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Effects of *Azorhizobium caulinodans* and *Piriformospora indica* Co-Inoculation on Growth and Fruit Quality of Tomato (*Solanum lycopersicum* L.) under Salt Stress

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Abstract: Salt stress is a worldwide environmental signal, reducing the growth and yield of crops. To improve crop tolerance to salt, several beneficial microbes are utilized. Here, nitrogen-fixing bacterium *Azorhizobium caulinodans* and root endophytic fungus *Piriformospora indica* were used to inoculate tomato (*Solanum lycopersicum*) under salt stress, and the effects of the co-inoculation were investigated. Results showed that *A. caulinodans* colonized in the intercellular space in stems and roots of tomato plants, while *P. indica* colonized in the root cortex. Two weeks following salt treatment, co-inoculated tomato plants grew substantially taller and had larger stem base diameters. Activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and reduced and oxidized ascorbate and glutathione (i.e., AsA, DHA, GSH, and GSSG, respectively) concentrations along with the ratios of AsA/(AsA + DHA) and GSH/(GSH + GSSG) increased in the leaves of co-inoculated plants under salt stress. The co-inoculation significantly increased soluble proteins and AsA in fruits; however, concentrations of soluble sugars and proanthocyanins did not show significant changes, compared with NaCl only treatment. Data suggest that *A. caulinodans* and *P. indica* co-inoculation boosted tomato growth and improved the quality of tomato fruits under salt stress. O-inoculation of *A. caulinodans* and *P. indica* might be employed to enhance tomato plant salt tolerance.

Keywords: tomato; *Azorhizobium caulinodans*; *Piriformospora indica*; salt stress; growth; fruit quality

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1. Introduction

Soil salinity affects more than 6% of the world's total land area (about 800 million hectares of land globally) [1], and roughly one billion hectares in more than 100 countries are affected (Food and Agriculture Organization, 2015). Poor irrigation techniques, incorrect fertilizer use, and industrial contamination have all contributed to the rise in this instance [2].

Among the various salts in the soil, NaCl is the most prevalent [3]. High salinity is caused by high concentrations of Na^+ and Cl^- in the soil solution, which causes hyperosmotic stress, preventing plant roots from absorbing water and nutrients from the soil [4]. However, most crops such as rice [5–7], wheat [8,9], tomato [10,11], and many other plants [12–15] are glycophytes, and their development and yield are affected by excessive NaCl.

Salt stress causes ionic, osmotic, and secondary stresses in plants, including oxidative damage [16]. Under salt stress, plants need to change their physiological and biochemical mechanisms that regulate ion and osmotic balance in response to salt stress, minimizing salt stress damage and enhancing detoxification [17]. They need to regulate signaling pathways to re-establish cellular ionic, osmotic, and reactive oxygen species (ROS) homeostasis [16,18]. For example, the Salt Overly Sensitive pathway plays a crucial role in maintaining ionic

homeostasis via extruding sodium ions into the apoplast [19–21]. Mitogen-activated protein kinase cascades also mediate ionic, osmotic, and ROS homeostasis [18,22,23]. SnRK2 (sucrose nonfermenting 1-related protein kinase 2) proteins, on the other hand, are involved in maintaining osmotic homeostasis [18,24].

In natural ecosystems, plants symbiose with microorganisms. These beneficial microorganisms, such as arbuscular mycorrhizal fungi [25–27], ectomycorrhizal fungi [28–30], ericoid mycorrhizal fungi [31], root endophytic fungi [32–34], and plant growth-promoting rhizobacteria [35–37], improve plant resistance to salt stress. These microorganisms alter physiological processes in their host plants. For instance, ectomycorrhizal fungi enhanced root NO_3^- uptake and mediated K^+/Na^+ homeostasis [38,39], and also improved water transport properties by regulating the role of aquaporins [40], increased biosynthesis of compatible osmolytes [41,42], and finally increased gas exchange and growth of their host plants under salt stress [41,43]. Other types of beneficial microorganisms show similar functions against excessive salt.

Nitrogen-fixing bacteria have a remarkable potential to promote plant development, notably in the Leguminosae family of plants. Since certain nitrogen-fixing bacteria can only colonize the roots of plants belonging to the Leguminosae family, nitrogen-fixing bacteria that can colonize non-legume plants might be important in agricultural practice. *Azorhizobium caulinodans,* in this sense, is a symbiotic nitrogen-fixing bacterium that colonizes in both roots and stems of the semi-aquatic (water stress-tolerant) tropical legume *Sesbania rostrate* [44], and it can also colonize other plant species which do not belong to the Leguminosae family, such as tomato [45], rice [46], and wheat [47,48]. Formation of stem nodules is via the crack entry mode at the site of adventitious root primordia located on the stems of *S. rostrata*. In wheat, the nitrogen-fixing bacterium may colonize a variety of tissues, including leaves, root hairs, lateral root connections, intercellular space in root and stem epidermis, and vascular bundles [48]. These organisms cause abnormal miRNA expression in tissue and time-dependent ways, and responsible miRNAs participate in plant-microbe interactions. On the other hand, a gene encoding c-di-GMP phosphodiesterase (Chp1) may modulate the motility of *A. caulinodans* in legume host *S. rostrata* and form the nodulation process by increasing the biosynthesis of extracellular polysaccharides, which could protect rhizobia against H_2O_2 [49]. Two chemotaxis response regulators, *A. caulinodans* CheY1 and CheY2, also regulate chemotaxis and competitive colonization of host plants [50]. Furthermore, Si et al. [51] identified and characterized an organic hydroperoxide resistance gene *ohr* (AZC_2977) and its regulator ohrR (AZC_3555) in *A. caulinodans*. *A. caulinodans* mutant Δ*ohr* showed less nodulation and reduced the nitrogenase activity; thus, *ohr* is essential for nodulation and nitrogen fixation. Transmembrane chemoreceptor TlpA1 and CheZ protein in *A. caulinodans* are also critical for colonization of the host plants [52,53]. Thus, *A. caulinodans* colonization helps host plants in many aspects, e.g., increased growth [54] and enhanced adaptability to Pb/Zn tailings [55,56]. However, the functions of *A. caulinodans* remain largely unknown under environmental stresses, particularly salt stress, since it is a halotolerant bacterium. *Piriformospora indica*, on the other hand, is a root endophytic fungus that can colonize the roots of most desert plants. Plant tolerance to environmental stressors, particularly salt stress, is improved by this fungus [57,58]. Yet, the impact of co-inoculation of *P. indica* with other beneficial bacteria on plant development and crop quality under salt stress received little attention.

Tomato (*Solanum lycopersicum* L.) is one of the most popular vegetable species globally because of its importance in agronomy [59]. Despite this, abiotic factors, particularly salt stress, regularly hinder its production and quality. High salt concentrations reduce tomato germination, leaf number, and area, slows down shoot and root growth, increases root/shoot ratio, induces leaf senescence, and ultimately impairs crop production [60]. Improving tomato plant tolerance and quality under salt stress is vital for tomato practice. Therefore, our aim in this work was (1) to investigate the impact of *A. caulinodans* and *P. indica* inoculation on tomato plant growth and biomass accumulation under salt stress,

and (2) to assess the impact of co-inoculation on the physiological status and tomato fruit quality under salt stress.

