Inoculation of Halotolerant Yeast Meyerozyma guilliermondii Regulates Tomato (Lycopersicon esculentum) Salt Tolerance

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Article

Inoculation of Halotolerant Yeast *Meyerozyma guilliermondii* Regulates Tomato (*Lycopersicon esculentum*) Salt Tolerance

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Abstract: How to improve plant tolerance and yield under salt stress is critical for ensuring sufficient food supply since plant survival and agricultural productivity are both affected by salinity. Some evidence has showed that beneficial microorganisms have a high ability to improve plant salt tolerance and increase crop yield. But few studies were involved in effects of halotolerant yeasts on plants under salt stress. In this present research, *Meyerozyma guilliermondii*, a halotolerant yeast, was inoculated with tomato plants followed by salt treatment of four different NaCl concentrations (0, 100, 200, and 300 mM). Our results showed that inoculation of *M. guilliermondii* increased the chlorophyll biosynthesis and photosynthetic machinery effectiveness under salt stress, contributing to biomass accumulation. Under salt treatment of 300 mM NaCl, the yeast inoculation significantly increased ascorbate concentrations in leaves, yet showed no effects on levels of glutathione and proline. Antioxidant enzymes were affected differently by the yeast inoculation. It was found that the yeast inoculation increased superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) activities under 300, 100, and 200 mM NaCl, respectively. Total soluble sugar levels increased in inoculated tomato plant leaves; however, there were no significant differences between different NaCl concentrations. Under 300 mM NaCl, the yeast inoculation significantly decreased H_2O_2 levels and reduced malondialdehyde levels. All together, our results showed that halotolerant yeast *M. guilliermondii* inoculation might be a strong candidate for regulating tomato growth under salt stress by increasing ability to scavenge reactive oxygen species and chlorophyll intactness, and by strengthening photosynthetic machinery.

Keywords: salt stress; photosynthesis; antioxidant enzymes; ROS scavenging; *Meyerozyma guilliermondii*

1. Introduction

Saline soils are widely distributed all around the world. It has been assessed that salinity influences about 20% of irrigated land (~ 45 million ha) and ~ 2.1% (~ 32 million ha) of dryland agricultural worldwide [1], and that more than 50% of the cultivated land would be salinized by the year 2050 [2]. Thus, soil salinization has become a significant menace to agricultural activity and ecosystems worldwide [3–5]. Soil salinization has been proven to have a deleterious impact on plant physiology, including ion toxicity, physiological drought, oxidative stress, and nutrient insufficiency [6,7]. A large amount of salt in the soil leads to the lower osmotic potential of the root-soil interface than that of the plant root cell interface, which will make it difficult for the plant to absorb water and eventually lead to physiological drought [8–10]. In such case, plants close stomata to reduce transpiration; however, this reduces the diffusion of CO_2 into leaf tissues as well. Salt stress also causes oxidative stress through the production of reactive oxygen species (ROS) [3]. When ROS accumulates in large amounts, these radicals lead to the peroxidation of bio-membrane lipids, DNA

breakage, protein degradation [11]. Therefore, cell activity is affected, thus inhibiting plant growth and development. High soil salinity also leads to a reduction in total chlorophyll concentrations and the degradation of chloroplast structure by minimizing grana [12]. The photosynthetic electron transport rates are also altered by salt stress [13]. Net photosynthetic rates and biomass accumulationare both reduced due to all these changes [14]. Therefore, the agricultural practice should consider limiting the damage caused by salt stress and increasing net photosynthetic rates and biomass accumulation.

Many beneficial microorganisms may increase the tolerance of their host plants tolerance to salt stress, such as arbuscular mycorrhizal fungi [12,15–17], ectomycorrhizal fungi [18–20], root endophytic fungi [21], dark septate endophytic fungi [22–24], and plant growth-promoting rhizobacteria [25–27]. These beneficial microorganisms are crucial for reducing salt stress damage and enhancing biomass accumulation and crop yield. Some yeast species, acting as endophytic fungi, show functions similar to these beneficial microorganisms. For instance, *Yarrowia lipolytica* produced secondary metabolites (e.g., high concentrations of indole-3-acetic acid, indole-3-acetamide, phenols, and flavonoids) to ameliorate the negative impacts of salt stress and promoted maize growth [28,29]. Saccharomyces cerevisiae enhanced the oxidative defense system of Linum usitatissimum seedlings and maintained plasma membrane integrity under salt stress [30]. The transgenic tomato (*Lycopersicon esculentum* Mill.) with the HAL1 gene from S. cerevisiae also showed increased growth by maintaining significantly high ratios of K^+/Na^+ [31]. All findings suggest that yeast species may improve plant tolerance to salt stress through several pathways.

Yeasts have evolved conserved physiological adaptation mechanisms involved in complex responses at the molecular level to cope with various environmental stress and are highly adaptive [32]. Some of them are halophilic, such as *Meyerozyma caribbica* [28,29,33] and *Meyerozyma guilliermondii* (henceforth Megu) [34,35]. Their wide distribution confers remarkable functions in ecosystems. Megu played crucial roles, such as biocontrol of postharvest fruit rot [36–39], degradation of pollutants [35], biodiesel feedstock potential [40], production of xylitol [41] and silver/silver chloride nanoparticles [42], and bioremediation of heavy metal (loids) [43,44]. As noted, Megu has a wide range of uses in biotechnological, agricultural, and ecological field [45]; hence, it might be used as a model microorganism in plant-microbe interaction research [46]. The draft genome sequence of Megu also improves related studies [47,48]. However, as a root endophytic fungus with the halophilic trait, it remains unclear whether Megu promotes plant tolerance and growth under salt stress, just as its compeer *M. caribbica* [28,29]. In this study, the effects of Megu inoculation on tomato (*Lycopersicon esculentum* Mill.), a model plant for studying the salt tolerance mechanism of plants, were studied under salt stress. Our aim was (1) to evaluate the effects of the inoculation on the growth and salt tolerance of tomato plants; (2) to investigate the physiological processes through which plant-microbe interaction promotes salt tolerance; (3) to establish Megu as a good candidate in agricultural practice against salt stress.

