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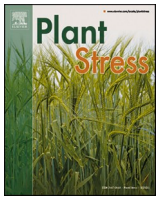

Salt stress mitigation and improvement in fruit nutritional characteristics of tomato plants: New opportunities from the exploitation of a halotorelant *Agrobacterium* strain

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ABSTRACT

Soil salinity is considered one of the most limiting factors in large agricultural areas, and tomato (*Solanum lycopersicum* L.) is highly sensitive to this abiotic stress, which exacerbates under drought. The use of halotolerant PGPR is a promising strategy to enhance tomato tolerance and productivity in saline soils. The present study tested the capability of the recently discovered bacterial strain PVr_9 to increase tolerance to salinity in tomato plants in *vitro*, greenhouse and open field conditions. When inoculated with PVr_9, *in vitro* seedlings showed a significant increase in primary root length, number of secondary roots and fresh weight both in absence of stress and when exposed to 150 mM NaCl. In plants exposed to salt treatment, PVr_9 upregulated the salt tolerance genes *SOS1* and *NHX1*, and the enzyme prolyl aminopeptidase involved in proline metabolism. Proteins associated with resistance against pathogens were also upregulated by PVr_9 in absence of salt stress. In greenhouse, PVr_9-inoculated tomato plants treated for 1-week with 150 mM NaCl showed a significant increase in shoot fresh biomass, chlorophyll, proline content, and APX activity. Tomato fruits from PVr_9 inoculated plants, exposed in the field to salt stress during the flowering stage, showed higher levels of carotenoids, lycopene, and L-ascorbic acid, as well as an increased antioxidant capacity compared to fruits of uninoculated plants. These results indicate PVr_9 as a potential biostimulant in tomato for better tackling soil salinization in a context of climate change and expansion of coastal salinity, while there is large scope for the improvement of the nutritional characteristics of tomato fruits.

1. Introduction

Salinity is being increasingly recognized as one of the most destructive environmental stressors globally, impacting negatively on the productivity and quality of many crops on a massive scale. According to the 2021 report from the Food and Agriculture Organization (FAO), it affects approximately 1257 million hectares of agricultural land (FAO, 2021). Salinization in agricultural fields refers to the accumulation of a high concentration of soluble ions, particularly sodium (Na) and chloride (Cl), in both the soil and the rhizosphere, which hinders the growth and development of plants by limiting/impeding water absorption through the roots (Shrivastava and Kumar, 2015). This limitation leads to a reduction in crop yield and overall productivity (El-Ramady et al., 2024). Soil is typically deemed saline when the electrical conductivity (EC) of the saturation extract in the root zone derives from a concentration of ions >40 mM at 25 °C, with 15 % unbound Na (Shrivastava and Kumar, 2015).

Salinity stress induces alterations in various plant physiological and metabolic processes, depending on the severity and duration of the stress, ultimately impeding plant growth. Elevated salinity exerts

Abbreviations: PGPRs, plant-growth promoting rhizobacteria; ESEM, environmental scanning electron microscope; ROS, scavenging reactive oxygen species; PAR, photosynthetically active radiation; RU, relative humidity; FDR, false discovery rate; CAT, catalase; APX, ascorbate peroxidase; TR, trolox; PCA, principal component analysis; PAP, prolyl aminopeptidase; SAR, systemic acquired resistance; IRS, induced systemic resistance; PRs, pathogenesis related proteins.

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multiple adverse effects on plants, including water stress, ion toxicity, nutritional imbalances, oxidative stress, disruptions to metabolic processes, membrane disorganization, diminished cell division and expansion, and genotoxicity (Ullah et al., 2021). Plants have developed adaptable systems to address salinity stress by undergoing changes at morphological, physiological, biochemical, and molecular levels. Salt tolerance is achieved through the activation of numerous salinity-stress-specific genes and transcription factors which regulate cytoplasmic ion content, osmotic adjustment, and antioxidant metabolism (Arif et al., 2020).

Tomato (*Solanum lycopersicum* L.) is a globally significant cultivated cash crop, its fruits being widely consumed as food in the fresh market, and it is also a model organism for studies on genetics, fruit developmental processes, and stress tolerance research (Rothan et al., 2019). Tomatoes are native to Western-South America, and their wild relatives have been adapted to severely saline coastal regions. However, cultivars have lost their salt resistance during domestication (Pailles et al., 2020), resulting in up to 50% yield losses due to salt stress (Shrivastava and Kumar, 2015).

One proposed approach to enhance tomato tolerance to soil salinity is the utilization of Plant Growth Promoting Rhizobacteria (PGPR). PGPR can colonize the rhizosphere of many plant species and have beneficial effects on both the host plant growth and susceptibility to plant diseases (Giannelli et al., 2023). In this mutualistic relationship, PGPR benefit from the wide variety of organic compounds produced by the plant and released into the soil through its roots (Prashar et al., 2014). Conversely, PGPR can enhance growth in a saline environment by triggering global changes in gene expression and metabolic pathways in host plants, thereby providing valuable insights into key plant-microbial regulatory mechanisms. The isolation and testing of halotolerant PGPR from *Bacillus, Enterobacter* and *Pseudomonas* genera as single strains or consortia on different tomato varieties have confirmed their capacity to promote plant growth and tolerance in soil salinization (Feng et al., 2024; Haque et al., 2022; Patani et al., 2023).

Interestingly, PGPR can also increase tomato fruit quality. Inoculation with different genera such as *Pseudomonas, Bacillus, Azotobacter, Enterobacter* and *Stenotrophomonas* has been shown to increase yield, fruit size, lycopene, antioxidants, K, P and N in tomato fruits (Hariprasad and Niranjana, 2009; Gashash et al., 2022; Tuong et al., 2022).

In a previous study, we demonstrated that PVr_9 bacterial strain can confer salt tolerance to the model plant *Arabidopsis thaliana*, by modulating salt tolerance responsive genes, thus protecting plants by Na toxicity (Giannelli et al., 2024).

The objectives of this study are to investigate the capacity of PVr_9: (i) to stimulate the growth of root system and shoot biomass of tomato plants; (ii) to alleviate salt stress symptoms in tomato plants under NaCl feeding solution; (iii) to identify possible plant molecular functions modulated by PVr_9 both under control and salt stress conditions; and (iv) to evaluate possible positive effects of PVr_9 inoculation on the nutritional quality of tomato fruit under both control and salt stress conditions. Our work was extended from controlled *in vitro* conditions to green house and in field experiments to obtain a more comprehensive overview of the PVr_9 beneficial effects under salt stress. Overall, the results obtained would allow validating PVr_9 as a potential biostimulant in tomato to confer both higher salt stress tolerance and an improvement in the nutritional characteristics of fruits.

2. Materials and methods

2.1. Bacterial growth

The bacterial strain used in this work was isolated from the rhizosphere of *Pteris vittata*, an arsenic hyperaccumulator fern (Antenozio et al., 2021); it showed homology with *Beijerinckia fluminensis* and has recently been reclassified as *Agrobacterium* spp. strain PVr_9 in the NCBI database (GenBank: MT013514.1). PVr_9 was previously tested in the plant model species *Arabidopsis thaliana* as both PGPR and salt stress tolerance inducer (Giannelli et al., 2022, 2024).

In this work, PVr_9 strain was grown overnight in 100 mL of liquid Luria and Bertani medium on shaking (130 rpm) at 28 °C. After measuring OD at 600 nm with a spectrophotometer (Varian Cary 50 UV–Visible), the bacterial cell suspension was diluted to 10^8 cells mL^{-1} with sterile double-distilled water and used in all the trials.

2.2. Plant growth conditions and treatments

Our work is divided into three types of experiments: a controlled *in vitro* system suitable for the evaluation of the possible gene and protein modulation by PVr_9 under control and salt stress conditions, then moving to conditions more similar to those in nature, in greenhouse for the evaluation of vegetative stress parameters, and in the open field for the study of fruit characteristics.

2.2.1. In vitro assay

A preliminary *in vitro* assay was performed to test the capacity of PVr_9 to increase salt tolerance in tomato seedlings. For this purpose, tomato seeds (*S. lycopersicum cv.* Riccio of Parma) were surface sterilized for 10 min with a 40 % v/v NaClO solution, then for 5 min with 70 % v/v EtOH, and finally rinsed four times with double distilled sterile water.

Seeds were placed on sterilized filter paper to dry under a laminar flow hood for 15 min. Seeds were then plated in ¼ MS (Murashige & Skoog, Duchefa Biochimie, Haarlem, The Netherlands) and 1 % plant agar (Duchefa) on Petri dishes, and incubated in an environmentally controlled growth chamber (24 °C; 16/8 h light/dark photoperiod; 120 μmol m^{-2} s^{-1} PAR, 75% RU) for germination. Uniformly germinated seedlings were transferred to sterilized 10-cm diameter 8-cm height glass pots containing half-strength MS medium with 1 % w/v sucrose and 1 % plant agar. Each pot accommodated four plants incubated in an environmentally controlled growth chamber (24 °C; 16/8 h light/dark photoperiod; 120 μmol m^{-2} s^{-1} PAR, 75 % RU). After formation of the first true leaf, roots dipping was performed in 20 mL bacterial cell suspension (10^8 cells mL^{-1}) for 15 min, then the plantlets were transferred to 1/2 MS, 1 % (w/v) sucrose, 1 % plant agar, both with and without 150 mM NaCl. Plants not treated with PVr_9 were also transferred to a fresh 1/2 MS, 1 % (w/v) sucrose, 1% plant agar, with and without 150 mM NaCl and returned to growth chamber conditions. In order to observe the actual root colonization by PVr_9 root samples were collected 48 h after the inoculum for ESEM analysis following the procedure described by Dal Cortivo et al. (2017). After 72 h treatment, root and shoot tissues were sampled and stored at −80 °C for molecular analyses (Real-time PCR and 2D-PAGE) considering three biological replicates for each sample and treatment (Control; PVr_9 inoculated; 150 mM NaCl; PVr_9 inoculated + 150 mM NaCl). In addition, fifteen plants per treatment (Control; PVr_9 inoculated; 150 mM NaCl; PVr_9 inoculated + 150 mM NaCl) were grown for additional five days to measure morphological and physiological parameters.