2. Materials and Methods

2.1. P. indica and A. caulinodans Cultures

Piriformospora indica DSM 11827 (syn. *Serendipita indica*, herein abbreviated as Piin) was acquired from Matthias-Schleiden Institute, University of Jena, Germany. The fungus was cultured on a Kafer medium, with the method introduced by Johnson et al. [61]. *Azorhizobium caulinodans* B81176 (i.e., ORS 571, ATCC 43989, abbreviated as Azca) was bought from Mingzhou Biotechnological Co., Ltd., Ningbo, China (10 June 2020). The bacterial strain was cultured in the modified arabinose gluconate medium/liter: 1.1 g 2-*N*-morpholinoethane sulfonic acid, 1.0 g yeast extract, 1.0 g arabinose, 1.3 g HEPES, 0.25 g Na_2SO_4, 1.0 g gluconic acid, 0.22 g KH_2PO_4, 12.0 mL NH_4Cl (16 g/100 mL), 1.0 mL $FeCl_3$ (0.679 g/100 mL), 1.0 mL $Na_2MoO_4{\cdot}2H_2O$ (1.0 g/100 mL), 0.1 mL $NiCl_2$ (1.2 g/100 mL), 1.0 mL $CaCl_2$ (1.5 g/100 mL), and 1.0 mL $MgSO_4{\cdot}7H_2O$ (18 g/100 mL). The pH of the medium was adjusted to 6.6 with KOH and autoclaved at 120 °C for 30 min.

2.1.1. Tomato Growth, Microbial Inoculation, and Salt Treatments

Seeds of tomato (*Solanum lycopersicum* L. cv. "Sunrise") were bought from Guanhe Seed Company, Shouguang, China. The seeds were sterilized (2 min) in the ethanol solution (75%) followed by a sodium hypochlorite step (NaClO, 0.75%) for 15 min, and then were washed several times with sterile distilled water. The sterilized seeds were sown in the sterilized culture mix (perlite: peat: coconut shell powder = 1:2:1) bought from Jinrun Biotechnological Co. Ltd., Jinan, China, in plastic pots (16 cm in height, 25 cm in diameter) (1.5 kg of culture mix per pot), and were grown in a greenhouse with 16 h light/8 h dark (PPFD 300–400 $\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$) period, 85%, relative humidity, and 25/20 °C (day/night). After germination, the seedlings with two true leaves and a similar height were selected; two seedlings were left per pot. These seedlings were watered according to the moisture of the growing mix in the pots.

Tomato seedlings were inoculated with microbial solutions two weeks after seed germination: 10 mL of *A. caulinodans* (5×10^5 bacteria ml^{-1}) or/and a suspension solution of *P. indica* hyphae (10 g/L) per pot. Two weeks after microbial inoculation, tomato seedlings were treated with 300 mM NaCl, 100 mL per pot. Thus, 7 treatments occurred, i.e., (1) CK treatment (without NaCl, not inoculated with *A. caulinodans* or *P. indica*); (2) Azca treatment (not treated with NaCl, inoculated only with *A. caulinodans*); (3) Piin treatment (not treated with NaCl, inoculated only with *P. indica*); (4) NaCl treatment (treated with 300 mM NaCl, not inoculated with *P. indica* or *A. caulinodans*); (5) NaCl + Azca treatment (treated with 300 mM and inoculated only with *A. caulinodans*); (6) NaCl + Piin treatment (treated with 300 mM and inoculated only with *P. indica*); (7) NaCl + Azca + Piin treatment (treated with 300 mM NaCl and inoculated with both *A. caulinodans* and *P. indica*), 20 pots for each treatment. The pots which were not inoculated with *A. caulinodans* or *P. indica* were treated with 10 mL of sterile water. After salt treatment, seedlings were watered with 100 mL of sterile water or 300 mM NaCl once every two days. Before 300 mM NaCl was added, pots were irrigated with 100 mL of sterile water (100 mL per pot) to avoid salt accumulation.

2.1.2. Micrography

Two weeks after inoculation, tomato roots were taken out from the pots and washed with tap water. To test effective *P. indica* infection in tomato roots, the roots were dyed with 0.05% trypan blue in lactophenol [61]. As for *A. caulinodans,* the roots and young stems were dyed with Gram staining solute and 0.05% trypan blue in lactophenol. Then, the photos were taken under a microscope (Nikon Ds-Ri2, Japan).

2.1.3. Determination of Height and Stem Base Diameters of Tomato Plants

The height of tomato plants was measured with a soft tape one week and two weeks after salt treatment, and their diameters were measured with a digital vernier caliper.

2.1.4. Determination of Chlorophyll Fluorescence

The chlorophyll fluorescence was measured using a portable pulse modulation fluorometer (Junior-PAM, Walz, Effeltrich, Germany) three weeks after salt treatment. Three leaves of the same age were chosen to determine chlorophyll fluorescence, as shown in the guidebook of the instrument. Specific chlorophyll fluorescence parameters were recorded after dark adaptation for 30 min, e.g., the quantum yield of the photosystem II (PSII) (Y(II)), electron transfer rate (ETR), the photochemical quenching (*qP*), and the maximum quantum yield of PSII (F_v/F_m). The biological significances of these parameters were introduced by Maxwell and Johnson [62].

2.1.5. Plant Harvesting and Shoot Biomass Determination

Tomato plants were collected ninety days after transplantation, and ten shoots were picked at random and weighed. The fresh leaves of the same ages and fruits with the same maturity were treated with liquid nitrogen and stored at −80 °C for biochemical analyses.

2.1.6. Determination of Photosynthetic Pigment Concentrations

Photosynthetic pigments (chlorophyll *a*, *b* and carotenoids) were extracted by homogenizing 1 g of fresh leaves in 5 mL of 96% ethanol. After centrifugation for 10 min at 3500× *g*, the concentrations of the pigments were measured spectrophotometrically at 470, 649, and 665 nm, as described by Kumar et al. [63].

2.1.7. Determination of Soluble Sugars and Proteins

Total soluble sugars were determined using anthrone colorimetry at 625 nm [64]. The total soluble proteins were determined as described by Bradford [65].

