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Halotolerant native bacteria *Enterobacter* 64S1 and *Pseudomonas* 42P4 alleviate saline stress in tomato plants

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Abstract

Salinity is one of the principal abiotic stresses that limit the growth and productivity of crops. The use of halotolerant plant growth-promoting rhizobacteria (PGPR) that increase the growth of salt-stressed crops is an environmentally friendly alternative to promote plant yield under salinity. The aim of this study was to test native PGPR, isolated according to their tolerance to NaCl, and to evaluate their influence on morphological, physiological, and biochemical traits promoted by salt stress in tomato plants. *Enterobacter* 64S1 and *Pseudomonas* 42P4 were selected as the most efficient strains in terms of salt tolerance. Both strains were classified as moderately resistant to salinity (NaCl) and maintained their plant growth-promoting activities, such as nitrogen fixation and phosphate solubilization, even in the presence of high levels of salt. The results of a greenhouse experiment demonstrated that PGPR inoculation increased root and shoot dry weight, stem diameter, plant height, and leaf area compared to control non-inoculated plants under non-saline stress conditions, reversing the effects of salinity. Inoculated plants showed increased tolerance to salt conditions by reducing electrolyte leakage (improved membrane stability) and lipid peroxidation and increasing chlorophyll quantum efficiency (Fv/Fm) and the performance index. Also, inoculation increased the accumulation of proline and antioxidant non-enzymatic compounds, such as carotenes and total phenolic compounds. The catalase and peroxidase activities increased with salinity, but the effect was reversed by *Enterobacter* 64S1. In conclusion, *Enterobacter* 64S1 and *Pseudomonas* 42P4 isolated from salt-affected regions have the potential to alleviate the deleterious effects of salt stress in tomato crops.

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1-Introduction

The increasing world population demands more agricultural land and higher yields per unit area, leading to soil degradation (Etesami & Maheshwari, 2018). Some crops have been moved to more marginal areas and different soil types (Etesami & Maheshwari, 2018). Salinity is one of the most relevant abiotic stresses that limit the growth and productivity of crops (Zörb et al., 2019); more than 424 million hectares of topsoil (0-30 cm) and 833 million hectares of subsoil (30-100 cm) are salt-affected (SAS). More than two-thirds of global salt-affected soils are found in arid and semi-arid climatic zones according to the Global Map of Salt-Affected Soils (GSASmap, The Food and Agriculture Organization of the United Nations, FAO 2021). The area of salt-affected soils continues to increase and the problem is aggravated even more by climate change (Kaushal & Wani, 2016; Machado & Serralheiro, 2017). In arid and semi-arid zones, every year, about 1-2% of agronomically productive lands are turned into unproductive areas as a consequence of salinization (Rasool et al., 2013).

Salinity affects the uptake of nutrients and water by plants, produces ion cytotoxicity (mainly due to Na^+, Cl^-, and SO_4^{2-}), and osmotic stress (Chinnusamy et al., 2005; Munns, 2002a; Tomaz et al., 2020). Osmotic stress produces physiological changes, such as membrane instability and nutrient imbalance, which affect the capacity to detoxify reactive oxygen species (ROS), to modify the antioxidant enzymes and to impair photosynthetic activity (Gupta & Huang, 2014). Consequently, the metabolic imbalance generates an accumulation of ROS, such as singlet oxygen, superoxide, hydroxyl radical, and hydrogen peroxide, and high levels of ROS may cause oxidative damage to membrane lipids, proteins and nucleic acids (Chinnusamy et al., 2005; Nadarajah, 2020). Therefore, salinity alters the physiological functions required for plant growth and development, leading to plant death in some cases (Zhu, 2001). One of the mechanisms involved in oxidative stress alleviation is the upregulation of different enzymatic antioxidants, such as catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), to scavenge the overproduction of ROS and the accumulation of compatible solutes, such as proline, glycine betaine or sugar (Noreen et al. 2010; Gupta & Huang, 2014). Proline has been widely reported as a multifunctional amino acid involved in osmotic adjustment, cell homeostasis and stress recovery (Forlani et al., 2019). Its accumulation is one of the main adaptive responses in plants against abiotic stresses, including salinity, and it can improve salt tolerance (El Moukhtari et al., 2020).

Tomato is the second most cultivated vegetable crop in the world (FAO). It is classified as a glycophyte (salt-sensitive plant) species with a salinity threshold of 2.5 dS m^{-1}, although it may be considered moderately salt-tolerant (Machado & Serralheiro, 2017). Argentina occupies the 17th place in tomato production ranking worldwide and Mendoza, located in a semi-arid region, is one of the main industrial tomato producers in the country (Argerich & Smith, 2020; WPTC, 2020). However, salinity is a serious constraint that affects tomato growth from germination to fruit production (Cuartero & Fernández-Muñoz, 1999). Moreover, the irrigation of tomato crops in semi-arid regions

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can increase soil salinity. Thus, sustainable alternatives need to be studied to improve tolerance to salt stress in tomato plants.

The use of halotolerant microorganisms that increase the growth of salinity-stressed crops is an interesting alternative to modifying salt tolerance in plants (Etesami & Glick, 2020). Among these microorganisms, plant growth-promoting rhizobacteria (PGPR) are effective candidates for stress amelioration in plants (Etesami & Maheshwari, 2018). The PGPR promote plant growth by direct mechanisms, including enhanced availability of nutrient, N fixation, phosphorous solubilization, siderophores and plant hormones production (such as indole acetic acid and abscisic acid) or by indirect mechanisms suppressing pathogens and inducing systemic resistance or tolerance to stress (Bottini et al. 2004; Cohen et al. 2009, 2015; Glick, 2012; Glick, 2014; Salomon et al. 2016). The PGPR are used in different crops to enhance their growth and protect them against various stress conditions (Ansari et al., 2021; Bhatt et al., 2020; Cohen et al., 2016; Funes Pinter et al., 2018). Plants exposed to salt stress inoculated with PGPR increase their antioxidant activity ameliorating the effect of salt stress (Islam et al., 2016). Other PGPRs have 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity or modify the plant hormone status to protect the plant from abiotic stress (Siddikee et al. 2011, Glick 2014, Cohen et al. 2015, Barnawal et al. 2017).

In a previous study, we isolated and identified four PGPR strains that increase tomato seedling growth (Pérez-Rodriguez et al., 2020). The aim of the present study was to characterize the native PGPR, isolated according to their salt tolerance, and to evaluate their physiological and biochemical ability to counteract salt stress in tomato plants.

2-Materials and methods

2.1-Tolerance of bacterial strains to NaCl

The NaCl tolerance was assessed in four bacterial strains (*Enterobacter* 64S1, *Pseudomonas* 42P4, *Ochrobactrum* 53F and *Cellulosimicrobium* 60I1) previously isolated from roots and the rhizosphere of tomato (Pérez-Rodriguez, et al. 2020). These bacteria are able to solubilize different sources of inorganic phosphate to fix N_2, to produce siderophores and indole acetic acid (Pérez-Rodriguez et al., 2020). Salt tolerance was determined in solid Luria-Broth medium (LB, Sigma Chem. Co.) supplemented with increasing concentrations of NaCl (from 11.70 to 190 g L^{-1} NaCl). The strains grew in liquid LB media in an orbital shaker at 28°C and 120 rpm for 24 h. Each bacterial culture was grown in duplicate and 10 µL of each one was seeded in Petri dishes. Then, they were incubated at 28°C for 48 h. The strains were classified according to the Larsen scale (1986) and defined as (1) non-resistant when a low salt concentration (11.70 g L^{-1} NaCl) inhibits their growth, (2) slightly resistant when they tolerate up to 70.13 g L^{-1}, (3) moderately resistant when they tolerate concentrations between 70.13 g L^{-1} and 190 g L^{-1}) and (4) extremely resistant when they can grow at concentrations higher than 190 g L^{-1}.