2.2.2. Greenhouse experiments

The ability of PVr_9 to increase salt stress tolerance in tomato plants was evaluated in plants grown in sterilized soil in a greenhouse at 24 °C for 52 days. Tomato seeds were sown on 4.5-cm wide, and 5.5-cm high pots filled with 0.1 L of sterilized horticultural soil. Three weeks after germination, 15 mL of diluted bacterial suspension (10^8 cells mL^{-1}) was added to the soil of each pot once a week for three weeks. On the remaining days, the same amount of water was added. The salt treatments were imposed immediately after the last bacterial inoculation. The plants were subjected to a salt stress treatment three times a week by watering them with the same volume (15 mL) of 150 mM NaCl solution (salt treatment) or water (control). Three days after the last NaCl treatment, the leaves were sampled and stored at −80 °C for the quantification of chlorophyll, proline, and total soluble sugars, APX and CAT activities. For the analysis of the mineral elements profile, the leaves

were dried at 65 °C for 72 h. Four biological replicates were obtained for each sample and treatment. The experiment was replicated twice.

2.2.3. Field experiment

The effects of PVr_9 inoculation on the nutritional and organoleptic characteristics of tomato fruits in control condition and in condition of salt stress were evaluated in plants grown in soil in field conditions. For this purpose, tomato seeds were sown on 4.5-cm wide, and 5.5-cm high pots filled with 0.1 L sterilized horticultural soil in greenhouse. Three weeks after germination, plants of uniform size were selected and inoculated or not with 15 mL diluted bacterial suspension (10^8 cells mL^{-1}) once a week for three weeks. After three weeks, the plants were transferred to 4-L volume pots filled with horticultural soil, one plant per pot, with three plants (replicates) per treatment. Ten days after the beginning of flowering, one month after transplanting, half the plants, both treated or not with PVr_9, were subjected to NaCl treatment following this procedure: a total of 10 treatments were carried out, one every three days. The first treatment was performed with 50 mM NaCl, the second with 100 mM NaCl, and the remaining eight treatments were performed with 150 mM NaCl. Each time, 1 L of the salt solution was applied per pot. The same amount of water was added to the control plants. At the end of the trial, the mature fruits were harvested and stored at −80 °C for the analyses of soluble solids, dry matter, ashes, mineral elements profile, and antioxidants content (*i.e.* total carotenoids, lycopene, β-carotene, ascorbic acid, total polyphenols, and total antioxidant capacity). Three biological replicates were set for each treatment.

2.3. Morphological parameters

The seedlings were sampled to measure the length of the primary root and the number of secondary roots, and the roots were washed in water, dried on blotting paper, and positioned along a graduated ruler.

For the shoot fresh biomass measurement, each plant was collected from *in vitro* vessels, and the total fresh weight taken. For plants grown in the greenhouse, only the shoot biomass was measured. For dry biomass determination, shoots were dried at 65 °C overnight before weight measurement. A total of 15 plants per treatment were analyzed for both *in vitro* and in greenhouse experiments. Results are expressed as means ± s.d.

2.4. Gene expression analyses by real-time PCR

Total RNA extraction was performed on root and shoot tissues of plants grown *in vitro* and inoculated or not with PVr_9 sampled 72 h after stress as compared with controls (Section 2.1). Extraction was performed following the EasyPure® Plant RNA Kit (TransGen Biotech Co, Beijing, China) protocol. RNA quality was assessed by electrophoresis on 1 % (w/v) agarose gels to test for DNA integrity, and quantified by Nanodrop ND1000 (Thermo Fisher Scientific, Waltham, MA, USA). Reverse-transcription of 1 μg of total RNA was performed with HiScript III RT SuperMix for qPCR (Vazyme, Nanjing, PRC) following the manufacturer's instructions (https://n-genetics.com/files/co/Documents/manual/vazyme_19524.pdf). cDNA was analyzed through qPCR using Universal SYBR Green kit and Quantstudio 3 (Thermo Fisher Scientific). Data were analyzed with the ΔΔCt method, and the results are expressed as Log2($2^{-\Delta\Delta Ct}$) of means. Primers were designed for *S. lycopersicum SOS1* gene codifying for a plasma membrane Na^+/H^+ antiporter (accession number NM_001247769.3 Fwd 5′-CAAGGGGCTGATGTCTCTGG-3′ and Rev 5′-GCTTTGATGACTCTCGCCCT-3′), and for *S. lycopersicum NHX1* gene, a vacuolar Na^+/H^+ antiporter (accession number NM_001246987.1 Fwd 5′- ACGCCCACTTCTCACTAACG-3′ and Rev 5′-ACAGGGGTCGCATAAAAGCA-3′). *S. lycopersicum* actin was used as housekeeping gene (accession number NM_001321306.1 Fwd 5′-TTCAAAGGGCGAGTACGACGAG-3′ and Rev 5′-CAGCAGACCCGAGTTCACTTTT-3′). Three biological and three technical replicates were performed for each treatment. Amplification was conducted as follows: 2 min 50 °C, 5 min 95 °C; 15 s 95 °C, 1 min 60 °C (40 cycles); dissociation curves were obtained with 15 s 95 °C, 1 min 60 °C, 1 s 95 °C.

2.5. Differentially expressed protein analysis by 2D-PAGE

Leaf samples weighing 200 mg from the *in vitro* trial were frozen in liquid nitrogen, ground into a powder and added to 200 μL lysis buffer [50 mM Tris–HCl pH 7.5, 2 M thiourea, 7 M urea, 2 % (v/v) Triton X-100, 1 % dithiothreitol (DTT), 2 % (w/v) soluble polyvinylpolypyrrolidone, 1 mM phenylmethylsulphonylfluoride, and 0.2 % (v/v) β-mercaptoethanol]. Total protein extracts were obtained and treated as described in Brambilla et al. (2023). A 2D-PAGE was conducted to identify differentially expressed proteins in leaves of *S. lycopersicum* plantlets inoculated with Pvr_9 strain and either exposed or not to 150 mM NaCl, according to Degola et al. (2017). Three independent runs of each sample for each of the three biological replicates were performed. The spots excised from the gel were dissolved in 12 μL of 3 % ACN/0.1 % FA, and peptides were separated in a 10 cm pico-frit column (75 μm ID, 15 μm Tip; New Objective) packed in-house with C18 material (Aeris Peptide 3.6 _m XB-C18, Phenomenex) using a nano-HPLC system (Ultimate 3000, Dionex—Thermo Fisher Scientific) coupled with an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific). H_2O/FA 0.1 % and ACN/FA 0.1 % were used as eluents A and B, respectively, and chromatographic separation of peptides was performed at a flow rate of 0.25 μL/min using a linear gradient of eluent B from 3 % to 40 % in 20 min. A Data Dependent Acquisition (DDA) method was used: a full scan between 300 and 1700 Da was conducted at high resolution (60,000) on the Orbitrap, and the 10 most intense ions were selected for CID fragmentation and MS/MS data acquisition at low resolution in the linear ion trap. Raw data files were analyzed with the software package Proteome Discoverer 1.4 (Thermo Fisher Scientific) interfaced with a Mascot Search Engine (version 2.2.4, Matrix Science). MS/MS spectra were searched against the *S. lycopersicum* UniProt database (version February 2024, 51,512 entries) using the following parameters: enzyme specificity was set to trypsin with one missed cleavage allowed; precursor and fragment ion tolerance were 10 ppm and 0.6 Da, respectively. Carbamidomethylcysteine and methionine oxidation were defined as fixed and variable modifications, respectively. The Percolator algorithm was used to assess FDR at the protein and peptide level. Proteins identified with at least two unique peptides with high confidence (FDR < 1 %) were considered positive hits. The precursor area node of Proteome Discoverer was used to estimate the abundance of the proteins in each sample.

2.6. Physiological and biochemical parameters

Physiological and biochemical parameters were analyzed on the leaf tissues of PVr_9 inoculated or uninoculated tomato in greenhouse-grown plants under control and salt stress conditions. The leaf chlorophyll content was measured following the method described by Zhang et al. (2020). In brief, 0.1 g fresh leaf tissue was ground into a fine powder using liquid nitrogen, followed by homogenization with 1 mL of 100 % N,N-Dimethylformamide (DMF). After centrifugation at 10,000 x *g* for 10 min, the supernatant was collected, and its optical density was measured at 664 nm and 647 nm using a spectrophotometer. The total chlorophyll content was calculated as previously described (Zhang et al. 2020).

