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Effects of arbuscular mycorrhizal fungi and plant growth-promoting rhizobacteria on growth and reactive oxygen metabolism of tomato fruits under low saline conditions

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Abstract: Land salinization is a major form of land degradation, which is not conducive to the growth and quality of fruits and vegetables. Plant salt tolerance can be enhanced by arbuscular mycorrhizal fungi (AMF) or plant growth-promoting rhizobacteria (PGPR). This study examined the effects of inoculation with PGPR singly or in combination with AMF, on the growth and quality of tomato fruits under low saline conditions. Tomatoes were cultivated in a greenhouse with sterilized soil, inoculated with PGPR, AMF, or co-inoculated with PGPR and AMF, and NaCl solution (1%) was added to the soil. The results indicated that AMF + PGPR decreased the roots and shoot biomass accumulation, and increased the number and fresh biomass in tomato fruits to a certain extent compared with non-inoculated plants. PGPR and AMF mediated the level of reactive oxygen and lipid peroxidation, the accumulation of antioxidants, and the activity of antioxidant enzymes, including proanthocyanidins, flavonoids, ascorbic acid, superoxide dismutase, peroxidase, and total antioxidant capacity. Furthermore, PGPR, AMF, and PGPR + AMF improved the overall osmotic adjustments and accumulation of soluble sugars and soluble proteins. Therefore, the AMF-*Funneliformis mosseae* and PGPR-*Bacillus subtilis* can potentially alleviate the adverse effects of salt stress and be applied as a biofertilizer in agricultural practice.

Abbreviations

AMF:	arbuscular mycorrhizal fungi
PGPR:	plant growth-promoting rhizobacteria
H_2O_2:	hydrogen peroxide
MDA:	malondialdehyde
SOD:	superoxide dismutase
POD:	peroxidase
T-AOC:	total antioxidant capacity
TSS:	total soluble sugar
TSP:	total soluble protein

Introduction

In the past decades, continuous deterioration of land salinization has led to a sharp decline in arable land worldwide (Gong *et al.*, 2020). It is estimated that salinization will threaten 50% of arable land in the next 30 years (Chandrasekaran *et al.*, 2014). In recent years, extensive utilization of chemical fertilizer has led to increasing land salinization and agricultural pollution (Mokhtar *et al.*, 2020), including vegetables such as tomatoes (*Solanum lycopersicum*). Tomato is one of the most nutritious fruits and vegetables consumed worldwide; it is an excellent source of vitamin C, sugar, and natural antioxidants (Chohan and Perveen, 2015). However, salt stress has a deleterious effect on tomato growth, which represses seed germination, reduces the survival rate, and decreases nutrient biomass (Egamberdieva *et al.*, 2019). Several studies have demonstrated colonization of microorganisms inoculated from symbiotic associations with host-plant, which is an effective strategy to alleviate the detrimental effects of salt stress (Kumar *et al.*, 2014; Mukhopadhyay *et al.*, 2021).

Arbuscular mycorrhizal fungi (AMF) are ubiquitous in different soil types, and can infect the roots of most plants including tomatoes, forming the AM structure. Then, a beneficial symbiotic association is established between AMF and the hos-plant (Shi *et al.*, 2021; Yang *et al.*, 2021). In this association, AMF colonization improves the roots of host-plant growth, promoting the uptake of nutrients, including nitrogen, phosphorus, potassium, and so on (Baum *et al.*, 2015). Under salt stress, AMF re-establishes ion homeostasis

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to remedy insufficient nutrients, enhancing the activity of antioxidant enzymes to stimulate the antioxidant defense system, and improve osmotic adjustment of the host-plant (He *et al.*, 2007; Li *et al.*, 2020; Pan *et al.*, 2020); thus, effectively enhancing the salt tolerance of plants. Meanwhile, AMF upgrades soil fertility and maintains the balance of soil pH by increasing or decreasing the mineral content of the saline soil (Zhang *et al.*, 2011).

Plant growth-promoting rhizobacteria (PGPR) generally include *Pseudomonas*, *Bacillus*, *Enterobacter*, *Rhizobia*, phosphate solubilizing bacteria, and *Erwinia*. PGPRs have been shown to promote plant growth and yield under abiotic as well as biotic conditions (Srividhya *et al.*, 2020; Kerbab *et al.*, 2021). Additionally, PGPRs are an important functional component of biofertilizers in laboratory research and agricultural production (Vessey, 2003; Salme *et al.*, 2017). Numerous studies have indicated the promotion mechanisms of PGPR, including increasing the activity of antioxidant enzymes and metabolite accumulation and regulating the content of plant endogenous hormones (Panwar *et al.*, 2016; Qi *et al.*, 2021). Apart from this, most of the phosphorus is present in solid form in the soil, which is difficult to be harnessed by plants; however, previous studies have reported that PGPR improve the plant uptake of mineral elements, especially phosphorus (Dilfuza *et al.*, 2017; Cordero *et al.*, 2018).

