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Bacterial-Mediated Induction of Systemic Tolerance to Salinity with Expression of Stress Alleviating Enzymes in Soybean (*Glycine max* L. Merrill)

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Abstract This study explored several features related to salt tolerance in soybean plants through plant growth-promoting rhizobacteria (PGPR; *Pseudomonas* sp. strain AK-1, and *Bacillus* sp. strain SJ-5). We report the significant effect of 1-aminocyclopropane-1-carboxylate deaminase, indole-3-acetic acid production and exopolysaccharide production from both bacterial strains on physical parameters and biochemical activities in *Glycine max* plants under salt stress. In this report, we investigated the leaf water content, osmolyte accumulation, and activities of stress-responsive enzymes in the absence and presence of salt stress. Control (plants devoid of bacterial strains) and PGPR-inoculated soybean plants were grown in half Murashige and Skoog medium subjected to saline and non-saline conditions. Results showed that PGPR-inoculated plants had superior tolerance against salt stress, as shown by their enhanced plant biomass (fresh weight), higher water content, higher photosynthesis activity, and lower osmotic stress injury. The increased proline accumulation and lipoxygenase activity in PGPR-inoculated plant roots contributed to increased plant tolerance to salt stress. SJ-5-inoculated plants (0.414 U/mg protein) and AK-1-inoculated plants (0.403 U/mg protein) showed higher LOX activity than control plants (0.366 U/mg protein). Proline content was higher in SJ-5-(120 μg/g f.w.) and AK-1-(135 μg/g f.w.)inoculated plants than control plants (90 μg/g f.w.). Peroxidase activity was also higher in PGPR-inoculated plant roots during salinity. These results suggest that, in PGPR-inoculated roots, lipoxygenase plays a role in mitigating the adverse effect of salt stress. Furthermore, enhanced proline maintains osmotic balance and a positive water potential for water entrance into the roots, and peroxidase enzyme reduces oxidative damage by lowering reactive oxygen species level under salt stress. Our results indicated that both *Pseudomonas* and *Bacillus* are multifunctional PGPR strains that can promote plant growth, development and reduce salinity stress. However, our *Bacillus* bacterium strain had more ACC deaminase, phosphate solubilization, and siderophore activity under salt stress as compared to the *Pseudomonas* strain.

Keywords Antioxidant enzymes · *Bacillus* · Native-PAGE · *Pseudomonas* · Salinity

Introduction

Crops grown in arid and semi-arid regions are frequently exposed by adverse abiotic environmental factors such as drought and high soil salinity. In these crops, legume plants, mostly soybeans, are highly affected by low levels of salinity. However, when this plant is inoculated with an arbuscular mycorrhizal (AM) fungus, the extent of growth suppression decreased, which helped plants to survive under adverse conditions (Auge and others 1992; Porcel and others 2004; Sharifi and others 2007). On the other hand, few reports are available on soybean with PGPR inoculation, which showed increased plant biomass, shoot, root length, and osmolytes with successful alleviation of salt