2.2-Bacterial growth curve under NaCl conditions

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The growth rate of the strains in liquid LB medium (control 10 g L^{-1}) and supplemented with different NaCl concentrations (30, 60 and 90 g L^{-1}) was monitored by spectrophotometric absorbance at 530 nm (OD_{530}, biomass production) in a UV-Vis spectrophotometer Cary 50 (Varian Inc.). One colony of the strains was cultured on LB medium at 28°C and 120 rpm until the stationary phase. Then, 50 µL of each bacterial culture was transferred to different tubes with 5 mL LB medium and the growth was evaluated by the change in OD_{530} until the stationary phase.

2.3-Evaluation of PGPR traits under NaCl conditions

Nitrogen fixation ability was determined in agar plates with N-free semisolid medium (Döbereiner, 1988) at different NaCl concentrations (0, 10, 20, 30 and 60 g L^{-1}). The bacteria grew in liquid LB medium during 24 h at 28°C and 120 rpm. Then, 1 mL of bacterial culture was centrifuged at 800 *g* for 2 min, the supernatant was removed and the pellet resuspended in physiological solution (0.85% NaCl), repeating the last procedure twice. An aliquot of 10 µL of the bacterial suspension was seeded on the NFb (nitrogen-free medium) plates in triplicate and incubated at 28°C for 7 days. The growth of the bacteria demonstrated the ability to fix N_2.

Phosphate solubilization capacity was checked according to Nautiyal (1999) in solid National Botanical Research Institute Phosphate (NBRIP) supplemented with different NaCl concentrations (0, 10, 20, 30 and 60 g L^{-1}). The bacteria were harvested and suspended in physiological solution as described previously. Then, 10 µL of the bacterial suspension was seeded on the NBRIP plates in triplicate and incubated at 28°C for 7 days. The growth of the bacteria and/or the presence of the halo of clearance around the colony were considered positive. In addition, the colony and halo diameters were determined and percentages of halo diameter formation were determined by the following equation:

Percentages of halo diameter = (halo diameter - colony diameter)/colony diameter.

2.4-Greenhouse experiment with PGPR bacteria under salt stress

2.4.1-Bacterial culture

One colony of each strain selected was pre-cultured in LB medium at 28°C and 120 rpm until a concentration of 10^8 CFU mL^{-1}. The bacteria cultures were centrifuged at 6850 *g* at 4°C for 10 min. The supernatants were discarded and the pellets were washed with sterile physiological solution (0.85% NaCl), centrifuged again, and diluted to a concentration of 10^7 CFU mL^{-1} with 0.85% NaCl for further inoculation.

2.4.2-Plant materials, growth conditions and plant inoculation

A pot experiment was conducted to evaluate the potential of the salt-tolerant PGPR in alleviating the salt stress effects in the host tomato plant (*Solanum lycopersicum* L.). The industrial variety UCO 14 (INTA, Mendoza, Argentina) was used in this study. Tomato seeds were surface sterilized with 70% ethanol for 1 min and washed with sterile distilled water. Then, they were sown in alveolar boxes of 60 mL containing the sterilized Kekkilä DSM 1 W growth medium (Kekkilä professional). The medium contained 70% brown and 30% dark *Sphagnum fuscum* dominant peat (N-P_2O_5-K_2O 15-12-29

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and microelements 0.6 kg m^{-3}, electrical conductivity (EC) 0.2 dS m^{-1}, pH 5.9). A basal fertilization treatment with 1 mL solution of 10.5 g L^{-1} with Hakaphos® Base 18-18-18 (COMPO) was applied to each alveolar box. The seedlings were cultured under greenhouse conditions at 24 ± 2°C and irrigated daily with distilled water to keep the soil water status close to field capacity. Fifteen days after sowing, the following treatments were applied on the soil surface: 1) Control (C): 1 mL of physiological solution; 2) *Cellulosimicrobium* 60I1: 1 mL of physiological solution with 10^7 CFU mL^{-1}; 3) *Ochrobactrum* 53F: 1 mL of physiological solution containing 10^7 CFU mL^{-1}; 4) *Enterobacter* 64S1: 1 mL of physiological solution with 10^7 CFU mL^{-1}; 5) *Pseudomonas* 42P4: 1 mL of physiological solution with 10^7 CFU mL^{-1}. Fifteen days after inoculation, the seedlings were transplanted to plastic pots (0.75 L) filled with the Kekkilä DSM 1 W growth medium and sand (1:1). After three days, the seedlings were re-inoculated with 2 mL of the respective bacterial inoculum (10^7 CFU mL^{-1}) or physiological solution (control). The pots were irrigated with distilled water during the first week after transplantation. During the rest of the experiments, the plants were watered twice a week with NaCl solution (150 mM) or distilled water (control). The experiment consisted of a factorial arrangement of 10 treatments (two levels of the salt stress factor per five levels of the bacteria factor) with 10 replicates (n=10). After one month, plants were carefully removed to determine the most effective strains in alleviating salt stress by evaluating the plant dry weight (PDW).

The experiment was performed again under the same conditions previously described with the two most promising strains, according to the improvements in PDW of the preliminary experiment. The experiment consisted of a factorial arrangement of six treatments (two levels of the salt stress factor per three levels of bacteria factor) with 20 replicates (n=20). The EC of the substrate at the end of the experiment was 1.5 dS m^{-1} for treatments irrigated with water and 10.5 dS m^{-1} for treatments irrigated with NaCl solution (150 mM). At the end of the experiment, physiological and biochemical parameters were evaluated.

2.4.3-Vegetative growth determinations

Plant height, stem height (from the base to the last branch), basal diameter, leaf area, root dry weight (RDW) and shoot dry weight (SDW) were determined at the end of the experiment. Leaf area was determined using the software ImageJ (v1.51 j, http://imagej.nih.gov/ij). Plants were carefully removed from the soil medium and the roots were washed with water to remove adhering soil. RDW and SDW were determined after drying the samples in a hot oven at 60°C for 7 days. In addition, the salt tolerance index (STI) was calculated according to Siddikee et al. (2011) as follows:

STI= DWS or DWB/DWC, where DWS is the dry weight of plants grown under salt stress, DWB is the dry weight of plants grown with PGPR inoculation under salt stress and DWC is the dry weight of plants grown under control conditions (without salt stress and inoculation of PGPR).

2.4.4-Photosynthetic pigments, photosynthetic efficiency and content of polyphenols and anthocyanin

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Photosynthetic pigments, polyphenols and anthocyanin leaf pigments were determined spectrophotometrically as described by Cohen et al. (2015). Total chlorophyll (Chl; Chl *a* + Chl *b*) and carotenoid levels were determined from 1 cm^2 leaf area, whereas total phenolic compounds (TPC_S) and anthocyanin levels were determined using two leaf discs of 1 cm^2 (from the third leaf). The maximum efficiency of photosystem II (Fv/Fm) and the performance index (PI_{abs}) were determined with a fluorometer (Hansatech Instruments LTD) as indicators of photosystem II damage and stress resistance capacity, respectively. The leaf was incubated in the dark with the leaf-clip placed on the third leaf (from the base) for 20 min before determination. The relative content of chlorophyll was determined using a portable chlorophyll meter (SPAD-502, Konica Minolta Sensing).

2.4.5-Water status

Leaf relative water content (RWC) was determined using the following equation: RWC = 100* (FW - DW)/(FTW - DW). The FW is the leaf fresh weight and the fully turgid weight (FTW) is the weight of the leaf determined after a period of 48 h immersion in distilled water. The dry leaf weight (DW) was obtained after oven-drying the leaves in an oven at 80°C until a constant mass was reached.