Proline was extracted and quantified as previously reported (Giannelli et al., 2024). Briefly, 50 mg of leaves were frozen in liquid nitrogen, ground, and homogenized with ethanol 95 % (v/v), then heated at 55 °C for 20 min. One mL of a reaction mixture composed of 1 % (w/v) ninhydrin (2,2-dihydroxyindane-1,3-dione) dissolved in a mixture of 60 % (v/v) acetic acid and 20 % (v/v) ethanol was added to 250 μL of the extract. The samples were heated at 95 °C for 20 min in the dark, then

centrifuged for 1 min at 7000 rpm. The proline concentration was evaluated by detecting the absorbance at 520 nm wavelength according to a standard curve made with a standard solution of L-proline ranging from 0.05 to 1 mM. Data are expressed as μmol mg^{-1} of fresh weight (FW). The quantification of total soluble sugars was evaluated as follows: 50 mg of fresh leaves were washed with 2 mL of 70 % ethanol (v/v), homogenized in 1.5 mL of 96 % ethanol, then placed in a water bath at 80 °C for 10 min. After cooling, the extract was centrifuged at 4000 rpm for 10 min and the supernatant stored on ice for measurement. The total concentration of soluble sugars was determined by reacting 100 μL of the ethanolic extract with 1 mL of freshly prepared anthrone reagent (150 mg anthrone plus 100 mL of 72 % sulfuric acid) and heating at 80 °C for 15 min. in order to measure the amount of total soluble sugars (μg mg^{-1} FW) after cooling, the absorbance was detected at 625 nm using a spectrophotometer using a glucose standard curve ranging from 0.01 mg mL^{-1} to 1 mg mL^{-1}. CAT and APX activities were determined as proxies for antioxidation power by using the method established by Maksimovic and Živanovic (2012) with some modifications. In brief, 100 mg of fresh leaves were ground in liquid nitrogen, suspended in 500 mL of 50 mM potassium phosphate buffer (pH 7 with 0.1 mM EDTA) and 1 mM phenyl-methane-sulfonyl-fluoride (PMSF) and centrifugated at 14,000 rpm at 4 °C for 20 min. Supernatant was utilized to determine CAT and APX enzyme activity according to Wang et al. (2009) and expressed as Units mg^{-1} protein. The protein content was estimated according to the Bradford method using BSA as standard. A total of four samples per treatment were analyzed.

2.7. Fruit chemical analyses

Homogenized samples of tomato fresh fruits coming from the field experiment were analyzed as soluble solids, dry matter, ashes, total carotenoids, lycopene, β-carotene, ascorbic acid, total polyphenols content, and total antioxidant capacity.

Soluble solids, dry matter, and ashes were determined according to IFU N.8 rev. 2017, UNI EN 12,145 1999, and IFU N.9 rev. 2005 methods, respectively.

Total carotenoids, expressed in g lycopene kg^{-1} dry matter, lycopene, and β-carotene were obtained using the UNI EN 12,136 1999 method. The ascorbic acid determination was carried out via a liquid chromatographic system HPLC-DAD (254 nm wavelength), consisting of Alliance 2695 Sep. Module, Alliance column heater, and 2996 photodiode array detector (Waters, Sesto San Giovanni, Milan, Italy). Samples diluted with 6 % v/v metaphosphoric acid, filtered on paper filter (Chemifarm, Parma, Italy) and 0.2 μm nylon filter (Millex-GN Millipore; Bedford, USA) by syringe drive, were injected into a 250 × 4.6 mm, 5 μm C18 Hypersil Gold column (Thermo Scientific) kept at 30 °C. Chromatographic separation was performed applying an isocratic elution with 1 mL/min flow, using metaphosphoric acid 0.3 % v/v as eluent. Data acquisition and chromatograms integration as well as management of chromatographic system were performed using Empower 2.0 software (Waters). The total polyphenolic content of the samples, expressed as mg kg^{-1} of (+)-catechin equivalents, was determined according to the Folin-Ciocalteu colorimetric assay described by Papagiannopoulos et al. (2004). Absorbance was measured at 720 nm using a UV–Vis spectrophotometer (Shimadzu UV-1900i). The antioxidant capacity was given as TR equivalents, in the units of μmol TRg^{-1}. The molar absorptivity of TR in Folin-Ciocalteu method is as follows: $\mathfrak{E}TR = 4.65 \times 103 Lmol^{-1}cm^{-1}$.

2.8. Plant and fruit mineral profile

Dried leaves (0.1 g), belonging to four biological replicates of the greenhouse experiment, and homogenized samples of tomato fresh fruits (1 g in triplicate) grown in field in different conditions were mineralized with 2 mL of 69% v/v ultrapure HNO_3 (Ultrex™ II, J.T. Baker™, Avantor, USA) through a high-pressure microwave-assisted digestion system (Ultrawave, Milestone s.r.l., Italy). The digested samples, spiked with Be, *Sc*, Rh and Bi as internal standards, opportunely diluted with ultrapure grade water (0.05 μS/cm, Purelab ULTRA Elga, UK), and filtered on 0.45 μm filters (Millex®-HA, Millipore, Merck, Germany) were introduced into a single quadrupole inductively coupled plasma mass spectrometer (Q-ICP-MS, iCAP™ RQ, Thermo Fisher Scientific, USA), using an ionomic approach to obtain the mineral elements profiles of the plant tissues. Macro- (Na, Mg, P, K, Ca, Fe) and micro- (Al, Cr, Mn, Co, Ni, Cu, Zn, As, Se, Mo, Cd, Pb) elements were analytically quantified by using customized multi-element standards (CPA Chem, Bulgaria), with concentrations ranging between 0 and 200 μg kg^{-1} and 0–20 mg kg^{-1} for micro and macro elements, respectively.

2.9. Statistics

One-way ANOVA and Two-way ANOVA in the Past 4.06b software (Hammer et al., 2001) were used for statistical analyses of tomato plant morphological and biochemical data. The Shapiro-Wilk test was used for normality evaluation, and Tukey's test for multiple comparisons; differences were considered significant at $p < 0.05$.

One-way ANOVA carried out on ionomic profiles and antioxidants content, and PCA of fruits describing differences related to mineral elements and antioxidants were performed via the SPSS software (IBM SPSS Statistic, V22.0).

The post-hoc Tukey's HSD test or T3 Dunnett's test for mineral elements with non-homogeneous variances were performed for multiple comparisons in One-way ANOVA. The Shapiro-Wilk test was used for normality evaluation; differences were considered significant at $p < 0.05$.

Before PCA analysis, data were autoscalated by subtracting the mean and dividing by the standard deviation within each variable, verifying the absence of outliers, and testing the sampling adequacy by Kaiser-Meyer-Olkin (KMO = 0.60) and variables correlation by Barlett's test of sphericity ($p < 0.05$)

3. Results

3.1. In vitro experiments

3.1.1. PVr_9 increases root length and biomass of tomato seedling

Roots of *in vitro* PVr_9 inoculated seedling were firstly analyzed via ESEM to evaluate the effective bacterial colonization. ESEM analysis was performed under both control and salt stress conditions, on 10-day old plantlets. In Fig. S1 bacterial colonization was noticeable on tomato roots after 48 h under both control (Fig. S1A) and salt treatment (Fig. S1B). The bacterial strain formed a biofilm colonizing the root diffusely, starting from the area above the root tip and continuing towards the root elongation zone and root hair area.

Morphological parameters as primary root elongation, number of secondary roots and fresh biomass were also assessed on tomato seedlings inoculated or not with PVr_9 and treated or not with 150 mM NaCl for five days. PVr_9 inoculated seedlings showed a significant increase in primary root length (Fig. 1A, B), and in the number of secondary roots (Fig. 1C), with a significant increase in total fresh weight (Fig. 1D) compared to uninoculated ones under both control and salt stress conditions.

3.1.2. PVr_9 modulated tomato salt tolerance genes and proteins linked to defense against pathogens

SOS1, and *NHX1* genes, were chosen and analyzed via qRT-PCR in both roots and leaves, in PVr_9 inoculated and uninoculated seedlings under control conditions and after 72 h of 150 mM NaCl treatment.