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stress in soybean plants (Han and Lee 2005; Naz and others 2009). Excess salinity in the soil, due to the high concentrations of Cl^- and Na^+ ions affects plant systems and decreases yield and crop quality (Mahajan and Tuteja 2005). Soil salinity induces production of reactive oxygen species (ROS) such as superoxide anion (O_2^-), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2) causing oxidative stress resulting in cellular damage to the plant. In response to oxidative stress, plants accumulate osmolytes (proline, glycinebetaine, sugars, and so on), proteins, antioxidative enzymes including superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POX, EC 1.11.1.7), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), and some defense enzymes such as polyphenol oxidase (PPO) and lipoxygenases (LOXs; EC 1.13.11.12). Peroxidase has a major importance in reducing oxidative stress and in defense mechanisms. It has higher affinity for H_2O_2 than catalase (CAT), making it an efficient scavenger of H_2O_2 in stressful conditions. Various POD isoforms have been found at different subcellular locations in plants. These are cytosolic, stromal, thylakoidal, mitochondrial, and peroxisomal isoforms. H_2O_2 generated in the intercellular space of the plant during stress and first diffuses into the cytosol where cytosolic POD scavenges it and only then diffuses into the peroxisome where CAT works as a scavenger. Therefore, peroxidase has a higher ability to protect plants against oxidative stress as compared with CAT (Lee and Lee 2000). Peroxidase is also involved in a wide range of metabolic processes such as lignin biosynthesis, degradation of IAA and wound healing (Sharma and others 2012). POD is reported to be enhanced by salinity as well as water stress, and this was positively correlated with stress tolerance (Khedr and others 2003; Banu and others 2009). SOD is the first defense enzyme that catalyzes the dismutation of O_2^- to H_2O_2 and O_2. PPO oxidizes phenolic compounds such as caffeic acid and catechol to their respective quinones. These quinones produce brown pigments by cross-linking with proteins, leading to damaged plant tissues. Based on the browning reactions, PPO has mainly been suggested as a defense enzyme against pests and pathogens (Mayer 2006). Proline (Pro) is the most common osmolyte accumulating in plant tissues during adverse conditions. It protects the plant in different ways including osmotic adjustment, stabilizing cellular structure, scavenging hydroxyl radicals, and reducing damage to the photosynthetic apparatus (Kishor and others 1995). LOX initiates the degradation of free fatty acids and esterifies lipids and produces an unsaturated fatty acid hydroperoxide. These hydroperoxides are further converted into a group of products such as oxylipins, jasmonic acid (JA), and methyl jasmonate (MeJA). Oxylipins are involved in a wide range of important physiological functions, such as growth, fertility, signal transduction, and biotic or abiotic stress response. In some reports, JAs and MeJAs have been shown to regulate interactions between roots and bacteria (Hause and Schaarschmidt 2009).

One of the most recently promising areas of research for future studies of counteracting the adverse effects of salinity on plant growth includes the implementation of salt-tolerant bacteria with growth-promoting abilities in such stress conditions. Plant growth-promoting rhizobacteria (PGPR) are a group of bacteria that colonize roots and enhance the growth of plants under salt stress (Mayak and others 2004a), drought stress (Vardharajula and others 2011), nutrient deficiency, and heavy metal contaminations (Sheng and others 2008). PGPR promote plant growth by enhancing the efficiency of water uptake and nutrients and maintaining a K^+/Na^+ ratio under salt stress (Mayak and others 2004a). PGPR-inoculated plants enhanced lateral root development due to higher indole-3-acetic acid (IAA) production, and reduced stress ethylene levels in plants by the production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase enzyme activity (Senthil and others 2009; Glick and others 2007).

Strains with plant growth-promoting activity have been identified from various genera of which *Pseudomonas* and *Bacillus* are the most extensively studied (Kumar and others 2011; Choudhary 2012; Glick 2012). In this study, we have reported that gram-positive *Bacillus* and gram-negative *Pseudomonas* strains possess ACC deaminase, IAA, phosphate solubilization, exopolysaccharide (EPS) production, and siderophore production activity that stimulated the growth of soybean under salinity stress in hydroponic conditions. *Bacillus* spp. reportedly has favorable effects on plant growth, higher yield, and tolerance (Compant and others 2005; Wahyudi and others 2011). Wu and others (2005) reported in their study that microbial inoculi of *Bacillus megaterium* and *Bacillus mucilaginous* not only increased plant growth, but also improved nutritional assimilation of plants (N, P, and K). Inoculation of *Bacillus* in hydroponic medium also reportedly enhanced plant growth and yield (Yan and others 2003; Maziah and others 2010; Ryu and others 2005). *Pseudomonas putida* GR12- 2 increased the length of seedling roots by IAA production and was capable of stimulating plant growth by producing the enzyme 1-aminocyclopropane- 1-carboxylic acid (ACC) deaminase which hydrolyzes ACC (Caron and others 1995; Glick and others 1997). In another study, *Pseudomonas fluorescens* TDK1 possessing ACC deaminase activity enhanced growth of groundnut under saline field conditions, compared to strains lacking the enzyme (Saravana and Samiyappan 2007). It has been reported that *Pseudomonas* in hydroponic conditions improved growth and yield parameters during stress condition (Yan and others 2003; Ryu and others 2005; Nadeem and others 2007; Farinati and others 2011). In addition to