2.1.8. Assay of Enzymes

About 0.5 g of fresh tomato leaves were mixed with 2 mL of 50 mM HEPS-KOH buffer (pH 7.8, containing 0.1 mM EDTA) and ground into a homogenate on ice. The final volume of the homogenate was 10 mL. The homogenate was then centrifuged at 15,000× *g* for 15 min under 4 °C. The supernatant was used for SOD, CAT, and POD activity assays. Superoxide dismutase (SOD) (EC 1.15.1.1) activity was measured by the inhibition of the photochemical reduction of NBT, as described by Becana et al. [66]. One unit of SOD was defined as the amount of enzyme activity that produced a 50% inhibition of NBT reduction under the assay conditions. SOD activities were calculated as SOD activity (U/g) = $(X_{CK} - X_E) \times V_T/(X_{CK} \times W \times V_S \times 0.5)$. Here, X_{CK} is the absorbance of control under light; X_E is the absorbance of sample; V_T is the total volume of enzyme extract; W is the weight of the sample; and V_S is the volume used for determining the enzyme activity. Catalase (CAT) (EC 1.11.1.6) activity was determined by directly measuring the decomposition of H_2O_2 at 240 nm, in the 50 mM potassium phosphate buffer, pH 7.0, containing 10 mM H_2O_2 and enzyme source (ca 35 μg protein) in a final volume of 1 mL at 25 °C, as described by Aebi [67]. The 0.1 unit change in the absorbance during 1 min was defined as 1 unit of CAT activity. CAT activity was calculated as: CAT activity (U/(g × min)) = $(\Delta A_{240} \times V_t)/(W \times V_S \times 0.1 \times t)$. $\Delta A_{240} = A_{C0} - (A_{C1} + A_{C2})/2$. V_T: the total volume of the enzyme extract (mL); V_S: the volume of the solution used for determining CAT activity (mL); t: reaction time (from the time that H_2O_2 was added into the cuvette to the time that the absorbance was recorded) (min); W: sample weight (g FW); A_{C0}: absorbance of control; and A_{C1} and A_{C2}: the two absorbance of a sample, obtained, respectively. Peroxidases (POD) (EC 1.11.1.7) were assayed by the method of Chance and Maehly [68], using guaiacol as the reductant. Guaiacol POD activity was measured by following the increase in the absorbance at 470 nm after the formation of tetraguaiacol

(26.6 mM^{-1} cm^{-1}). The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.0, 0.25% (18 mM) guaiacol, 5 mM H_2O_2 and extract (ca 0.14 µg protein) in a final volume of 1 mL at 25 °C. The change of 0.01 unit in the absorbance during 1 min was defined as 1 unit of POD activity. POD activity was calculated as: POD activity $(U/(g \times min)) = (\Delta A_{470} \times V_t)/(W \times V_S \times 0.01 \times t)$. Here, ΔA_{470} is the change in the absorbance during the reaction time; V_t is the total volume of enzyme extract; W is the weight of the sample (g FW); V.s is the solution volume used for determining the POD activity (mL); and t is the reaction time (min).

2.2. Assay of Reduced and Oxidized Glutathione

Non-protein thiols were extracted by homogenizing 0.3 g of leaves in 3 mL of 0.1 N HCl (pH 2) and 1 g polyvinylpyrrolidone (PVP). After centrifugation at 10,000× *g* for 10 min at 4 °C, the supernatants were used for the analysis. Total glutathione, i.e., reduced glutathione and oxidized glutathione (GSH and GSSG), were determined in the homogenates spectrophotometrically at 412 nm, using yeast-glutathione reductase 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and NADPH. GSSG was determined by the same method in 2-vinyl pyridine, and GSH concentration was calculated from the difference between total glutathione and the GSSG [69].

2.3. Determination of Ascorbate Concentrations

Reduced ascorbate (AsA) and dehydroascorbate (DHA) were determined as described by Law et al. [70]. One g of fresh leaves were homogenized in 10% (*w*/*v*) TCA, and the supernatants were used for the assay. For the concentration, half of a sample of preparation was assayed for the total AsA concentration, and the other half was assayed for AsA only; DHA concentration was then deduced from the difference. Sodium hydroxide (10 µL, 5 M) was added to 400 µL of extract, were mixed, and the mixture was centrifuged for 2 min at 3500× *g*. Obtained supernatants (200 µL) were added to 200 µL of 150 mM NaH_2PO_4 buffer, pH 7.4, and 200 µL of water. 200 µL of buffer and 100 µL of 10 mM dithiothreitol were added to another half (200 µL) of the supernatant, and, after thorough mixing and incubation at room temperature for 15 min, 100 µL of 0.5% (*w*/*v*) *N*-ethylmaleimide were added. Both samples were vortex-mixed and incubated at room temperature for 30 s. 400 µL of 10% (*w*/*v*) TCA, 400 µL of 44% (*v*/*v*) H_3PO_4, 400 µL of 4% (*w*/*v*) bipyridyl in 70% (*v*/*v*) ethanol and 200 µL of 3% (*w*/*v*) $FeCl_3$ was then added to each sample. After vortex-mixing, samples were incubated at 37 °C for 60 min, and the absorbance at 525 nm was recorded.

2.4. Determination of Malondialdehyde (MDA) Levels

The MDA concentration was determined according to the method introduced by Fazeli et al. [71].

2.5. Determination of Total Proanthocyanidin Concentrations

Total proanthocyanins' concentration in tomato fruits was determined using the modified acid-vanillin method [72,73]. First, 1 g of tomato leaves was ground with 10 mL of methanol (70%, *v*/*v*), and the homogenate was centrifuged at 15,000× *g* for 10 min under 4 °C. The supernatants were used for the measurement of the total proanthocyanins. Then, 10 µL of the extract was added to the test tubes and then 990 µL distilled water to dilute the extracts as well as 2 mL vanillin solution (2%, *w*/*v*) in H_2SO_4 (70%, *v*/*v*). The solutions were left in the dark for 15 min; subsequently, absorbances were determined at 500 nm, using a standard curve prepared with catechin solution.

2.6. Statistical Analysis

Statistical analyses of all the experimental data were carried out using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The mean comparison was performed for differ-

ent physiological parameters from all the treatments with the LSD test at a significance level $p = 0.05$.

3. Results

3.1. Colonization of A. caulinodans and P. indica

Stem nodules occurred on tomato seedlings inoculated with *A. caulinodans*, and the bacterium was observed in the intercellular space in the stem cortex (Figure 1A) and root cortex (Figure 1B). The root endophytic fungus *P. indica* colonized in the roots of tomato seedlings, and its spores were found in the root cortex cells of tomato seedlings (Figure 1C).

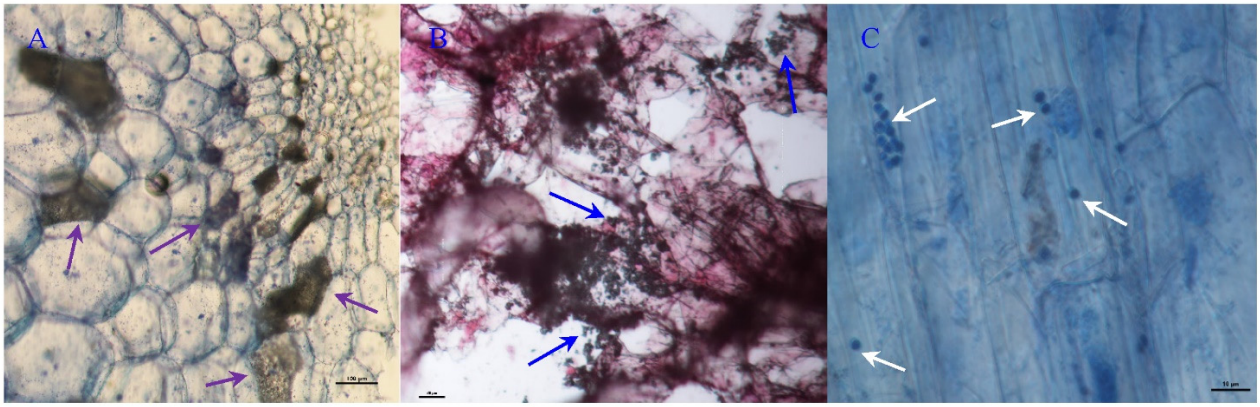

Figure 1. Infection of *A. caulinodans* in tomato stems (**A**) and roots (**B**), and infection of *P. indica* in tomato roots (**C**). In panel A, the arrows indicate *A. caulinodans* located in intercellular space in tomato stems, stained with trypan blue; bar = 100 µm. In panel B, the arrows indicate *A. caulinodans* located in the cortex intercellular space in tomato roots, stained with Gram staining solution; bar = 20 µm. In panel C, the arrows indicate spores of *P. indica* stained with trypan blue; bar = 10 µm.

