 3500 x *g* during 5 min to separate phases, then the aqueous phase was dried under nitrogen flow and stored at −20°C under an inert atmosphere. The sample was resuspended in 0.5 mL of milli-Q water, sonicated, and filtered with nylon filter (0.22 µm) and suitably diluted. Soluble carbohydrates were quantified by ion chromatography following the method proposed by Palma et al. (2014) with some modifications on a Dionex ICS-3000 chromatograph (Dionex Corp.). Glucose, fructose and sucrose were separated in a CarboPac PA20 column with eluent A (distilled water) and eluent B (NaOH 0.2 M), the gradient profile was applied as follow (t (min); %A): (0; 80%), (8; 80%), (9; 0%), (15; 0%), (16; 80%), (21; 80%) flow rate 0.5 mL/min. Polyols were separated in a CarboPac MA1 column with eluent A (distilled water) and eluent B (NaOH 0.6 M); the gradient profile was applied as follows (t (min); %A): (0; 100%), (15; 40%), (26.5; 0%), (35; 0%), (37; 100%), flow rate 0.4 mL/min. Results were calculated by standard curves.

2.4.7 | Determination of amino acids

The amino acids were extracted from lyophilized material with a mix of ethanol/chloroform/water (12/5/1; v/v/v). Norvaline and sarcosine were used as internal standards. The extract was centrifuged at 3500 x *g* during 10 min at 4°C and the supernatant was separated into chloroform and aqueous phases by the addition of HCl 0.1 N and chloroform. The mixture was centrifuged at 3500 x *g* during 5 min to separate phases; then, the aqueous phase was dried under nitrogen flow and stored at −20°C under an inert atmosphere. The dried samples were resuspended in 0.9 mL of HCl 0.1 N, sonicated and filtered with nylon filter (0.22 µm) and suitably diluted. Combining o-phthalaldehyde (OPA) and fluorenylmethyl chloroformate (FMOC) chemistries was used for pre-column derivatization of amino acids.Chromatographic analysis was done by following the method proposed by Palma et al. (2019) with some modifications. The amino acids were quantified by HPLC (Agilent 1260

Strain	0 mM	100 mM	200 mM	430 mM	855 mM	1283 mM	2053 mM
B22	+++	++	++	++	+	–	–
P6	+++	+++	+++	+++	++	+	–
N3	+++	+++	++	++	+	–	–
B23	++	++	+++	+++	+	+	+

TABLE 1 Growth of PGPB isolates at different concentrations of NaCl (mM).

Symbols: Growth evaluation by means of measure of halo diameter (–) No growth; (+, <1 cm) Little growth; (++, 1–1,5 cm) Moderate growth; (+++, > 1,5 cm) Excellent growth.

Infinity) with an ACE 5 C18-PFP 4.6 mm × 250 mm column and a fluorometer using excitation and emission wavelengths of 340 and 450 nm (0–15 min) and 260 and 325 nm (16–33 min). Amino acids were eluted at a flow rate of 1 mL/min using an elution gradient with sodium acetate buffer 25 mM pH 6.8 (A) and acetonitrile/methanol/water mix (45/45/10, v/v/v) (B). The gradient profile, expressed as (t [min]; %A), was: (0; 80%), (20; 40%), (24; 40%), (26; 0%), (31; 0%) and (33; 80%).

2.5 | Statistical analysis

Statistical analysis was carried out using GraphPad Prism 7.04 program. Data normality was assessed using the Shapiro–Wilk test or the D'Agostino and Pearson test. After normalizing them, they were analyzed by simple ANOVA using the Tukey test for the comparison of means, with a confidence interval of 95%. Data were normalized and scaled for principal components analysis (PCA) using MetaboAnalyst 5.0. To measure the linear correlation between the studied variables, a correlation matrix based on Pearson Correlation Coefficient was performed using MetaboAnalyst 5.0.

3 | RESULTS

3.1 | Plant beneficial traits of bacterial isolates

The four plant growth-promoting bacteria (PGPB) used in this study were selecected based on their ability to produce siderophores, as well as enzymes such as lipases, acid and alkaline phosphatases, DNase and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Rodríguez et al., 2020; Rodríguez et al., 2022). All bacterial isolates were able to grow with concentrations of NaCl in the range of 0–855 mM (Table 1). The P6 and B23 strains also grew at 1283 mM of NaCl. The B23 strain grew at all the concentrations of NaCl tested in this study including 2053 mM (Table 1). These results demonstrate that the PGPB used in this study are capable of growing in a wide range of salt concentrations and all the strains are halotolerant.

3.2 | Effect of bacterial inoculations on the growth of tomato under non-salinity and salinity conditions

The inoculation with the 4 PGPB showed a positive effect on the growth of tomato plants 28 d after the inoculation (Figure 1A). The inoculation with the strain *P. segetis* P6 showed the highest increases in biomass (61%) and length (33%) of plants under non-saline conditions. To investigate the PGP activity of the 4 halotolerant bacteria after 28 d of salt treatment (100 mM NaCl), their agro-morphological attributes were measured. The plant dry weight was significantly reduced under salinity stress compared to the unstressed plants (Figure 1A). However, the dry weight of tomato plants significantly increased under salinity stress when PGPB were inoculated compared to the control without inoculation. Plant height also decreased upon salt stress. This decrease was only restored by *Peribacillus castrilensis* N3 strain inoculation (Figure 1B), while treatment with the other strains did not significantly mitigate the reductions in plant height induced by NaCl. A similar trend in stem diameter was observed, where the tomato plants inoculated with N3 strain showed the highest values of stem diameter under 100 mM NaCl (Figure 1C).