2.4.6-Protein content and antioxidant enzyme activities

According to Berli et al. (2010), 130 mg of fresh weight leaves were ground and homogenized (Ultra-Turrax, T 10 basic; IKA) with 5 mL of extraction solution (100 mM potassium phosphate buffer pH 7.5, 0.1% Triton X-100, 1 mM EDTA and 0.5 mM ascorbic acid) in the presence of 0.25 g of polyvinylpolypyrrolidone (PVPP) at 5°C and centrifuged at 9300 *g* for 5 min. Then, the supernatants were collected in 1.5 mL tubes and stored at -20°C until being assayed for protein and antioxidant enzymatic activity. The absorbance determinations were realized with 10 mm optical path quartz cells in a Cary-50 UV-vis spectrophotometer. The total protein content (PC) was assessed following Bradford’s technique (1976) by measuring absorbance at 595 nm, with bovine serum albumin as standard. The catalase activity (CAT) was determined according to Azevedo et al. (1998) by assessment of H_2O_2 consumption at 240 nm in a 2.5 mL reaction mixture containing 100 mM potassium phosphate buffer (pH 7.5) and 100 µL of sample. The ascorbate peroxidase activity (APX) was determined as described by Barka (2001). The decrease in ascorbate absorbance at 290 nm was monitored in a 2.5 mL reaction mixture containing 50 mM potassium phosphate buffer pH 7.0, 100 mM EDTA, 50 mM ascorbic acid and 1 mM H_2O_2. The total peroxidase activity (POX) was determined by monitoring the oxidation of guaiacol to tetraguaiacol at 470 nm in 2.5 mL of reaction mixture containing 50 mM potassium phosphate buffer pH 6.0, 2.4 mM H_2O_2 and 20 mM guaiacol (Zhang & Kirkham, 1994).

2.4.7-Proline content, electrolyte leakage and lipid peroxidation

The proline content was determined as described by Bates et al. (1973) and modified by Berli et al. (2013). For that, 0.5 g of leaf sample were ground and homogenized in 2.5 ml of 3% aqueous sulfosalicylic acid solution. To the extracts were added 250 mg of insoluble PVPP, vigorously vortex-mixed for 30 s and centrifuged at 9300 *g* for 10 min (Eppendor Centrifuge 5804R, with rotor F34-6-

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38, Hamburg, Germany). Then 2 ml of glacial acetic acid and 2 ml of 2.5% acid ninhydrin solution were added to the supernatants and kept at 100°C for 1h. The reaction was chilled in an ice bath and extracted with 4 ml of toluene (vortex-mixing vigorously during 1 min). Finally, the OD of the toluene phase was recorded at 520 nm in 10 mm optical path cells. Proline content was determined from a standard curve and calculated on the basis of leaf FW. The electrolyte leakage was determined according to Shi et al. (2006). For that, 10 leaf discs (10 mm in diameter) from leaves of the third ramification (from the base) were placed in 50 mL tubes and washed with deionized water to remove surface-adhered electrolytes. Then the discs were immersed in 30 mL of deionized water in the dark and at room temperature for 24 h and the electrical conductivity (EC1) was determined using a conductivity meter (Ohaus, ST3100M-F). The tubes were then heated in a temperature-controlled water bath at 95°C for 20 min and then cooled to room temperature and the EC (EC2) was determined. The electrolyte leakage was determined according to the equation: EC1/EC2 * 100. The malondialdehyde (MDA) content was determined as an indicator of oxidative damage following the protocol described by Heath & Packer (1968). Samples of 0.1 mg were homogenized using a mortar and pestle with 0.1 mL of 0.1% trichloroacetic acid solution (TCA) and the extract was incubated at 4°C. Then the extract was centrifuged at 9300 *g* for 5 min, and 0.5 mL of supernatant was collected and combined with stock solution (20% TCA and 0.5% thiobarbituric acid), vortexed during 15 s and incubated in a water bath at 95°C for 60 min. The reaction was chilled in an ice bath and then the extract solution was centrifuged at 9300 *g* for 10 min. Finally, the supernatant absorbance was assessed at 532 nm and the nonspecific absorption at 600 nm was subtracted. MDA content was calculated considering the molar coefficient extinction as 155 mM^{-1} cm^{-1}.

2.4.8-Statistical analysis

All statistical analyses were performed with InfoStat software (InfoStat version 2018v. Grupo InfoStat). The effect of the "bacteria" and "salt stress" factors and their interaction was evaluated by multifactorial ANOVA analysis, LSD Fisher comparison, and 0.05 of significance.

3-Results

3.1-Bacterial strains are tolerant to NaCl but salinity delays their growth

All strains were salt resistant, *Ochrobactrum* 53F and *Cellulosimicrobium* 60 I1 grew up to 60 g L^{-1} NaCl, while *Pseudomonas* 42P4 and *Enterobacter* 64S1 grew up to 80 g L^{-1} and 90 g L^{-1} NaCl, respectively, in solid medium. According to Larsen (1986), *Ochrobactrum* 53F and *Cellulosimicrobium* 60 I1 strains can be classified as slightly resistant, whereas *Pseudomonas* 42P4 and *Enterobacter* 64S1 strains are moderately resistant (Table 1). The different concentrations of salt in liquid LB medium affected the growth curve of the cultures of four strains, reducing, and in some cases inhibiting, their growth (Fig. 1). The growth of *Enterobacter* 64S1 strain was similar in the LB medium supplemented with 30 g L^{-1} NaCl than to control conditions (10 g L^{-1}), whereas *Pseudomonas* 42P4 and *Ochrobactrum* 53F strains started the exponential phase 1-2 hours later, reaching the same

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OD_{530} in the stationary phase. Increasing the salt concentration up to 60 g L^{-1} delayed the onset of the exponential growth phase and slowed the growth rate of the four strains compared with the control; however, *Enterobacter* 64S1 took less time to reach the exponential phase than the other strains. Only *Enterobacter* 64S1 grew at 90 g L^{-1} with a longer latency phase, around 10-12 h.

3.2-PGPR have N_2 fixation and phosphate solubilization ability under salt conditions

The plant growth-promoting traits of the salt-tolerant bacteria were also tested under different NaCl concentrations. *Enterobacter* 64S1 and *Pseudomonas* 42P4 fixed N_2 in the salt concentration tested (0 – 60 g L^{-1}); however, the final pH reached in the medium was different. This was assessed by the color change in the bromothymol blue indicator (blue color reveals pH greater than 7.6). In contrast, *Ochrobactrum* 53F and *Cellulosimicrobium* 60 I1 were capable of fixing N_2 up to 10 g L^{-1} NaCl (Fig. S1).

The four strains grew in the NBRIP medium indicating their ability to solubilize phosphate; however, the addition of NaCl affected the solubilization halo size (Fig. S2). The increase in NaCl concentration decreased and, in some cases, inhibited this capacity. None of the strains grew in the NBRIP plates supplemented with 60 g L^{-1} of NaCl. *Enterobacter* 64S1, *Pseudomonas* 42P4 and *Ochrobactrum* 53F were capable of solubilizing phosphate in the plates amended with 10 g L^{-1} of NaCl (Table 2); however, *Cellulosimicrobium* 60I1 grew in these conditions but did not present a clear solubilization halo. *Enterobacter* 64S1 exhibited the highest percentage of the solubilization halo in all concentrations of NaCl from 1.41% (at 10 g L^{-1} of NaCl) to 1.14% (at 30 g L^{-1} of NaCl) compared to the other strains. Only *Enterobacter* 64S1 and *Pseudomonas* 42P4 were able to solubilize P at 30 g L^{-1} of NaCl, whereas *Ochrobactrum* 53F presented the lowest percentage (0.03%) of the solubilization halo in the NBRIP plates supplemented with 20 g L^{-1} of NaCl.

3.3-PGPR inoculation increased vegetative growth under salt stress and non-stress conditions

A greenhouse experiment was conducted to select the most effective strains in alleviating salinity stress. *Enterobacter* 64S1 increased the dry weight (DW) of non-stressed tomato plants (Fig. 2A,B). Salinity strongly reduced the DW of tomato plants compared to control conditions, whereas *Enterobacter* 64S1 and *Pseudomonas* 42P4 reversed the effect in plants exposed to salinity. These strains were therefore selected for additional experiments.