3.2. Effects of Inoculations on Tomato Plant Growth under Different Treatments

The shoot height of tomato seedlings treated with 300 mM NaCl only was considerably lower than that of tomato plants treated with all other treatments one week later (Figure 2A), and there were no significant variations in plant height among all other treatments (Figure 2A). Inoculation of *A. caulinodans* and *P. indica*, alone or combined, significantly enhanced stem base diameters under salt stress a week after NaCl treatment, compared to NaCl only treatment (Figure 2A).

Two weeks after salt treatment, NaCl only treatment resulted in a significant reduction in plant height, compared with all the other treatments (Figure 2B). However, co-inoculation of *A. caulinodans* and *P. indica* significantly increased the height of tomato plants under salt stress, compared with NaCl only (Figure 2B). Two weeks after salt treatment, inoculation of *P. indica* only and co-inoculation with *A. caulinodans* resulted in a significant increase in the stem base diameters under salt stress, compared to NaCl only treatment (Figure 2B). Two weeks after salt stress, the height of tomato plants treated with NaCl + Azca, NaCl + Piin, and NaC + Azca + Piin increased 15.21%, 13.57%, and 23.55%, respectively, compared to NaCl only treatment (Figure 2B).

Tomato plants under different treatments showed phenotypic differences in the growth and maturity of tomato fruits at the end of the tomato growth period (Figure 3). The shoot fresh weight data also showed significant differences (Figure 4). Under no-salt stress conditions, *A. caulinodans* or *P. indica* only inoculations significantly increased the shoot fresh weight of tomato plants (Figure 4). In contrast, inoculation of *A. caulinodans* and *P. indica*, alone or together, significantly increased the shoot fresh weight of tomato plants under salt stress (Figure 4). On the other hand, the shoot fresh weight of plants treated with NaCl + Azca, NaCl + Piin, and NaC + Azca + Piin increased by 68.04%, 88.69%, and 112.28%, respectively, compared to NaCl only treatment (Figure 4).

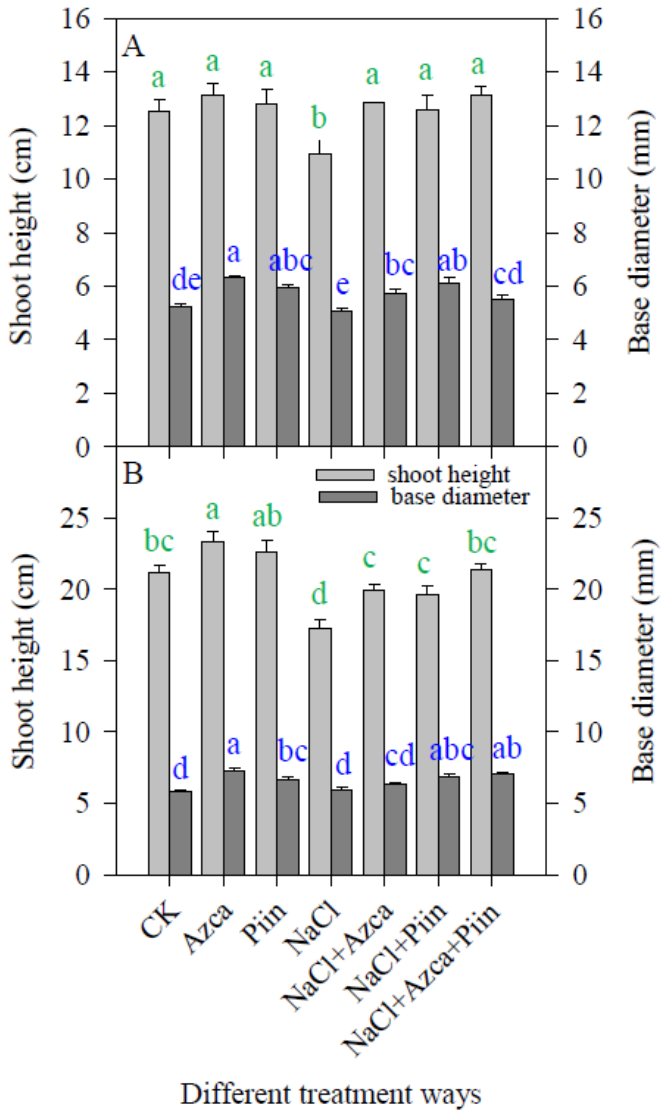

Figure 2. Changes in height and stem diameters of tomato plants under different treatments. (**A**): a week after salt treatment; and (**B**): two weeks after salt treatment. Results were shown as mean ± SE (n = 3). Green and blue lowercases indicate statistical differences in shoot height and base diameters, respectively. The means with identical lowercase indicate insignificant differences ($p > 0.05$).

3.3. Effects of Inoculations on the Photosynthetic Pigments

The inoculation of *A. caulinodans* or *P. indica* only increased the concentration of Chl *a* and Chl *b* under no-salt stress conditions, compared to the CK treatment (Table 1). NaCl-only treatment resulted in the lowest concentrations of Chl *a* and Chl *b* (Table 1). The inoculation with *A. caulinodans* and *P. indica*, alone or together, increased concentrations of Chl *a* and Chl *b* under salt stress, compared with NaCl only treatment; however, there were no significant differences (Table 1). The *A. caulinodans* (i.e., NaCl + Azca) only inoculation and the co-inoculation of the two organisms (i.e., NaCl + Azca + Piin) significantly increased the total chlorophyll concentrations under salt stress, compared to NaCl only treatment (Table 1). Under salt stress, *A. caulinodans* and *P. indica* inoculation, alone or together, increased the concentrations of carotenoids, yet, there were no significant differences compared with NaCl only treatment (Table 1).