3.3 | Contribution of *P. castrilensis* N3 to growth performance and salt tolerance of tomato plant in presence of EPS

Considering the previous results observed in this study, *P. castrilensis* N3 was used to evaluate the role of the inoculation of this PGPB with the exopolysaccharide (EPS) produced by an halophilic bacterium, mauran, in preventing salt stress in tomato plant. Biomass was evaluated as dry weight of plants. N3 combined with EPS significantly increased the biomass under non-stress and stress conditions. Under salt stress, the plant dry weight significantly increased by 1.9-fold when N3 was inoculatedin combination to EPS compared to the control treatment (non-inoculated) (Figure 2A). A similar trend was observed for plant height and stem diameter (Figure 2B,C): Both parameters significantly increased with the inoculation of N3 with EPS under control and salt conditions with respect to the non-inoculated control and to the inoculated-only plants (data not shown).

The sodium content in tomato plants' shoots significantly increased with the combined treatment of N3 and EPS under salt stress conditions compared to the non-inoculated control (Figure 3B). However, this treatment did not significantly change the Na^+ content in plants' roots under saline conditions (Figure 3A).

The salt stress-responsive gene *SlSOS1* was over-expressed by 2-fold upon N3 inoculation with EPS compared to the non-inoculated plants under saline stress (Figure 3C).

The expression of *NHX4* was also induced by N3 + EPS inoculation, especially under salinity (1.75-fold higher in N3 + EPS inoculation than non-inoculated plants (Figure 3D).

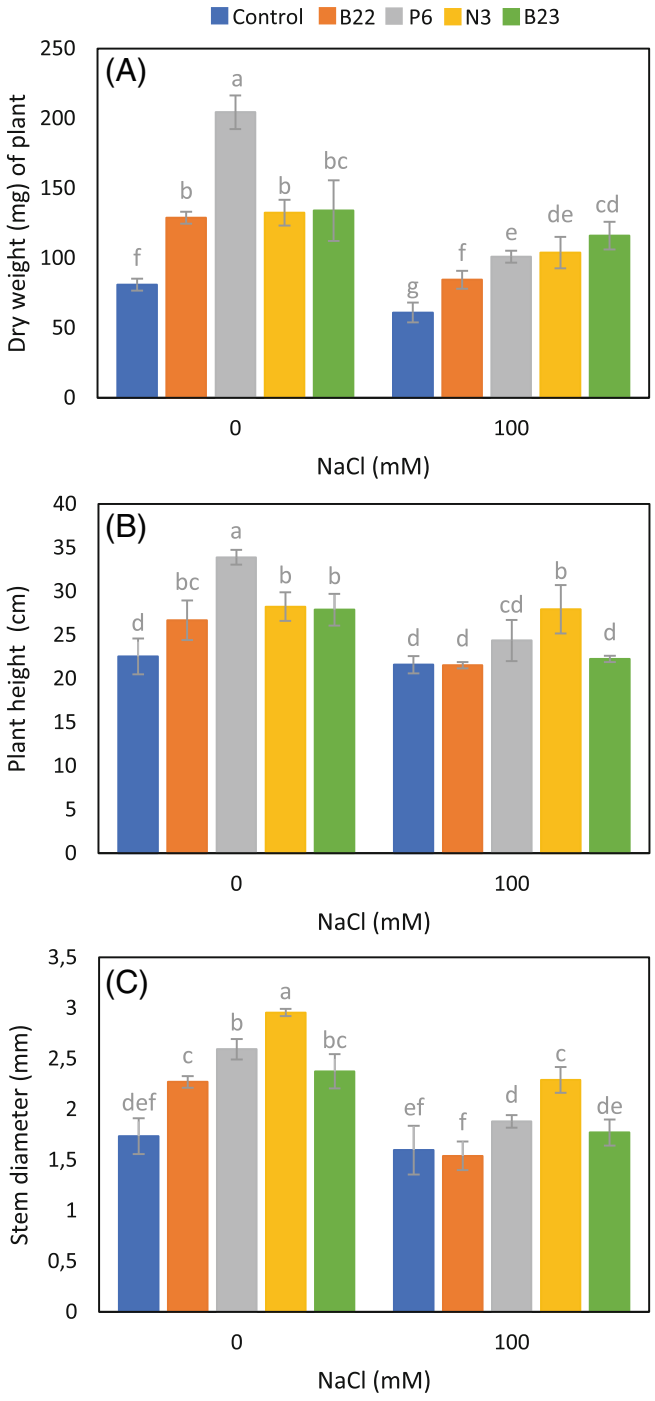

FIGURE 1 Effects of PGPB inoculation on dry weight (A), plant height (B) and stem diameter (C) of tomato plants under 0 and 100 mM NaCl. Data presented are means ± SE (n = 3 triplicate, each pooling the results of 3 plants). Different letters indicate significant differences according to Tukey's test ($p < 0.05$).

