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Research Article

Dual inoculation of *Bradyrhizobium* and *Enterobacter* alleviates the adverse effect of salinity on *Glycine max* seedling

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Abstract

The aid of beneficial microbes, which is a well-accepted strategy, may improve plant salt tolerance. However, the mechanisms that underpin it are unclear. In this study, seedling experiments were carried out to assess the effect of *Bradyrhizobium* and *Enterobacter* on the germination, growth, nonenzymatic and enzymatic content in soybean (*Glycine max* L.) under salt stress. Water was sprayed on the seeds as a control, and with 75 mM, 150 mM NaCl as salt stress. The findings demonstrate that salt stress (75, 150 mM) caused a significant decrease in germination, morphological criteria, and membrane stability index (MSI) when compared to control seeds but increased lipid peroxidation (MDA), electrolyte leakage (EL), osmotic pressure, proline, citric acid, sugar content, antioxidant enzymes. Furthermore, endophytic *Bradyrhizobium* and *Enterobacter* inoculation resulted in a significant rise in all of the above metrics.; however, these treatments resulted in significant reductions in ROS, EL, and MDA in stressed plants. Finally, the findings showed that combining *Bradyrhizobium* and *Enterobacter* was the most efficient in reducing the harmful effects of salt on soybean plants by boosting antioxidant up-regulation and lowering membrane leakage and ROS.

Keywords: antioxidant enzymes; endophytic; osmotic pressure; soybean

Introduction

Soil salinity harms plant growth and has become one of the major limiting factors in agricultural production throughout the globe. High salinity disturbs the cellular osmotic equilibrium by decreasing the water potential within cells, such as drought and freezing stresses (Arif *et al.*, 2020). Ion poisoning is also caused by prolonged salt stress due to increasing Na^+ and Cl^- ions concentrations. Such adverse circumstances cause oxidative stress by producing reactive oxygen species (ROS), such as hydrogen peroxide, superoxide, singlet oxygen, and hydroxyl radicals, all of which are harmful to cell survival (Silva *et al.*, 2020). In NaCl-polluted soil, microbe (bio-augmentation) is an ecologically viable method of soil decontamination, which does not change

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soil properties. In addition, many microorganisms can remove NaCl from the soil through absorption and/or precipitation. However, two factors often hamper this approach: (I) microorganism tolerance to high saline levels. (II) nutrient shortage and poor growth circumstances in these saline-stressed (Sofy *et al.*, 2020a).

The nutrient deficit may be addressed by adding and enhancing appropriate growing conditions, but the biggest challenge is finding resistant microorganisms to high NaCl levels. Although some researchers believe that microbial domestication takes a long period (Komaresofla *et al.*, 2019), soil organisms may be workable to avoid mutant bacteria in harsh environments.

Egypt often faces significant fresh irrigation water shortages, particularly after completing the Grand Ethiopian Renaissance Dam. As a result, it's critical to employ ecologically friendly methods to assist plants in withstanding salt-water usage in irrigation (Gebresenbet and Wondemagegnehu, 2021).

Foliar spraying of plants or treatment of crop seeds with plant growth-promoting bacteria (PGPB) seems to be a vital ingredient that should be verified to increase crop production under different abiotic stresses in such a justifiable manner by using beneficial microorganisms in agricultural operations (Ngalimat *et al.*, 2021). In addition, PGPB may assist plants in coping with salt water (Kumar *et al.*, 2020). The first is that it reduces osmotic stress by increasing osmolyte concentrations and phytohormone signals like indole-3-acetic acid (IAA) and gibberellic acid (GA_3) (Zhang *et al.*, 2020). The second goal is to achieve ion homeostasis and improve nutritional absorption in order to reduce ion stress and nutrient deficiency (Assaha *et al.*, 2017; Megahed *et al.*, 2013). The third objective is to reduce oxidative stress by increasing photosynthetic capacity (Wang *et al.*, 2020). Finally, PGPB may improve plant performance under abiotic and biotic stress conditions (Brilli *et al.,* 2019; Megahed *et al.*, 2012).

Soybean (*Glycine max*) is a significant agricultural commodity that is extensively used as human food and animal due to its high protein (18%) and oil (38%) content (Yasmin *et al.*, 2020). However, it is also known for its partial salt sensitivity, which causes a 20-40% decrease in yield as salinity stress increases (Chung *et al.*, 2020). In addition, growth, seed quantity, quality, and nodulation are all harmed by high salt stress (Adhikari *et al.*, 2020).

This research aims to investigate the effects of *Bradyrhizobium* and *Enterobacter* inoculation on the growth, antioxidant system, and osmolytes accumulation in Glycine max seedlings under salt stress.

Materials and Methods

The isolated endophytic bacterium, *Bradyrhizobium japonicum* (*B. japonicum*; accession number EMCC No.1112), was obtained from an Egyptian agent for the American Type Culture Collection "Mircen", Ain Shams University, Cairo, Egypt. In addition, the isolated endophytic *bacterium, Bacillus amyloliquefaciens* (Accession number MG214652; *named as MAP3*), *Brevibacillus parabrevis* (Accession number MG214653; *named as MAP4*), *Rhizobium leguminosarum* (Accession number MG214656; *named as MAP7*), and *Pseudomonas hibiscicola* (Accession number MG21465; *named as MAP8*) were isolated from Vicia bean nodules. In addition, *Enterobacter cloacae* (Accession number MT012829; *named as DeltaPSK*) and *Bacillus paraflexus* (Accession number MT012831; *named as Delta YSK*) were isolated from *Lotus glaber* nodules, and *Pseudomonas aeruginosa* (Accession number MH580294; *named as ASA235*) was isolated from sea sediment. These isolates were used in this study were obtained from a previous study conducted in the Botany Department, Faculty of Science, Mansoura University.

Salt tolerance capacity: the isolates' salt tolerance was assessed by observing their capacity to grow on NA media supplemented with various amounts of NaCl: 0 mM, 150 mM, 430 mM, 860 mM, 1290 mM, 1720 mM, and 2000 mM. The cultures were incubated at 37 ± 2 °C for 2 days in a rotating shaker incubator at 150 rpm. Following incubation, the growth of bacteria was measured by spectrophotometry at 600n.m (Hmaeid *et al.*, 2019).

Plant growth-promoting criteria: The endophytic bacteria were evaluated *in vitro* for plant growth promotion with and without salt NaCl, like with indole 3-acetic acid (IAA) synthesis (Gordon and Weber, 1951), gibberellic acid (GA_3) production (Holbrook *et al.,* 1961), ammonia production (Cappuccino and Natalie, 2005).