In roots, *SOS1* expression increased by 2.5 times in plants treated with 150 mM NaCl compared to the controls regardless of PVr_9 inoculation (Fig. 2A). In leaves, *SOS1* showed a 10-fold increase in the transcript in PVr_9 inoculated seedlings treated with 150 mM NaCl

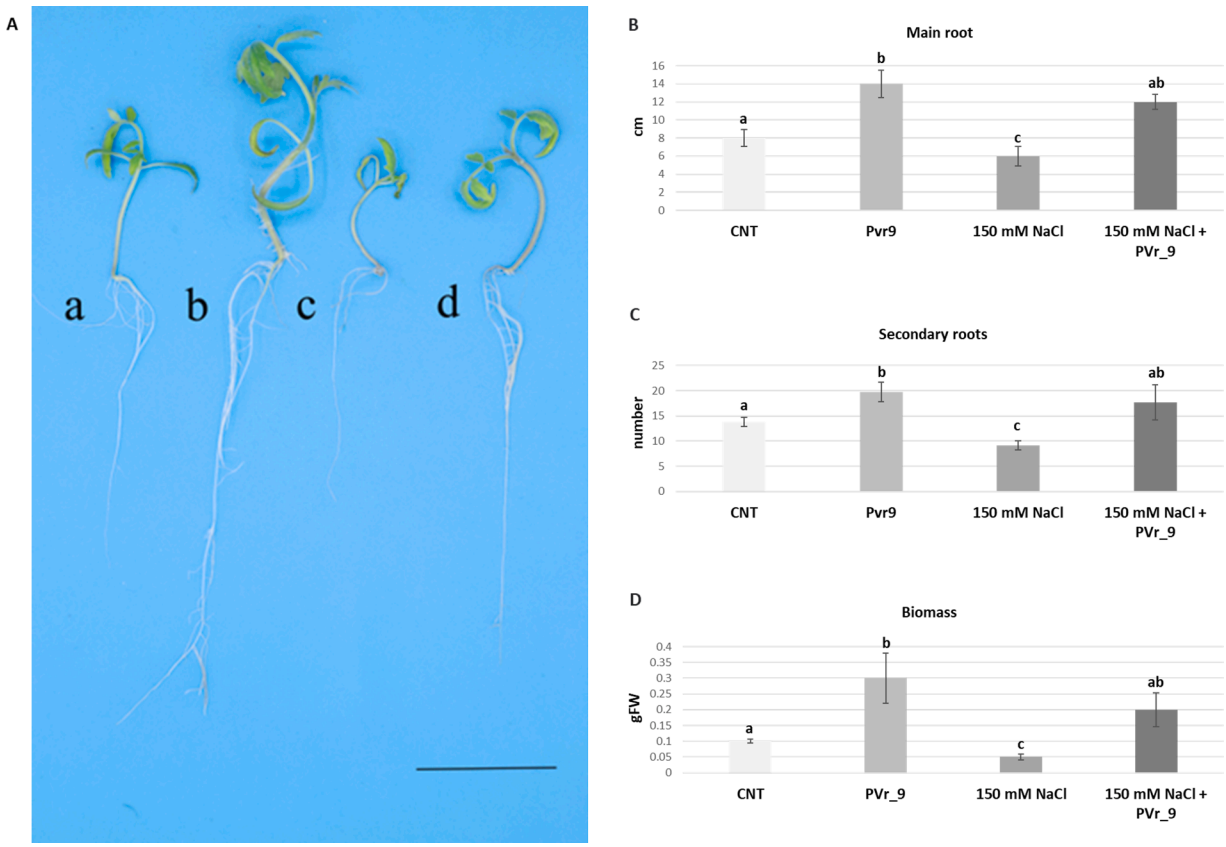

Fig. 1. **(A)** *In vitro* experiment: representative photographs of *Solanum lycopersicum* plants inoculated or not with PVr_9 bacterial strain without NaCl or after five days of 150 mM NaCl treatment; **(a)** Control, **(b)** PVr_9, **(c)** 150 mM NaCl, **(d)** 150 mM NaCl + PVr_9; Black bars in photograph correspond to 6 cm length. **(B)** Primary root length, **(C)** Number of secondary roots, and **(D)** seedling fresh weight both in control conditions and under 150 mM NaCl stress. Data were expressed as mean ± s.D. ($n = 15$ biological replicates). Different letters represent significant differences among treatments (Two-way ANOVA and Tukey's test, $p \leq 0.05$).

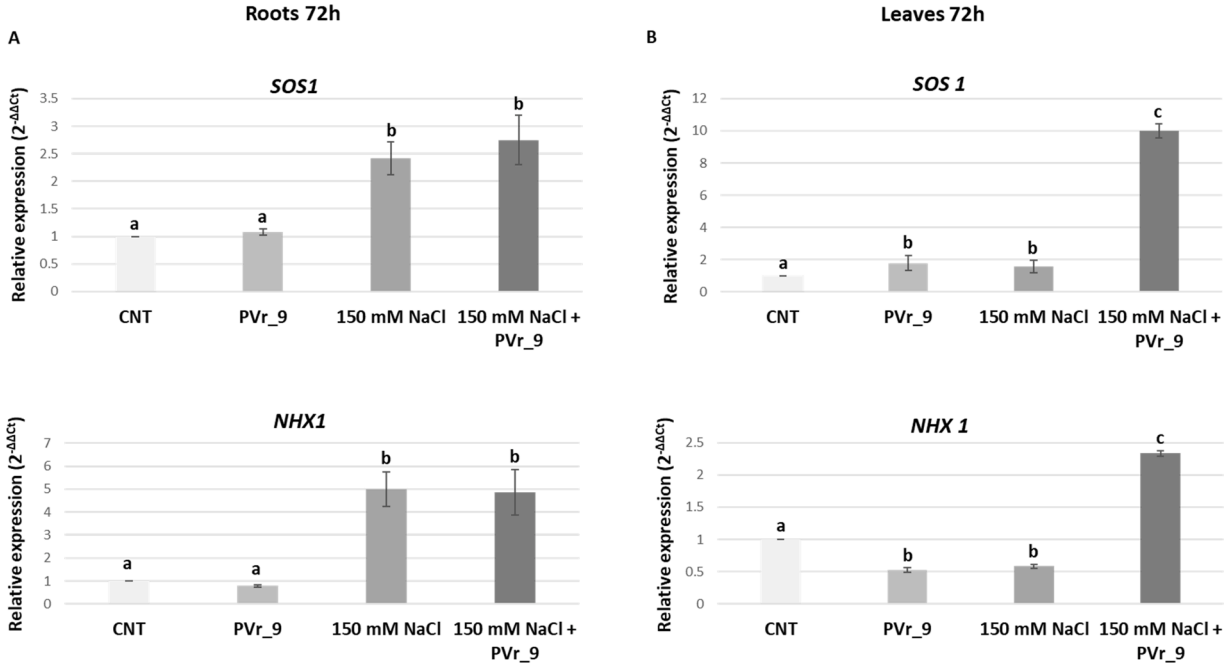

Fig. 2. *In vitro* experiment: qRT-PCR determination of relative expression levels of *Solanum lycopersicum* salt stress tolerance genes. *SOS1* (Salt Overly Sensitive 1); *NHX1* (Sodium Hydrogen Exchanger 1) in roots **(A)** and leaves **(B)** of *Solanum lycopersicum* plants treated with 150 mM NaCl inoculated or uninoculated with PVr_9. RNA was extracted after 72 h of treatment. Data are means ± s.D. ($n = 3$ biological replicates). Different letters represent significant differences among treatments (Two-way ANOVA and Tukey's test, $p \leq 0.05$).

compared to the control, while only a small increase in the transcript was observed in control condition between PVr_9 inoculated *vs.* uninoculated seedlings (Fig. 2B).

A similar trend was observed for the expression of *NHX1*. Indeed, in roots, *NHX1* showed a 5-fold increase in the transcript in seedlings treated with 150 mM NaCl, regardless of the inoculation treatment, compared to the control (Fig. 2A). In leaves, a 2-fold increase in *NHX1* transcript was also observed in PVr_9 inoculated seedlings treated with 150 mM NaCl while a decrease in the transcript was observed in Pvr_9 and 150 mM NaCl treated samples compared to the control (Fig. 2B).

The 2D-GE analysis, used to investigate which proteins were modulated in leaves of tomato seedlings inoculated with PVr_9 and exposed or not to 150 mM NaCl, revealed that, in control conditions (without NaCl), four proteins involved in microbial pathogen stress response were upregulated, namely, PR10, chitinase, steroid 16 alpha-hydroxylase and heme-binding protein 2 (Fig. S2 and Table 1).

On the other hand, under salt stress condition, the inoculation of PVr_9 showed an upregulation of PAP, a key enzyme involved in proline accumulation in plant tissues under water stress (Fig. S2 and Table 1), while in the same condition there was a downregulation of proteins generically related to stress response (such as chloroplastic molecular chaperons) and enzymes specifically induced by salt stress (namely cysteine synthase and phosphoribosylamine-glycine ligase) (Fig. S2 and Table 1).

Table 1
In vitro experiment: 2D-GE protein identification by LTQ-Orbitrap XL mass spectrometer and relative abundance (fold) in leaves of plants either inoculated or not with PVr_9 strain, in presence or not (CNT) of 150 mM NaCl, ($n = 3$ biological replicates).