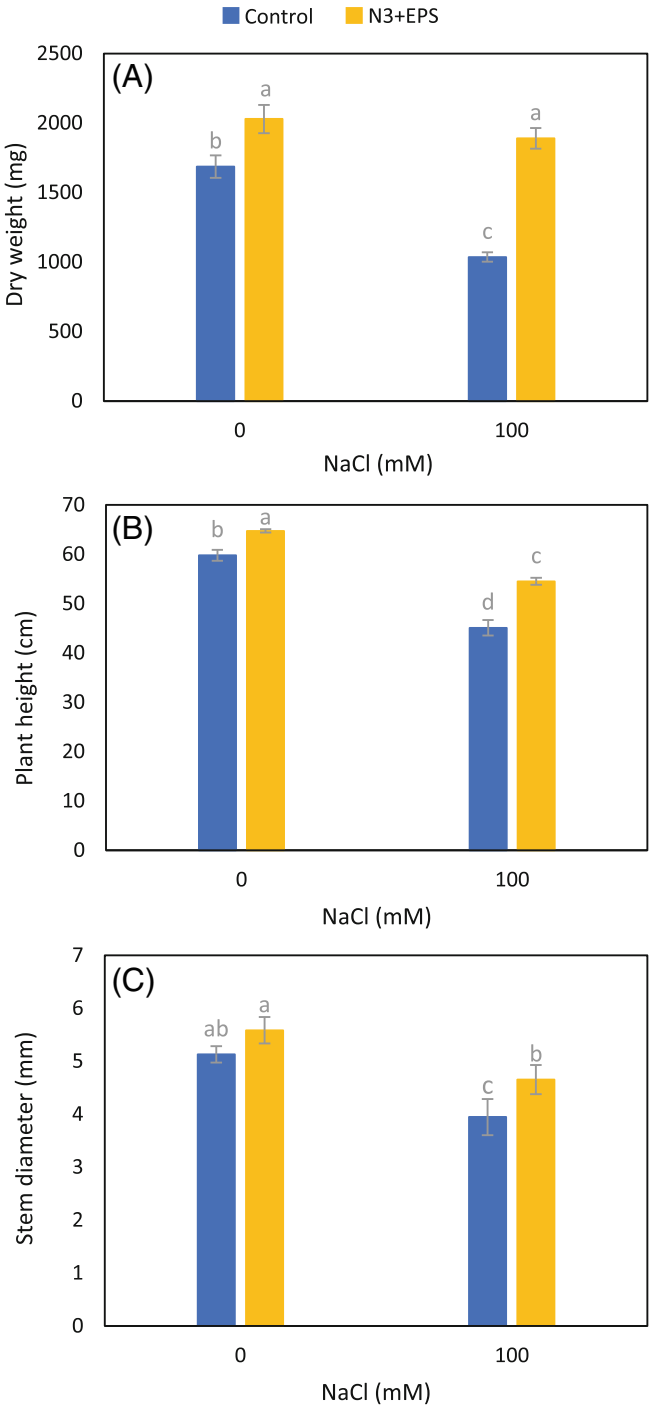

FIGURE 2 Effects of N3 inoculation and EPS (mauran) on dry weight (A), plant height (B) and stem diameter (C) of tomato plants under 0 and 100 mM of NaCl. Data presented are means ± SE of triplicate samples of 3 plants each. Different letters indicate significant differences according to Tukey's test ($p < 0.05$).

3.4 | Effects of *P. castrilensis* N3 and EPS on osmolytes of tomato leaves under non-salinity and salinity conditions

The content of soluble sugars (glucose, fructose and sucrose) in tomato leaves did not change significantly in N3-EPS-inoculated plants compared to un-inoculated plants under non-salinity conditions. However, under 100 mM NaCl, the treatment with N3-EPS showed promising enhancement compared to non-inoculated plants for all the soluble sugars included in this study (glucose: 64%; fructose: 58% and sucrose: 41%; Figure 4A-C). The same trend was observed for myo-inositol, whose content increased by 15% in

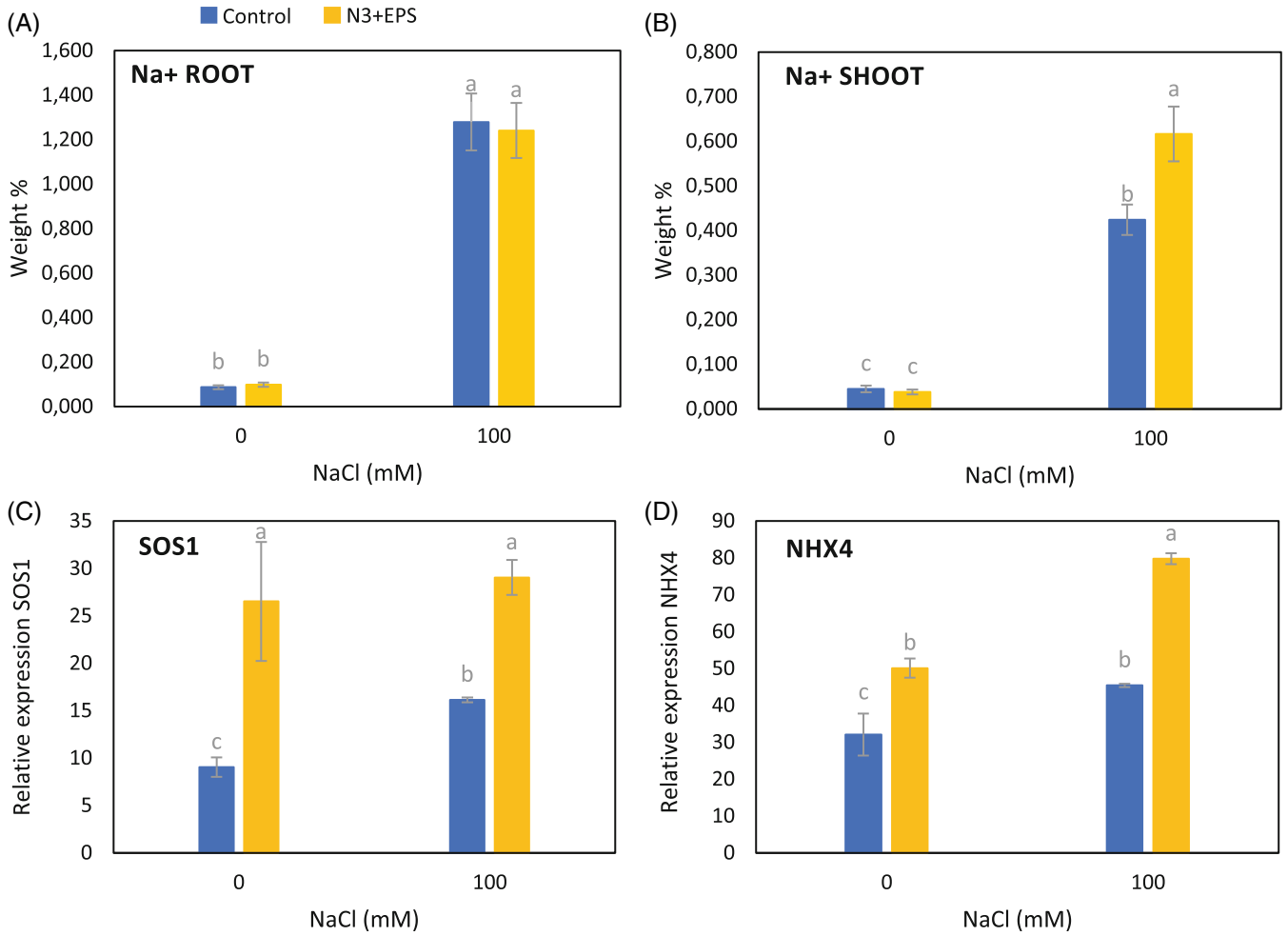

FIGURE 3 Changes in the content of sodium in root (A) and shoot (B) of tomato plants treated with 0 and 100 mM of NaCl and inoculated with N3 and EPS; expression of salt stress-responsive gene *SlSOS1* (C) and *NHK4* (D).

inoculated tomato plants compared to non-inoculated ones (Figure 4D).