Siderophores production was assessed by inoculating MM9 broth medium (15 ml) with the bacterial isolates. At 28 °C for two days. The content of siderophores was estimated using the formula below (Schwyn and Neilands, 1987):

$$\text{Siderophores units (\%)} = \frac{Ar - As}{Ar} x\ 100$$

Ar = Absorbance at 630 nm (CAS reagent)

As = Absorbance of the sample at 630 nm.

All endophytic bacterial applications can grow at various NaCl concentrations (1–11.62%).

Preparation of bacterial inoculum:

By comparing the bacterial inoculum concentration to a 0.5 Mc-Farland turbidity standard (1.5× 108 CFU/mL), the bacterial inoculum concentration was adjusted (Sandrasagaran *et al.*, 2014). Both bacterial inoculum production and seed bacterization were performed in accordance with Rajendra et al. (2006), with an inoculum concentration equal to an application rate of 10^6 CFU mL^{-1}. 100 mL of culture suspension was added to previously sterilized prepared broth medium for each endophytic bacterial strain and incubated overnight at 30 °C in a shaker incubator (100 rpm).

Experimental design

Soybean homogeneous seeds (*Glycine max* var. 'Giza11') were gained from the Agricultural Research Center, Giza, Egypt, and the experiments were conducted in the Botany Department, Faculty of Science, Mansoura University.

The seeds were surface-sterilized for 2-3 minutes with 4 % NaOCl; after carefully rinsing with sterile water, the embryo was hydro-primed for 8 hours to activate it. The broth containing a 10% sugar solution was used to inoculate the seeds. The seeds were thoroughly agitated until a fine coating developed on them. Under laboratory settings, inoculated seeds were put overnight for drying. At 25 ± 2 °C, seeds were then germinated in Petri dishes with three layers of filter paper Whatman 1. Germination tests were carried out following the International Seed Testing Association's rules (Matthews *et al.*, 2012). A NaCl solution of 0, 75, and 150 mM was sprayed on the seeds, and samples were collected from seedlings 10 days old. During the study, the ambient temperature, CO_2, and relative humidity levels were 18-22 °C, 300-410 μmol mol^{-1}, and 53-57 percent, respectively. In a completely randomized design (CRD), fifteen treatments with 10 repetitions were used:

The treatments were designed as follows upon the salt tolerance and plant growth-promoting criteria coming in results of the tested isolates:

The first group: seeds sprayed with tap water besides these treatments

T1: control with tap water only

T2*: B. Japonicum*

T3: *B. Japonicum + MAP3*

T4: *B. Japonicum + MAP8*

T5: *B. Japonicum + Delta PSK*

The second group: seeds sprayed with 75 mM NaCl

T6: (75 mM NaCl)

T7: *B. Japonicum*

T8: *B. Japonicum + MAP3*

T9: *B. Japonicum + MAP8*

T10: *B. Japonicum + Delta PSK*

The third group: seeds sprayed with 150 mM NaCl

T11: (150 mM NaCl)
T12: *B. Japonicum*
T13*: B. Japonicum + MAP3*
T14: *B. Japonicum + MAP8*
T15*: B. Japonicum + Delta PSK*

Estimation of germination parameters

Seedling length, seedling biomass (fresh and dry weight), and Germination percent (GP) were determined. Also, some indices were calculated as follows;

The mean germination time (MGT) was computed using (Matthews *et al.*, 2012).

$$\mathrm{MGT} = \frac{\sum \mathrm{F} \times \mathrm{X}}{\sum \mathrm{F}}$$

F: the number of newly germinated seeds at the time of X

X: the number of days elapsed since sowing.

Evaluations of Pick Value (PV), Mean Daily Germination (MDG), and germination Value (GV) Germination rate were calculated by the following equations (Reyhaneh *et al.*, 2013).

$$\mathrm{PV} = \frac{\text{Maximum germinated seed number at one day}}{\text{Day number}}$$

$$\mathrm{MDG} = \frac{\text{Germination\%}}{\text{Total experiment days}}$$

$$\mathrm{GV} = \mathrm{PV} \times \mathrm{MDG}$$

Germination rate (GR) was determined according to (Vashisth and Nagarajan, 2010):

Germination rate (GR) = (a/1)+(b-a/2)+(c-b/3)+.....+(n-n-1/N)

Where a, b, c, ...,n are numbers of germinated seeds after 1, 2, 3, ..., N days from the start of imbibition.

The seedling vigor's were calculated using (Maguire, 1962):

$$\text{Vigor index I} = \text{Germination\%} \times \text{Seedling length (cm)}$$

$$\text{Vigor index II} = \text{Germination\%} \times \text{Seedling weight (g)}$$

Estimation of membrane features

The membrane features determined here in fresh soybean seedlings contain electrolyte leakage (EL), membrane stability index (MSI), and lipid peroxidation (MDA).

Szalai *et al.* (1996) used to measure the seedling's electrolyte leakage (EL). First, the EC of shoot discs was measured after placing them in tubes containing 10 mL of boiling distilled water (EC1). Next, the EC was determined after heating the tubes in a water bath for 30 minutes at 45-55 °C (EC2). The sample was then boiled for 10 minutes at 100 °C to measure the EC (EC3).

The following formula was used to calculate EL:

$$\text{Electrolyte leakage (\%)} = \frac{\mathrm{EC2} - \mathrm{EC1}}{\mathrm{EC3}} x\ 100$$

Sairam (1994) calculated the membrane stability index (MSI) utilizing 200 mg of shoot tissue in a test tube containing 10 mL distilled water in two sets. The first set was heated on boiling water for 30 minutes at 40 °C, and the conductivity bridge measured the solution's electrical conductivity (C1). The second batch was heated for 10 minutes in 100 °C boiling water, and its conductivity was also defined (C2).

The following formula was used to calculate the MSI:

MSI %={1-(C1/C2)}×100

Hernández and Almansa (2002) defined malondialdehyde (MDA) to assess and quantify lipid peroxidation products. First, one gram of the shoot was soaked in 5 ml of 0.1 percent trichloroacetic acid (TCA) and centrifuged for 5 minutes at 10,000 rpm. Then, 4 ml of 20 percentage TCA including 0.5 percentage thiobarbituric acid was put to each ml of supernatant, then incubated for 30 minutes at 95°C,

centrifuged again, and the absorbance was measured at 532 and 600 nm. Using an extinction coefficient of 155×10-3 M^{-1} cm^{-1}.