This is a significant amount of important information on the physiology and genetics of the tomato, which is effectively cultivated as a model crop in the study of saline land reclamation and utilization of biofertilizers for sustainable agriculture. (Cuartero and Fernández-Muñoz, 1998; Garcia-Gonzalez and Sommerfeld, 2016). However, there has not been much focus on the influence of the inoculation of beneficial microorganisms on tomato fruits in saline soil, especially under low saline conditions. Therefore, this work aimed to (i) compare the effects on fruit growth, antioxidant capacity, and accumulation of soluble substances under different inoculations and (ii) recognize the most effective inoculation treatment of PGPR-*Bacillus subtilis* and AMF-*Funneliformis mosseae* under a low saline condition in a pot experiment.

Materials and Methods

Plant materials and growth conditions

Tomato (cultivar Dongshenghong; provided by the company of Beijing Dongsheng Seed Industry, China) seeds of consistent size were selected, surface sterilized with 75% ethanol solution for 1 min, and rinsed with sterile distilled water three times. Three seeds were sown in separate disinfected pots filled with 300 g autoclaved soil (0.11 MPa, 121°C, 2 h). The pots were randomly placed in a greenhouse, and the temperature was maintained in the region of 25°C. Seedlings were reduced to one seedling per pot 1.5 weeks after emergence.

Microbial inoculum

The inocula of *B. subtilis* (PGPR; 10^8 CFU mL^{-1}) and *F. mosseae* (AMF) were preserved in our lab. The AMF inoculum was a mixture of vermiculite with spores, hyphae, and root residues. For the inoculation treatments, 10 g AMF inoculum (The spore density of 2000 per 10 g inoculum) was mixed well with soil.

Experimental design

The experiment comprised two treatment factors: inoculation (non-inoculation control, AMF, PGPR, and AMF + PGPR treatment) and salinity (control and salt treatments). A completely randomized block design with four replicates was used. For salt treatments, 180 mL (1%) NaCl solution was added to the soil on three consecutive days after three weeks, and sterile distilled water of the same volume was applied as the control. The pots were irrigated regularly with sterile distilled water and 1/2 Hoagland nutrient solution (three times a week) to orient illumination (>500 μmol m^{-2} s^{-1} photosynthetic active), temperature (25/16 ± 2°C day/night) and humidity (60%).

Parameter measurements

After three months, the tomato plants were harvested. First, plant height was measured, and the fruits were picked to determine their number and fresh mass. The shoots and roots were washed with deionized water and dried in a high-temperature oven at 80°C for three days. Then the dry mass of fruits, shoots, and roots was measured.

Fresh fruits were homogenized in 5 mL phosphate buffer (0.1 mol L^{-1}, pH 7.8) and centrifuged at 10,000 × g for 20 min at 4°C, and the supernatant extract was used for assays to estimate hydrogen peroxide (H_2O_2), malondialdehyde (MDA), proanthocyanidins, flavonoids, ascorbic acid, superoxide dismutase (SOD), peroxidase (POD), total antioxidant capacity (T-AOC), sucrose, fructose, total soluble sugar (TSS) and total soluble protein (TSP) content.

The H_2O_2 content was determined with the test described by Zhang and Qu (2004). The extract (1 mL) was mixed with 0.1 mL titanium sulphate solution (5%) and 0.2 mL concentrated ammonia. The mixture formed sediment after centrifugation at 3,000 × g for 10 min, and the supernatant was discarded. Sediment was washed with acetone solution until no plant pigment was observed, then 5 mL H_2SO_4 (2 mol L^{-1}) was added. The mixed solution and washing liquid were transferred to a volumetric flask (10 mL). The absorbance was read by a spectrophotometer (Shimadzu Corporation, Kyoto, Japan; the same as below) at 415 nm to calculate H_2O_2 content.

The MDA content was determined with the thiobarbituric acid (TBA) test described by Zhang and Qu (2004). One milliliter extract was added to 2 mL 0.6% TBA, which was placed in a boiling water bath for 15 min, then cooled rapidly, and centrifuged to obtain the supernatant. Absorbance was read at 600, 532, and 452 nm. Tissue MDA concentration was calculated according to the formula: MDA (μmol g^{-1} fw) = $(6.45 \times (D_{532} - D_{600}) - 0.56 \times D_{450}) \times 0.015/W$.