Soil salinity also influences the levels of osmoprotectant solutes in tomato leaves. Free amino acids, free phenols, the non-protein amino acid GABA and proline content increased drastically with the application of 100 mM of NaCl. However, if we compare the content of these osmoprotectants under saline conditions between inoculated and non-inoculated plants, the application of N3-EPS significantly increased total amino acids by 12% (Figure 4E), total phenols by 9% (Figure 4F) and GABA by 13% (Figure 4G). The highest increase under salt stress was observed in the proline content of tomato leaves following inoculation with N3 + EPS (39%) (Figure 4H).

Arginine content in non-inoculated plants increased by 1.5-fold under salinity. By contrast, in N3-EPS inoculated treatment, no significant differences were observed during salt stress (Figure 5A). However, a significant increase of the polyamine putrescine was observed with N3-EPS inoculation under salinity conditions (Figure 5B). A different trend was observed for spermidine and spermine, whose levels decreased in the presence of salt in non-inoculated and inoculated treatments (Figure 5C,D). However, the lowest levels of both polyamines were detected in the leaves of tomato plants inoculated with N3 + EPS.

The components of principal component analysis (PCA) produce a total variation of 48.7%. PC1 and 2 accounted for 27 and 21.7% of the total variability, respectively (Figure 6). The treatment that consists of the inoculation of N3 together with the application of mauran under salt stress appeared clearly separated from 100 mM NaCl, whereas control, N3 + EPS and N3 + EPS + NaCl grouped together. The separation between tomato plants treated with N3 + EPS + NaCl and 100 mM NaCl treatment revealed the importance of PC2.

Pearson's correlation analysis was performed among different osmoprotectants and physiological parameters of *Solanum lycopersicum* (Figure 7). The dendrogram showed a cluster composed of proline, stem diameter and dry weight proline. These parameters had a strong inverse correlation with arginine content. Sucrose and fructose also appeared in the same cluster as proline. GABA showed a direct correlation with putrescine; both metabolites are in the same cluster.

These physiological parameters showed a strong inverse correlation with arginine content. We also found a strong inverse correlation