Salt stress significantly reduced the vegetative parameters assessed. *Enterobacter* 64S1 and *Pseudomonas* 42P4 significantly increased plant growth under non-stress conditions and reversed the effect of salinity (Fig. 3 and 4). The RDW, SDW, stem height and diameter, plant height and leaf area were increased by *Enterobacter* 64S1 and *Pseudomonas* 42P4 treatment with respect to the control plants under both non-saline stress and saline stress conditions. Saline stress decreased these parameters but there was no interaction between bacteria x stress; however, the stem diameter was also affected by these factors (P(b)<0.0001 and P(s)=0.0004) and their interaction (P(bxs)=0.0196). *Enterobacter* 64S1 and *Pseudomonas* 42P4 treatments increased RDW and SDW by 47-48% and 25-

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26%, respectively, in non-saline conditions. Salt stress reduced RDW and SDW parameters with respect to the non-stressed control plants, but inoculation reversed the effect of salt stress (Fig. 3A,B). *Enterobacter* 64S1 and *Pseudomonas* 42P4 also had higher RDW (53 and 59%, respectively) and SDW (28 and 36%, respectively) than the non-inoculated controls under salt conditions. Also, these RDW and SDW were similar to the non-stressed control plants.

Under non-saline stress conditions, the plant height, stem height and diameter increased after treatment with the strains by 13-15%, 11-12% and 7-8% (Fig. 3C-E), respectively, but the leaf area did not increase with inoculation (Fig. 3F). On the other hand, plants exposed to salinity presented a reduction in these parameters, which was reversed by the PGPR inoculation. The increments in plant height, stem height and diameter, and leaf area were 16%, 21-23%, 18% and 24-27% by *Enterobacter* 64S1 and *Pseudomonas* 42P4, respectively, compared to the control plants with saline stress. In addition, the salt tolerance index increased with inoculation compared to non-inoculated plants (Table 3). Plants inoculated with *Enterobacter* 64S1 and *Pseudomonas* 42P4 had 1.41- and 1.34-fold higher tolerance index compared to the control plants.

3.4-PGPR inoculation increased photosynthetic pigments, total phenolic compounds, photosynthetic efficiency under salt stress

The plants inoculated with *Pseudomonas* 42P4 strains had more Chl content in comparison with the control plants under both non-saline stress (45%) and saline stress (76%) (Fig. 5A). Moreover, the plants inoculated under saline conditions presented higher Chl content than under non-saline conditions and inoculation with *Enterobacter* 64S1 only increased the Chl levels under saline stress. Under non-saline conditions, the carotenoid levels in the inoculated plants were similar to the control plants, whereas inoculation with *Enterobacter* 64S1 and *Pseudomonas* 42P4 increased the carotenoids by about 37 and 53%, respectively, under saline stress compared to control (Fig. 5B).

There was no difference between treatments in the total phenolic compounds under non-stress conditions; however, it increased under stress conditions upon inoculation with *Enterobacter* 64S1 and *Pseudomonas* 42P4 by more than 50% over the control (Fig. 5C). The anthocyanin levels in leaves were not affected by the different treatments (Fig. 5D).

As observed in Fig. 6A, the Fv/Fm was affected by the bacteria (P(b)<0.0001) and salt (P(s)<0.0001) factors and their interaction (P(bxs)<0.0042). Under non-stress conditions, there were no differences between treatments and the Fv/Fm ranged between 0.80-0.81, while in the control plants under saline stress, the value of Fv/Fm was reduced by up to 0.69. However, plants inoculated with *Enterobacter* 64S1 and *Pseudomonas* 42P4 had an higher Fv/Fm (between 0.79 and 0.77). Similar behavior was found with the PI index, which was significantly reduced in the control plants under salt stress conditions. The inoculated plants had higher PI with both strains, with similar values to the non-stressed control plants (Fig. 6B). The SPAD Index was similar in the inoculated and non-inoculated plants under non-saline stress conditions and it decreased in the control plants exposed to salt stress (Fig. 6C). However, inoculation with *Enterobacter* 64S1 and *Pseudomonas* 42P4 increased the SPAD

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index by 11 and 19%, respectively. Leaf RWC values decreased in salt-stressed plants; however, the inoculated plants showed higher values than the control plants under stress conditions, without any significant differences between treatments, but similar to the non-saline stress control plants (Fig. 6D).

3.5-The antioxidant activity is modified according to the bacterial strain and the enzyme analyzed.

The protein content was only affected by the bacteria factor ($P<0.05$), while no significant effects of salt or factor interactions were observed (Fig. 7A). *Pseudomonas* 42P4 inoculation increased the protein content in respect to the control plants under non-saline conditions. However, the inoculated stressed plants exhibited a tendency to increase the protein content with respect to the stressed control plants. The treatments did not affect CAT activity, although CAT activity in stressed control plants tended to increase with respect to the non-stressed control. In addition, under saline stress, *Enterobacter* 64S1 reduced CAT activity with respect to the control plants (Fig. 7B). POX activity was affected by salinity. The stressed control plants exhibited higher antioxidant activity (higher POX activity) than the non-stressed control plants. Under the non-stress conditions, there were no differences between treatments. However, under saline stress, *Enterobacter* 64S1 induced a reduction in POX activity in respect to the control and the plants inoculated with *Pseudomonas* 42P4 (Fig. 7C). APX activity was only affected by salinity stress that induced an increase in the antioxidant activity. Under saline stress, the control plants had a higher APX activity than the inoculated plants (Fig. 7D).

3.6-PGPR inoculation reduces electrolyte leakage and MDA levels, and increases proline levels under salt stress conditions

Electrolyte leakage was affected by the salt factor and the interaction between bacteria and salt, and the MDA levels were affected by the bacteria and salt factors and their interactions. Salt stress significantly increased the percentage of electrolyte leakage (Fig. 8A) and MDA levels (Fig. 8B). However, electrolyte leakage significantly decreased with *Enterobacter* 64S1 and *Pseudomonas* 42P4 inoculation (9 and 18%, respectively) compared to the stressed control plants (Fig. 8A). Also, the plants inoculated with *Pseudomonas* 42P4 presented the lowest MDA level under non-stressed conditions, followed by *Enterobacter* 64S1, as compared to the control unstressed plants. Furthermore, while salinity incremented the MDA level, inoculation with *Enterobacter* 64S1 and *Pseudomonas* 42P4 decreased it by 64 and 58%, respectively, under saline stress conditions (Fig. 8B).

The proline level was affected by the bacteria and salt factors and their interactions. Tomato plants accumulated higher proline levels under salt stress than under non-saline conditions (Fig. 8C). In addition, *Enterobacter* 64S1 and *Pseudomonas* 42P4 inoculation increased proline levels by 53 and 67%, respectively, over the stressed plants, whereas only plants treated with *Pseudomonas* 42P4 accumulated more proline under non-stress conditions.

4-Discussion

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Climate change has exacerbated the severity of environmental stresses where salinity is one of the major abiotic stresses affecting plant growth and crop yield (Chinnusamy et al., 2005; Ilangumaran & Smith, 2017). Although plants regulate salinity tolerance through different mechanisms depending on inherent genetic traits (Munns, 2002b), the PGPR may enhance the mitigation of salt stress, thus supporting better plant growth development under stressful conditions (Numan et al., 2018). However, different stress factors influence the performance of microorganisms (Grover et al., 2011). Native PGPR isolated from saline soils are the best adapted to such environmental conditions, with advantages in competition, establishment and survival as compared to non-native PGPR (Etesami & Glick, 2020).

In this context, we characterized four native PGPR previously isolated from the rhizosphere of tomato growing in saline soil (Pérez-Rodriguez et al., 2020). In this study, we evaluated their salt tolerance and demonstrated the effectiveness of two of them in reducing the negative effects of salinity in tomato plants by inducing systemic tolerance. *Enterobacter* 64S1 and *Pseudomonas* 42P4 inoculation increased plant tolerance by reducing the electrolyte leakage, lipid peroxidation and increasing chlorophyll production, Fv/Fm and PI_{abs}. Moreover, inoculation increased the proline levels and accumulation of antioxidant non-enzymatic compounds, such as carotenes and total phenolic compounds. However, the antioxidant activity varied according to the bacterial strain and the enzyme analyzed.