2. Materials and Methods

2.1. Yeast and plant culture

Meyerozyma guilliermondii BNCC337334 (ATCC 6260) was purchased from Beina Lianchuang Institute of Biotechnology, Beijing. Megu was cultured in YM solid medium (yeast extract 5 g, malt extract 3 g, glucose 10 g, peptone 5 g, agar 20 g in distilled water 1 L, pH 6.0-6.4) for 72 h at 25℃. Viable blocks were selected to culture in YM liquid medium (without agar) in a constant temperature shaker (150 r • min^{-1} for 72 h at 25℃). The seeds of tomato (*Lycopersicon esculentum* cv. "Sunrise") were purchased from Guanhe Seed Company, Shouguang, Shandong province, China. The seeds were sterilized in an ethanol solution (75%) for 2 min, followed by sodium hypochlorite solution (NaClO, 0.75%active chlorine) for 15 min, then were washed several times with sterile distilled water. These sterilized tomato seeds were sowed in sterilized cultivation substance bought from Jinrun Biotechnological Co. Ltd., Shandong province, China, in plastic pots (16 cm in height, 25 cm in diameter), and were cultured in the greenhouse with 16 h light/8 h dark (PPFD 300–400 μmol • m^{-2} •

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s^{-1}), relative humidity of 85%, and 25/20°C (day/night). After germination, the seedlings with two true leaves were selected (2 seedlings per pot). These seedlings were watered according to the moisture of the cultivation substance. 25 d after germination, tomato seedlings were inoculated with the hyphal suspension solution (10 g • L^{-1}) of cultured Megu (10 ml per pot). 30 d after germination, tomato plants were treated with 0, 100, 200, and 300 mM NaCl. The treatments were divided into eight groups: (1) -Megu + 0 mM NaCl; (2) -Megu + 100 mM NaCl; (3) -Megu + 200 mM NaCl; (4) -Megu + 300 mM NaCl; (5) +Megu + 0 mM NaCl; (6) +Megu + 100 mM NaCl; (7) +Megu + 200 mM NaCl; (8) +Megu + 300 mM NaCl. Ten pots were used for each treatment (a total of 80 pots). Thirty days after inoculation, roots of tomato plants inoculated with Megu suspension solution were examined by micrography. The remaining tomato plants continued to be cultured until they produced fruits.

2.2. Harvesting

Whole tomato plants were removed from soil at 90 d of growth and then washed with tap water. Plants were divided into above- and below-ground parts at root-shoot joints. Fresh weight of shoot, roots, and fruits were recorded. Leaves were immersed in liquid nitrogen and then stored at -80°C for further analysis.

2.3. Experimental methods

2.3.1. Chlorophyll concentrations and fluorescence determination

On 85 d of growth, five pots were randomly selected from each treatment. Three leaves with the same age per pot were selected for chlorophyll fluorescence measurement using a pulse-amplitude-modulated fluorometer (Junior-PAM, Walz, Germany), with a PAR of 190 μmol•m^{-2}•s-1. Chlorophyll concentrations of the leaves of the same age were determined as described by Lichtenthaler and Wellburn [49].

2.3.2. Fungal growth and root infection under different NaCl concentrations

Megu was cultured in YM solid medium containing 0, 100, 200, and 300 mM at 25°C for 24 and 48 h. The developmental photos of Megu were captured using a digital camera at 24 and 48 h. The photos were then analyzed with the software ImageJ (https://imagej.nih.gov/ij) and the fungal areas were obtained. Megu infection in tomato roots exposed to different NaCl concentrations was investigated under a microscope by the method of Vahabi et al. [50].

2.3.3. Determination of ascorbate and glutathione concentrations

Ascorbic acid concentrations were determined using the spectrophotometric method [51]. About 1 g of fresh leaves of the plants was placed in a mortar, 4 ml of oxalic acid–EDTA extracting solution was added, followed by 1 ml of orthophosphoric acid and 1 ml of 5% tetraoxosulphate (vi) acid. 2 ml of ammonium molybdate was added to the resulting mixture, followed by 3 ml of deionized water. Leaves were ground to homogenate. The homogenate was centrifuged for 10 min at 4°C (5000 rpm). The absorbance of the supernatant was measured at 760 nm. The concentrations of the ascorbic acid in the samples were then calculated from a standard curve.

0.5 of fresh tomato leaves was mixed with three mL extraction buffer containing 1 mM EDTA in 5% metaphosphoric acid in a cooled mortar and then was ground to ho-mogenate with a pestle. The homogenate was centrifuged at 11,500 × *g* for 12 min at 4°C. Aliquots (0.2 mL) of supernatant were neutralized with 0.3 mL phosphate buffer (0.5 M, pH 7.0) followed by reduction of the oxidized fraction with 0.1 M dithiothreitol. For as-saying GSH, 0.2 mL supernatant was neutralized. Proper oxidation and reduction of GSH were confirmed in the presence of GR observed for 1 min at 412 nm to determine total GSH content [52].

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2.3.4. Soluble sugar and protein determination

Levels of soluble sugars were determined by anthrone colorimetry [53]. Concentrations of soluble proteins were determined according to the method introduced by Bradford [54].

2.3.5. Determination of antioxidant enzyme activities

0.5 g of leaves was weighed into a cooled mortar, liquid nitrogen was added, and the leaves was ground quickly with extraction buffer, and centrifuged at 4°C at 12,000 rpm. Superoxide dismutase (SOD) activity was measured following the method of Beau-champand Fridovich by monitoring the photoreduction of nitroblue tetrazolium at 560 nm [55]. One unit of the enzyme was determined as the amount of enzyme reducing 50% of the substrate. The enzyme activity is expressed as unit • g^{-1} FW. Catalase (CAT) activity was measured based on the reduction of H_2O_2, according to the method of Velikova et al. [56], by monitoring the decrease in the absorbance at 240 nm for 1 min. Peroxidase (POD) activity was measured according to the method of Lin and Kao [57].