The SOD activity was measured according to Bai *et al.* (1996), based on the inhibition of SOD to reduce nitroblue tetrazolium (NBT) by photochemically generated superoxide radicals. The reaction mixture contained 50 mM phosphate buffer at pH 7.8, 14 mM methionine, 75 μM NBT, 0.1 μM EDTA, 4 μM riboflavin, and the required amount of extract. One unit of SOD was defined as the amount of extract required to inhibit the reduction rate of NBT by 50% at 25°C.

The POD activity was determined by guaiacol oxidation (Bai *et al.*, 1996) in a reaction mixture containing 100 mL phosphate buffer (0.1 mol L^{-1}, pH 6.0), 56 μL guaiacol, and 38 μL H_2O_2 (30%). Three milliliters reaction solution was

added to 1 mL extract. Compared with phosphate buffer (0.1 mol L^{-1}, pH 7.8) as the control group, the absorbance was recorded immediately at 470 nm per 1 min three times. According to variation of values in absorbance and standard curve, POD activity was calculated.

The content of proanthocyanidins, flavonoids, ascorbic acid, T-AOC, sucrose, and fructose was determined according to the instructions provided in the testing kits (Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China).

TSS content was determined by the anthrone method (Zhang and Qu, 2004) using distilled water as the standard. The reaction mixture contained 1 mL extract and 5 mL anthrone agent (100 mg anthrone + 100 mL 76% H_2SO_4). This was placed in a boiling water bath for 10 min, cooled, and the absorbance was read at 620 nm. The soluble sugar content was calculated according to the formula: C (μg g^{-1}) = A × N/W.

TSP content was determined using the Coomassie brilliant blue G-250 method (Zhang and Qu, 2004). The reaction mixture contained 0.1 mL extract and 5 mL G-250 protein solution agent (100 mg G-250 + 50 mL 95% ethanol + 100 mL 85% orthophosphoric acid + sufficient distilled water; 1000 mL in total). The absorbance was read at 595 nm. The soluble protein content was calculated according to the formula: C (μg) = C × V/W × a.

Statistical analysis

The experimental data were assessed with correlation analysis and one-factor ANOVA using SPSS (version 22.0 for windows). The significance of differences among means was tested using Duncan's test at a 5% level.

Results

Plant growth and yield

The treatments did not have any significant effect on the height of tomato plants (Fig. 1a). Under non-salt stress condition, the dry mass of shoot and root of single inoculation with PGPR or AMF was higher than that of the control; nevertheless, the opposite was observed under salt stress condition, and the control treatment produced the highest shoot and root dry mass (Figs. 1b and 1c). Although their fruit dry mass was higher than those in the inoculation treatments, only one fruit was observed in a sample plant of non- and salt stress control treatments (Fig. 1d). Single inoculation with PGPR and co-inoculation of PGPR and AMF produced a higher fruit number (by 19%, 39%, respectively) and fresh mass (by 30%, 25%, respectively) (Figs. 1e and 1f). Instead, fruit number and fresh mass in AMF inoculated samples were the lowest among all treatments; meanwhile, those of the control was higher than single inoculation under non-salt stress condition.

Reactive oxygen and lipid peroxidation

Under the salt stress condition, fruit H_2O_2 content was lower in samples inoculated with PGPR (by 13%) treatments than in the control (CK, Fig. 2a). Salt stress increased H_2O_2 content except in PGPR inoculated samples by 21%, 83%, and 44%, respectively, in CK, BS, GM and BS+GM. Compared with the control, single inoculation and co-inoculation of PGPR and AMF reduced fruit MDA content under non-stress condition by 15%, 19%, and 39%, respectively (Fig. 2b). Under salt-stress, the MDA content of only PGPR inoculated (by 8%) and co-inoculated (by 12.5%) samples were lower than in the control.

Antioxidants

Inoculation with AMF produced the highest fruit proanthocyanidin and flavonoid content under salt-stress but the lowest ascorbic acid content (Figs. 3a–3c). Interestingly, completely opposite results were observed in fruit ascorbic acid and flavonoid content after PGPR inoculation; those in co-inoculated samples were lower than the non- and salt stress control except for ascorbic acid content.

Under salt stress, SOD activity in the fruit was enhanced (Fig. 3d). Compared with the control, AMF inoculation markedly increased SOD and POD activity by 22% and 61.5%, respectively, under salt-stress conditions. Under the non-salt stress condition, inoculation treatments enhanced SOD activity more than that in the control. However, under the salt-stress condition, POD activity of single- and co-inoculation with PGPR was lower than the control; meanwhile, SOD activity was similar (Fig. 3e). Under salt stress, the enzyme activity of inoculation treatments was in the order of AMF treatment > co-inoculation treatment > PGPR treatment.