SSP	Protein name	Accession number	MASCOTT score	% Coverage	Number of protein	Number of unique peptides	Number of peptides	Number of PSMs	Number of AAs	MW (kDa)	pI	Fold (PVr_9/ CNT)
5402	*Oxygen-evolving enhancer protein 1*	A0A1D3L5W7	2606.72	48.94	1	18	18	95	329	35.0	6.79	0.3
5507	*Ribulose bisphosphate carboxylase/ oxygenase activase, chloroplastic*	A0A3Q7IN43	12,377.60	65.90	1	8	31	400	434	47.7	7.74	0.4
5509	*Ribulose bisphosphate carboxylase/ oxygenase activase, chloroplastic*	A0A3Q7IN43	2642.76	62.67	1	7	23	59	434	47.7	7.74	0.4
5605	*RuBisCO large subunit-binding protein subunit beta, chloroplastic*	A0A3Q7ECG0	3272.23	72.79	1	36	43	90	599	63.0	5.87	0.4
6203	*PR10 protein*	K4CWC4	246.97	27.50	1	3	3	5	160	17.4	5.74	3.2
6303	*Chitinase*	Q7Y0S1	1574.04	61.26	1	8	8	65	253	27.6	6.32	7.3
6412	*Steroid 16alpha-hydroxylase*	Q40131	615.95	34.21	1	12	12	20	342	38.2	5.64	11.9
6603	*ATP synthase subunit beta, chloroplastic*	A0A0C5CEC7	1336.74	50.20	1	18	19	36	498	53.4	5.39	2.4
8304	*Heme-binding protein 2*	A0A3Q7HG98	601.20	21.40	1	5	5	26	229	25.8	7.72	2.4
8405	*NAD-dependent epimerase/ dehydratase domain-containing protein*	A0A3Q7I5A7	741.63	39.31	2	13	13	26	407	43.9	7.55	2.2
8415	*NAD-dependent epimerase/ dehydratase domain-containing protein*	A0A3Q7I5A7	1650.94	45.45	2	15	15	57	407	43.9	7.55	115.7

SSP	Putative identification	Accession number	Score	Coverage	Number of protein	Number of unique peptides	Number of peptides	Number of PSMs	Number of AAs	MW (kDa)	pI	Fold (NaCl + PVr_9/ NaCl)
1505	*STI1/HOP DP domain-containing protein*	A0A3Q7EQ90	1005.50	52.44	1	16	16	32	349	37.1	4.51	0.3
3302	*Uncharacterized protein*	A0A3Q7GE81	417.74	48.90	1	14	14	16	317	34.0	5.85	0.4
3605	*RuBisCO large subunit-binding protein subunit alpha, chloroplastic*	A0A3Q7J0I3	10,196.46	78.23	1	35	35	275	588	61.9	5.30	0.5
3701	*Phosphoribosylamine–glycine ligase*	A0A3Q7EQ38	6234.94	29.33	1	34	34	179	1265	135.0	5.43	0.3
4609	*Prolyl aminopeptidase*	A0A5H1ZRW2	2456.11	36.87	1	2	14	62	537	56.6	6.13	2.1
5509	*Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic*	A0A3Q7IN43	2642.76	62.67	1	7	23	59	434	47.7	7.74	0.3
5606	*ATP synthase subunit beta, chloroplastic*	A0A0C5CEC7	1417.05	48.39	1	17	18	50	498	53.4	5.39	0.4
6204	*ATP synthase epsilon chain, chloroplastic*	A0A0C5C9T7	766.68	79.70	1	9	9	31	133	14.6	5.54	0.4
7405	*Cysteine synthase*	A0A3Q7I6B9	957.94	43.38	1	13	13	28	325	34.2	6.15	0.4

3.2. Greenhouse experiments

3.2.1. Pvr_9 increases plant biomass and modulated osmolytes content and antioxidant enzymatic activity under soil salinization

To confirm the positive effects of PVr_9 inoculation on tomato plants in more realistic conditions, an experiment was set up in a growth chamber by using sterilized universal soil. In Fig. 3A the positive effects on growth of PVr_9 inoculated plants compared to uninoculated ones, under both control and salt stress conditions, were ascertained along with the increase in size of the leaf blade (visual evaluation). Plants inoculated with PVr_9 showed a significant increase in shoot fresh weight (Fig. 3B) and chlorophyll content (Fig. 3D) compared to uninoculated ones under both control and salt stress conditions, while a significant increase in dry weight by PVr_9 was only evident in unstressed plants *vs.* the control (Fig. 3C).

Proline and total soluble sugars were assessed in greenhouse-grown tomato plants. No difference in proline content (Fig. 4A) was observed under control condition between PVr_9 inoculated and uninoculated plants, while PVr_9 led to significant increase in total soluble sugars in absence of salt stress (Fig. 4B).

As expected, salt treatment increased the amount of proline and soluble sugars both in PVr_9 inoculated and uninoculated plants. Inoculated stressed plants had the greatest increase in proline content (Fig. 4A), while total sugars content was similar in PVr_9 inoculated *vs.* uninoculated stressed plants (Fig. 4B).

The activity of antioxidant enzymes APX and CAT was also analyzed as a further indicator of PVr_9 salt stress-induced tolerance. No difference was observed in the activities of APX and CAT between PVr_9 inoculated *vs.* uninoculated plants under control conditions (Fig. 4C, D). The APX activity showed a significant increase in salt stressed plants compared to those grown under control conditions, with PVr_9 inoculated plants showing the highest activity (Fig. 4C). As far as CAT is concerned, salt stress decreased its activity both in inoculated and uninoculated plants to the same extent (Fig. 4D).

3.2.2. PVr_9 and salt modulated ion contents in tomato shoots

The content of Na, K, P, Fe and Ca along with micronutrients was evaluated in tomato shoots of greenhouse-grown plants, both inoculated and uninoculated with PVr_9, under control and 150 mM NaCl conditions to determine whether the presence of the bacterium modifies the ion homeostasis. No significant difference in macronutrients was observed in leaves of PVr_9 inoculated *vs.* uninoculated plants under control condition, except for a significant increase in Mg without salt treatment (Table S1). NaCl treatment increased the content of Na to the same extent (2/3-fold) in both PVr_9 inoculated and uninoculated plants while decreasing K content. No difference was observed in the amount of other macro elements under salt stress conditions (Table S1). As far as micronutrients are concerned, a significant increase in Cu and Zn was observed in PVr_9 inoculated plants compared to uninoculated ones while a decrease in contaminants such as Cr and Ni was observed under control conditions (Table S1).

3.3. PVr_9 affects nutritional and mineral characteristics of tomato fruits

An open field experiment was conducted to evaluate some nutritional characteristics of tomato fruits obtained from plants inoculated or not with PVr_9, in control conditions and subjected to salt stress during flowering. To this purpose, fruits of plants grown in pots in the field were harvested at maturity, and dry matter, soluble solids, ashes, and various antioxidant molecules were evaluated along with macro and micronutrients. In control conditions (absence of NaCl), a significant decrease in dry matter (6.05 *vs.* 6.82 g/100 g) and in soluble solids (4.67 *vs.* 5.24 g/100 g) in fruits deriving from PVr_9 plants compared to uninoculated ones was observed, while no significant differences were observed

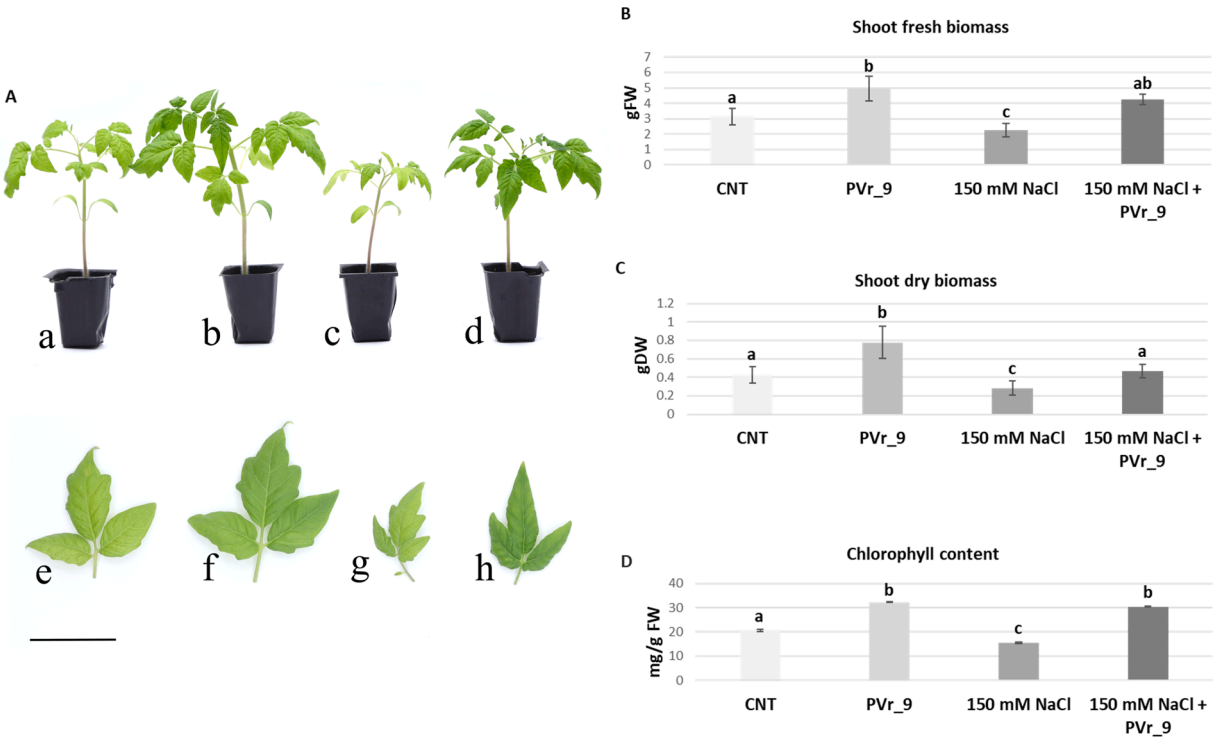

Fig. 3. Greenhouse experiment: **(A)** representative photographs of *Solanum lycopersicum* plants inoculated or not with PVr_9 and exposed or not to 150 mM of NaCl one week after the inoculation **(a)** Control, **(b)** PVr_9 inoculated plants, **(c)** 150 mM NaCl treated plants, **(d)** Pvr_9 inoculated and 150 mM NaCl treated plants, **(e)** leaf of control plants, **(f)** leaf of PVr_9 inoculated plants, **(g)** leaf of 150 mM NaCl treated plants **(h)** leaf of PVr_9 inoculated and 150 mM NaCl treated plants. Shoot fresh weight **(B)**, shoot dry weight **(C)**, and leaf chlorophyll content **(D)** under control conditions and salt stress. Black bars in photographs **(A)** correspond to 6 cm length. Data were expressed as mean ± s.D. ($n = 4$ biological replicates). Different letters represent significant differences between treatments (Two-way ANOVA and Tukey's test, $p \leq 0.05$).