understanding the properties of plant growth promotion, colonization mechanisms and strategies also represent an important aspect of the plant rhizobacterial interaction. Successful colonization of PGPRs plays a key role in the promotion of plant growth and health. According to research reviews, *Pseudomonas* and *Bacillus* bacterial strains are involved in both rhizoplanic and endophytic colonization (Choudhary 2012; Lugtenberg and Kamilova 2009; Compant and others 2010). In the present study, we examined the beneficial interaction and effects of PGPRs on *Glycine max* grown in hydroponic conditions (MS medium) under saline and non-saline conditions. We studied the mechanism of plant growth promotion at the molecular level in response to expression of antioxidant enzymes and proline content.

Materials and Methods

Bacterial Strain and Growth

Pseudomonas sp. strain AK-1 (NCBI accession no. KJ511869), and *Bacillus* sp. strain SJ-5 (NCBI accession no. KJ184312) (isolated from soybean rhizosphere) were used in the present study and maintained on nutrient broth (NB, Himedia, India) amended with 50 % glycerol at −20 °C. These bacterial strains were grown in nutrient broth amended with different concentrations of NaCl (40 mM-200 mM) and examined for their tolerance level (Jha and others 2010). Inoculum for the seed treatment was prepared by harvesting bacterial cells from 24-h cultures on nutrient agar plates at 26 °C. The inoculum was suspended in sterile distilled water to yield 10^8 colony-forming units (CFU) per ml. We have grown seeds of soybean variety PK9305 at different salt concentrations to check their susceptibility against salt stress and found that seedling growth was retarded at the 100 mM NaCl concentration. On the basis of the above-described reason, we have selected the 100 mM NaCl concentration for further study.

Plant Growth-Promoting Traits

Plant growth properties (IAA assay, ACC deaminase activity, EPS production, siderophore production, and Pi solubilization) of both strains were examined under normal and salinity stressed (100 mM) conditions.

IAA assay

IAA production was detected according to a modified method of Kasotia and others(2012). The bacterial culture was inoculated into NB supplemented with 500 ppm filter-sterilized L-tryptophan in the presence of the NaCl concentration and incubated on an orbital shaker at 150 rpm for 48 h. After 48 h, bacterial cells were centrifuged at 6000 rpm for 10 min and the supernatant was taken for IAA assay. The concentration of IAA produced by the bacterial culture was measured by a standard curve of IAA in the range of 10–100 ppm.

ACC Deaminase Activity

ACC deaminase activity of rhizobacteria was analyzed according to a modified method of Penrose and Glick (2003). Bacteria were grown on DF (Dworkin and Foster 1958) salt minimal media supplemented with 3 mM of ACC or 0.1 M of $(NH_4)_2SO_4$ for 48 h. The bacterial cells were harvested by centrifugation at 10,000 rpm at 4 °C for 10 min, washed twice with 0.1 M Tris–HCl (pH 7.5), resuspended in 1 ml of 0.1 M Tris–HCl (pH 8.5) and labialized by 5 % toluene (v/v) and then vortex at the highest speed for 1 min.

Pi Solubilization Assay

Bacterial strains were tested for phosphate solubilization by an agar assay method using sterilized Picovskaya's media (Nautiyal 1999) supplemented with NaCl. The medium was poured into sterilized Petri plates and after solidification was inoculated with SJ-5 and AK-1 bacterial strains. Plates were then incubated at 28 °C for 5 days. After incubation, plates were observed for transformed halo zone formed around the bacterial colony; the diameters of the colony and halo zone were measured. The ability of the bacterial strain to solubilized insoluble phosphate was measured by the following formula.

$$\text{Phosphate solubilization index} = \frac{\text{Total diameter (Colony + Halozone)}}{\text{Diameter of bacterial colony}}$$

Siderophore Production

Siderophore production of the bacterial strain was detected as described by Ahmad and others (2008) using chrome azurol S (CAS) at 100 mM NaCl concentration. The autoclaved CAS medium was poured into Petri plates and spot inoculated with bacterial isolates and incubated for 5 days at 28 ± 2 °C. Development of a clear yellow zone around the bacterial colony was considered as positive for the production of siderophore.