The concentration of MDA is expressed in mol g^{-1} F wt

Determination of oxidative damage marker

A known weight of plant shoot was homogenized in 1% (w/v) trichloroacetic acid (TCA) and centrifuged for 15 minutes at 12,000 rpm to measure hydrogen peroxide (H_2O_2). 0.5 mL of the supernatant was combined with 0.5 mL of 100 mM K phosphate buffer (pH 7.0) and 2 mL reagent (1 M KI w/v in distilled water H_2O). In the absence of seedling extract, the blank probe comprised of 1% TCA. The reaction was let to develop in the dark for 1 hour, and the absorbance was assessed at 390 nm. With a molar extinction value of 0.28 mol cm^{-1}, the amount of hydrogen peroxide was estimated (Alexieva *et al.*, 2001).

To directly express osmotic pressure, the Electrical conductivity (EC) of plant-water extracts was assessed. For proline determination, 1 mL of extract, ninhydrin reagent, and glacial acetic acid were reacted in a hot water bath for an hour, and 1 mL of the acid was added, accompanied by chilling, increasing to 5 mL with the acid, then reading at 510 nm (Bates *et al.*, 1973). For citric acid determination, 15 Ml of a de-proteinizing solution (3 g of each of $ZnSO_4$ with $HgCl_2$ in 100 ml of water) was incorporated into 5 mL of the extract and remaining overnight before filtering. Thus, 4 mL of 10 N HCl with one mL of 6.2 percent $FeCl_3$ were combined, and the absorbance at 445 nm was measured (Kolthoff and Sandell, 1948). Total soluble sugars were calculated by reacting 3 ml of anthrone reagent and 0.1 ml of seedling extract for 10 minutes, then measured the samples were at 625 nm (Irigoyen *et al.*, 1992).

Estimation of antioxidant enzymes activity

Antioxidant enzyme activity was measured in extracts produced by chilling 2 g of fresh tissue with 20 ml of 0.1 M phosphate buffer and centrifuge cooling for 20 minutes at 10,000 rpm. In addition, catalase, peroxidase, and superoxide dismutase were extracted using a pH 6.8 buffer (Agarwal and Shaheen, 2007).

Catalase (CAT) activity was measured in 3 mL of a reaction mixture comprising potassium phosphate buffer, pH 7.0, 11 mM H_2O_2, at 25 °C. UV spectrophotometry at 240 nm was used to assess activity by monitoring the time required for a drop-in from 0.45 to 0.40 (Mishra *et al.*, 1993*)*. Peroxidase (POX.) assay: 3 mL of 0.05 M pyrogallol were combined with 0.1 mL of extract and 0.5 mL of 1% H_2O_2, and the rise in absorbance at 420 nm was measured (ADAMS, 1978). Polyphenol oxidase (PPO), The rise in absorbance at 420 nm was measured after mixing two ml of 0.02 M phosphate buffer at pH7, one ml of 0.05 M pyrogallol, and one ml of the extract (Oktay *et al.*, 1995).

Statistical analysis

The study used a completely randomized design (CRD) with fifteen treatments and ten replicates. Thus statistical analysis was done using the COSTAT software (798 Lighthouse Ave. PMB 329, Monterey, CA, 93940, USA) (Gomez and Gomez, 1984). The two-way ANOVA using Fisher's at a 95% confidence level. XLSTAT Version 2016 was used to conduct Principal Component Analysis (PCA). Unfortunately, graph prism (version 9.1.1) drowned the graphs.

Results

Salt tolerance capacity

The salinity tolerance of the eight isolates was determined by evaluating their potential to grow in the presence of different concentrations of NaCl. As illustrated in Figure 1, results showed a strong negative relation between salt concentrations of media & bacterial growth. First, there was a gradual decrease in the bacterial growth, accompanied by an increase of NaCl concentration until the maximum concentration of 860 mM of salt peaked. Then, the growth decreased sharply. Showing that osmo-adaptive bacteria reached the

maximum available tolerance against the salt concentration of the media *in MAP3, MAP8, DeltaPSK, B. japonicum, and ASA235. MAP7* and *DeltaYSK,* the maximum concentration was 430mM, while in MAP4, the maximum concentration was 150 mM (Figure 1).

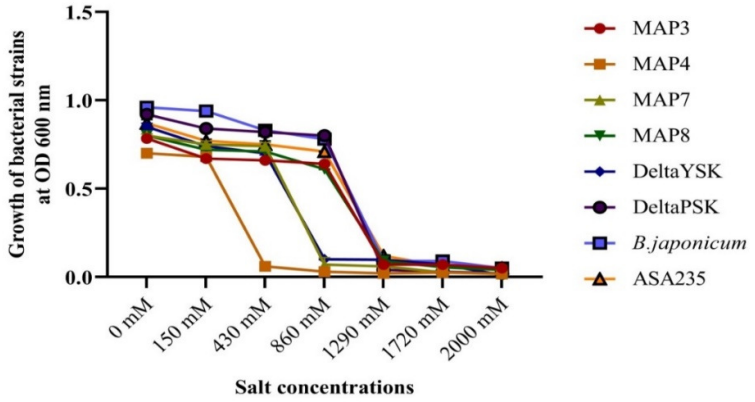

Figure 1. Effect of different salt concentrations (0, 0.87, 2.5, 5, 7.5, 10 and 11.62%) on growth of bacterial strains at OD600 nm after 48 hrs

Plant growth-promoting criteria of bacterial strain

Of all 8 isolates, 4 were effective endophytic bacteria: *MAP3, MAP8, Delta PSK, and B. japonicum* showed the most potent result. The total amounts of IAA produced without salt were 88.3, 82.1, 131.5, and 110.2 μg mL^{-1} and with salt were 71.3, 69.1, 94.1, and 85.4 μg mL^{-1} for *MAP3, MAP8, Delta PSK, and B. japonicum*, respectively, with GA_3 production amounts around 740.5, 783.1, 930.7, and 855.4 μg mL^{-1} without salt, and 602.4, 608.1, 701.2 and 654.1 μg mL^{-1} with salt.