Under salt stress conditions, the fruit total antioxidant capacity of PGPR inoculated samples increased significantly compared with that of the control fruits ($P < 0.05$). Additionally, when compared with salt stress control, AMF and co-inoculation increased fruit total antioxidant capacity as well, by 154% and 110%, respectively, although the difference was not significant (Fig. 3f).

Soluble substances

Under salt stress, only inoculation with AMF obviously increased sucrose and fructose content by 19% and 12.4% compared with those in the control, respectively (Figs. 4a and 4b). However, PGPR and PGPR+AMF treatments showed a modest increase (by 3.5% and 5.2%, respectively) in sucrose content and a decrease in fructose content. Fruit total soluble sugar increased (in CK, BS, GM, BS+GM) in response to salt stress by 16%, 66.4%, 35.7%, and 39.5%, respectively, and a similar improvement in total soluble protein was observed except in PGPR treatment (Fig. 4c). Under salt stress, inoculation with AMF significantly increased soluble protein content compared with that of the control ($P < 0.05$) (Fig. 4d). Besides, soluble protein content in inoculated samples was higher than that in the control under non-salt stress condition while inoculation with only AMF increased soluble sugar content. Generally, inoculation treatments produced higher total content of soluble sugar or protein than those in the control under both non-salt and salt stress conditions.

Discussion

Salt stress is one of the most significant abiotic stresses that affect the growth and productivity of crops and reduces the arable land. Utilization of PGPR and AMF may alleviate adverse effects of salt stress in many plant species and more positive effects than adverse effects were observed with