2.3.6. Determination of H_2O_2, proline, and MDA concentrations

To estimate H_2O_2, leaves were mixed with phosphate buffer (pH 6.5) and ground to homogenate. The homogenate was centrifuged for 10 min under 4°C at 12,000 rpm. Later the supernatant and reaction mixture (0.1% $TiCl_4$ in 20% H_2SO_4 (v/v)) was mixed at a 1:3 ratio and left at room temperature for 10 min. Prior to determination of the absorbance of the colored supernatant at 410 nm, the mixture was again centrifuged at 11,500 × *g* for 12 min. H_2O_2 concentrations were calculated using the extinction coefficient of 0.28 $\mu M^{-1} \bullet cm^{-1}$ and expressed as nmol•g^{-1} FW [58].

Free proline concentrations were determined according to the method described by Bates et al. [59]. About 0.5 g of leaves was ground into a fine powder in liquid nitrogen and then homogenized in 2 mL of 3% aqueous sulfosalicylic acid. After centrifugation, 0.3 mL of supernatant was mixed with 0.3 mL cold acetic acid and 0.3 mL of acid ninhydrin solution and then boiled in a water bath for 1 h. After cooling in an ice bath, the mixture was mixed with 0.6 mL toluene and vortexed for 1 min. The chromophore-containing toluene was separated from the aqueous phase, and its absorbance was measured at 520 nm against toluene. The proline concentration was determined based on the standard curve of proline and calculated as μg • g^{-1} FW.

Malondialdehyde (MDA) was determined according to Heath and Packer [60]. Fresh leaves (0.2 g) were homogenized with 5 mL 0.1 % (m/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 *g* for 5 min at 25°C. 4 mL of TCA (20 %) containing 0.5 % (m/v) thiobarbituric acid (TBA) was added to 1 mL of supernatant. The mixture was heated at 95°C for 30 min and then quickly cooled on ice. The mixture was centrifuged at 10,000 *g* for 10 min at 25°C. The absorbance of the supernatant was measured at 532 nm. The value for nonspecific absorption at 600 nm was subtracted. The amount of MDA-TBA complex was calculated from the extinction coefficient of 155 $mM^{-1} \bullet cm^{-1}$.

2.4. Statistical analysis

Statistical analyses of the data were carried out using SPSS 17.0 (IBM, Chicago, IL, USA). Datawith three repetitions were subjected to to analysis of variance (ANOVA) and compared by the least significant difference (LSD) at $p < 0.05$. The relationships among all items were analyzed by Pearson correlation.

3. Results

3.1. Yeast growth and infection under different NaCl concentrations

Megu was incubated in YM solid media containing 0, 100, 200, and 300 mM NaCl at 25°C for 24 and 48 h, and their growths were recorded (Figure 1 and Figure S1). When cultured in YM solid medium for 24 hours, Megu growth reduced as NaCl concentrations increased (Figure 1). However, when incubation continued up to 48 h, Megu growth showed the highest performance under 100 mM

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NaCl (Figure 1 and Figure S1). Megu was able to infect tomato roots at concentrations of 0, 100, 200, and 300 mM NaCl, with the maximum infection rate occurring at 100 mM (Figure 2).

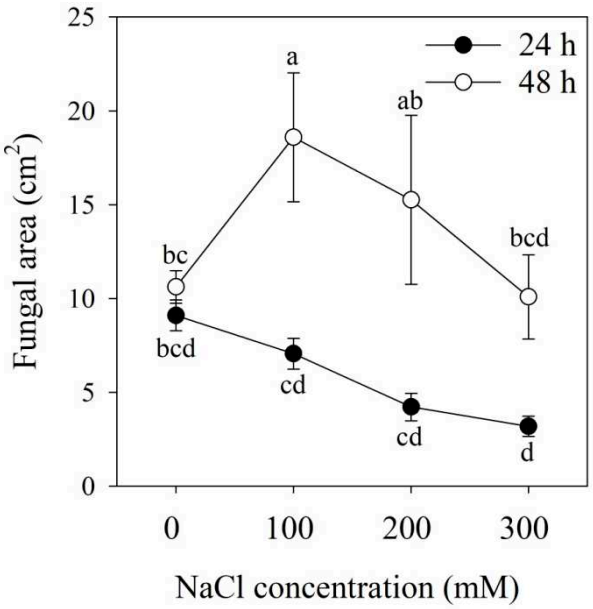

Figure 1. The growth of Megu under different NaCl concentrations. Megu was cultured in YM solid mediums containing 0, 100, 200, and 300 mM NaCl. The growth photos of the yeast were taken using a digital camera at 24 and 48 h. The photos were analyzed with the software ImageJ (https://imagej.nih.gov/ij) to obtain growth areas. Data were shown as mean ± SD. Data of the same day with different lowercases were significantly different (n=15, $p < 0.05$, LSD).