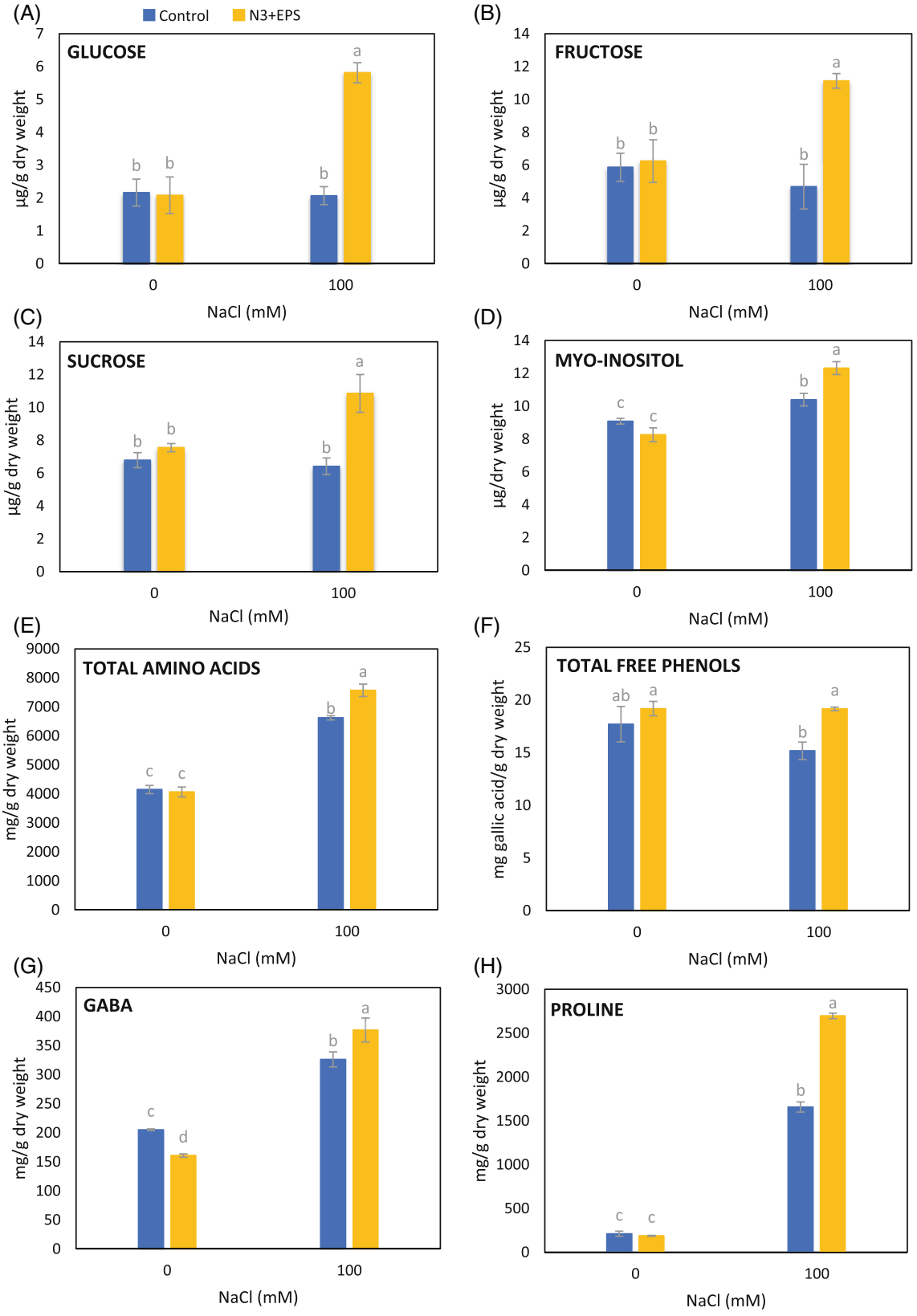

FIGURE 4 Changes in the content of glucose (A), fructose (B), sucrose (C), myo-inositol (D), total amino acids (E), total phenols (F), GABA (G) and proline (H) in tomato plants treated with 0 and 100 mM of NaCl and inoculated with N3 and EPS. Means ± SE, n = 9. Different letters indicate significant differences according to Tukey's test ($p < 0.05$).

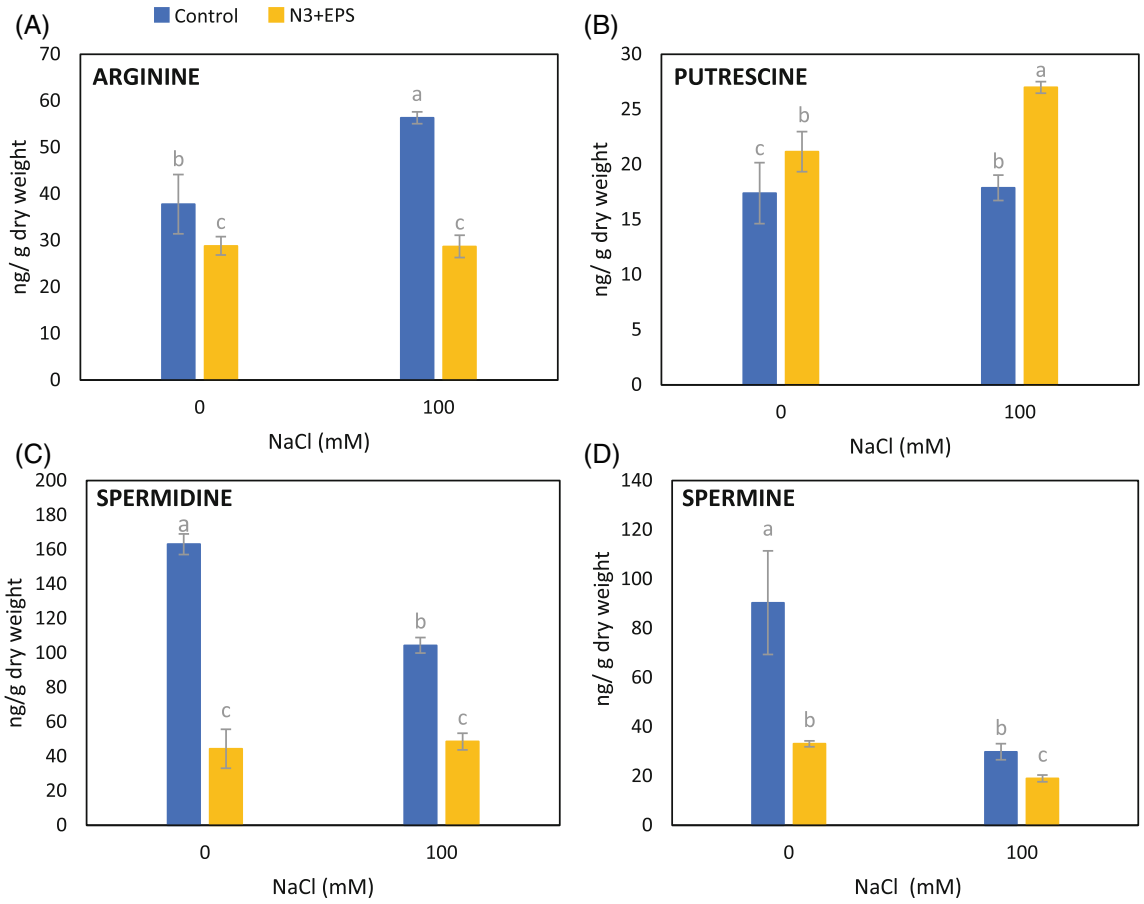

FIGURE 5 Changes in the content of arginine (A), putrescine (B), espermidine (C) and espermine (D) in tomato plants treated with 0 and 100 mM of NaCl and inoculated with N3 and EPS. Means ± SE, n = 9. Different letters indicate significant differences according to Tukey's test ($p < 0.05$).

between arginine and proline. However, a strong correlation was evident between GABA and putrescine.

4 | DISCUSSION

The present study was conducted to select halotolerant PGPB enhancing tomato plant tolerance to salt stress. Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops and is a model plant for stress tolerance research. The harmful effect of salinity in tomato was evident in the range of 100–150 mM NaCl, as shown by a decrease in plant height, dry weight and fruit production (Kiferle et al., 2022). Using PGPB could help to mitigate some abiotic stresses, such as salinity and to reduce the use of chemical inputs in agriculture through direct and indirect mechanisms.

The 4 PGPB used in this study could promote plant growth using direct mechanisms, including the sequestration of iron by siderophores and indirect mechanisms, including the production of ACC deaminase. Previous studies showed the relevance of both mechanisms in the PGP activity of bacteria under adverse abiotic stress (Belimov et al., 2015). All the PGPB tested in this study were able to improve plant dry weight under salinity stress. Among them, *P. castrilensis* N3 was the most efficient in promoting plant growth under this condition, also considering the stem diameter and plant height (Figure 1). These results are in accordance with previous reports describing the ability to provide salinity stress tolerance of other strains from the genus *Peribacillus* such *P. frigoritolerans* in *Medicago polymorpha* plants (Gil et al., 2023).