In addition, these four isolates could produce ammonia production by 27.5, 26.7, 38.8, and 18.4 mg/ml and 19, 14.3, 16.4, and 18.1 mg/ml without and with salt respectively, and produced siderophores by 53.7, 46.4, 81.6, and 64.4 units and 45, 32.9, 68 and 44.8 units without and with salt respectively (Table 1).

Table 1. plant growth-promoting criteria of bacterial strains

Bacterial strains	IAA (μg/ml)		GA_3 (μg/ml)		Ammonia production (mg/ml)		Siderophores (units)	
	0 mM	150 mM	0 mM	150 mM	0 mM	150 mM	0 mM	150 mM
MAP3	88.3±1.3c	71.3±0.98c	740.5±0.9d	602.4±1.8d	27.5±1.2^{b}	19±1.8^{b}	53.7±0.3^{d}	45±1.2^{de}
MAP4	65.5±1.2d	40.4±1.3d	722.9±0.8e	542.6±1.2e	15.3±1.8^{c}	9.7±0.2^{c}	47.4±1.6^{c}	33.2±0.9^{e}
MAP7	77.8±0.5c	32.4±1.3e	630.6±1.6f	471.1±1.2f	15.2±0.8^{c}	9.2±0.8^{c}	84.3±0.8^{b}	48±1.3^{d}
MAP8	82.1±0.98c	69.1±1.03c	783.1±1.2c	608.1±1.5c	26.7±1^{b}	14.3±0.7^{b}	46.4±0.5^{c}	32.9±1.7^{c}
DeltaYSK	24.3±1.6f	9.16±1.4g	606.7±1.5f	447.4±1.5f	9.2±0.9^{d}	6.1±0.6^{d}	26.4±1.7^{f}	16.6±1.2^{f}
DeltaPSK	131.5±1.1a	94.1±1.43a	930.7±1.5a	701.2±1.5a	38.8±1^{a}	16.4±0.1^{ab}	81.6±0.9^{b}	68±1.1^{a}
B. japonicum	110.2±1.5b	85.4±1.6b	855.4±1.4b	654.1±1.8b	18.4±0.3^{ab}	18.1±0.4^{a}	64.4±0.6^{c}	44.8±1.4^{de}
ASA235	29.1±1.8e	22.6±0.8f	582.6±1.5g	294.6±1.6g	13±0.96^{c}	8.8±0.5^{c}	95.1±0.8^{a}	56.1±1.70^{a}

The means (±SE) accompanied by a different letter in each column indicate significantly different p-value < 0.05 by the Fisher test.

Germination and morphological criteria

Figures (2a–e) demonstrate some of the germination parameters of soybean plants, such as germination %, MGT, and plant morphology, such as seedling length and seedlings' fresh and dry weight, after being treated with NaCl along with different bacterial treatments with 75 or 150 mM NaCl, which significantly reduced germination and morphological criteria. Germination% and MGT reduced by 14.27%, 44.10%, 11.14%, and

41.98%, respectively, when grown under 75 and 150 mM NaCl respectively. As seeds were sprayed with 75 and 150 mM of NaCl, seedling length fell by approximately 25.92% and 40.95%, respectively, comparing to non-salt plants. The treatment with endophytic bacteria (*B. japonicum, B. japonicum + MAP3, B. japonicum + MAP8, and B. japonicum + Delta PSK*) significantly increased germination and morphological criteria in salt-stressed plants compared to non-stressed plants. The most noticeable rises were observed in plants applied with *B. japonicum + Delta PSK* (T5), in which shoot length increased by t 34.98%, 51.52% for 75, and 150 mM for NaCl, respectively. With an increase in NaCl content, the seedling's fresh and dry weight decreased. So, when soybean plants were sprayed with 75 or 150 mM NaCl solutions, the fresh and dry weight of seedlings reduced by 47.62%, 71.43%, 45.16%, and 70.97%, respectively, whereas treatment with *B. japonicum + Delta PSK* showed increased fresh and dry weight of seedlings by 992.73%, 116.67%, 70.59%, and 200% respectively (Figure 2 d,e).

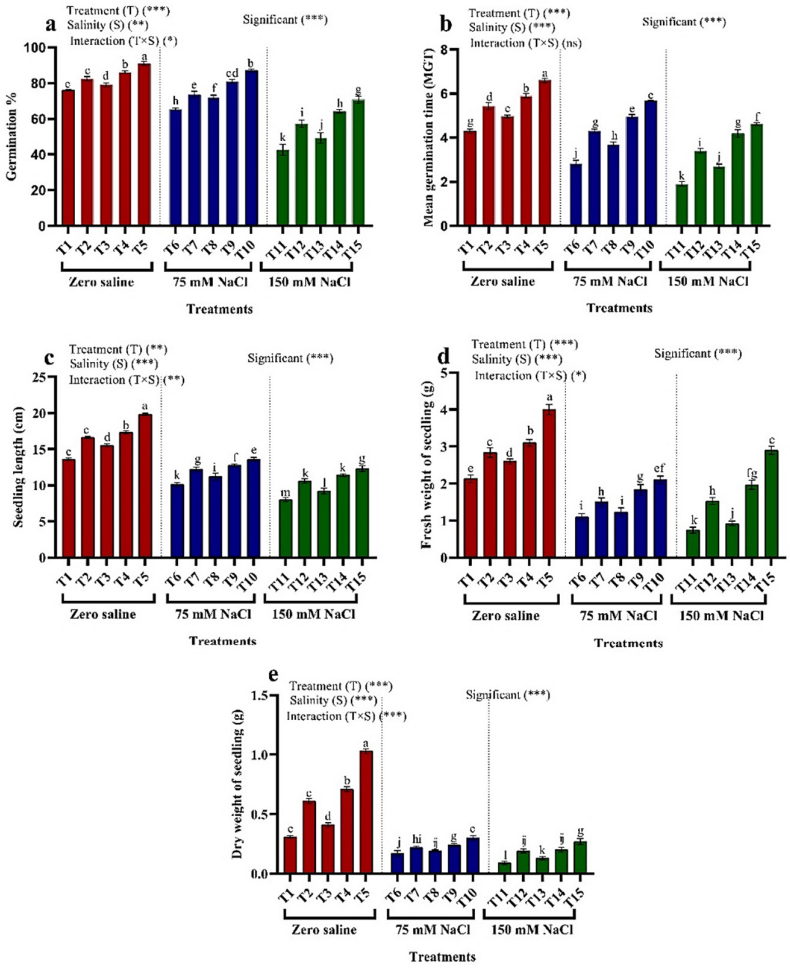

Figure 2. Effect of endophytic bacteria treated on soybean plant germination and morphological criteria under NaCl stress (75, 150 mM)