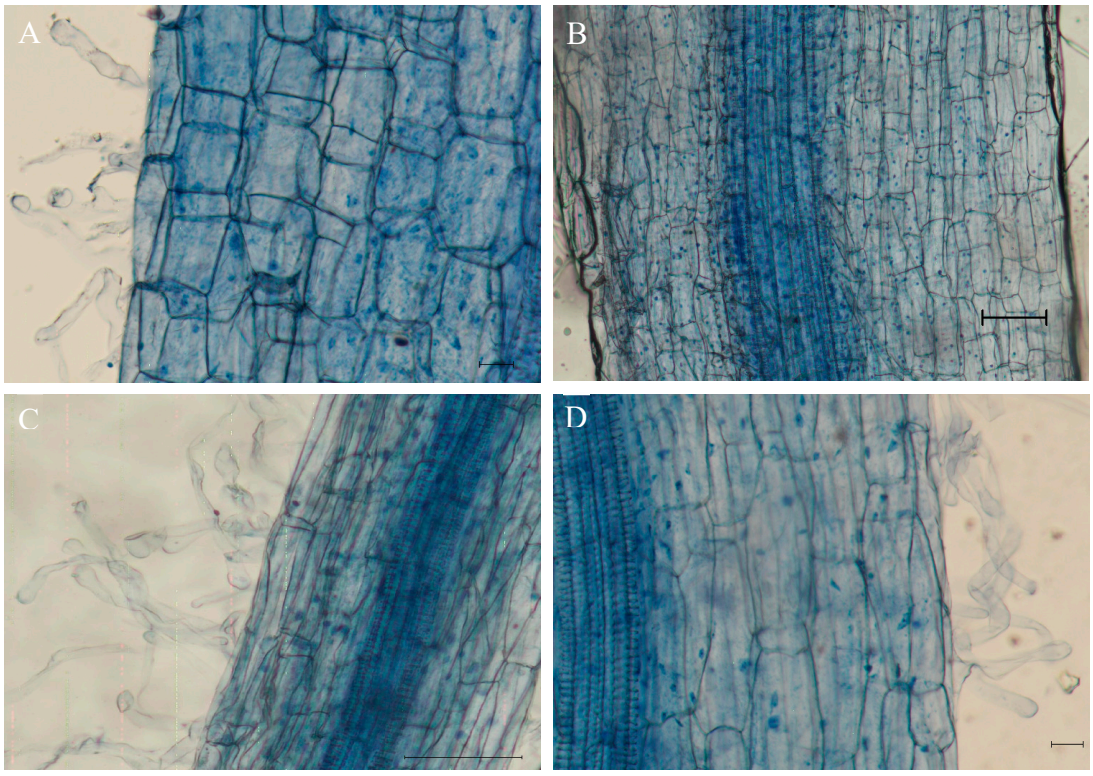

Figure 2. The infection ofMegu in roots of tomato plants under different NaCl concentrations. (A) 0 mM NaCl, bar = 10 μm; (B) 100 mM NaCl, bar = 100 μm; (C) 200 mM NaCl, bar = 100 μm; (D) 300 mM NaCl, bar = 10 μm. Blue arrows indicate Megu hypha on the root surface of tomato plants, and white arrows indicate spores of Megu.

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3.2. Megu effects on chlorophyll concentrations and fluorescence parameters in tomato plants under salt stress

The concentrations of Chl a, Chl b, and total chlorophyll were higher in Megu-inoculated tomato plants than those in plants without Megu inoculation under higher NaCl concentrations (200 and 300 mM), however, no significant differences occurred between them (Table 1, $p > 0.05$). Similarly, concentrations of carotenoids were higher in Megu-inoculated plants than those in plants without Megu inoculation under 300 mM NaCl (Table 1).

Table 1. The effects of *M. guilliermondii* (Megu) inoculation on concentrations of photosynthetic pigments of tomato plants under different NaCl concentrations.

Pigment level (mg/g FW)		NaCl concentrations (mM)			
		0	100	200	300
Chl *a*	N	17.29 a	18.58a	18.06a	16.41ab
	Y	17.92 a	14.16 b	18.98a	18.05 a
Chl *b*	N	7.31abc	8.95a	7.94 ab	7.09bc
	Y	7.54ab	5.74c	8.51 ab	7.97 ab
Carotenoids	N	2.89ab	2.84ab	3.10a	2.83 ab
	Y	3.08 a	2.52 b	3.02 a	2.92 ab
Total Chl	N	24.60 a	27.53a	26.00 a	23.51 ab
	Y	25.46 a	19.90b	27.49 a	26.01a

Note: N indicates non-inoculated; Y indicates inoculated plants. Each value represents the mean of three replicates of each treatment. For each biochemical parameter, data with different lowercases show significant difference (n=3, $P< 0.05$, LSD).

The actual photochemical quantum yield Y(II) and electron transport rate (ETR) in Megu-inoculated plants were significantly higher under higher salinity (200 and 300 mM NaCl), compared with plants without Megu inoculation (Table 2, $p < 0.05$). Under NaCl concentrations of 0-200 mM, the photochemical quenching (qP) was significantly higher in tomato plants inoculated with Megu, compared with plants without Megu inoculation (Table 2, $p < 0.05$). However, under 300 mM NaCl, there was no significant difference in qP between plants with and without Megu inoculation (Table 2, $p > 0.05$). qL in tomato plants inoculated with Megu showed significantly higher levels under all the NaCl treatments, compared to the non-inoculated plants (Table 2, $p < 0.05$). Under higher salinity (200 and 300 mM NaCl), the quantum yield of nonregulated energy dissipation Y(NO), was significantly lower in inoculated plants, compared to the no inoculated plants (Table 2, $p < 0.05$). The fluorescence parameter, Fv/Fm, was significantly higher in inoculated tomato plants under higher salinity (200 and 300 mM NaCl), compared with the non-inoculated plants (Table 2, $p < 0.05$).

Table 2. The effects of Megu inoculation on fluorescence parameters of tomato plants under different NaCl concentrations.