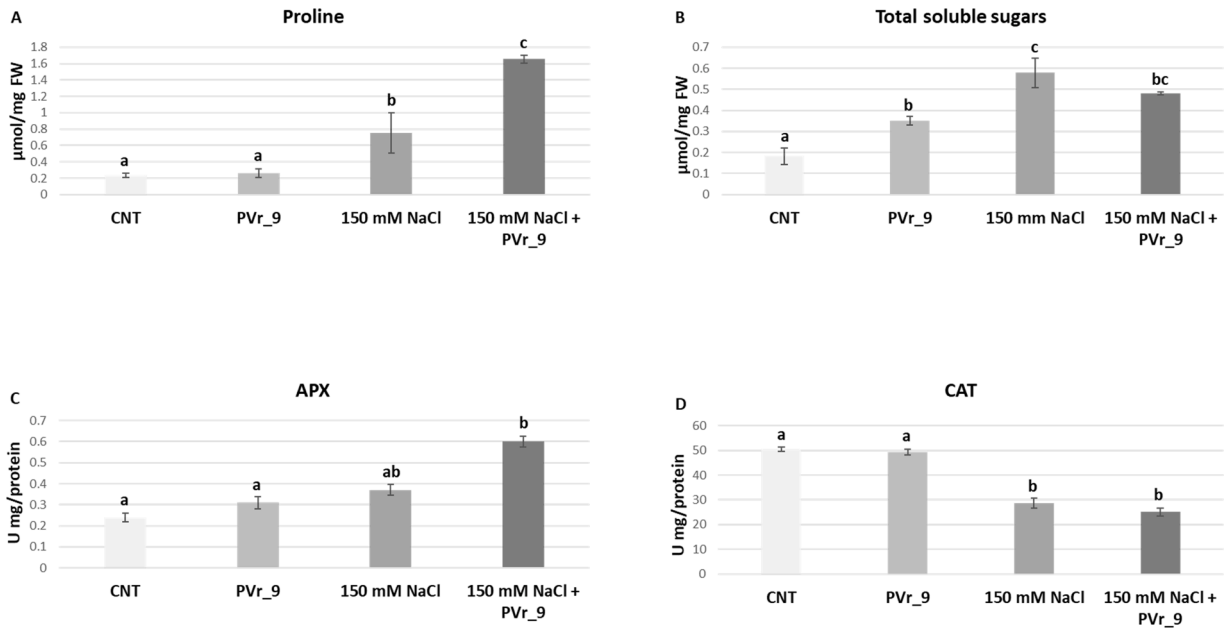

Fig. 4. Greenhouse experiment: salt stress markers accumulation and antioxidant enzyme activity. **(A)** Proline content, **(B)** Total soluble sugars, **(C)** APX, and **(D)** CAT activity in *Solanum lycopersicum* leaves inoculated or not with PVr_9 and exposed to 150 mM of NaCl starting from one week after the last soil bacterial inoculation. Data were expressed as mean ± s.D. ($n = 4$ biological replicates). Different letters represent significant differences between treatments (Two-way ANOVA and Tukey's test, $p \leq 0.05$).

between dry matter (6.72 *vs.* 6.85 g/100 g) and soluble solids (5.09 *vs.* 5.17 g/100 g) in fruits of PVr_9 inoculated and uninoculated plants, in stressed plants, respectively.

The level of carotenoids, β-carotene, lycopene, and L-ascorbic acid increased significantly in fruits of PVr_9 inoculated plants in control conditions *vs* uninoculated ones (Fig. 5A, B, C, D). Following the salt stress treatment, the levels of some nutraceutical compounds (β-carotene, L-ascorbic acid) decreased significantly compared to unstressed plants, but tomato fruit of PVr_9 inoculated plants showed a significantly lower decrease in L-ascorbic acid *vs.* fruit derived from uninoculated plants. Conversely, the overall polyphenol content was higher in the fruit of plants subjected to salt stress treatment (Fig. 5E). Moreover, fruits from PVr_9 inoculated plants showed a lower content of total polyphenols compared to uninoculated plants, under both control and salt stress conditions (Fig. 5E). In terms of total antioxidant capacity, results revealed that in the absence of salt stress the antioxidant level of fruits deriving from PVr_9 inoculated plants was lower than control, while in salt-stressed plants PVr_9 increased fruit antioxidant levels compared to that of uninoculated salt-stressed plants (Fig. 5F). As far as fruit mineral contents, PVr_9 inoculation determines a significant increase in Na in tomato fruits under both control and salt stress conditions, while Ca content increased significantly only in the absence of salt stress (Table S2). Interestingly, as far as trace elements accumulation, PVr_9 determines a significant decrease in Cd and Pb in both control and salt-stressed plants (Table S2). PCA performed on fruit traits analysed revealed a clear distinction between groups (Fig. 6). The synthetic variable PC1 explained the largest variability (52.93 % *vs.* 30.83 % of PC2) and allowed for separating salt-stressed treatments in the left-hand quadrants (Fig. 6) *vs.* unstressed plants set in the right-hand quadrants of the graph. PCA indicates that unstressed plants (no NaCl) possessed higher amounts of L-ascorbic acid, K- and β-carotene, while stressed plants showed higher Na and polyphenol contents. PC2 allowed for an efficient separation of inoculated *vs.* uninoculated tomato plants with PVr_9: uninoculated controls showed high Pb and Cd content, and inoculated plants high lycopene contents in fruits.

4. Discussion

4.1. Beneficial effects of PVr_9 strain on physiological and biochemical characteristics of tomato plants

Salinity variously impacts different aspects of plant development, encompassing germination, vegetative growth, and reproductive processes. Soil salinity introduces plant growth challenges such as ion toxicity, osmotic stress, deficiencies in essential nutrients (N, Ca, K, P, Fe, Zn), and oxidative stress; it also restricts the ability of plants to absorb water from the soil (Shrivastava and Kumar, 2015). Amongst the strategies intended to alleviate the detrimental effects of salinity on crop yield and quality, the use of PGPR is regarded as one of the most promising, being both effective and environmentally friendly (Kohler et al., 2009). In a previous study, the halotolerant bacterial strain PVr_9 showed several PGP traits such as IAA and siderophore production, biofilm formation, the ability to enhance the growth of the model species *A. thaliana,* as well as the inhibition of fungal phytopathogen development (Giannelli et al., 2022). In addition, PVr_9 was active in reducing growth impairment of *A. thaliana* under salt stress. More specifically, under salt-stress conditions, a reduced content of ROS, 8-oxo-dG, osmolytes and ABA was found in PVr_9 inoculated plants, accompanied by a modulation in the SOD, CAT and APX activity. Additionally, an increase in the expression of *SOS1* and *NHX1*, which are involved in Na translocation and compartmentation, was observed (Giannelli et al., 2024).

This study evaluated the capacity of PVr_9 to alleviate salt stress in *S. lycopersicum* under different growth conditions, by performing *in vitro*, greenhouse and open field experiments.

Morphological analyses showed beneficial effects of inoculation under both control and salt stress conditions (Figs. 1, 3); in addition, the bacterial strain seemed to be able to attenuate the significant decrease in the leaf chlorophyll content of plants exposed to 150 mM NaCl, but also to increase chlorophyll even in the absence of salinity, a common effect of PGPR (Mahmood et al., 2016; Panwar et al., 2016). Under salt stress condition, PVr_9 was also able to increase proline content in tomato leaves (Fig. 4A). Accumulation of osmotic regulators which maintain intracellular osmotic pressure homeostasis represents a crucial