On the other hand, it has been previously observed that the secretion of bacterial EPS in the rhizospheric soil of plants enhanced the chelation of a harmful concentration of Na^+. Based on these observations, we studied the PGP activity of N3 in combination with the EPS produced by the halophilic bacterium *H. maura* under non-salinity and salinity conditions. *H. maura* is a moderately halophilic bacterium with the ability to excrete large quantities of a well-characterized EPS known as mauran. Previous studies described that the co-inoculation of *H. maura* and *Ensifer meliloti* enhances alfalfa productivity in saline soils (Martínez et al., 2015). The inoculation of N3 and the application of mauran under salt stress significantly ameliorated the dry weight of tomato plants compared to control plants (Figure 2A). These results agree with other studies where, for example, the EPS secreted by the strain AK-1 ameliorates the binding of Na^+ from the soil and alleviates the toxic effect of salt on soybean plants (Kasotia et al., 2016). In fact, these results demonstrated the

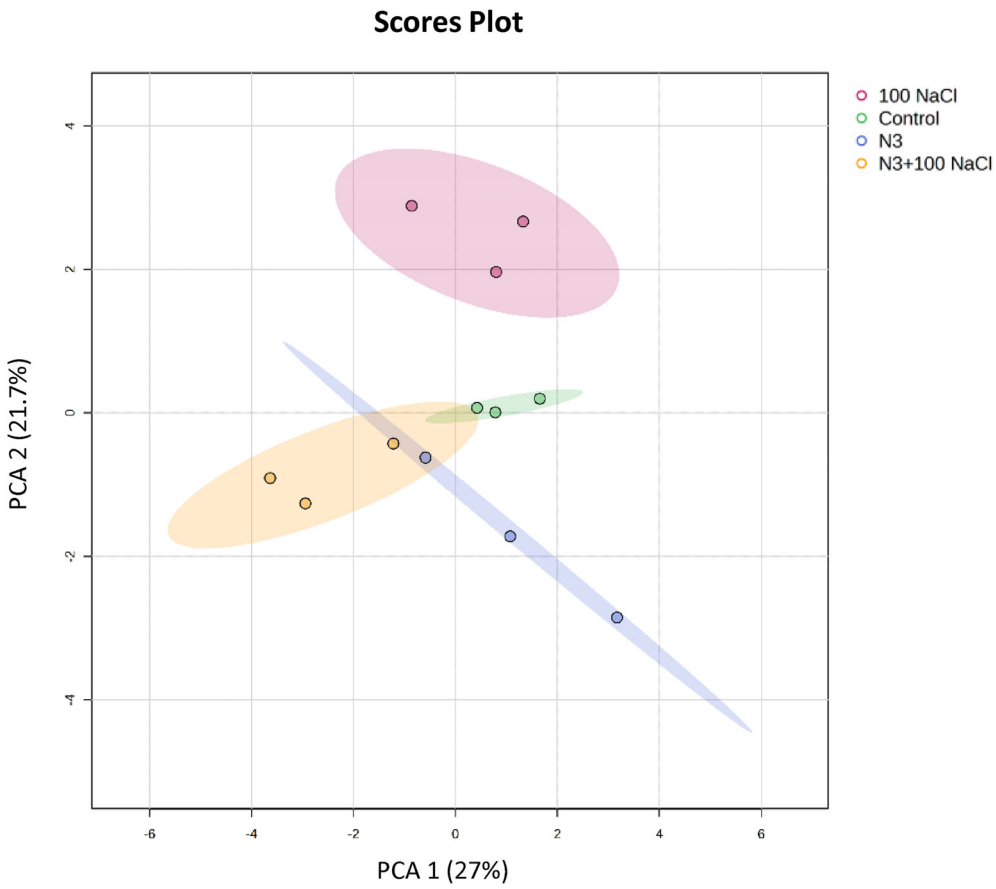

FIGURE 6 Principal component analysis (PCA) of physiological parameters and osmolytes in tomato plants under 0 and 100 mM of NaCl. Values in parentheses are the percentage of variance explained by each principal component (PC). Three biological replicates (3 plant each) were used (n = 3).

osmotic adjustment in tomato plants through the accumulation of energetically cheap inorganic ions, such as sodium, and the compartmentalization between ions in the vacuoles and osmolytes in the cytoplasm. The application of the halotolerant bacterium N3 and the EPS mauran has great potential as an economically viable and safe biotechnology, where the use of low levels of mauran (0.3% p/v) increase plants' salt tolerance. Furthermore, previous results demonstrated that the injection of closely related EPS is safe and well tolerated in mice (Ruiz-Ruiz et al., 2011) and the strain N3 is proved to be nonvirulent against the crustacean *Artemia salina*. Moreover, toxicity of cell-free supernatant from 48 h-cultures of strain N3 was negative in a bioluminiscence-based assay using as reporter *Aliivibrio fischeri* (personal communication).

Changes in the expression of *SlSOS1* and *NHX4* genes was observed in tomato plants under saline stress, mitigating the stress. The gene *SlSOS1* (*SALT OVERLY SENSITIVE 1*) was chosen as it encodes a Na^+/K^+ antiporter mediating sodium extrusion in root epidermal cells to reduce sodium accumulation in plants and in the parenchyma cells of root and shoot xylems to promote sodium translocation from root to shoot (Zhu et al., 2016). *SlSOS1* mutants were clearly more sensitive to salt stress than wild-type plants, which indicates that this gene plays a crucial role in salt tolerance in tomato (Wang et al., 2020). *SlSOS1* was overexpressed in N3 + EPS-treated plants under saline stress (Figure 3C), as previous studies have shown (Haroon et al., 2022).