Fluorescence parameter		NaCl concentrations (mM)			
		0	100	200	300
Y(II)	N	0.56 e	0.61 b	0.58 d	0.58 d
	Y	0.59 c	0.58 d	0.61 b	0.62 a
ETR	N	29.51 g	31.88 c	30.51 d	30.30 de
	Y	30.19 e	29.79 f	32.15 b	32.71 a
qP	N	0.90 g	0.95 d	0.91 f	0.93 c
	Y	0.93 e	1.00 a	0.98 b	0.96 c
qL	N	0.78 f	0.86 d	0.79 f	0.84 e
	Y	0.85 de	1.01 a	0.94 b	0.90 c
Y(NO)	N	0.42 a	0.37 c	0.40 b	0.40 b
	Y	0.41a	0.40 b	0.37 c	0.36 d
Fv/Fm	N	0.62 c	0.63 b	0.62 c	0.62 c
	Y	0.60d	0.60 d	0.64 a	0.64 a

Note: N indicates n on-inoculated; Y indicates inoculated plants. Each value represents the mean of three replicates of each treatment. For each fluorescence parameter, data with different lowercases show significant difference (n=25~30, $p < 0.05$, LSD).

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3.3. Effects of Megu inoculation on biomass accumulation in tomato plants under salt stress

Under normal conditions (i.e., 0 mM NaCl), inoculation of Megu significantly in-creased root fresh weight of tomato plants, by 42.27%, compared to the tomato plants without inoculation (Figure 3A and Figure S2, $p < 0.05$). Megu inoculation increased root fresh weight, by 26.24%, 46.91%, and 64.80% under 100, 200, and 300 mM NaCl, respectively. However, no significant changes occurred between inoculated and non-inoculated tomato plants under corresponding NaCl concentrations (Figure 3A, $p > 0.05$). Similar results were observed for the shoot and fruit fresh weights (Figure 3B, C, and Figure S3). Shoot biomass increased by 20.72%, 25.81%, and 42.81%, and fruit biomass increased by 43.65%, 82.68%, and 42.88% under 100, 200, and 300 mM NaCl, respectively.

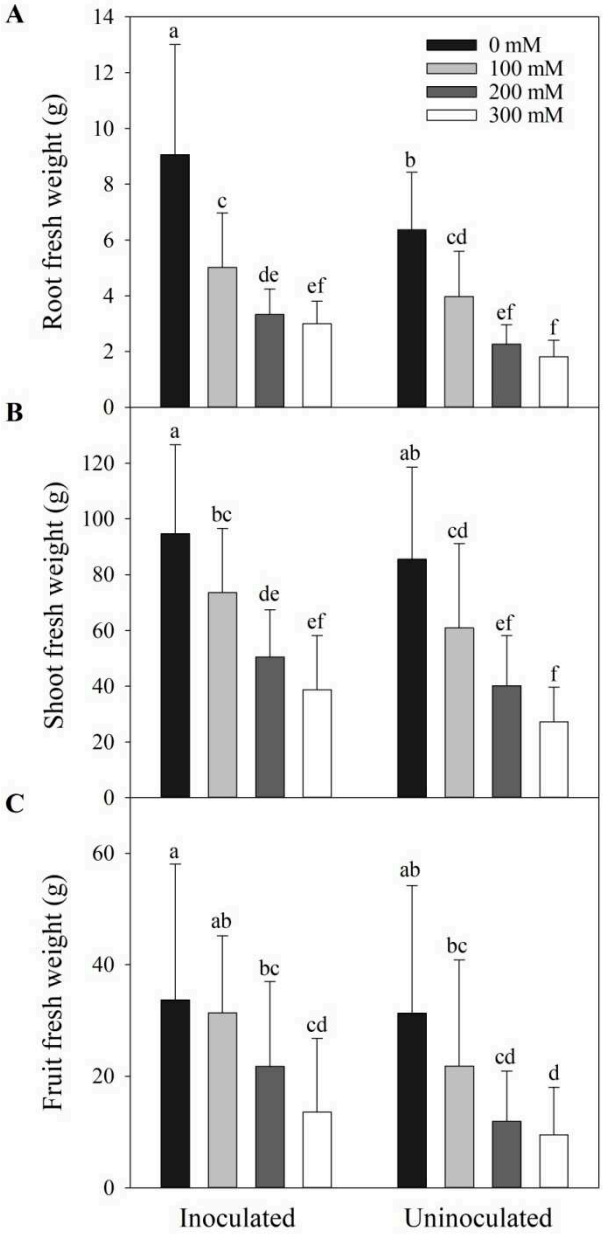

Figure 3. The effects of Megu inoculation on fresh weight of tomato plants under different NaCl concentrations. (A) Root fresh weight; (B) Shoot fresh weight; (C) Fruit fresh weight. Data were shown as mean ± SD. Data of the same parameter with different lowercases were significantly different (n=15, $p < 0.05$, LSD).

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3.4. Megu inoculation regulated levels of antioxidants and osmolytes in tomat plants under salt stress

No significant differences were observed from 0 to 200 mM NaCl in ascorbate concentrations between inoculated and non-inoculated tomato plants (Figure 4A, $p > 0.05$). However, under 300 mM NaCl, ascorbate was significantly higher in inoculated tomato plants, compared with no inoculation (Figure 4A, $p < 0.05$). Regardless of inoculation, salt stress significantly increased glutathione concentrations, compared with those under 0 mM NaCl (Figure 4B, $p < 0.05$), however, no significant changes in glutathione concentrations occurred between inoculated and non-inoculated tomato plants under salt stress (Figure 4B, $p > 0.05$). Salt stress significantly increased proline levels in both inoculated and non-inoculated plants, compared with plants under 0 mM NaCl (Figure 4C, $p < 0.05$), however, proline levels showed no significant changes between inoculated and non-inoculated plants under corresponding NaCl concentrations (Figure 4C, $p > 0.05$).