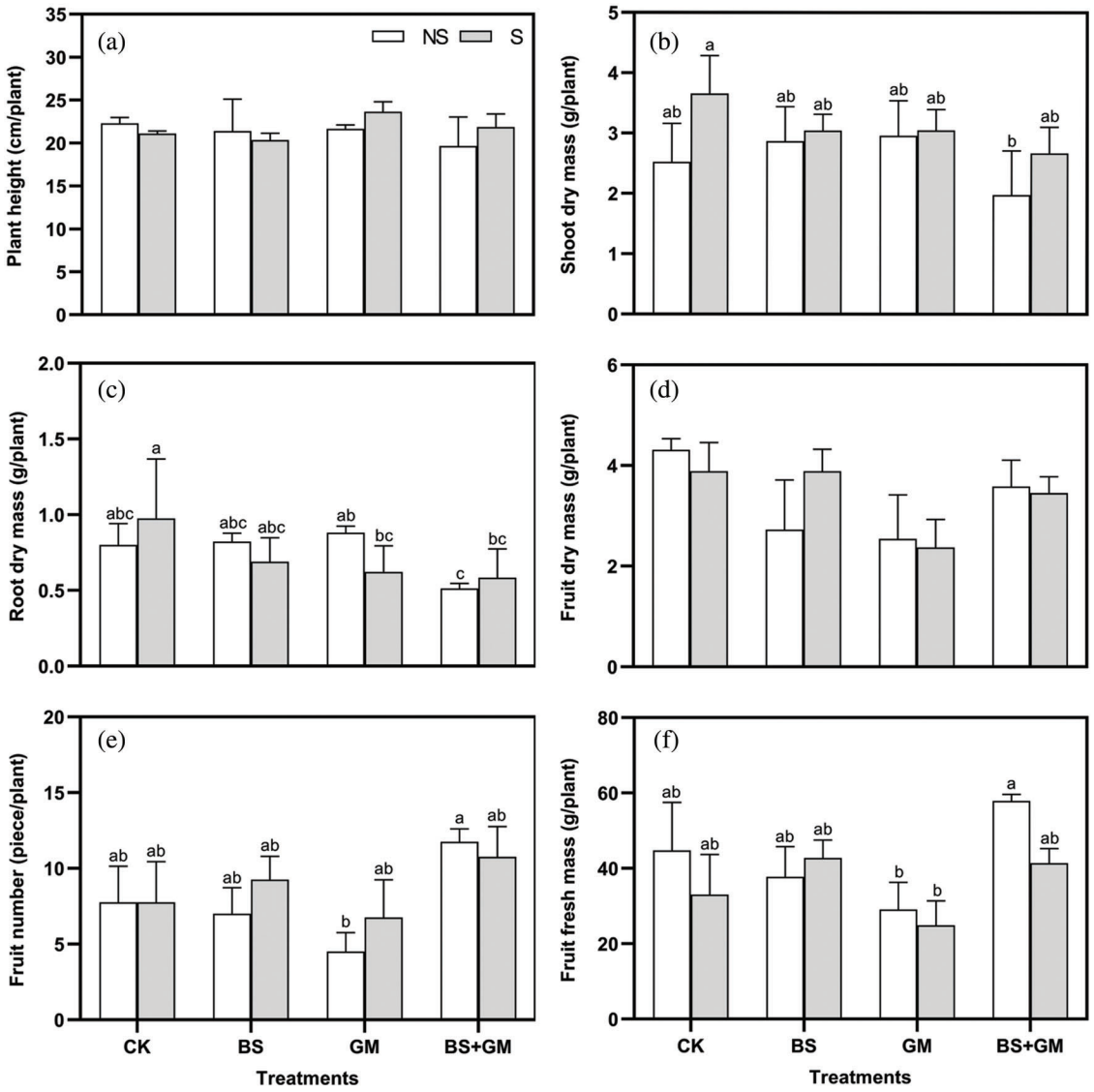

FIGURE 1. Effects of non-inoculation (CK) and inoculation with the PGPR-*Bacillus subtilis* (BS), AMF- *Funneliformis mosseae* (GM), and co-inoculation (BS+GM) on plant height (a), shoot (b), root (c) and fruit (d) dry mass, fruit number (e), and fruit fresh mass (f) under non- (NS) and salt stress (S) conditions. Different letters indicate significant differences ($P < 0.05$) among treatments by Duncan's test; n = 4.

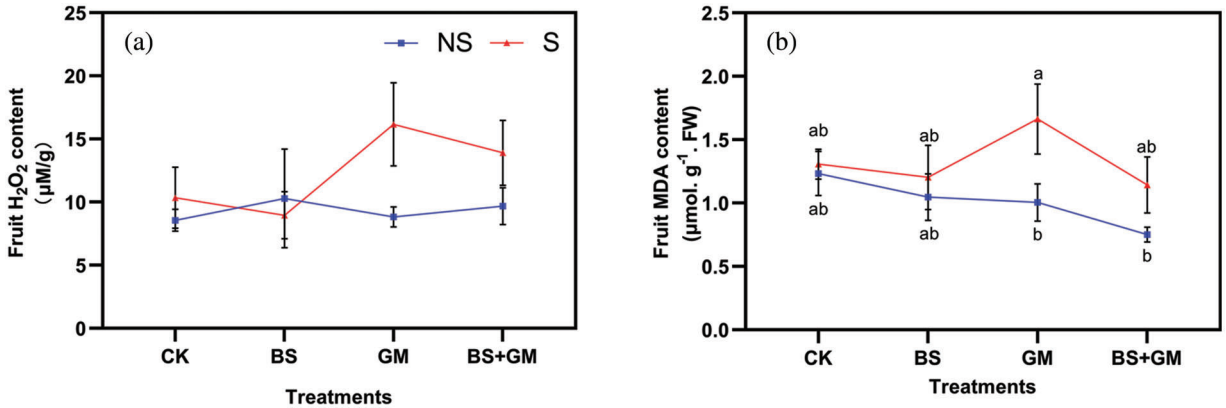

FIGURE 2. Effects of non-inoculation (CK) and inoculation with the PGPR-*Bacillus subtilis* (BS), AMF- *Funneliformis mosseae* (GM), and co-inoculation (BS+GM) on fruit H_2O_2 (a) and MDA content (b) under non- (NS) and salt stress (S) conditions. Different letters indicate significant differences ($P < 0.05$) among treatments by Duncan's test; n = 4.

inoculation treatments in these studies (Hajiboland *et al.*, 2010; Upadhyay *et al.*, 2012; Srividhya *et al.*, 2020). Our results also showed some negative effects under the low saline condition on the growth and reactive oxygen metabolism of tomatoes. Single inoculation and co-inoculation with PGPR and AMF have their advantages.