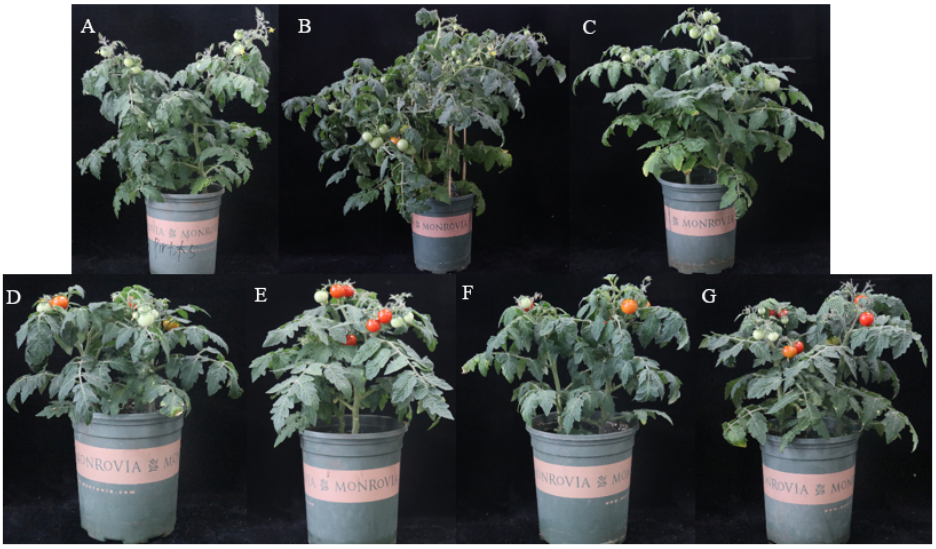

Figure 3. Growth status of tomato plants under different treatments. (**A**): CK treatment; (**B**): Azca treatment; (**C**): Piin treatment; (**D**): NaCl treatment; (**E**): NaCl + Azca treatment; (**F**): NaCl + Piin treatment; and (**G**): NaCl + Azca + Piin treatment.

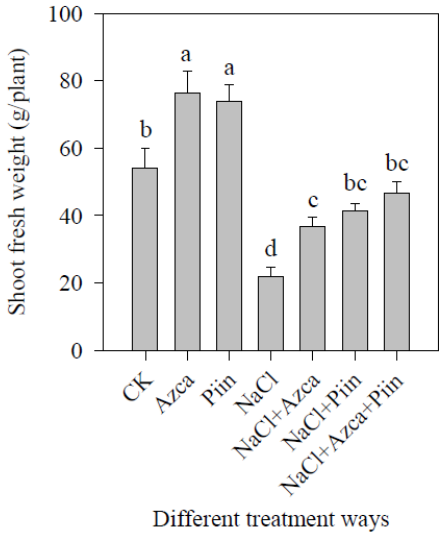

Figure 4. Shoot fresh weight of tomato plants under different treatments at the harvest. Shoots included stems, leaves, and fruits. Results were shown as mean ± SE (n = 10). The means with the same lowercases indicate that the differences were insignificant ($p > 0.05$).

Table 1. Photosynthetic pigment concentrations in leaves of tomato plants under different treatments.

	Chl *a* (mg/g FW)	Chl *b* (mg/g FW)	Car (mg/g FW)	Total Chlorophyll
CK	17.65 ± 0.63 abc	6.75 ± 0.34 a	3.45 ± 0.30 ab	27.84 ± 0.56 abc
Azac	18.61 ± 0.73 ab	7.95 ± 0.39 a	4.16 ± 0.10 a	30.72 ± 1.22 a
Piin	19.07 ± 1.13 a	7.67 ± 1.14 a	3.60 ± 0.60 ab	30.34 ± 0.79 ab
NaCl	15.11 ± 0.41 c	6.50 ± 0.25 a	2.52 ± 0.20 b	24.13 ± 0.80 d
NaCl + Azca	16.90 ± 0.96 abc	7.29 ± 0.54 a	3.25 ± 0.19 ab	27.44 ± 0.58 bc
NaCl + Piin	15.80 ± 0.90 bc	7.21 ± 1.22 a	3.20 ± 0.51 ab	26.22 ± 1.61 cd
NaCl + Azca + Piin	17.56 ± 0.92 abc	7.50 ± 0.45 a	3.63 ± 0.25 ab	28.68 ± 0.78 abc

Note: Data (means ± SD, n = 3) in the same column followed by different letters mean significant differences ($p < 0.05$; LSD test).

3.4. Effects of Inoculations on the Chlorophyll Fluorescence Parameters

Chlorophyll fluorescence parameters reflect the photosynthetic process dynamics and are valuable markers for assessing the impact of environmental stressors [74–76].

Under non-salt stress, *A. caulinodans* or *P. indica* only inoculation significantly increased the Y(II) and ETR, compared to CK treatment (Table 2). Y(II) showed the most significant value in the leaves of tomatoes treated with NaCl + Piin under salt stress among all four treatments. Y(II) was significantly higher than those in the NaCl + Azca + Piin group (Table 2). The two treatments, NaCl + Azca and NaCl + Piin, significantly increased the ETR under salt stress; however, co-inoculation significantly reduced the ETR, compared with NaCl only treatment (Table 2). Under salt stress, inoculation of *A. caulinodans* and *P. indica*, alone or together, significantly reduced the chlorophyll fluorescence parameter *qP*, compared to NaCl only treatment (Table 2). Inoculation of *A. caulinodans* and *P. indica*, alone or together, significantly increased the F_v/F_m under salt stress, compared to NaCl only treatment (Table 2).

Table 2. Chlorophyll fluorescence parameters of tomato leaves under different treatments.

	Y(II)	ETR	*qP*	F_v/F_m
CK	0.55 ± 0.00 f	29.00 ± 0.08 f	0.88 ± 0.00 d	0.5591 ± 0.00 e
Azca	0.62 ± 0.00 b	32.55 ± 0.10 b	0.94 ± 0.00 b	0.6171 ± 0.00 d
Piin	0.60 ± 0.00 d	31.36 ± 0.17 d	0.90 ± 0.00 d	0.6397 ± 0.00 c
NaCl	0.56 ± 0.00 e	29.55 ± 0.38 e	1.00 ± 0.05 a	0.4140 ± 0.01 f
NaCl + Azca	0.61 ± 0.00 c	32.01 ± 0.11 c	0.90 ± 0.00 cd	0.6768 ± 0.00 b
NaCl + Piin	0.64 ± 0.00 a	33.82 ± 0.13 a	0.93 ± 0.00 bc	0.6848 ± 0.00 a
NaCl + Azca + Piin	0.55 ± 0.00 f	28.61 ± 0.11 f	0.85 ± 0.040 e	0.6436 ± 0.00 c

Note: Data (means ± SD, $n = 30$) in the same column with different letters mean significant differences ($p < 0.05$; LSD test).

3.5. Effects of Inoculations on the Soluble Sugars and Proteins

The inoculation of *A. caulinodans* or *P. indica* significantly increased soluble sugars and proteins concentrations under non-salt stress (Figure 5). Additionally, compared to NaCl only treatment, *A. caulinodans*, and *P. indica*, alone and together, significantly increased the concentrations of soluble proteins (Figure 5).