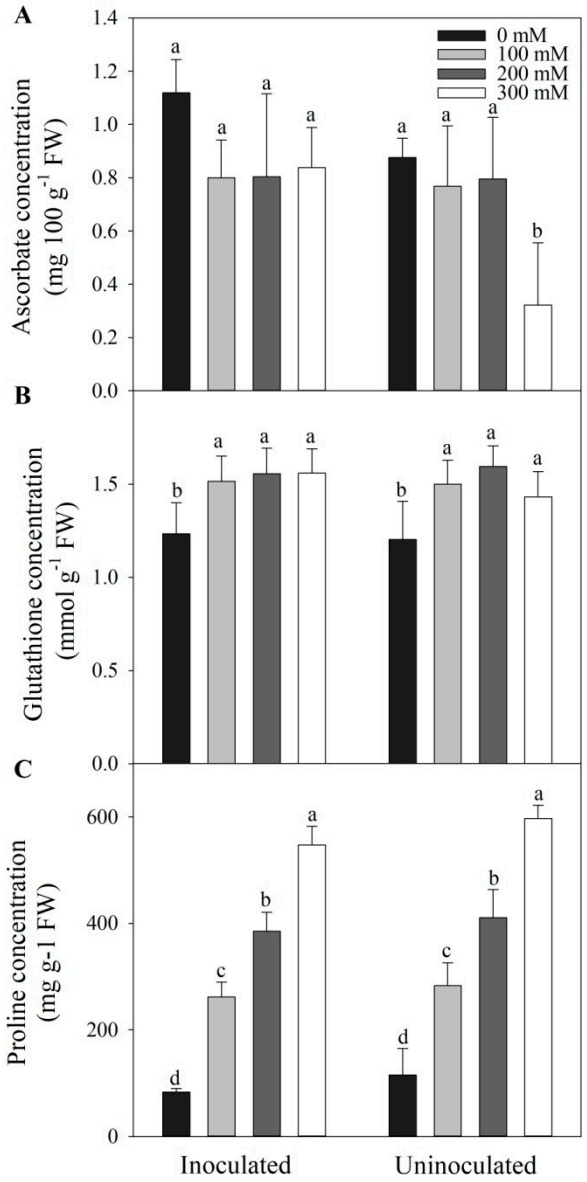

Figure 4. The effects of Megu inoculation on concentrations of ascorbate (AsA, A), glutathione (GSH, B), and proline (C) in the leaves of tomato plants under different NaCl concentrations. Data were shown as mean ± SD. Data the same parameter with different lowercases were significantly different (n=3, $p < 0.05$, LSD).

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3.5. Megu increased antioxidant enzyme activities in tomato plants under salt stress

Megu inoculation increased SOD activities under salt stress, however, only under 300 mM NaCl, Megu-inoculated tomato plants showed significantly higher SOD activity than that of non-inoculated tomato plants (Figure 5A, $p < 0.05$). CAT activities showed the highest value in inoculated tomato plants under 100 mM NaCl, significantly higher than those under other salt treatments (Figure 5B, $p < 0.05$). However, CAT activities showed no significant changes under higher salinity (200 and 300 mM) between inoculated and non-inoculated plants (Figure 5B, $p > 0.05$). Under 200 mM NaCl, POD activity in inoculated tomato plants was significantly higher than that of non-inoculated tomato plants (Figure 5C, $p < 0.05$), however, no significant changes occurred between inoculated and non-inoculated plants under the other NaCl concentrations (i.e., 0, 100, and 300 mM NaCl) (Figure 5C, $p > 0.05$).

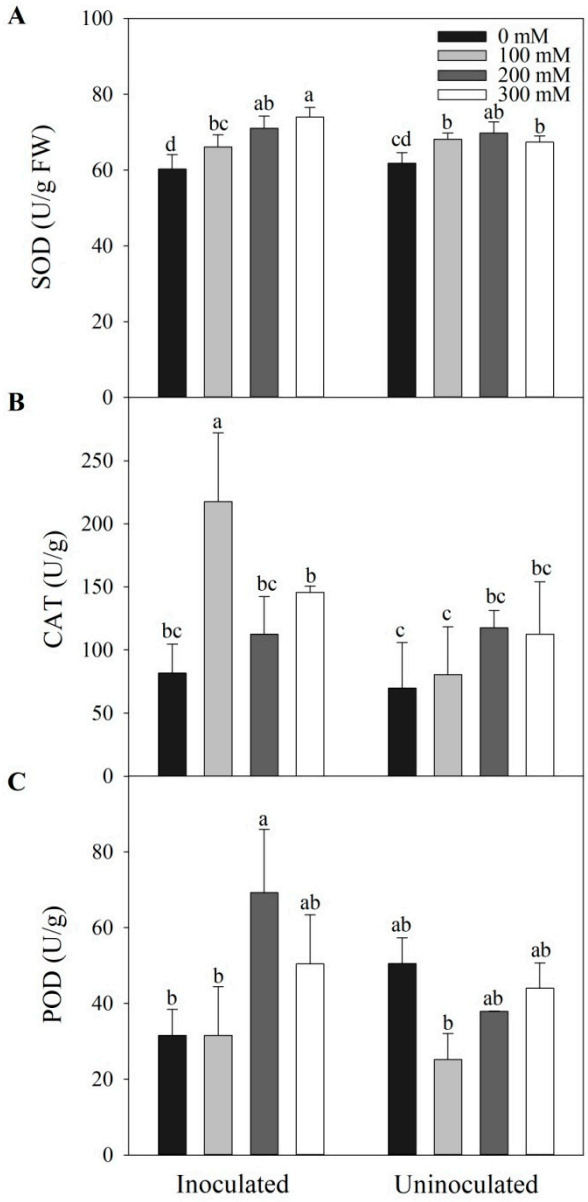

Figure 5. The effects of Megu inoculation on activities of superoxide dismutases (SOD, A), catalases (CAT, B), and peroxidases (POD, C) in the leaves of tomato plants under different NaCl concentrations. Data were shown as mean ± SD. Data of the same parameter with different lowercases were significantly different (n=3, $p < 0.05$, LSD).