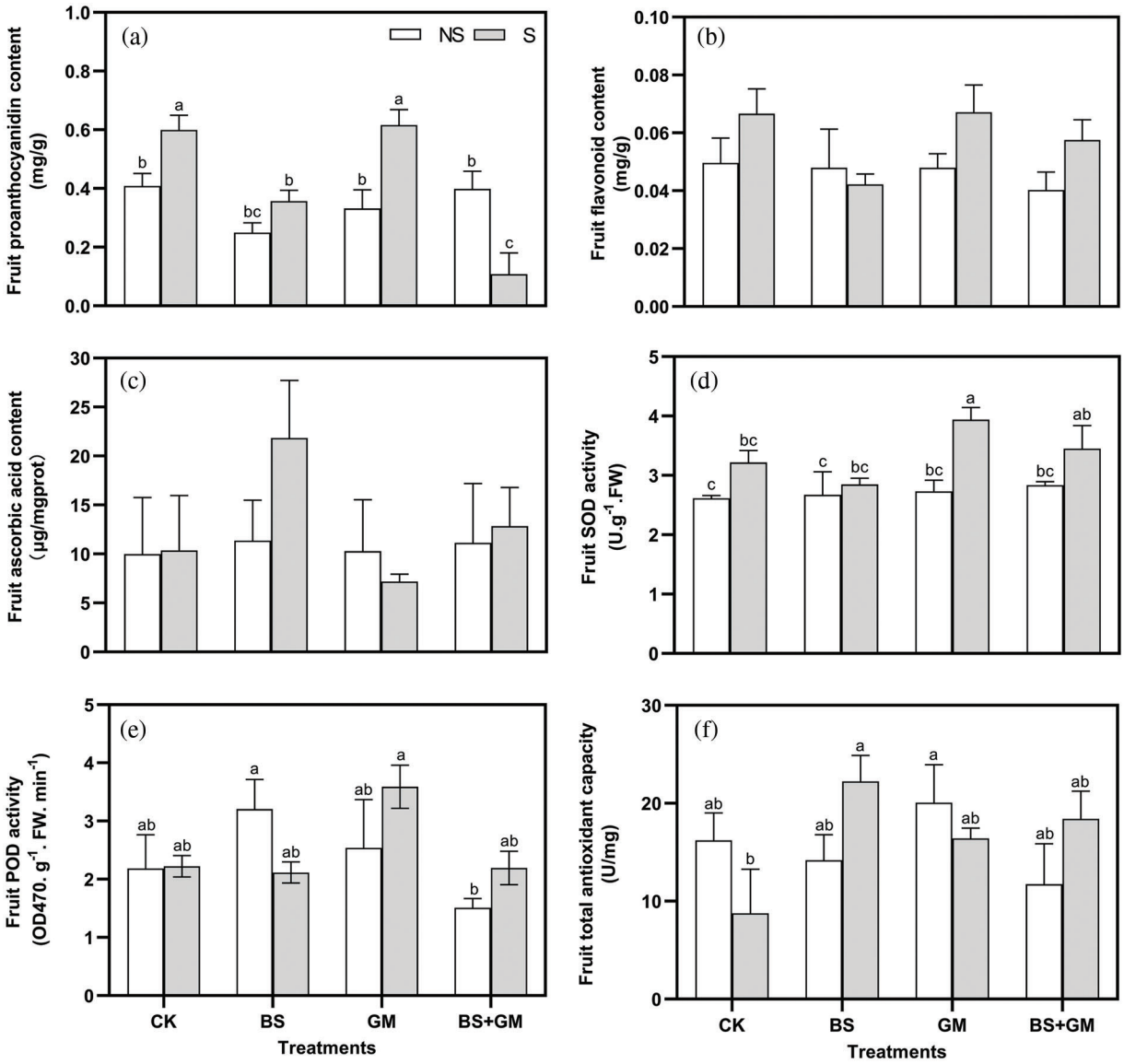

FIGURE 3. Effects of non-inoculation (CK) and inoculation with the PGPR-*Bacillus subtilis* (BS), AMF-*Funneliformis mosseae* (GM), and co-inoculation (BS+GM) on fruit proanthocyanidin (a), flavonoids (b), ascorbic acid content (c), SOD (d) and POD (e) activities, and total antioxidant capacity (f) under non- (NS) and salt stress (S) conditions. Different letters indicate significant differences ($P < 0.05$) among treatments by Duncan's test; n = 4.

This may be a result of resource distribution in symbionts, in which both microorganisms and host plants gain water and nutrients from the experimental environment.

Under stress conditions, the plant must adjust its morphological variables to propagate in adverse environments (Pan *et al.*, 2020). Our study showed that single inoculation with PGPR or AMF produced a higher shoot and root dry mass than those in the control with no salt stress, which was consistent with Xun *et al.* (2015) who reported that single inoculation with PGPR or AMF improved the growth of oat plant in saline-alkali soil. However, in our study, contrary results were observed under salt stress, and the improvement in plant height was not obvious between non- and inoculation treatments. This may be due to the concentration of NaCl, which finally inhibits microbial function.

Fruit number, fresh mass, and dry mass are considered important standards to measure the yield of tomatoes (Luitel *et al.*, 2012). Although co-inoculation led to the lowest shoot and root dry mass among treatments, the fruit number and fresh mass of single inoculation with PGPR and especially co-inoculation treatment were higher than those in the control under salt stress. This may be due to the relationship between nourishment and reproductive growth, and also implies the ability of inoculation to increase tomato production. However, under AMF inoculation treatment, shoot and root dry mass, and the fruit number were lower than in the control under both non- and salt stress conditions, possibly because of resource deficiency as reported by Adesemoye *et al.* (2009), who suggested that insufficient fertilizer content limited AMF to promote the growth of tomato. In the process of tomato cultivation, "single fruit" (only one fruit in a plant) was observed only in non-inoculation treatments, not in inoculation treatments. This may be due to the effects of PGPR and AMF on the number of flower buds, flowers and pollen fertility, and it would be interesting to study this further.