Regarding salt tolerance, two of the strains evaluated, *Enterobacter* 64S1 and *Pseudomonas* 42P4, were moderately resistant to salinity stress, while the other two, *Ochrobactrum* 53F and *Cellulosimicrobium* 60 I1, were classified as only slightly resistant (Table 1). A reduction in the growth of each strain was noted with the increase in NaCl concentration, mainly 60 and 90 g L^{-1} of NaCl (Fig. 1). The reduction in growth could be related to a hyper-osmotic pressure exerted on the cell membrane (Tank & Saraf, 2010). In addition, some authors reported a reduction in PGP (plant growth-promotion) activities under salinity stress (Karimzadeh et al., 2020). In our study, the isolated PGPR maintained their PGP activity, such as N_2 fixation and phosphate solubilization, even when evaluated in the presence of high levels of salt, with *Enterobacter* 64S1 and *Pseudomonas* 42P4 being the most efficient as they maintained these traits at 60 g L^{-1} and 30 g L^{-1} NaCl (Table 2, Fig. S1 and S2). In other studies, Karimzadeh et al. (2020) and Liu et al. (2016) reported that PGPR are capable of solubilizing phosphates under 40 and 50 g L^{-1} NaCl conditions. The mechanisms involved in salt tolerance by PGPR are related to their ability to produce EPS (exopolysaccharides), excretion of Na^+ from cells, intracellular accumulation of compatible soluble compounds, adaptation of proteins to high concentrations of soluble ions and accumulation of K^+, among others (Etesami & Glick, 2020).

In the greenhouse experiments under salinity conditions, the most efficient strains were *Enterobacter* 64S1 and *Pseudomonas* 42P4 (Fig. 2A,B). The control plants without salinity exhibited higher growth (RDW, SDW, stem diameter, plant height and leaf area) compared to the stressed control plants. However, the inoculation of both strains improved morphological parameters, such as biomass (RDW

and SDW), stem diameter, plant height and leaf area, and it alleviated salt stress, showing similar results to the non-saline stress control (Fig. 3). The inoculated plants accumulated more photoassimilates, reflected in the RDW and SDW, with respect to the control plants under saline and non-saline stress conditions. Similar results were presented by Mayak et al. (2004), with the inoculation of *Achromobacter piechaudii* in tomato seedlings in the presence of salinity. Likewise, Tank & Saraf (2010) reported increases in the root and stem length, as well as a greater number of leaves and lateral roots, in tomato plants subjected to 20 g L^{-1} NaCl and inoculated with PGPR. In the present study, it was also shown that the tolerance index to salinity of inoculated plants was higher than that of non-inoculated plants under stress, which demonstrates the positive effect of inoculation with the selected PGPR (Table 3). A higher root and aerial DW may mean longer and "stronger" roots and shoots, thus increasing the chances of plants to resist to saline stress (Siddikee et al., 2011).

One of the effects of salt is the reduction of water uptake by plants (Munns, 2002a). It is known that bacterial auxin (IAA) promotes root growth and exudation, and the formation of lateral roots that enables plants to uptake more nutrients, and it improves the exploitation of soil water under saline situations (Etesami & Glick, 2020; Yasmeen et al., 2020). IAA has a fundamental role in resistance to salt and in the promotion of plant growth (Numan et al., 2018). In the present study, increases in radical development of tomato plants may be related to IAA production by the PGPR. In fact, we previously demonstrated that *Enterobacter* 64S1 and *Pseudomonas* 42P4 produce IAA in culture medium (Pérez-Rodríguez et al. 2020). Similar findings of salt stress alleviation have also been reported in other crops, such as maize (Aslam & Ali, 2018), soybean (Egamberdieva et al., 2017) and cotton (Yao et al., 2010), after application of auxin-producing PGPR. The increase in nutrient uptake may be partially attributed to superior root development in the inoculated plants (Cordero et al. 2018; Khalilpour et al. 2021). In general, the leaf RWC is reduced in salt stress since water leaves the cell in response to the osmotic gradient generated (Cordero et al., 2018). In our study, as expected, the control plants exposed to salinity presented lower RWC than when were inoculated, although the differences were not statically significant (Fig. 6D). In addition, PGPR may enhance the RWC of plants exposed to stress conditions by decreasing electrolyte leakage and improving the stability of plant cell membranes (Asghari et al., 2020), as was demonstrated with our results.

Salinity decreased the photosynthetic efficiency and the PI_{abs} and Fv/Fm values, producing photoinhibition or other damage to the components of photosystem II (Lucas et al., 2014) due to oxidative stress damages (Iseki et al., 2015). The increase in PI_{abs} can be used as an indicator of the plants' ability to resist environmental stress. Inoculation with *Enterobacter* 64S1 and *Pseudomonas* 42P4 strains reversed the negative effect of salinity on Fv/Fm and PI_{abs} (Fig. 6A,B). These results agree with those of Barnawal et al. (2017), Gururani et al. (2013) and Funes Pinter et al. (2018) regarding the improvement of this index under salinity, heavy metals and metalloids conditions after PGPR inoculation. In this study, although there were no differences in the total Chl of control plants, with and without saline stress, there was an increase in Chl content in the inoculated stressed plants (Fig.

5A). Similar results were presented by Kumar et al. (2017). The increase in Chl levels raises the photosynthetic rate and starch production, which allows plants to grow under salinity (Kaushal & Wani, 2016).

On the other hand, there is a delicate balance between ROS production and their detoxification. ROS are necessary for plant growth as essential secondary messengers for cell metabolism (Martinez et al., 2018; Munns & Tester, 2008). However, salinity stress disrupts the equilibrium of ROS in cells, inducing their accumulation, which leads to oxidative stress. The ROS imbalance produces lipid peroxidation in cellular membranes, protein denaturation, DNA damage, carbohydrate oxidation, pigment breakdown, and impairment of enzymatic activity (Bose et al., 2014). The main molecules that maintain ROS equilibrium include carotenoids, flavonoids, and other phenolic compounds and enzymes, such as SOD, glutathione reductase, CAT and POX (Martinez et al., 2018). Under salinity stress, the increased activities of antioxidants are positively correlated with plant salt stress tolerance (Gururani et al., 2013; Kaushal & Wani, 2016).

Increases in antioxidant enzyme activities have been reported for PGPR inoculation under stress conditions (Saberi-Riseh et al., 2020; Yasmeen et al., 2020). In this study, the antioxidant activity was modified according to the bacterial strain and the enzyme analyzed (Fig. 7). The POX and CAT activities increased in stressed control plants compared to the non-stressed, while there was a reduction in these enzyme activities with the inoculation of *Enterobacter* 64S1 compared to the stressed control plants (Fig. 7B,C). The APX activity tended to increase in the control plants under saline stress (Fig. 7D). In agreement with our results, Fukami et al. (2018) and Han & Lee (2005) reported a decrease in the activities of antioxidant enzymes compared to the control plants under salinity stress in maize and lettuce, respectively. In addition, Asghari et al. (2020) reported a reduction in antioxidant enzymes' activity in PGPR inoculated pennyroyal plants as compared to the control plants under drought stress. In agreement with Sandhya et al. (2010) and Ansari et al. (2021), we assume that plants inoculated with PGPR had less stress compared to the non-inoculated plants. According to Kaushal & Wani (2016) and our previous results, different factors, such as PGPR strain, host plant, type and duration of stress, might be responsible for such variations in enzymatic activity.

Salt stress negatively affects the acquisition and homeostasis of essential nutrients, such as K and Ca, and consequently produces more electrolyte discharge through the misplacement of Ca associated with membranes. Thus, the integrity and permeability of the membrane are affected and a higher efflux of electrolytes accumulates inside the plant cells or tissue (Ilyas et al., 2020). The MDA content is an indicator of the level of cell membrane damage since it is an end product of polyunsaturated fatty acid oxygenation (Fukami et al., 2018). In the present study, we observed increased MDA content and electrolyte leakage in leaves of plants exposed to salinity, whereas inoculation with both strains decreased damage in leaves (Fig. 8A,B), similar to that reported by Habib et al. (2016), Ilyas et al. (2020) and Sarkar et al. (2018) in orka, maize and rice, respectively.