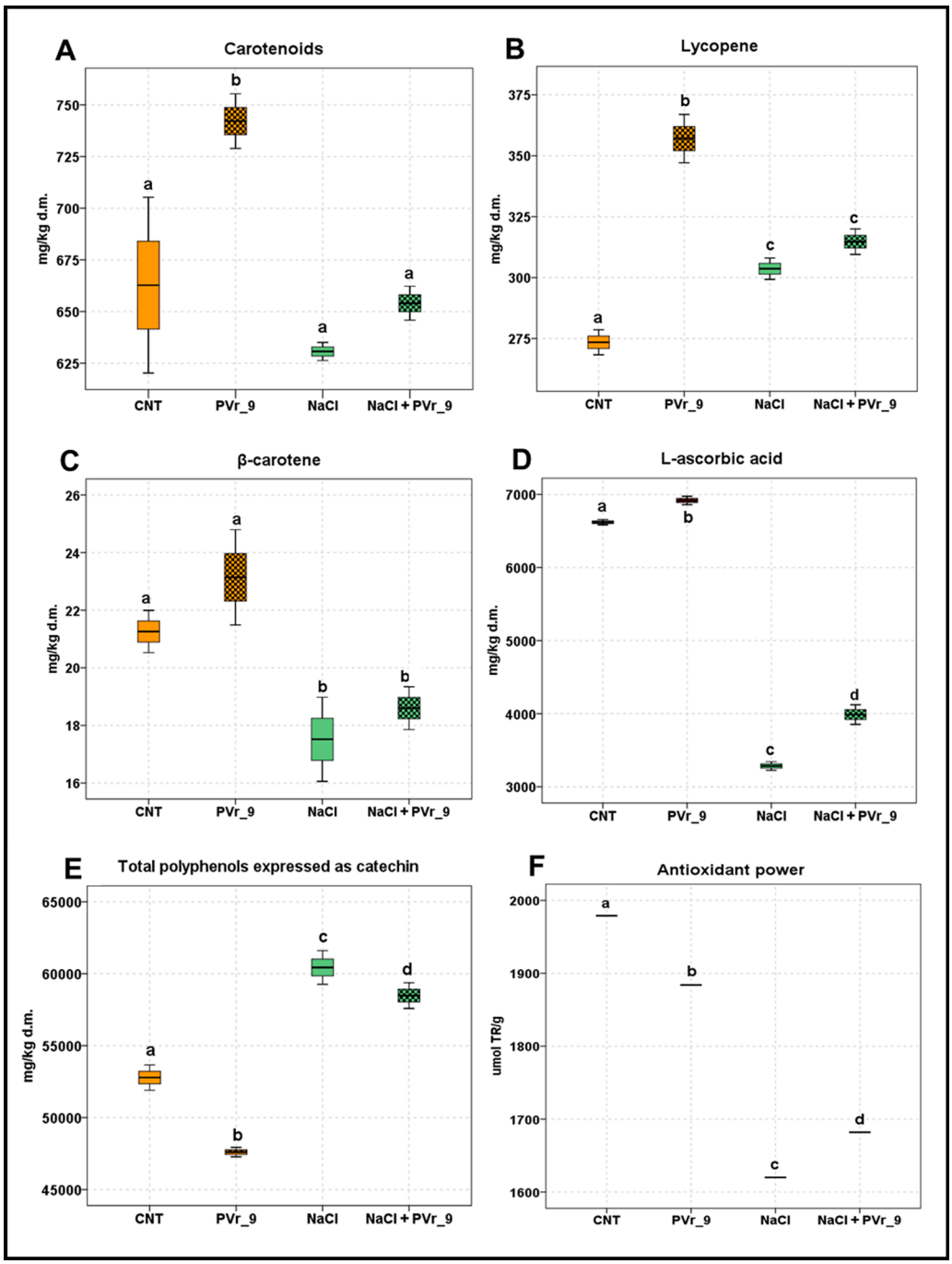

Fig. 5. Open field experiment: values of antioxidant molecules (carotenoids, lycopene, β-carotene, L-ascorbic acid), polyphenols and antioxidant power of tomato fruits presented as box plot. Plant treatments: Control (CNT), PVr_9 inoculated tomato plants (PVr_9), exposed to NaCl, and exposed to NaCl together with PVr_9 inoculation (NaCl+PVr_9). Data were expressed as mean ± S.D. ($n = 3$ biological replicates). Boxes marked with different letter are significantly different (Tukey's test, $p \leq 0.05$).

mechanism for plant tolerance to salt stress (Chen and Murata, 2002; Patani et al., 2023). Proline is also known to play a crucial role in strengthening the plant's antioxidant defense system, facilitating energy restoration (Desoky et al., 2019). The abundant presence of proline helps to mitigate damage caused by ROS, contributing to plant tolerance to salinity stress (Hasanuzzaman et al., 2021). PVr_9 was also able to significantly increase the levels of APX in salt-stressed plants, thereby suggesting a possible contribution of this enzyme in diminishing the overall ROS content in PVr_9 inoculated plants. Indeed, ROS generation due to salt stress can harm plants by inducing oxidative stress. APX and CAT are antioxidant enzymes crucial for safeguarding plants from oxidative stress (Caverzan et al., 2012). Numerous studies have demonstrated the capacity of PGPR to regulate the activity of antioxidant enzymes, including APX and CAT (Patani et al., 2023).

Contrary to APX, leaf CAT activity was found to be inhibited by salt, regardless of bacterial inoculation (Fig. 4C, D), a result already observed in other plant species (Tejera García et al., 2007).

Despite the modulation of biochemical response due to the presence of PVr_9, no significant differences in Na content was observed between PVr_9 inoculated *vs.* uninoculated plants in tomato leaves under salt stress condition (Table S1). Salt treatment resulted in a 2–3-fold increase in Na in leaf tissues, leading to a decrease in K content in both inoculated

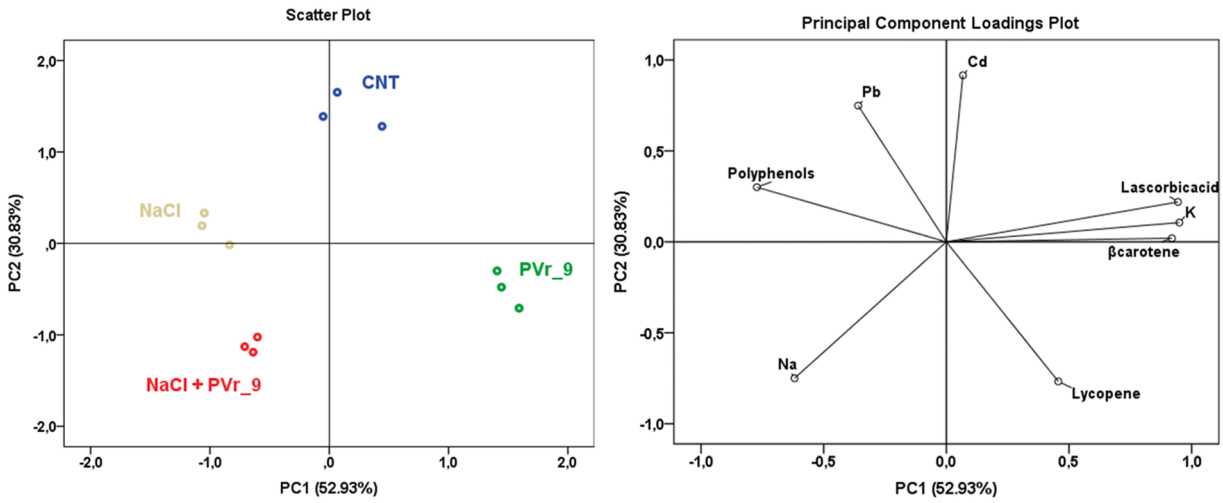

Fig. 6. Open field experiment: Principal component analysis (PCA) with synthetic variables PC1 and PC2. Scatter Plot (left) and Loading Plot (right) for treatment classification using nutraceutical characteristics and macro and micronutrients of tomato fruits. Treatments: Control (CNT), PVr_9 inoculated tomato plants (PVr_9), exposed to NaCl, and exposed to NaCl together with PVr_9 inoculation (NaCl+PVr_9).

and uninoculated plants, a condition usually reported in literature (Shi et al., 2022; Giannelli et al., 2024), whereas no other macro-element diminished in plant leaves due to salt stress in our experimental condition. On the contrary, previous results on *in vitro* grown *Arabidopsis* plantlets subjected to salt stress showed a strong reduction in all the macronutrients, even though in that case too no differences in the amount of such elements were observed between inoculated *vs.* uninoculated plants (Giannelli et al., 2024). Interestingly, despite Na treatment, PVr_9 inoculation increased the accumulation in leaves of essential micronutrients such as Zn and Cu, and decreased the accumulation of contaminants such as Cr and Ni compared to uninoculated plants, suggesting a possible role of this bacterial strain in selected mineral biofortification, by limiting metal contaminants.

4.2. *PVr_9 upregulated gene and proteins in tomato plants associated with biotic and abiotic stress tolerance*

The negative impact of salinity on plant productivity can be mitigated by maintaining the appropriate concentration of ions in the cytosol, a regulatory process that is facilitated by the activity of Na^+/H^+ antiporters NHX1 and SOS1 (Rao et al., 2021). Antiporters are recognised as playing a crucial role in plant ion homeostasis by controlling the cellular levels of Na ions: NHX1 reduces the accumulation of Na ions in the cytosol by actively transporting them through the tonoplast into the vacuole; while SOS1, which is located in the plasma membrane, is responsible for Na ions export from the cytosol to the apoplast, as described by Zhang et al. (2017). The upregulation of *NHX1* and *SOS1* transcript levels in various species under salinity stress has been associated with enhanced plant tolerance to high NaCl concentrations (Ali et al., 2021). This study reveals a significantly higher level of *NHX1* and *SOS1* transcripts in the leaves of PVr_9 inoculated plants after 72 h of salt treatment compared to uninoculated ones (Fig. 2), in accordance with results obtained in the model species *A. thaliana* (Giannelli et al., 2024), thus reinforcing the potential of PVr_9 in determining a less stressed phenotype in tomato plants.