On the other hand, the vacuolar *NHX*-type Na^+/H^+ antiporters mediate the sequestration of excessive sodium under salt stress and facilitate the transport of sodium into the vacuoles, lowering cytosolic sodium concentrations and protecting important enzymatic reactions in the cytoplasm from excess sodium levels (Horie and Schroeder, 2004). In this study, the inoculation of N3 combined with EPS could have triggered the plants to overproduce the *NHX4* gene expression involved in salt stress alleviation (Figure 3D). Same results were previously described by Vaishnav et al. (2020) and Sahu et al. (2021). The overexpression of *NHX4* in N3 + EPS-treated plants indicates the enhanced efficiency of the vacuole to compartmentalize sodium. In relation to the increased sodium levels observed in plant shoots of inoculated plants compared to the control (Figure 3B), N3 + EPS treatment could be a promising strategy to overcome both sodium toxicity and osmotic effect caused by high salinity (Vera-Estrella et al., 2005).

In plant cells, salinity causes water deficit and ionic imbalance, leading to osmotic stress (Zhao et al., 2021). The accumulation of different osmoprotectants, including carbohydrates, proteinogenic amino acids (e.g., proline) and non-protein amino acids (e.g., GABA), is found to be an adaptative mechanism used by plants to respond to this abiotic factor (Khan et al., 2018; Wu et al., 2020). The accumulation of osmoprotectant also plays an important role in scavenging ROS to counteract the oxidative stress generated by NaCl (Alhaithloul et al., 2019). The efficiency of N3 in combination with EPS to provide salt stress tolerance to tomato plants was confirmed by analyzing the induction of soluble sugars, amino acids, polyamines or phenols (Figures 4 and 5).

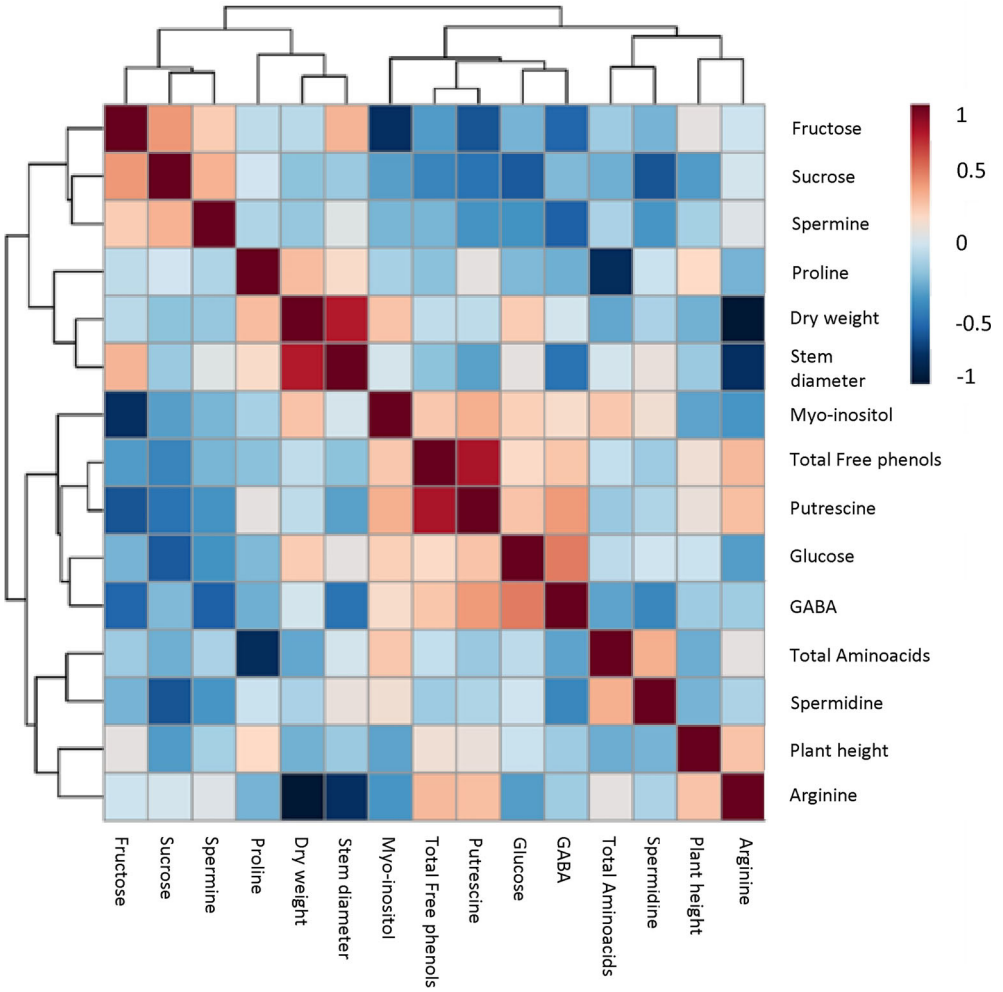

FIGURE 7 Correlation matrix based on Pearson Correlation Coefficient with a result range from −1 (indicating a strong inverse correlation) to 1 (indicating a strong direct correlation) among different osmoprotectans and physiological parameters in tomato plants with dendrogram.

All the soluble sugars included in this study (glucose, fructose, sucrose and myo-inositol) significantly increased under salinity conditions with the treatment N3 + EPS, certainly leading (Figure 4A-D) to osmotic adjustment and the cell membrane protection from oxidative stress as described earlier (Kazerooni et al., 2022). In fact, Kazerooni et al. (2022) have demonstrated that a fungal strain reduces symptoms of salinity in tomato plants due to the accumulation of glucose, fructose and sucrose. Myo-inositol metabolism is also important for the response to environmental stresses. It has been reported that mutants exhibiting reduced myo-inositol synthesis are much more sensitive to stresses (Murphy et al., 2008). Exogenous myo-inositol alleviates salt stress in plants by enhancing antioxidants, osmolytes and membrane stability via the upregulation of stress-responsive genes (Al-Mushhin et al., 2021).