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It is known that salt stress increases the osmotic pressure in the rhizosphere, thereby reducing the water flow toward roots. In this sense, plants accumulate compatible osmolytes, such as proline, to maintain root water uptake, reducing the negative impact of salt stress (Porcel & Ruiz-Lozano, 2004). Furthermore, proline, a compatible solute and osmoprotectant, plays a role in non-enzymatic antioxidant activities and is considered as a scavenger of hydroxyl radicals (El Moukhtari et al., 2020). In the present study, proline was increased in inoculated tomato plants under salt stress (Fig. 8C). These results are in line with Ilyas et al. (2020), who reported an increase in the level of proline in wheat leaves under saline stress after PGPR inoculation.

Our results showed that carotenoid and the total phenolic compounds in leaves increased in the inoculated plants exposed to salinity over the control non-inoculated plants (Fig. 5B,C), in agreement with Yasmeen et al. (2020). Carotenoids, lipid-soluble antioxidants, are involved in a multitude of functions in plant metabolism, including oxidative stress tolerance (Bose et al. 2014; Gill & Tuteja, 2010). Kerbab et al. (2021) reported that the carotenoid content significantly increased in plants treated with bacteria under stress conditions compared to non-inoculated control. Moreover, phenolic compounds are potent antioxidants necessary for scavenging ROS and protecting the lipid membranes from oxidative stress (Chiappero et al., 2019; Ha-tran et al., 2021). Therefore, the increment of non-enzymatic antioxidant contents in PGPR-inoculated tomato plants helps to withstand the negative effects of salt stress.

In general, our results demonstrated that *Enterobacter* 64S1 and *Pseudomonas* 42P4 alleviate the adverse effects of saline stress in tomato plants by improving physical and biochemical attributes. These bacteria were able to promote growth, improve photosynthetic parameters and increase the proline levels and non-enzymatic antioxidant contents, such as carotenoids and total phenolic compounds. Therefore, these native PGPR adapted to saline conditions can be used for the development of biofertilizers for salt-stressed areas. However, further investigation is necessary to evaluate their performance under field conditions.

Author contributions

Conception and design of the experiments: M.M.P.R., A.C.C. and M.P. Performance of the experiments and analysis of the data: M.M.P.R. with collaboration of I.F.P. (bacteria), M.A.L.U. (biochemical determination), M.G.G (morphological determination). Contribution of reagents/materials/analysis tools: A.C.C., M.P. and P.P., and M.M.P.R. and A.C wrote the paper. All authors critically reviewed and modified the paper.

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Data availability statement

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

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure Legends

Figure 1. Effect of different NaCl concentrations (10, 30, 60 and 90 g L^{-1}) on the growth of PGPR in LB medium. (A) *Enterobacter* 64S1, (B) *Pseudomonas* 42P4, (C) *Ochrobactrum* 53F and (D) *Cellulosimicrobium* 60 I1

Figure 2. (A) Representative photograph and (B) plant dry weight (DW) of inoculated and control plants under saline (150 mM NaCl) or non-saline stress conditions. Values are means ± SE (n=10 individual plants grown at the same time). Different letters indicate significant differences according to LSD Fisher test ($P \leq 0.05$).

Figure 3. (A) Root dry weight (RDW, mg $plant^{-1}$), (B) shoot dry weight (SDW, mg $plant^{-1}$), (C) plant height (cm), (D) stem height (cm), (E) stem diameter (mm) and (F) leaf area (cm^2) of tomato plants in non-saline stress or saline stress conditions (150 mM NaCl), non-inoculated (Control) or inoculated with *Enterobacter* 64S1 and *Pseudomonas* 42P4. Data are means ± SE (n= 10 individual plants grown at the same time). Different letters indicate significant differences according to LSD Fisher test ($P \leq 0.05$). *P*(b), *Enterobacter* 64S1 and *Pseudomonas* 42P4 effect; *P*(s), saline stress effect; *P*(bxs), *Enterobacter* 64S1 and *Pseudomonas* 42P4 x saline stress interaction effect.

Figure 4. (A-B) Photograph of representative tomato plants cultivated in pot under non-saline stress or saline stress conditions (150 mM NaCl), non-inoculated (Control) or inoculated with *Enterobacter* 64S1 (64S1) and *Pseudomonas* 42P4 (42P4): (A) Top view of plants and (B) View of aerial parts and roots of plants.

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Figure 5. (A) Levels of total chlorophyll (Chl), (B) carotenoids, (C) total phenolic compounds (TPC) and (D) anthocyanins measured in tomato plants under non-saline or saline stress conditions (150 mM NaCl), non-inoculated (Control) or inoculated with *Enterobacter* 64S1 and *Pseudomonas* 42P4. Data are means ± SE (n=4 individual plants grown at the same time). Different letters indicate significant differences according to LSD Fisher test ($P \leq 0.05$). *P*(b), *Enterobacter* 64S1 and *Pseudomonas* 42P4 effect; *P*(s), saline stress effect; *P*(bxs), *Enterobacter* 64S1 and *Pseudomonas* 42P4 x saline stress interaction effect.

Figure 6. (A) Maximum quantum efficiency of PSII (Fv/Fm), (B) performance index (PI), (C) SPAD Index and (D) relative water content (RWC) of tomato plants under non-saline stress or saline stress conditions (150 mM NaCl), non-inoculated (Control) or inoculated with *Enterobacter* 64S1 and *Pseudomonas* 42P4. Data are means ± SE (n= 10 individual plants grown at the same time). Different letters indicate significant differences according to LSD Fisher test ($P \leq 0.05$). *P*(b), *Enterobacter* 64S1 and *Pseudomonas* 42P4 effect; *P*(s), saline stress effect; *P*(bxs), *Enterobacter* 64S1 and *Pseudomonas* 42P4 x saline stress interaction effect.

Figure 7. (A) Protein Content, B) catalase (CAT) activity, (C) peroxidase (POX) activity, and (D) ascorbate peroxidase (APX) activity assessed in leaves of tomato plants under non-saline stress or saline stress conditions (150 mM NaCl), non-inoculated (Control) or inoculated with *Enterobacter* 64S1 and *Pseudomonas* 42P4. Data are means ± SE (n= 5 individual plants grown at the same time). Different letters indicate significant differences according to LSD Fisher test ($P \leq 0.05$). *P*(b), *Enterobacter* 64S1 and *Pseudomonas* 42P4 effect; *P*(s), saline stress effect; *P*(bxs), *Enterobacter* 64S1 and *Pseudomonas* 42P4 x saline stress interaction effect.

Figure 8. (A) Electrolyte leakage, (B) MDA and (C) proline content assessed in leaves of tomato plants under non-saline stress and saline stress conditions (150 mM NaCl), non-inoculated (Control) and inoculated with *Enterobacter* 64S1 and *Pseudomonas* 42P4. Data are means ± SE (n= 5 individual plants grown at the same time). Different letters indicate significant differences according to LSD Fisher test ($P \leq 0.05$). *P*(b), *Enterobacter* 64S1 and *Pseudomonas* 42P4 effect; *P*(s), saline stress effect; *P*(bxs), *Enterobacter* 64S1 and *Pseudomonas* 42P4 x saline stress interaction effect.