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3.6. Inoculation effects on soluble sugar and protein levels in tomato plants under salt stress

Salt stress induced increase in soluble sugar concentrations, however, Megu inoculation did not lead to significant changes in soluble sugar concentrations under salt stress (Figure 6A, $p > 0.05$). Under all the NaCl concentrations, no significant changes in soluble proteins were found between inoculated and non-inoculated tomato plants, comparing the corresponding NaCl concentrations (Figure 6B, $p > 0.05$).

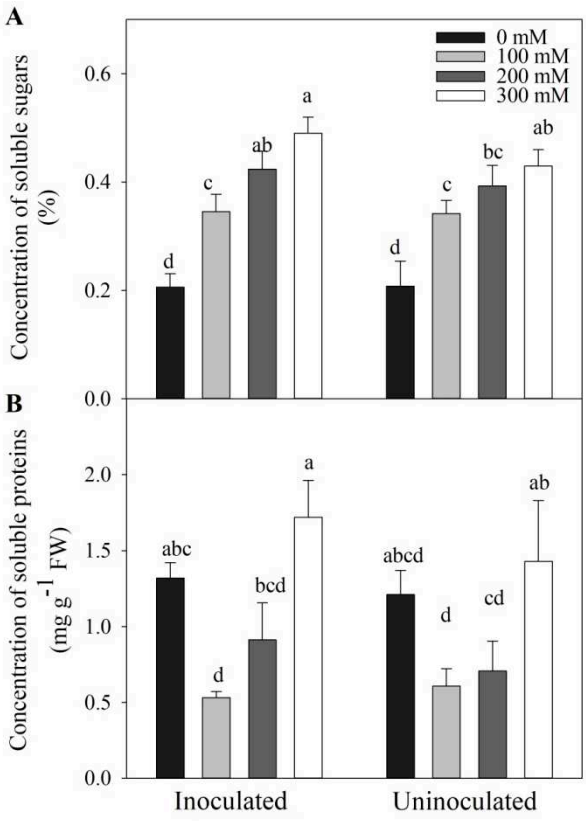

Figure 6. The effects of Megu inoculation on concentrations of total soluble sugars (A) and total soluble proteins (B) in the leaves of tomato plants under different NaCl concentrations. Data were shown as mean ± SD. Data of the same parameter with different lowercases were significantly different (n=3, $p < 0.05$, LSD).

3.7. Effects of Megu inoculation on H_2O_2 and MDA in tomato plants under salt stress

Under lower NaCl concentrations (i.e., 0, 100, 200 mM NaCl), H_2O_2 levels showed no significant changes between inoculated and non-inoculated tomato plants (Figure 7A, $p > 0.05$). However, under 300 mM NaCl, H_2O_2 level was significantly lower in inoculated plants than that in non-inoculated plants (Figure 7A, $p < 0.05$). MDA levels were lower in Megu inoculation than no-inoculation under all the NaCl concentrations, however, no significant changes occurred between inoculated and non-inoculated plants under all the NaCl concentrations (Figure 7B, $p > 0.05$).

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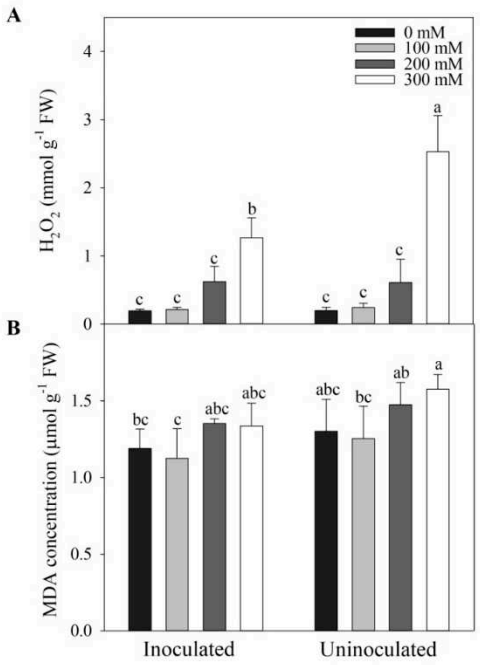

Figure 7. The effects of Megu inoculation on concentrations of H_2O_2 (A) and MDA (B) in the leaves of tomato plants under different NaCl concentrations. Data were shown as mean ± SD. Data of the same parameter with different lowercases were significantly different (n=3, $p < 0.05$, LSD).

4. Discussion

4.1. Megu inoculation regulated ROS scavenging in tomato plants under salt stress

Reactive oxygen species (ROS) have strong oxidizing power and can cause cell membrane damage, irreversible metabolic disorders, and eventually cell death. Environmental stresses cause a great amount of ROS in plants, including salt stress [25,61,62]. Under normal growth conditions, plants maintain a dynamic balance between formation and scavenging of ROS. However, when excessive ROS occurs under environmental stresses, the dynamic balance is disrupted, and ROS levels increase, leading to oxidative damage to plant cells [63] Antioxidants and antioxidant enzymes function in scavenging ROS and maintaining ROS homeostasis in plants under salt stress. In the present study, Megu inoculation significantly increased the ascorbate levels in leaves of tomato plants under 300 mM NaCl (Figure 4A). Reduced ascorbate is considered the most powerful ROS scavenger. It acts as electron donors in non-enzymatic ROS scavenging reactions and participates in the enzymatic reactions (such as ascorbate peroxidase) [11]. Under 300 mM NaCl, increased ascorbate levels caused by Megu inoculation was possibly helpful for ROS scavenging. As for another antioxidant glutathione, Megu inoculation showed no effect on the glutathione levels in the leaves of tomato plants under all the four NaCl treatments (Figure 4B), suggesting that the inoculation was not directly involved in glutathione biosynthesis or reduction reactions from oxidated form.