T1 = control with tap water only; T2 = *B. Japonicum*; T3 = *B. Japonicum + MAP3*; T4 = *B. Japonicum + MAP8*; T5 = *B. Japonicum + Delta PSK*; T6 = 75 mM NaCl; T7 = *B. Japonicum* + 75 mM NaCl; T8 = *B. Japonicum + MAP3* + 75 mM NaCl; T9 = *B. Japonicum + MAP8* + 75 mM NaCl; T10 = *B. Japonicum + Delta PSK* + 75 mM NaCl; T11 = 150 mM NaCl; T12 = *B. Japonicum* + 150 mM NaCl; T13 = *B. Japonicum + MAP3* + 150 mM NaCl; T14 = *B. Japonicum + MAP8*+ 150 mM NaCl; T15 = *B. Japonicum + Delta PSK*+ 150 mM NaCl. The Fisher test revealed different letters differ significantly from each bar (p-value < 0.05). ns denotes not significant; *, **, and *** denote (p-value < 0.01).

The positive impact of endophytic bacteria; *B. japonicum, B. japonicum + MAP3, B. japonicum + MAP8, and B. japonicum + Delta PSK* on soybean seedling growth under saline water (75, 150 mM NaCl) was determined by measuring the PV, MDG, GV, GR, Vigor index I, Vigor index II and the results are shown in Figure (3). The PV, MDG, GV, GR Vigor index I, Vigor index II of soybean seedlings exposed to salty conditions were significantly reduced. When compared to control plants, *B. japonicum + Delta PSK* significantly improved the seedling's PV (37.22%), MDG (53.49%), GV (164.89%), GR (35.66%), Vigor index I (79.69%), Vigor index II (311.27%).

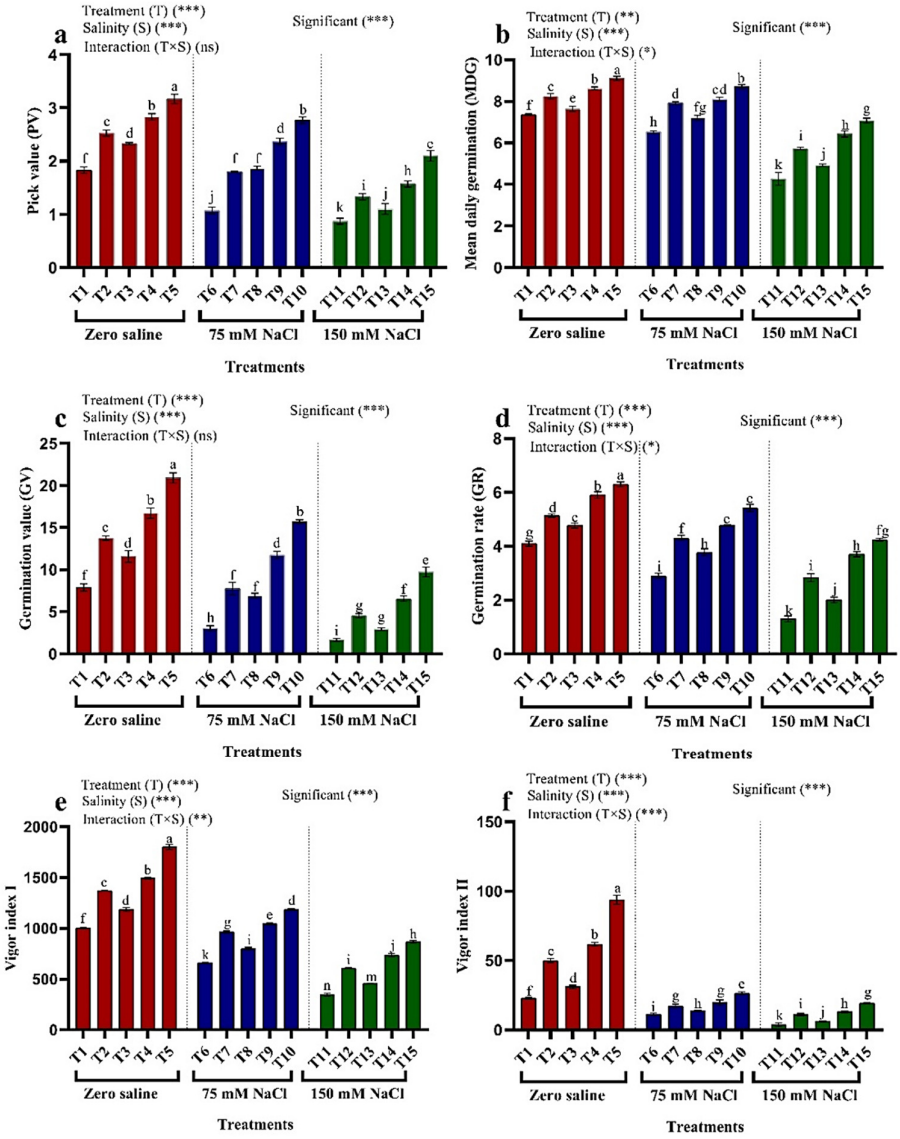

Figure 3. Effect of endophytic bacteria treated on PV, MDG, GV, GR, Vigor index I, Vigor index II of soybean plant under NaCl stress (75, 150 mM)

T1 = control with tap water only; T2 = *B. Japonicum*; T3 = *B. Japonicum + MAP3*; T4 = *B. Japonicum + MAP8*; T5 = *B. Japonicum + Delta PSK*; T6 = 75 mM NaCl; T7 = *B. Japonicum* + 75 mM NaCl; T8 = *B. Japonicum + MAP3* + 75 mM NaCl; T9 = *B. Japonicum + MAP8* + 75 mM NaCl; T10 = *B. Japonicum + Delta PSK* + 75 mM NaCl; T11 = 150 mM NaCl; T12 = *B. Japonicum* + 150 mM NaCl; T13 = *B. Japonicum + MAP3* + 150 mM NaCl; T14 = *B. Japonicum + MAP8*+ 150 mM NaCl; T15 = *B. Japonicum + Delta PSK*+ 150 mM NaCl. The Fisher test revealed different letters differ significantly from each bar (p-value < 0.05). ns denotes not significant; *, **, and *** denote (p-value < 0.01).