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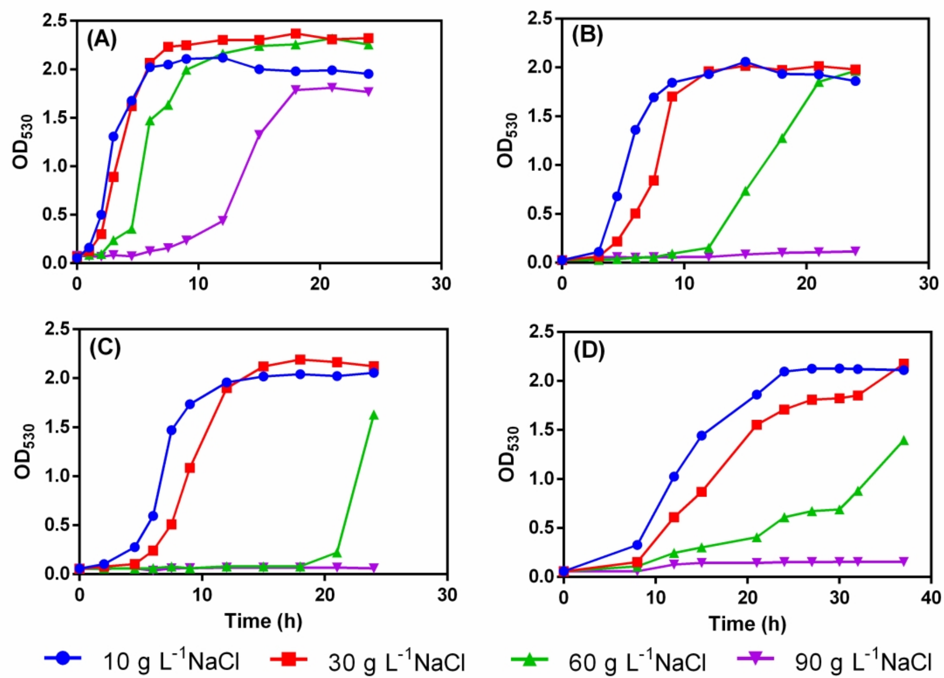

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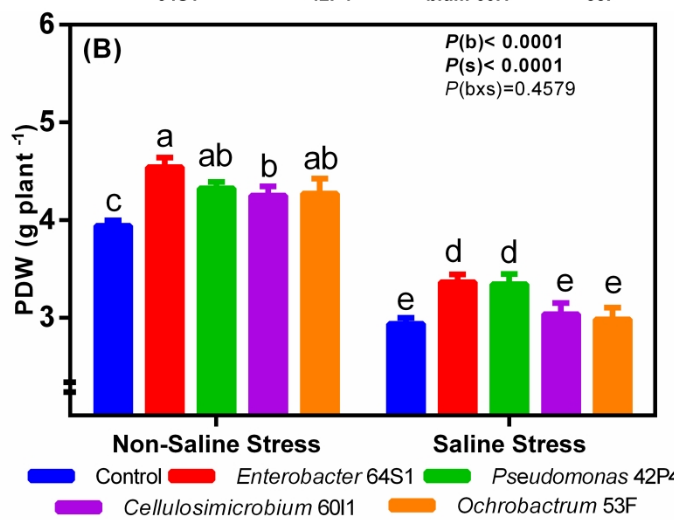

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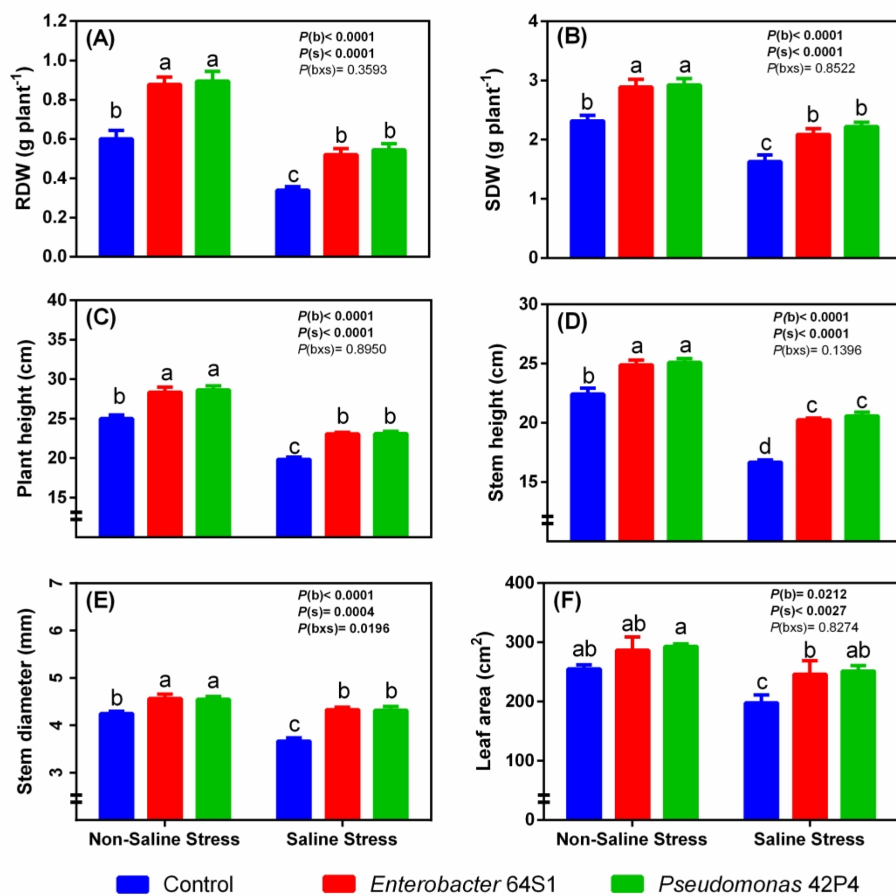

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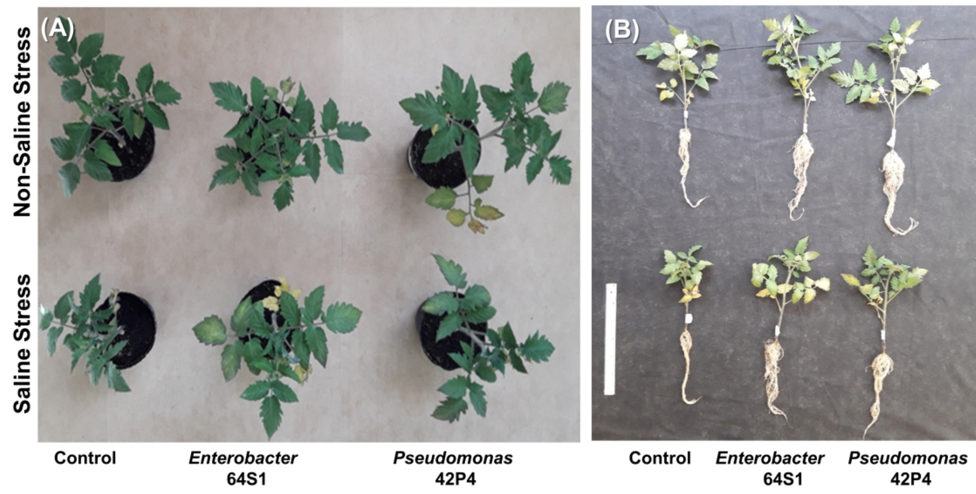

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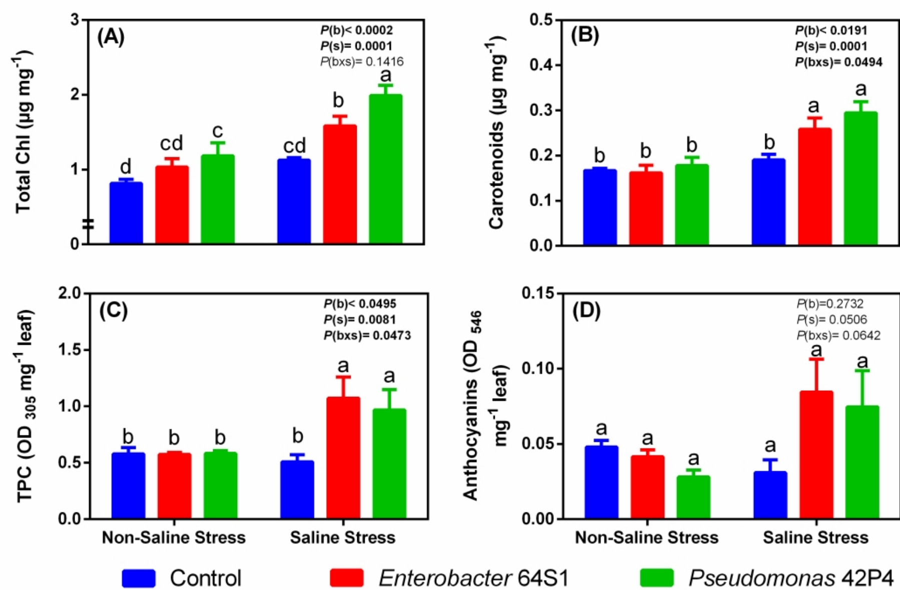