Beyond being an osmoprotectant, proline is also considered a potent nonenzymatic antioxidant in plants [11]. Under NaCl treatments, proline levels increased under treatments of NaCl concentrations; however, inoculation of Megu did not promote proline biosynthesis (Figure 4C), suggesting that inoculation did not increase salt tolerance via triggering proline biosynthesis. Carotenoids are also listed among strong antioxidants [11]. We found that the levels of carotenoids in the leaves of inoculated tomato plants showed no significant differences from those in non-inoculated plants (Table 1), suggesting that Megu inoculation induced no effects on carotenoid biosynthesis under salt stress.

SOD activities significantly increased under salt stress, compared to control (0 mM NaCl), and showed no significant differences between inoculated and non-inoculated tomato plants under lower NaCl concentrations (i.e., 100 and 200 mM, Figure 5A); how-ever, under 300 mM NaCl, Megu

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inoculation triggered SOD activities significantly (Figure 5A), suggesting that the inoculation promotes SOD under heavy salt stress. Similarly, under higher NaCl concentrations, the inoculation increased CAT and POD activity, but the significant effect was observed under heavy salt stress (200 and 300 mM NaCl, Figure 5B, C). The findings indicate that Megu inoculation increased antioxidant enzyme activities under heavy salt stress. Under 300 mM NaCl, H_2O_2 levels were significantly lower in the leaves of inoculated tomato plants, compared with non-inoculated plants (Figure 7A), suggesting that increase in levels of the antioxidants and activities of the antioxidant enzymes resulted in a reduction in H_2O_2 levels under heavy salt stress (300 mM NaCl). Under salt stress, the Megu inoculation also resulted in a reduction in MDA levels (Figure 7B). These data indicate that Megu inoculation increased resistance against oxidative stress during salt stress.

4.2. Effect of Megu inoculation on osmoregulation of plants under salt stress

Plants maintain their normal physiological activities by maintaining cell turgor pressure. As a result, under salt stress, plants accumulate more osmolytes, such as free proline, soluble carbohydrates, and soluble proteins, to balance the osmotic potential, [64,65]. Proline levels in the leaves of inoculated tomato plants were nearly equivalent to those in non-inoculated tomato plants (Figure 4C), suggesting that Megu inoculation had no effect on proline biosynthesis and accumulation, thus the inoculation cannot improve osmoregulation via proline biosynthesis. Verma et al. [66] reported that MYC2, a bHLH transcription factor, represses the expression of the P5CS1 gene, which encodes delta1-pyrroline-5-carboxylate synthase1, one of the core enzymes in the proline biosynthesis, thus negatively regulating proline biosynthesis in plant cells. Based on the results [65], it can be speculated that Megu inoculationmight not regulate the MYC2 protein activity and upstream mitogen-activated protein kinase (MAPK) cascade. In addition, some members of the genus *Trichoderma*, such as *T. harzianum*, *T. virens*, and *T. atroviride*, increased proline biosynthesis in salt-stressed plants [67,68]. Compared with these root endophytic fungi, Megu shows a weak role/minor function in promoting proline biosynthesis.

Total soluble sugar levels significantly increased in inoculated and non-inoculated tomato plants under salt stress, compared to tomato plants not exposed to NaCl (Figure 6A). This result was consistent with earlier researches [65,69,70]. However, under lower NaCl concentrations (100 and 200 mM), levels of soluble sugars in Megu-inoculated tomato plants were almost equal to those in non-inoculated plants (Figure 6A). Under higher NaCl concentration (300 mM), levels of soluble in Megu-inoculated tomato plants was higher than that in non-inoculated plants (Figure 6A), suggesting that Megu inoculation played role in improving biosynthesis of soluble sugars under heavy salt stress. A similar case occurred in levels of total soluble proteins (Figure 6B). Altogether, Megu inoculation might improve salt tolerance of tomato plants under heavy salt stress via regulating osmolyte biosynthesis. In fact, expression of genes involved in biosynthesis of osmolytes should be investigated in future and elucidate the mechanisms that Megu inoculation regulates their biosynthesis.

4.3. Effects of Megu inoculation on growth of tomato plants under salt stress

Salt stress finally affects plant growth and development. Based on our data, the inoculation of Megu increased the fresh weight of roots, shoots, and fruits of tomato plants under salt stress (Figure 3, Figure S2, and Figure S3). Increased biomass accumulation might be related to photosynthetic activities. Some studies showed that symbiosis between root endophytic fungi and plants improve plant growth and biomass accumulation under environmental stresses, such as heavy metal stress [71,72] and drought stress [73]. Under drought stress, inoculation of endophytic fungi led to increased net photosynthesis, compared to endophyte-free plants [73]. Increased net photosynthesis should be related to increased levels of photosynthetic pigments and chlorophyll florescence parameters. Under heavy metal stress, inoculation of the root endophytic fungus, *Piriformospora indica*, increased levels of Chl a and Chl b and fluorescence parameters (Fv/Fm and ETR) [71]. In our study, Megu inoculation also increased levels of Chl a and Chl b under heavy salt stress (200 and 300 mM NaCl) (Table 1), meanwhile the inoculation also increased levels of chlorophyll fluorescence parameters (Y(II), ETR,

qP, and Fv/Fm) under 200 and 300 mM NaCl (Table 2). Thus, our results are consistent with those from Shababivand et al [71]. Improvement of photosynthetic pigments and chlorophyll fluorescence parameters caused by inoculation of endophytic fungi helps increase in net photosynthesis and biomass accumulation (Figure 3, [74]).

Conclusions: inoculation of the halotolerant yeast *M. guilliermondii* improved salt tolerance of tomato plants via increasing ROS scavenging and accumulation of osmolytes and promoted growth via strengthening photosynthetic machinery.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1. The growth of *M. guilliermondii* (Megu) under different NaCl concentrations for 24 and 48 h; Figure S2. The photos showing root growth of un-inoculated and inoculated tomato plants with Megu under different NaCl concentrations; Figure S3. The photos showing growth of non-inoculated and Megu inoculated tomato plants subjected to different NaCl concentrations.

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