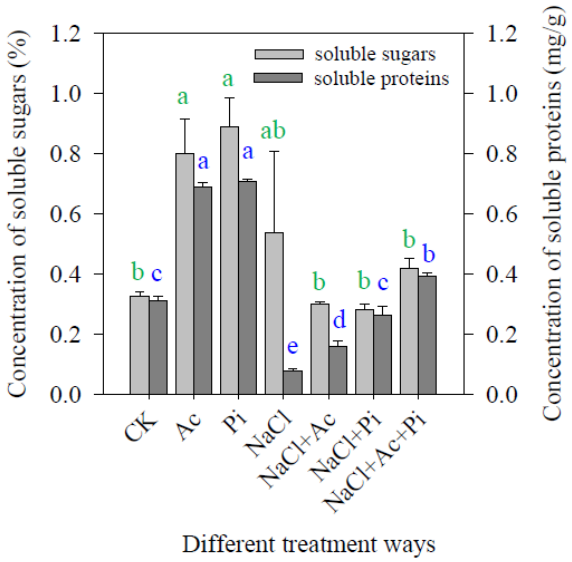

Figure 5. Concentrations of soluble sugars and proteins in leaves of tomato plants under different treatments. Results were shown as mean ± SE ($n = 3$). The lower-cases of green and blue indicate statistical differences in soluble sugar and soluble protein concentrations, respectively. The means with the same lowercases indicate that the differences were insignificant ($p > 0.05$).

3.6. Effects of Inoculations on the Antioxidant Enzyme Activities

Under non-salt stress, the inoculation of *A. caulinodans* or *P. indica* increased the SOD activity; however, there were no significant differences compared to CK treatment

(Figure 6A), whereas co-inoculation significantly increased the SOD activities compared to NaCl-only treatment (Figure 6A). *A. caulinodans* or *P. indica* significantly increased the CAT activity (Figure 6B) under non-salt stress, yet, inoculation of *A. caulinodans* and *P. indica*, alone and together, did not show a significant effect on CAT activities under salt stress (Figure 6B). On the other hand, *P. indica* inoculation increased the POD activity significantly under non-salt stress (Figure 6C) while *A. caulinodans* did not, compared with CK treatment (Figure 6C). The two treatments, i.e., NaCl + Piin and NaCl + Azca + Piin, significantly increased the POD activity, compared with NaCl only treatment under salt stress (Figure 6C).

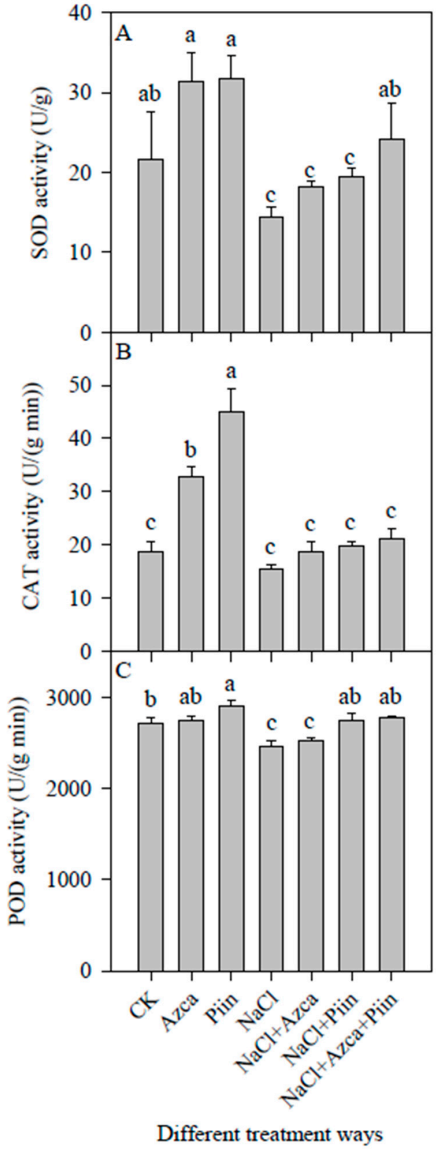

Figure 6. Changes in antioxidant enzymes in leaves of tomato plants under different treatments. (**A**): SOD activity; (**B**): CAT activity; and (**C**): POD activity. Results were shown as mean ± SE (n = 3). The means with the same lowercases indicate that the differences were insignificant ($p > 0.05$).

3.7. Effects of Inoculations on the Antioxidants

The inoculation of each microorganism significantly increased concentrations of reduced ascorbate under non-salt stress (AsA, Figure 7A), yet did not affect dehydroascorbate (DHA) concentrations compared with CK (Figure 7A). Similarly, the inoculation of *A. caulinodans* and *P. indica*, alone or in combination, increased AsA concentrations

compared to NaCl only treatment (Figure 7A). The two treatments, i.e., NaCl + Piin and NaCl + Azca + Piin, significantly increased DHA concentrations under salt stress (Figure 7A).

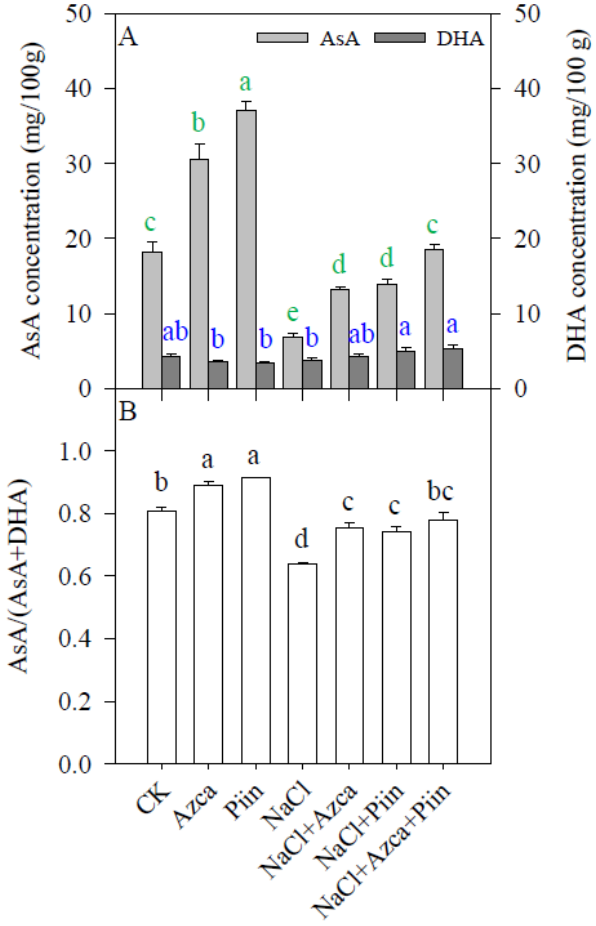

Figure 7. Changes in AsA and DHA concentrations and the ratios of AsA/(AsA + DHA) in leaves of tomato plants under different treatments. (**A**): AsA and DHA concentrations; and (**B**): the ratios of AsA/(AsA + DHA). Results were shown as mean ± SE (n = 3). Green and blue lowercases indicate statistical differences of AsA and DHA concentrations, respectively. The means with the same lowercases indicate that the differences were insignificant ($p > 0.05$).

Similarly, inoculation of *A. caulinodans* and *P. indica* affected concentrations of reduced glutathione (GSH) and oxidized glutathione (GSSG). Under both non-salt stress and salt stress, inoculation of *A. caulinodans* or *P. indica* significantly increased the GSH concentrations, compared with CK treatment and NaCl only treatment, respectively (Figure 8A). However, *A. caulinodans* and *P. indica* inoculation alone and in combination did not affect GSSG concentrations under salt stress (Figure 8A).