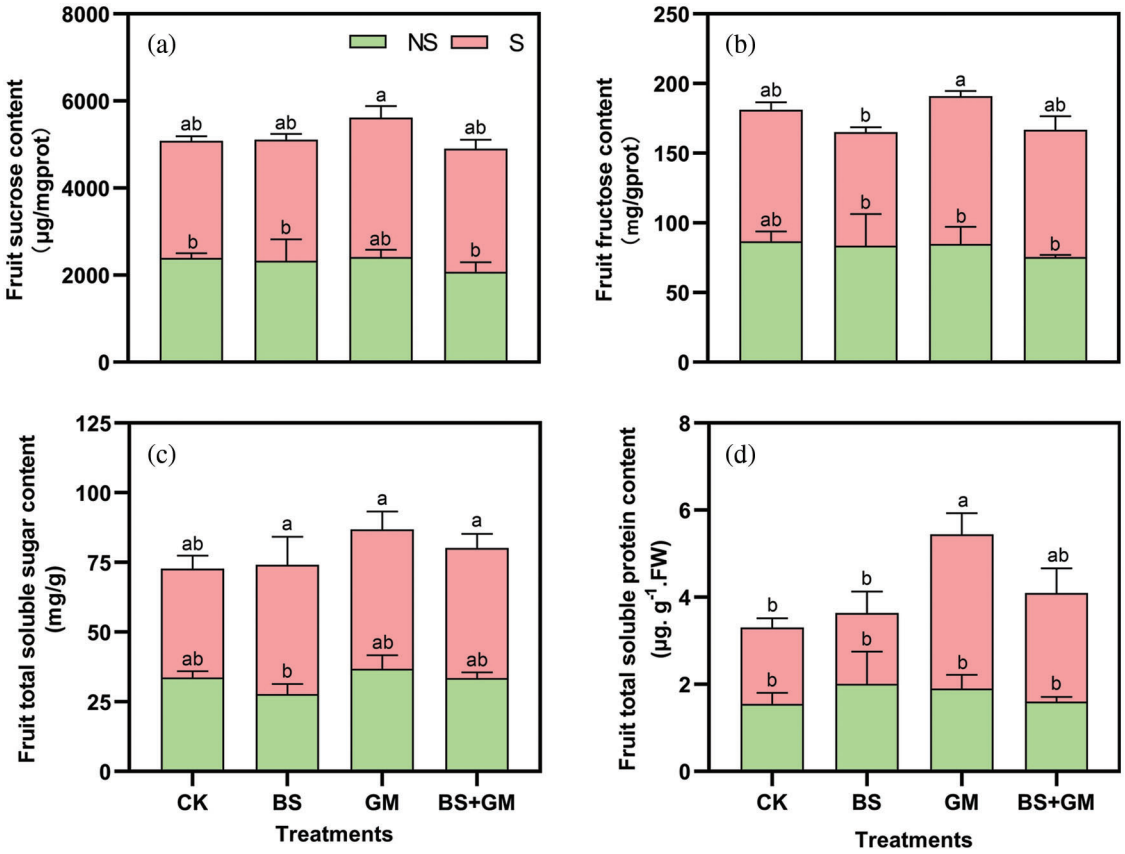

FIGURE 4. Effects of non-inoculation (CK) and inoculation with the PGPR-*Bacillus subtilis* (BS), AMF- *Funneliformis mosseae* (GM), and co-inoculation (BS+GM) on fruit sucrose content (a), fructose content (b), total soluble sugar content (c) and total soluble protein content (d) under non-salt (NS) and salt stress (S) conditions. Different letters indicate significant differences ($P < 0.05$) among treatments by Duncan's test; n = 4.