Estimation of membrane features

The electrolyte leakage (EL) significantly increased in seedlings under NaCl stress compared with non-saline plants but showed a significant ($P < 0.05$) decrease when seedling was treated with *B. japonicum + Delta PSK* under NaCl stress (Figure 4 a). However, NaCl stress caused a significant rise in MDA content in seedlings compared with control plants. Inoculating *B. japonicum + Delta PSK* with NaCl significantly ($P < 0.05$) decreased the MDA content. *B. japonicum + Delta PSK* inoculation improved MSI in soybean plants during favorable control conditions compared with saline and non-saline conditions. salinity reduced MDA (23.72%) for 75 mM NaCl and (46.73%) for 75 mM NaCl (Figure 4 b).

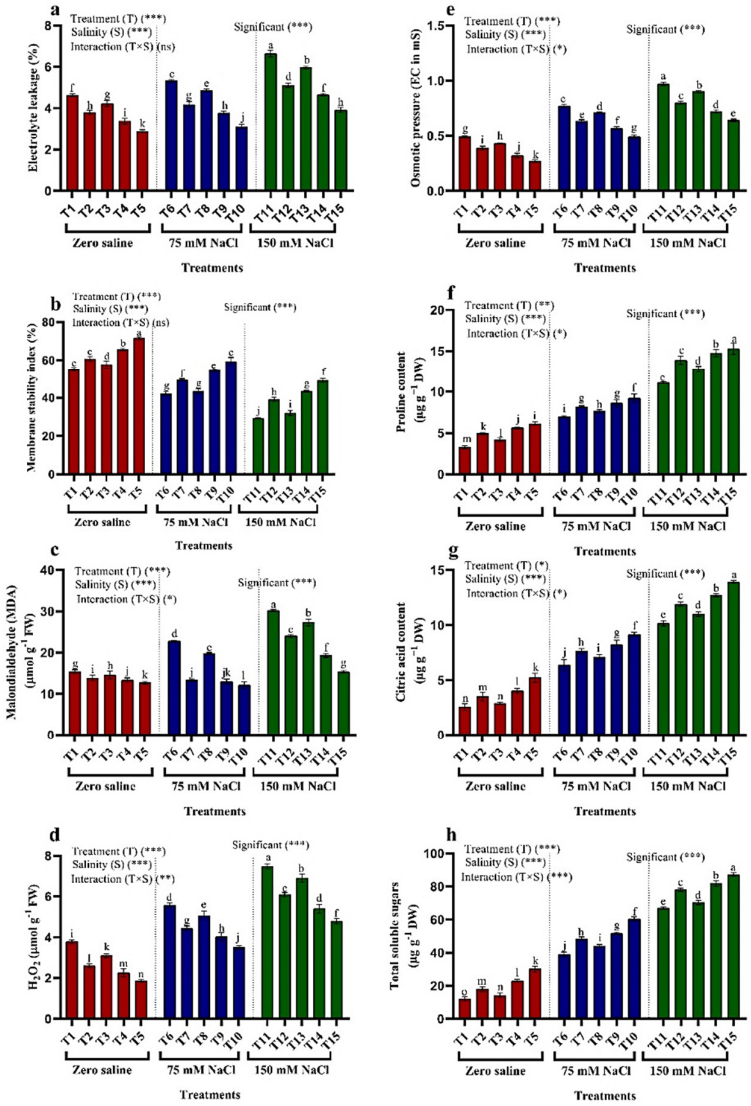

Figure 4. Effect of endophytic bacteria treated on membrane features and oxidative damage marker of soybean plant under NaCl stress (75, 150 mM)

T1 = control with tap water only; T2 = *B. Japonicum*; T3 = *B. Japonicum + MAP3*; T4 = *B. Japonicum + MAP8*; T5 = *B. Japonicum + Delta PSK*; T6 = 75 mM NaCl; T7 = *B. Japonicum* + 75 mM NaCl; T8 = *B. Japonicum + MAP3* + 75 mM NaCl; T9 = *B. Japonicum + MAP8* + 75 mM NaCl; T10 = *B. Japonicum + Delta PSK* + 75 mM NaCl; T11 = 150 mM NaCl; T12 = *B. Japonicum* + 150 mM NaCl; T13 = *B. Japonicum + MAP3* + 150 mM NaCl; T14 = *B. Japonicum + MAP8*+ 150 mM NaCl; T15 = *B. Japonicum + Delta PSK*+ 150 mM NaCl. The Fisher test revealed different letters differ significantly from each bar (p-value < 0.05). ns denotes not significant; *, **, and *** denote (p-value < 0.01).

Oxidative damage marker

One of the harmful effects of salt stress is oxidative damage to cell membranes. To examine the role of endophytic bacteria in oxidative damage, we next measured the reactive oxygen species production in soybean seedling sprayed with NaCl (75, 150 mM). Under salt stress (75, 150 mM) the H_2O_2 (46.97%, 97.36%,), osmatic pressure (57.14%, 97.96%,), proline (114.64%, 241.54%,), citric acid (148.83%, 296.88%,) and total soluble sugar contents (225%, 458.3%,) were increased compared to 75, 150 mM NaCl, respectively (Figure 4 d-h). Meanwhile, treatment with *B. japonicum + Delta PSK* significantly decreased the H_2O_2 content and osmatic pressure, while treatment *with B. japonicum + Delta PSK* significantly increased the proline, citric acid, and total soluble sugar contents as compared to salt stress.

Antioxidant enzymes activity

Under salt stress and non-stress circumstances, the activities of antioxidant enzymes were evaluated to assess the positive impact of endophytic bacteria; *B. japonicum, B. japonicum + MAP3, B. japonicum + MAP8, and B. japonicum + Delta PSK* (Figure 5). Furthermore, the impact of endophytic bacteria under saline or non-saline stress increased the activities of antioxidant enzymes such as CAT, POX, and PPO in plants.