Co-inoculation also affected the ratios of AsA/(AsA + DHA) and GSH/(GSH + GSSG) (Figures 7B and 8B). Under both non-salt stress and salt stress, *A. caulinodans* or *P. indica* inoculation significantly increased the AsA/(AsA + DHA) values compared to CK and NaCl only, respectively (Figure 7B). The inoculation also considerably increased the GSH/(GSH + GSSG), compared with NaCl-only treatment (Figure 8B).

3.8. Effect of Inoculations on the Malondialdehyde Concentrations

Salt stress-induced decomposition of biomembrane in tomato leaves. Under non-salt stress, *A. caulinodans* or *P. indica* inoculation reduced malondialdehyde (MDA) concentrations significantly compared to CK treatment (Figure 9). Under salt stress, *A. caulinodans*,

and *P. indica*, alone and together, also significantly reduced the MDA concentrations, compared with NaCl only group of plants (Figure 9).

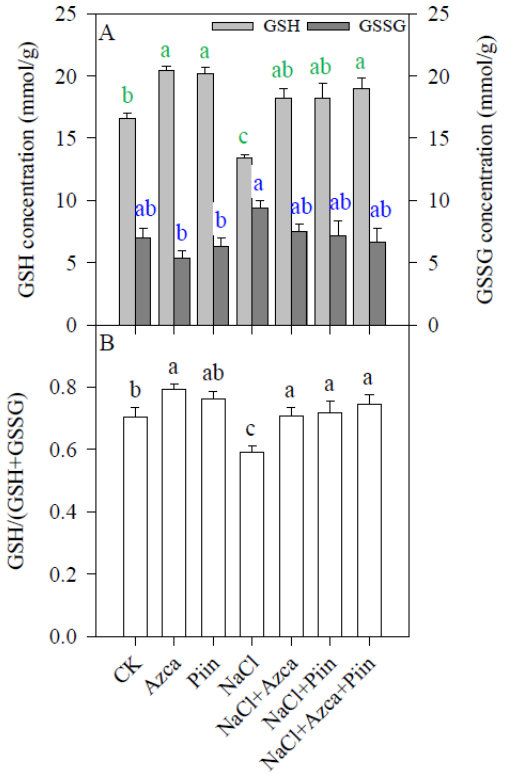

Figure 8. Changes in GSH and GSSG concentrations and the ratios of GSH/(GSH + GSSG) in leaves of tomato plants under different treatments. (**A**): GSH and GSSG concentrations; and (**B**): the ratios of GSH/(GSH + GSSG). Results were shown as mean ± SE (n = 3). Green and blue lowercases indicate statistical differences in GSH and GSSG concentrations, respectively. The means with the same lowercases indicate that the differences were insignificant ($p > 0.05$).

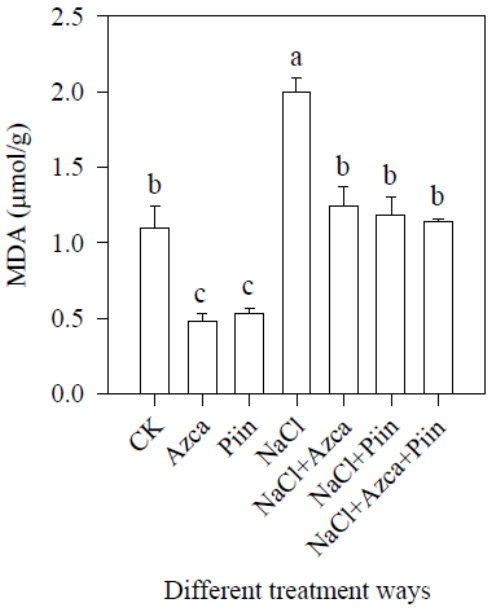

Figure 9. Changes in MDA concentrations in leaves of tomato plants under different treatments. Results were shown as mean ± SE (n = 3). The means with identical lowercase indicate insignificant differences ($p > 0.05$).

3.9. Effects of Inoculations on the Fruit Quality

The concentrations of soluble sugars and proteins in tomato fruits remarkably increased by *A. caulinodans* or *P. indica* under non-salt stress compared with CK treatment (Figure 10A,B). However, among three microbial treatments, only single inoculation of *P. indica* (i.e., NaCl + Piin treatment) increased the concentrations of soluble sugars under salt stress, compared with NaCl-only treatment (Figure 10A), whereas NaCl + Piin and NaC + Azca + Piin treatment significantly increased concentrations of soluble proteins (Figure 10B).

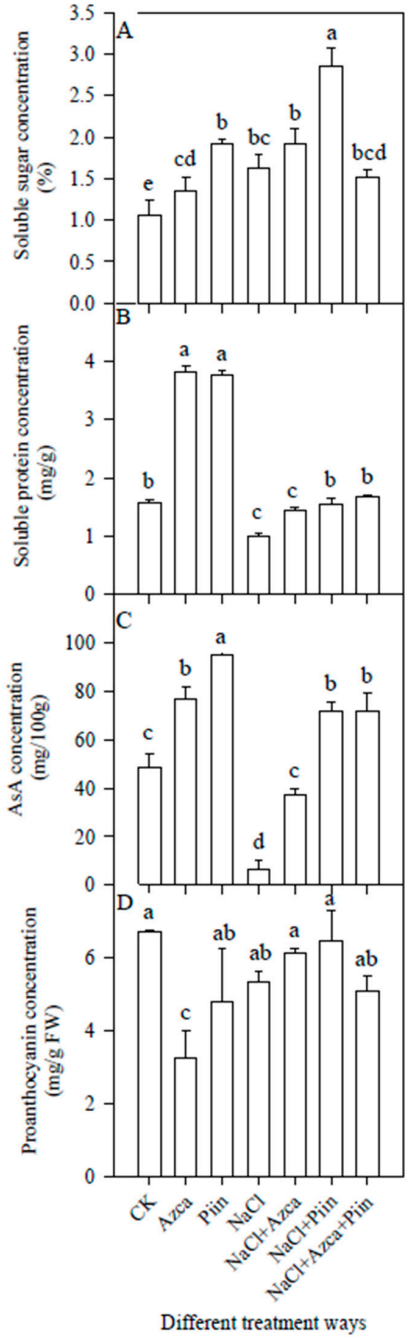

Figure 10. Changes in nutrition of tomato fruits under different treatments. (**A**): soluble sugars; (**B**): soluble proteins; (**C**): ascorbate; and (**D**): total proanthocyanins. Results were shown as mean ± SE ($n = 3$). The means with the same lowercases indicate that the differences were not significant ($p > 0.05$).

Under non-salt stress and salt stress conditions, inoculation of *A. caulinodans* and *P. indica*, alone and together, significantly increased ascorbate concentrations, compared with CK and NaCl-only treatment, respectively (Figure 10C). *A. caulinodans* and *P. indica* showed different effects on proanthocyanin biosynthesis in tomato fruits under non-salt stress. The inoculation of *A. caulinodans* reduced the pro anthocyanin concentrations in tomato fruits (Figure 10D); however, proanthocyanin concentrations in fruits of tomato plants inoculated with *P. indica* did not show significant changes (Figure 10D), compared with CK treatment. However, alone and together, both *A. caulinodans* and *P. indica* did not affect the proanthocyanin concentrations in tomato fruits under salt stress (Figure 10D).