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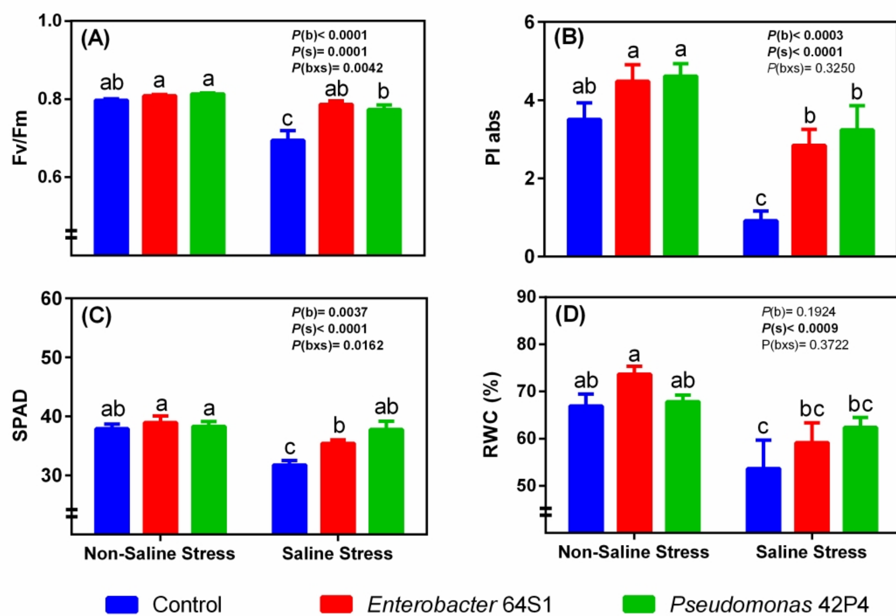

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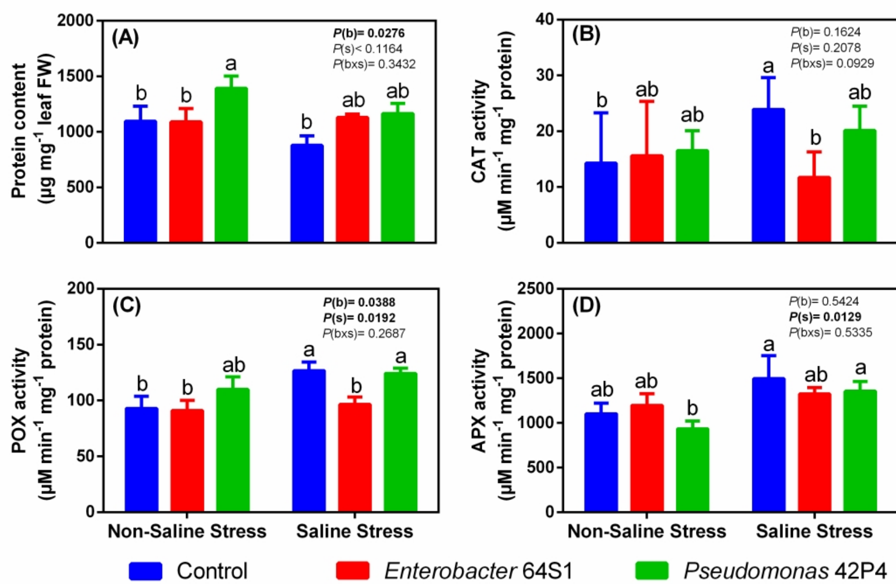

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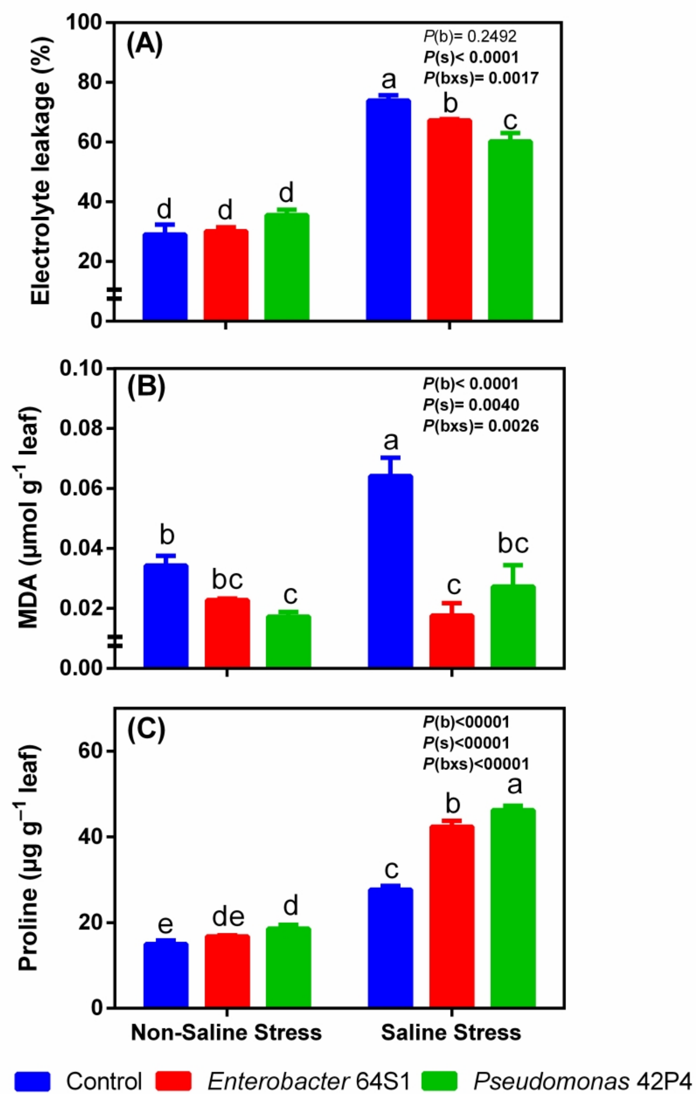

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Table 1. Tolerance of *Pseudomonas* 42P4, *Ochrobactrum* 53F, *Cellulosimicrobium* 60I1 and *Enterobacter* 64S1 strains to NaCl

Strain	Maximum NaCl concentration (g L^{-1}) tolerable	Classification
Pseudomonas 42P4	80	moderately resistant
Ochrobactrum 53F	60	slightly resistant
Cellulosimicrobium 60I1	60	slightly resistant
Enterobacter 64S1	90	moderately resistant

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Table 2. Phosphate solubilization halo produced by *Enterobacter* 64S1, *Pseudomonas* 42P4, *Ochrobactrum* 53F and *Cellulosimicrobium* 60I1 at different NaCl concentration (g L^{-1}). Values are means ± SE (n = 3). The different letters indicate significant differences using Fisher's LSD test at $P < 0.05$.

NaCl (g L^{-1})	*Enterobacter* 64S1	*Pseudomonas* 42P4	*Ochrobactrum* 53F	*Cellulosimicrobium* 60I1
	% Halo	% Halo	% Halo	% Halo
0	1.41+ 0.01 a	0.33 + 0.02 d	0.25 + 0.02 e	0.03 + 0.01 h
10	1.28+ 0.01 b	0.33 + 0.02 d	0.17 + 0.04 f	-
20	1.15 + 0.01 c	0.31 + 0.01 d	0.03 +0.01 h	-
30	1.14 + 0.01 c	0.08 + 0.01 g	-	-

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Table 3. Salt tolerance index of tomato plants non-inoculated (Control) and inoculated with *Enterobacter* 64S1 and *Pseudomonas* 42P4. Values are means ± SE (n = 10 individual plants grown at the same time). The different letters indicate significant differences using Fisher's LSD test at $P < 0.05$.

Treatment	Salt Tolerance Index
Salt Stress	
Control	0.68 ± 0.05 b
Enterobacter 64S1	0.91 ± 0.06 a
Pseudomonas 42P4	0.96 ± 0.06 a

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