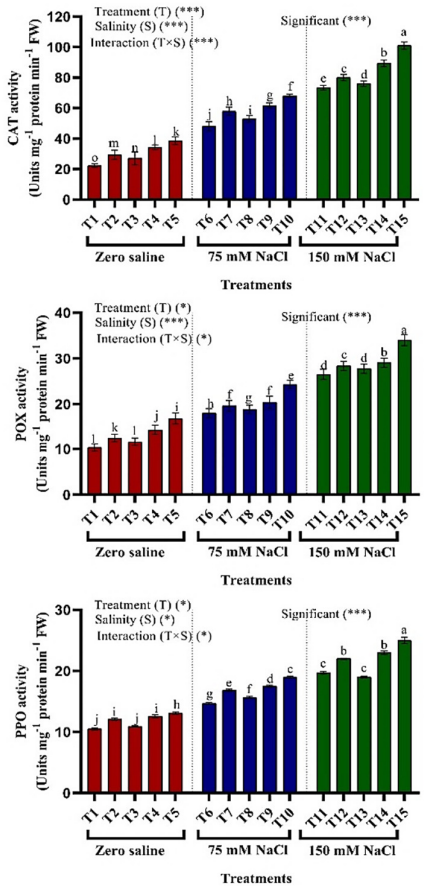

Figure 5. Effect of endophytic bacteria treated on the antioxidant enzyme of soybean plant under NaCl stress (75, 150 mM)

T1 = control with tap water only; T2 = *B. Japonicum*; T3 = *B. Japonicum + MAP3*; T4 = *B. Japonicum + MAP8*; T5 = *B. Japonicum + Delta PSK*; T6 = 75 mM NaCl; T7 = *B. Japonicum* + 75 mM NaCl; T8 = *B. Japonicum + MAP3* + 75 mM NaCl; T9 = *B. Japonicum + MAP8* + 75 mM NaCl; T10 = *B. Japonicum + Delta PSK* + 75 mM NaCl; T11 = 150 mM NaCl; T12 = *B. Japonicum* + 150 mM NaCl; T13 = *B. Japonicum + MAP3* + 150 mM NaCl; T14 = *B. Japonicum + MAP8*+ 150 mM NaCl; T15 = *B. Japonicum + Delta PSK*+ 150 mM NaCl. The Fisher test revealed different letters differ significantly from each bar (p-value < 0.05). *, and *** denote (p-value < 0.01)

Principal component analysis (PCA)

To explain the multi-factorial impacts of our treatments on all variables (germination growth, enzymatic and nonenzymatic oxidants) of soybean plants under NaCl stress and non-saline conditions, principal component analysis (PCA) was used (Figure 6). The cross-validation technique requires the use of two major components to describe the variation of the studied features. The two factors (PC1 and PC2) were derived from the eigenvalues of the covariance matrix. They account for 90.37% of data variability (75.61% and 14.76% for PC1 and PC2, respectively).

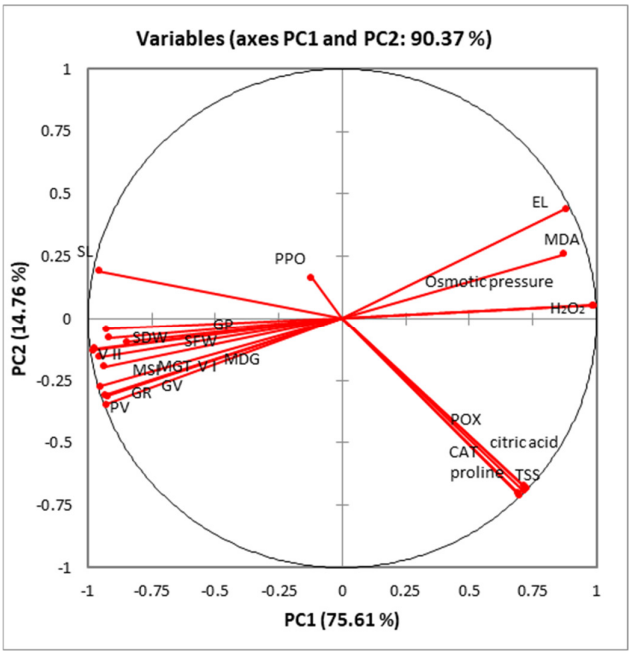

Figure 6. Principal component analysis was used to understand variable treatment relationships in soybean plants better

The variables contain SFW (fresh shoot weight), SDW (shoot dry weight), SL (shoot length), MGT (mean Germination time), PV (Pick Value), GV (Germination Value), MDG (Mean Daily Germination), GR (Germination rate), VI (Vigor index I), MDA (malondialdehyde), H2O2 (hydrogen peroxide), EL (electrolyte leakage), PPO (Polyphenol oxidase), CAT (catalase), POX (peroxidase) and TSS (total soluble sugar).

Discussion

Salt is one of the fundamental soil degradation issues and caused crop production loss (Sofy *et al.*, 2021c). On the other hand, the preventive effect of salinity on plant improvement maybe because of the excessive osmotic potential, ion toxicity, the close of stomata, which reduces CO_2 absorption and limits photosynthetic apparatus *(Mbarki et al.*, 2018). Therefore, substituting salinity-responsible ions is deemed worthy of improving saline soils, either synthetically or by incorporating microbial communities or carbon-based substances (Ilangumaran and Smith, 2017).

Endophytic bacteria may solubilize micro and macro elements for plants and enhance macronutrient mobilization and absorption by complex solubilizing compounds in the soil and transferring them to their plant (Etesami, 2020). The utilization of microbes in NaCl-polluted soil is an ecologically friendly way to decontaminate the soil without changing its properties. Absorption and/or precipitation by a variety of microorganisms may decontaminate NaCl from the soil. Using soil organisms in severe environments to avoid mutant microbes may be viable (Etesami and Alikhani, 2019).

Under both unstressed and salt-stressed conditions, treatment with endophytic bacteria; *B. japonicum, B. japonicum + MAP3, B. japonicum + MAP8, and B. japonicum + Delta PSK* increased seedling germination and morphological criteria (germination percent, MGT, Seedling length, fresh and dry weight of seedling, PV, MDG, GV, GR, Vigor (Figures 2,3).

Previous research (Yadav *et al.*, 2018; Alraey *et al.*, 2019; Nawaz *et al.*, 2020) showed many bacterial sp. from the genera Pseudomonas, Exiguobacteriumd Bacillus play an essential role in plant growth promotion under growth-limiting circumstances.

These results are in line with those of Sofy *et al.* (2021d), who discovered that salt stress reduced the fresh and dry shoot weight as well as the root weight of pea plants. However, bacterial treatments have a significant increase in the root, shoot lengths, fresh, dry root, and shoot weights under salinity conditions. Yasmin *et al.* (2020) also demonstrated that application with PGPB caused a highly significant increase in morphological criteria of soybean plants under salt stress. Valenzuela-Aragon *et al.* (2019) found co-inoculation of wheat plants with Enterobacter cloacae, the plant growth-promoting bacteria, has increased morphological criteria.