4. Discussion

4.1. Co-Inoculation of A. caulinodans and P. indica Promotes Growth and Fruit Quality

A. caulinodans and *P. indica* possess a wide range of plant hosts, including tomato [45,49,50,77,78]. In our study, the two microorganisms were found to colonize in tomato plants (Figure 1). *A. caulinodans* was detected in the roots and stems of tomato plants (Figure 1A,B), while *P. indica* spores were found only in roots (Figure 1C). Their inoculation improved tomato growth and development when salt stress was present in the environment. Improved phenotypic characteristics (Figure 2B) and shoot fresh weight (Figure 4) of inoculated plants under salt stress suggest that co-inoculation with *A. caulinodans* and *P. indica* possessed the most significant effect on tomato physiology. These data were in accordance with the latest research dealing with the plants co-inoculated with mycorrhizal fungi and PGPRs or root endophytic fungi under salt and drought stress [78–80]. Increased biomass accumulation might be attributed to the protected photosynthetic machinery under salt stress since co-inoculation of *A. caulinodans,* and *P. indica* increased the intactness of chlorophylls and carotenoid concentrations under salt stress (Table 1).

A. caulinodans functions as a nitrogen-fixing bacterium and can colonize in non-legume plants. No other research has been reported on the functions of *A. caulinodans* under salt stress to the best of our knowledge. Under non-salt stress and salt stress, inoculation of *A. caulinodans* only significantly increased the shoot fresh weight of tomato plants (Figure 4), suggesting that the *A. caulinodans* play a critical role in the biomass development of tomato plants as a nitrogen-fixer. However, the changes in nitrogen metabolism of host plants inoculated with *A. caulinodans* remain unclear and need to be addressed at the biochemical and molecular levels.

Robust evidence proved that the root endophytic fungus *P. indica* increased biomass accumulation under salt stress in several previous studies [10,81,82]. The increase in *P. indica*-induced biomass might be related to its multiple functions under salt stress: (1) increased concentrations of photosynthetic pigments and improved gas exchange, and subsequent photochemical dynamics [10]; (2) maintenance of K^+/Na^+ homeostasis [58,77]; (3) increased abundance of photosynthetic proteins [83]; and (4) changes in fatty acid composition in phospholipids in the leaves and better maintenance of membrane stability [84]. Our data are in accordance with some of this evidence (Tables 1 and 2, and Figure 4).

Tomato fruits contain not only proteins, sugars, organic acids, or lycopene, but also several vitamins (e.g., vitamins A, C, E) and other nutrients that are important for a balanced diet for human health and can reduce the risk of cancer and heart disease [85]. The antioxidant AsA is, therefore, might be essential for maintaining a balanced diet and preventing human health risks to a certain extent. Here, in our work, the inoculation of *A. caulinodans* and *P. indica* significantly increased AsA concentrations under both non-salt and salt stress conditions (Figure 10C). Anthocyanins, on the other hand, are also essential antioxidants [86,87]. However, inoculation of *A. caulinodans* and *P. indica* showed contrary effects on proanthocyanin accumulation in tomato fruits when salt stress is not present in the environment (Figure 10D). Yet, these contrary effects resulted in no meaningful differences in proanthocyanin accumulation in fruits of tomato plants inoculated with *A. caulinodans* and *P. indica*, alone and together, compared with NaCl-only treatment under salt stress (Figure 10D). In addition, under non-salt and salt stress conditions, inocula-

tion of *A. caulinodans* and *P. indica*, alone and in combination, significantly increased the concentrations of soluble proteins in tomato fruits, compared with CK and NaCl-only treatment, respectively (Figure 10C). Therefore, all the data suggest that co-inoculation with *A. caulinodans* and *P. indica* improves the quality of tomato fruits produced under excessive salt.

Interestingly, salt stress promoted the precocity of tomato fruits, presumably due to the hormonal changes, and inoculation of *A. caulinodans* and *P. indica*, alone and together, showed a more substantial effect on the plants than NaCl-only treatment (Figure 3). The precocity of tomato fruits is beneficial for earlier harvest and continuous fruit-setting because tomato possesses the characteristic of constant flowering and fruiting.

4.2. Co-Inoculation of A. caulinodans and P. indica Enhances Salt Tolerance

Under abiotic stresses, reactive oxygen species (ROS) might be produced by several different pathways cross-talking each other. Plants have evolved multiple strategies to respond to this oxidative stress, especially various antioxidants and antioxidant enzymes are in charge of scavenging cellular ROS in the plant. Our results showed that the triggered activities of SOD and POD were significant in the leaves of tomato plants treated with NaCl + Azca + Piin (Figure 6A,C). This combination significantly increased the concentrations of reduced ascorbate and glutathione (Figures 7A and 8A). These data suggest that the co-inoculation of *A. caulinodans* and *P. indica* improves the ability to scavenge ROS in salt-stressed tomato plants. Increased activities of antioxidant enzymes and concentrations of antioxidants resulted in a significant reduction in the peroxidation of the biomembranes of the leaves of tomato plants under salt stress (Figure 9). These results were found under the latest studies [84,88,89]. For instance, Baltruschat et al. [84] showed that the inoculation of *P. indica* significantly elevated the amount of ascorbate in quantity and increased the activities of several antioxidant enzymes in the roots of barley (*Hordeum vulgare*) under salt stress.

High ratios of AsA/(AsA + DHA) and GSH/(GSH + GSSG) are beneficial for ROS scavenging. Consequently, during the ROS scavenging processes, lower concentrations of oxidized ascorbate and glutathione (Figures 7A and 8A) and higher ratios of AsA/(AsA + DHA) and GSH/(GSH + GSSG) (Figures 7B and 8B) measured under salt stress indicate that the antioxidant enzymes, dehydroascorbate reductase (DHAR) and glutathione reductase (GR), played essential roles in the transformation of oxidized ascorbate and glutathione to the reduced form of ascorbate and glutathione, respectively. Accordingly, increased activities of antioxidant enzymes and AsA and GSH concentrations resulted in significantly lower MDA in the leaves of tomato plants inoculated with *A. caulinodans* and *P. indica*, alone and together, under salt stress (Figure 9). The findings imply that co-inoculation with *A. caulinodans* and *P. indica* improved tomato plant salt tolerance.

5. Conclusions

Co-inoculation of the two microorganisms, *A. caulinodans* and *P. indica*, improved tomato plants' growth and fruit quality under salt stress. The combined inoculation alleviated the deteriorating physiological effects caused by excessive NaCl through improving enzymatic and non-enzymatic antioxidants.

Author Contributions: C.W. planned the whole experiments and revised the manuscript. Z.X. carried out all the experimental work and wrote the rough manuscript. N.P. and A.G. reviewed and revised the rough manuscript. All authors have read and agreed to the published version of the manuscript.

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