H_2O_2 is a toxic reactive oxygen species. Excessive H_2O_2 reduces cellular membrane permeability and adversely affects metabolic pathways (Hashem *et al.*, 2018). As shown in this study, salt stress increased H_2O_2 content, which finally damaged the cell membrane system. However, H_2O_2 content was no significant decline except for single inoculation with PGPR. This may be due to the decline in other components of ROS, such as superoxide ions and hydroxyl and peroxide radicals. MDA is an end product of lipid peroxidation and reflects the degree of damage due to stress (Zhu *et al.*, 2010). PGPR and AMF were found to alleviate adverse effects by reducing MDA content when subjected to salt stress (Xun *et al.*, 2015; Hashem *et al.*, 2018). However, our results were not completely concurrent with these, as we observed that single inoculation and co-inoculation decreased MDA content in the absence of salt stress, while the content was higher than in the control after AMF under salt stress. Similar results were observed in the previous study wherein AMF and rhizobacteria affected the physiology and performance of *Sulla coronaria* plants under salt stress (Hidri *et al.*, 2019); this may be due to the specific type and combination of PGPR and AMF.

The antioxidant defense system can be enhanced to alleviate salt stress (He *et al.*, 2007). Proanthocyanidin, flavonoid, and ascorbic acid are important antioxidants that enhance salt stress tolerance by neutralizing oxygen free radicals (Koes *et al.*, 1994; Li *et al.*, 2021; Raiola *et al.*, 2015). In our study, we observed that single inoculation of AMF increased proanthocyanidin and flavonoid content but decreased ascorbic acid content under salt stress. Instead, PGPR inoculation treatment simply increased ascorbic acid content. This may be because of different regulatory mechanisms of non-enzymatic antioxidants in PGPR and AMF inoculations.

Moreover, SOD can dismutate superoxide radicals to H_2O_2 and oxygen, and POD is involved in converting H_2O_2 into water and oxygen. Our results showed that inoculation with PGPR singly increased the SOD and POD activities in tomato fruit compared with those in the control only under no-salt stress. This may be due to different fruit setting stages under inoculation conditions, as documented in tomato graft treatment with *B. subtilis*, higher activity was observed on day 28 after grafting (Padró *et al.*, 2021). We also found that SOD and POD activities were higher in AMF-only inoculation than in the control under both non-salt and salt stress conditions, which was in line with Huang *et al.* (2010), who suggested induction in SOD and POD activities in AM symbiosis compared to those in non-AM plants. It implies that AMF inoculation could alleviate injuries due to oxidation to finally enhance the salt stress tolerance of tomatoes. The positive effects of co-inoculation treatments were not obvious, which may be due to the type of PGPR. The T-AOC was measured considering the antioxidant defense system, including enzymatic and non-enzymatic systems. As shown in the results, inoculation with PGPR or AMF significantly enhanced the enzymatic

and non-enzymatic systems; this was in agreement with previous reports on *Cucumis sativus* L. (Hashem *et al*., 2018) and *Vigna radiata* L. (Panwar *et al*., 2016).

Under salt stress, the plant undergoes osmotic adjustments by synthesizing a high concentration of soluble sugar and soluble protein, finally increasing intracellular osmotic (Janah *et al*., 2021). In plant tissues, sucrose and fructose are common soluble sugars, and we found that AMF enriched their content over the control under salt stress. Similar results were observed in the *Oryza sativa* L. *subsp*. *indica* under water deficit condition (Tisarum *et al*., 2019). But PGPR single and co-inoculation treatments led to low or negative increments; likewise, in the present study, fruit total soluble sugar and total soluble protein content increased under salt stress condition, except for PGPR inoculation treatment. This is probably because of different experimental designs such as different types of inocula, the combinations, and so on. Nevertheless, the results were consistent with the investigation conducted by Fernandez *et al*. (2012) who observed an increase in total soluble sugar content under cold stress. This may be due to plant self-rescue mechanisms triggered by adverse environments, and AMF markedly enhanced the total soluble sugar and protein content than in the control. This agrees with the previous study on *Poncirus trifoliata* in low-zinc soil (Chen *et al*., 2017). We also found that inoculations improved the total TSS and TSP content than the non-inoculation tomato under non-salt and salt stress conditions, respectively. It implies that single inoculation and co-inoculation with PGPR and especially AMF can possibly ameliorate tomato fruit quality.

To conclude, in the present study, we observed that single inoculation and co-inoculation with PGPR and AMF do not exert exactly the same effects on tomato growth and fruit quality. Inoculation with AMF led to better fruit quality, while PGPR induced higher antioxidant capacity, and co-inoculation gained more yields. Obviously, it is necessary to conduct in-depth studies on more kinds of microbial inoculums under different levels of salt-stress for applications in agriculture production.

Authors' Contribution: The authors confirm their contribution to the article as follows: WZ conducted the experiment, statistical analysis, and original draft writing. MMZ and KZT designed the idea and helped with manuscript drafting. All activities took place under the guidance and assistance of XCZ. All authors contributed to the experiment and article and finally approved the submitted version.

Availability of Data and Materials: All data generated or analyzed during this study are included in the article.

Ethics Approval: Not applicable.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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