Due to osmotic stress and ion toxicity, salinity dramatically changes plant biochemical and physiological consequences, resulting in a severe decrease in root water uptake (Sofy *et al.,* 2020b).

MDA and electrolyte leakage are the most frequently used parameters to evaluate the degree of salt damage and determine plant salt tolerance (Mansour *et al.*, 2020). In addition, EL and MDA showed a dose-response relationship with NaCl in the current research, as EL and MDA increase when the NaCl levels increase (Figure 4). On the contrary, plants treated with *B. japonicum + Delta PSK* depicted a reduction in EL and MDA. Cen *et al.* (2020) found a comparable decrease in EL and MDA in alfalfa plants due to salt exposure. Thus, endophytic bacteria reduced the harmful effects of salt stress on maize by increasing plant tolerance to NaCl, as shown by lower EL and MDA levels (Li *et al.*, 2020). However, Mahmoud *et al.* (2020); Sofy *et al.* (2020c) suggested that high EL could signify high potassium content in the plant. Our findings stated that an increase in EL % primarily owes cell membrane injury problems due to excess oxidative stress caused by NaCl. A further source of evidence for our theory, the EL% is the significant decrease in MSI (Figure 4 a,b), which shows a dose-response relationship to salinity as MSI reduced to its lowest recorded value at 150 mM NaCl. On the other hand, higher MSI values were observed in plants that received endophytic bacteria, particularly in *B. japonicum + Delta PSK,* which exhibited a significant increase in MSI and membrane integrity.

Extensive reactive oxygen species (ROS) development under salinity stress is like other stressful circumstances. Considerable amounts of oxidative biomarkers like H_2O_2 were significantly increased under environmental stresses and used as a positive parameter to evaluate plants' tolerance to saline stress (Desoky *et al.*, 2020; Sofy *et al.*, 2021a). This study showed that all oxidative biomarkers were relatively low by inoculation of endophytic bacteria concerning the levels reported in salt-stressed plants. The current study's findings prove the observational data reported recently (Gupta and Pandey, 2020). From a physiological perspective, the increased prevalence of H_2O_2 content in soybean plants has been related to increased plasma membrane destruction and dehydration cytoplasm resulting from severe salinity stress. H_2O_2 is responsible for the increased solute degradation related to oxidative stress (Silva *et al.*, 2020; Mohamed *et al.*, 2012,2016). Lastochkina *et al.* (2020) noted the pattern of clarification close to our observation. Dual inoculation with *B. japonicum and Delta PSK* can be regarded as a promising source for preserving the integrity of the plasma membrane and regulating water intake, and increasing water use efficiency, thus minimizing oxidative stress (Harman *et al.*, 2019). Egamberdieva *et al.* (2017) state that it may benefit from *B. japonicum + Pseudomonas putida* to increase water availability and decrease soil salinity. The potential benefit of *B. japonicum* is increased crop yield, and biomass production in bean plants under salt stress conditions decreased the quantity of $O_2^{\bullet -}$, MDA, and H_2O_2 (Meena *et al.*, 2020; El-Beltagi *et al.*, 2019).

Lastochkina (2019) reported that PGPB was proposed to reduce MDA to a minimum level to counteract salinity conditions. Proline reduces the detrimental effects of salinity by mediating ROS scavenging to maintain proteins and other essential biomolecular structures, in addition to the crucial function of proline,

citric acid, and total soluble sugar levels in the maintenance of cell water balance (Abu-Shahba *et al.*, 2021). Other studies support the proline synthesis observed on the PGPR-inoculated plants in this study. For example, Mowafy *et al.* (2021) found that PGPR enhanced proline aggregation, resulting in better water absorption, water quality, and photosynthetic efficiency in maize plants.

ROS molecules must be detoxified to reduce injury under stressful conditions. In addition, the efficient destruction of ROS requires the coordination of many antioxidant enzymes (Sofy *et al.*, 2021b). Multiple scavenging enzymes, like CAT, POX, and PPO, reduce the impact of ROS. On the other hand, many antioxidant enzymes are fully dedicated to maintaining ROS homeostasis, while others are involved in growth, redox control of target proteins, and detoxification processes (El-Beltagi *et al.*, 2018; El-Beltagi *et al.*, 2020). Under salt stress, the activity of antioxidant enzymes including CAT, POX, and PPO significantly increased, helping to control ROS accumulation (Figure 5). High levels of antioxidant activity have been observed in plants under stress irrigated with 150 mM NaCl alone or in combination with endophytic bacteria; *B. japonicum, B. japonicum + MAP3, B. japonicum + MAP8, and B. japonicum + Delta PSK* compared to control plants and salt-stressed plants. Increasing enzyme activity in stressed soybean plants is an effective strategy for coping with the harmful effects of salt stress and scavenging reactive oxygen species that cause oxidative stress in plants. The role of PGPB in alleviating salt stress could be attributable to an auxiliary mechanism (Zhang *et al.*, 2019). Antioxidant enzymes increase, implying that more and more ROS is needed, and such ROS effects are decreased. CAT plays an essential role in the elimination of H_2O_2 from the cell's various organelles. POX is also engaged in H_2O_2 scavenging and plays an essential function in stress tolerance (Sharaf *et al.*, 2009; Mohamed *et al.*, 2018). ROS-scavenging enzymes like CAT and PPO were significantly increased in different crops inoculated by PGP microbial isolates in saline conditions (Hmaeid *et al.*, 2019).

Conclusions

Endophytic bacteria are recognized for being osmoregulated, its accumulation in plant tissues, on the other hand, depends on plant species and agriculture methods. This protection is closely linked to improving germination and morphological criteria, MSI, MDA osmolytes, by reducing oxidative stress caused by ROS and regulating the activity of antioxidant enzymes (CAT, POX, and PPO). In addition, the inoculation of B. *japonicum + Delta PSK* in NaCl may increase the tolerance of plants to salt stress by reducing the oxidative damage caused by ROS production. However, the results appear that the dual inoculation of *B. japonicum and Delta PSK* is more effective than others. This result paved the way for the use of such a combination in a pot experiment to verify its potentiality to overcome salinity stress on soybean yield.

Authors' Contributions

Conceptualization: MSA, MAA, SAH and AMM; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization: MSA, MAA, SAH and AMM; Writing - original draft; Writing - review and editing. MSA, MAA, SAH, MRS and AMM. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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