A similar trend to that described above for soluble sugars has been observed for total amino acids, GABA and proline content (Figure 4E, G, H). Amino acids' accumulation has been shown to improve plant tolerance to environmental challenges, including salt stress. Different mechanisms have been proposed for the role of amino acids in plant tolerance to salinity, including acting as compatible solutes against osmotic changes. The increase in the proline content in tomato plants inoculated with N3 + EPS supports the fact that this proteinogenic amino acid counteracts the osmotic stress caused by salinity, helping the hydration and preventing protein dehydration and denaturation under salt stress (Hao et al., 2021). Generally, a strong correlation is established between abiotic stress (such as high salinity, drought or low temperatures) and proline accumulation (Khan et al., 2018; Wu et al., 2020). On the other hand, the increased GABA in N3 + EPS-treated tomato can be related to previous studies where the application of exogenous GABA increased the osmotic adjustment capacity to resist water loss and neutralized the excessive NaCl in the vacuoles in tomato leaves (Wu et al., 2020). The beneficial effect of GABA treatment and the role of GABA shunt activation under salt stress are well-documented in plants (Dabravolski and Isayenkov, 2023). GABA acts as the central hub metabolite, connecting different pathways (glucose, fructose, sucrose, myo-inositol, proline, antioxidant defence, etc.) impaired by salt stress and providing an alternative metabolic pathway (GABA shunt) to increase energy production and alleviate the negative effects of salt stress (Dabravolski and Isayenkov, 2023). Additionally, other research showed that GABA plays a major role in the accumulation of phenolics. Both mild salt stress and GABA treatment increased total phenolics and flavonoid content, 4-coumarate coenzyme A ligase, cinnamic acid 4-hydroxylase and phenylalanine ammonia lyase mRNA levels and protein activities (Wang et al., 2021). Buffagni et al. (2022) reported

that polyamines treatment under salt stress also affected the accumulation of secondary metabolites involved in the redox balance, including phenols or flavonoids. The present study demonstrates the increase of phenols in the tomato plant treated with salt in the presence of N3 + EPS (Figure 4F). Similar results were previously described in the foliar application of copper nanoparticles that induce salt tolerance of tomato by stimulating the antioxidant mechanism such as phenols (Pérez-Labrada et al., 2019).

Other interesting metabolites in plant responses to salt stress are polyamines. Polyamines are organic polycations involved in many processes of plant growth and development, including antioxidant systems and ionic homeostasis or photosynthetic pigment defense (Shao et al., 2022). Polyamine biosynthetic pathway included the first polyamine synthesized via arginine descarboxylase or ornithine descarboxylase called putrescine. In this study, we found a significant decrease in arginine and no changes in ornithine levels (data not shown) with N3 + EPS compared to the non-inoculated plant in salt-stress conditions. The increase of the putrescine levels in plants inoculated with N3 + EPS under salinity conditions demonstrated the role of the arginine descarboxylase in the salt tolerance of tomato plants in this case. In the polyamine pathway, putrescine is subsequently converted to spermidine and spermine. However, in this study, it has been observed that only putrescine increases significantly in plants treated with N3 + EPS under saline conditions, while the levels of spermidine and spermine were reduced in this treatment (Figure 5). Similar results were found, for example, in the treatment of apple callus with salt (Liu, 2006). Conversion of arginine to putrescine improves salt tolerance in tomato, likely due to alleviation of salt stress by this polyamine. Putrescine has the ability to restore root development by improving the transcript level of protein related to plant defensive mechanisms and carbohydrate and amino acid metabolism, which leads to stress tolerance (Yuan et al., 2016). Various studies found that treatment with exogenous putrescine induced the production of osmotic substances (carbohydrates, amino acids or proline), stimulated different physiological processes (Mohammadi et al., 2018) and elevated the ethylene synthesis in salt-stressed plants (Quinet et al., 2010). Previous reports also demonstrated that putrescine improves photosynthesis in cucumber plants by improving photochemical efficiency, hence ameliorating the adverse effects of salt (Zhang et al., 2009). The high levels of putrescine found in N3 + EPS-treated tomato are very important in salinity tolerance because putrescine can increase light energy utilization and is an efficient stimulator of ATP synthesis in comparison to spermidine and spermine in terms of maximal % stimulation (Ioannidis et al., 2006). Verma and Mishra (2005) also reported that putrescine reversed the salinity-induced reductions in seedling growth and biomass accumulation in leaf tissues of *Brassica juncea*, suggesting that putrescine might activate antioxidant enzymes and elevating antioxidants helping to prevent membrane peroxidation and denaturation. In this study, the results of PCA analysis revealed an excellent discrimination between different samples under salinity (Figure 6). In fact, the plants inoculated with N3 + EPS were separated from non-inoculated plants in these stress conditions and all inoculated plants were grouped together. Moreover, some osmoprotectants, such as proline, reached a high content in N3 + EPS-treated plants under salinity and revealed a direct correlation with plant dry weight. Both parameters are also elements of the same cluster along with the diameter of the stem and sucrose and fructose (Figure 7). This response shows the relevance of osmoprotectants for plant growth under salt stress conditions.

5 | CONCLUSIONS

This study proposes a way to enhance salt tolerance of tomato plants by the inoculation of halotolerant PGPB and application of exopolysacharides (EPS) and reveals some salt-tolerance mechanisms induced by the application of PGPB with EPS. The synergy of the bacterium *Peribacillus castrilensis* N3 with the EPS mauran confers salt stress tolerance in tomato plants by increasing sodium transporter genes expression and osmoprotectants content, including soluble sugars, polyols, proline, GABA, phenols and the polyamine putrescine. The results obtained suggest that this synergy has great potential as an inductor of salinity tolerance in the agriculture sector. Further investigation into the abiotic stresses mechanisms induced by this synergy, as well of the use of other synergies, is warranted.

AUTHOR CONTRIBUTIONS

I.S. and F.P. conceived and supervised the study; I.S. and F.P. designed the experiments; P.S., A.C.C., S.S., I.S. and F.P. performed the experiments; P.S. and F.P. analyzed the data; I.S. prepared the figures and wrote the manuscript; P.S. and D.G. performed the gene expression studies; P.S., A.C.C., S.S., D.G., I.LL., I.S. and F.P. edited the manuscript and reviewed the literature.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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