Proteomic data also supported the capability of the PVr_9 bacteria strain to reduce the detrimental effects of salt exposure in tomato (Table 1; Fig. S2). Compared to uninoculated plants, the presence of 150 mM NaCl in inoculated plants determined the upregulation of leaf PAP, which catalyses the cleavage of proline or hydroxyproline from the N-terminus of polypeptides (Li et al., 2010): in numerous studies the upregulation of PAPs was found to be often associated with the increase in proline content, an important osmoprotector against drought and salt stress in plants (Szawłowska et al., 2011; Ghifari et al., 2020). Accordingly, the increase in PAP observed in our conditions in association with a proline content enhancement (Fig. 4A) suggests a direct involvement of the PVr_9 strain in modulating the inner proline levels in salt-exposed tomato plants, and, in turn, in reducing the plant sensitivity to salinity.

On the other hand, a cysteine synthase involved in cysteine biosynthesis (Wirtz et al., 2006) was found to be downregulated in PVr_9 inoculated salt-stressed plants. Its overexpression was reported to be associated with conditions that imply an increased demand for cysteine -such as heavy metal or oxidative stress – as cysteine has been supposed to act as an antioxidant, indirectly relieving oxidative stress (Noji et al., 2001). Here, the observation that PVr_9 inoculated plants exposed to NaCl showed a reduction in cysteine synthase when compared to uninoculated ones suggests a lower demand for antioxidant agents, being also coupled with higher levels of proline and APX (Fig. 4C).

The downregulation of three proteins related to protein folding processes, namely STI1/HOP DP domain-containing protein, Rubisco large subunit-binding protein (α subunit) and Ribulose bisphosphate carboxylase/oxygenase activase in plants treated with PVr_9 (Fig. S2; Table 1), corroborates the hypothesis of stress mitigation exerted by this bacterial strain in tomato plants. Indeed, under severe abiotic stress, plants cells are prone to accumulating high levels of oxidized and misfolded peptides, and the production of proteins with protective function tends to increase (Kosová et al., 2013). STI1/HOP, a co-chaperone that connects and regulates the Hsp70-Hsp90 chaperone machinery (Schmid et al., 2012), has also been associated with the plant response to biotic and abiotic stress (Toribio et al., 2020), and an increase in STI1 and HOP-like protein transcripts was detected in rice panicles and soybean under stressful conditions, respectively (Dooki et al., 2006; Zhang et al., 2003).

In addition, the expression of both the α subunit of RuBisCO large subunit-binding protein and RuBisCO activase was found to be upregulated in different plant species under salt stress (Chen, 2018): in higher plants, the former is involved in folding the RuBisCO large subunits in the chloroplast (Liu et al., 2010), and the latter is essential for the activation and maintenance of RuBisCO catalytic activity (Parry et al., 2008). Since salinity suppresses the activity of photosynthetic enzymes by reducing the efficiency of operational and structural integrity of photosynthetic enzymes (Pan et al., 2021), the upregulation of these two proteins has been considered a positive response of the stressed plants aimed at reducing salt-depended damage to the photosynthetic system. In our *in vitro* trial, under salt stress condition PVr_9 inoculated plants showed a significant decrease in both these proteins

related to the activity of RuBisCO (Table 1), thereby suggesting that the presence of the strain could have counteracted the negative effects of salt on the photosynthetic apparatus of treated tomato plants, which, in turn, would require a lower RuBisCO turnover.

The proteome analysis also provided interesting cues on the interaction between PVr_9 strain and tomato plants under non-saline conditions: when comparing inoculated plants with uninoculated ones, plant defense proteins such as PR-10, chitinase, and Heme-binding protein 2 (HBP2) were found to be upregulated 3.2-, 7.3-, and 2.4-fold, respectively (Table 1). PGPRs are known to stimulate plant defense mechanisms, for instance by promoting the activation of SAR and ISR (Kaur et al., 2022). Pathogenesis related-10 protein and chitinase belong to the family of PRs – a group of highly variable molecules involved in SAR and hypersensitive response that are induced by pathogens and defense-related signaling molecules; they are known to accumulate in infected as well as in surrounding tissues and provide protection against the pathogen's spread (Ali et al., 2018). Overexpression of PR proteins, individually or in combination, greatly increases the efficacy of plant defense response, and overexpression of chitinase and/or PR10 was demonstrated to increase disease resistance against a wide range of pathogens (Gupta et al., 2013; Fan et al., 2015).

The antimicrobial properties of protein HBP2 isolated from the apoplast of *S. lycopersicum* against plant pathogens such as *Pseudomonas syringae* pv. tomato, *Xhanotomonas vesicatoria, Clavibacter michiganensis* sp. *Michiganensis*, and *Bacillus cinerea* has recently been assessed *in vitro* by Farvardin et al. (2023). Taken together, these findings strongly support a possible role of PVr_9 in actively stimulating plant biotic and biotic defense mechanisms.

4.3. PVr_9 increases nutritional characteristics of tomato fruits

The greatest differences in quality characteristics of tomato fruits are observed in plants inoculated with PVr_9 not subjected to salt treatment (Fig. 5). PVr_9 increases carotenoids, lycopene, β-carotene, and L-ascorbic acid (Fig. 5, Table S2). Other authors obtained similar results on the beneficial effects of different PGPR in increasing quality characteristics of tomato fruits. Ordookhani et al. (2010) showed that a combined inoculation of *Pseudomonas, Azotobacter* and *Azosprillum* in tomato plants increased lycopene antioxidant activity and K contents in tomato fruit (Ordookhani et al., 2010). Antioxidant compounds, *i.e.*, L-ascorbic acid, β-carotene, and lycopene were shown to increase significantly in tomato fruits deriving from plants fertilized with a compost containing the beneficial fungus *Trichoderma harzianum* T22 and an increase in mineral elements such as P, K, Ca, Mg, Cu, Fe, Mn and Zn (Khan et al., 2017) was seen too. *Bacillus subtilis* and *Bacillus amyloliquefaciens* can also improve tomato fruit yield and quality, as revealed by an increased number of fruits per plant, fruit size, total soluble solids, and L-ascorbic acid contents compared to uninoculated controls. N, P, and K contents in fruits were also increased by bacterial inoculation (Gashash et al., 2022). Poor information is found in the literature regarding the possible positive effects of PGPR in increasing yield and quality traits of fruits deriving from plants exposed to salt stress treatment. The application of *Bacillus amyloliquefaciens* IT45 to salt-stressed strawberry plants increased fruit weight and sweetness, while reducing total organic acids compared to uninoculated ones (Ntanos et al., 2021). *Stenotrophomonas* sp. SRS1 increased the total fruit number under non-saline conditions, enhanced fruit weight under both normal and salt stress conditions and increased the number of mature (red) fruits in three MicroTom tomato cultivars (Tuong et al., 2022). Our results suggest that PVr_9 limits the negative effects of NaCl-salinity in tomato fruit minimizing the reduction in all the nutritional compounds tested here, thus maintaining a higher antioxidant power compared to uninoculated stressed plants (Fig. 5). In addition, PVr_9 significantly increases Na contents in tomato fruits, under both control and salt stress conditions suggesting a possible mechanism in compartmentalizing Na ions into structures that are less harmful to the plant (Fig. 6; Table S2). The ability of PVr_9 to limit the accumulation of contaminants such as Pb and Cd in the fruit (Fig. 6) makes this bacterium strain interesting not only for biofortification purposes but also for food safety.

5. Conclusions

This study has demonstrated that the application of the halotolerant strain PVr_9 confers salt tolerance traits to tomato plants, confirming previous results obtained in the plant model *Arabidopsis thaliana*. PVr_9 can increase root growth, shoot biomass, proline content and APX activity in tomato plants exposed to salt stress, which are all indicators of better plant wellbeing. These results match the molecular data showing an increase in the expression of salt-tolerant genes *SOS1* and *NHX1*, an upregulation of PAP and a downregulation of cysteine synthase due to PVr_9 inoculation, which correlates with a less stressed plant phenotype. The upregulation of proteins involved in defense mechanisms against fungal pathogens in PVr_9 inoculated plants suggest its role in priming against phytopathogens, which could be exploited even in the absence of salinity. Here it was innovatively found that the PVr_9 bacterial strain can improve the nutraceutical characteristics of tomato fruits by increasing carotenoids, lycopene, β-carotene and L-ascorbic acid, and some macro- and micro-elements along with a decrease in the amounts of some important contaminants. All these data open up the possibility of utilizing PVr_9 as a viable biostimulant and bio-pesticide in field trials of important agronomical crops such as tomato and possibly other species. Further investigations in open field trials should be carried out to define the most efficient inoculation in the target crops, by identifying suitable bacterial doses, application method and timing, and maximizing the benefits of this PGPR.

CRediT authorship contribution statement

Silvia Potestio: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Gianluigi Giannelli:** Methodology, Conceptualization. **Francesca Degola:** Writing – review & editing, Formal analysis. **Teofilo Vamerali:** Writing – review & editing, Funding acquisition. **Rosaria Fragni:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Emanuela Cocconi:** Methodology, Formal analysis. **Luca Sandei:** Funding acquisition. **Giovanna Visioli:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Data availability

All data are present in the paper and in the supplementary information file.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2024.100558.

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