EPS production assay

For quantitative determination of EPS, NaCl was added to a 100-ml flask containing 25 ml of NB medium. The medium was inoculated with 300 µl of overnight bacterial culture (OD_{600nm} 0.3) and incubated on a 150 rpm shaker for 48 h at 27 °C. EPS was extracted by the Qurashi and Sabri (2011) method with some modification. After the incubation period, bacterial cultures were centrifuged at 10,000 rpm for 15 min at 4 °C. The EPS fraction from the bacterial supernatant was precipitated using three volumes of pre-chilled acetone. After 48 h, precipitated EPS was separated by filtration with the help of Whatman filter paper. The filter paper was allowed to dry overnight at 58 °C and was reweighed after overnight drying. The increase in the weight of the filter paper was the EPS produced.

Plant Growth and Stress Induction

Soybean seedlings were grown in a plant growth chamber under a 16 h/8 h light/dark photoperiod at 26 ± 2 °C temperature. Seeds of the soybean variety PK9305 were surface sterilized by the modified method of Amprayn and others (2012). Seeds were surface sterilized with 0.1 % $HgCl_2$ for 1 min and 70 % ethanol for 3 min with repetitive washing after each treatment with Milli Q water (Millipore, Germany). Surface sterilized seeds were germinated in the petriplates containing two layers of moist filter paper in the incubator at 25 °C for 3 days. To study the effect of isolates on the physiological and biochemical parameters, we selected 3-day-old germinated seedlings and transferred them to a flask containing half MS media and bacterial suspension (A_{600} nm = 0.3). For controls, seeds were transferred to a flask containing only half MS media. Salinity treatment was started by adding 100 mM NaCl to the MS media of 7-day-old seedlings. There were six experimental groups with three replicates [Control (C), control + 100 mM NaCl (CS), AK-1-inoculated (AK), AK-1 + 100 mM NaCl (AKS), SJ-5-inoculated (S), and SJ-5 + 100 mM NaCl (SS)] based on the bacterial inoculation and salt stress treatment. The plants were harvested after 7 days of salt treatment, and divided into shoot, root, and leaf for analysis of different growth parameters and antioxidant enzyme activity in all treatments.

Chlorophyll Estimation

Chlorophyll estimation was performed according to a modified Wellburn (1994) method. Shoot and leaf tissues (1 g) were soaked in 10 ml 80 % acetone and incubated for 24 h in the dark. After incubation, the absorbance of the green solution was read at 662 and 645 nm. Total chlorophyll content was calculated using the following formula (Lichtenthaler and Wellburn 1985), and the amount of pigment was expressed as µg/g f.w. Chlorophyll a (Chl a) = [(ABS_{662} × 11.75)–(ABS645 × 2.35)], Chlorophyll b (Chl b) = [(ABS_{645} × 18.61)–(ABS662 × 3.96)]. Chlorophyll content = Chl a + Chl b.

Determination of Proline Content

Total free proline content was measured by a modified method of Bates and others (1973). According to this method, leaf samples (0.1 g) were homogenized in 5 ml of 3 % sulfosalicylic acid and then filtered. Two ml of filtrate was mixed with 2 ml of acid ninhydrin reagent (1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid) and 2 ml of glacial acetic acid. The reaction mixture was heated at 100 °C for 1 h and then placed on ice for 20 min, and 4 ml of toluene was added into it, its absorbance was measured at 520 nm and the amount of proline was determined in µg/g f.w from a standard curve.

Stress Enzymes Activity Assay

Crude enzyme extraction from the plant tissues was carried out according to the modified method of Qureshi and others (2013). One gram of shoot and root tissue was homogenized in 4 ml of 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetate (EDTA), 2 % polyvinylpyrrolidone, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 15,000×*g* for 20 min at 4 °C, and the supernatant was collected. The supernatant was used as an enzyme extract for SOD, PPO, POD, LOX, and CAT activity assays and protein estimation.

SOD Activity

The activity of SOD was determined spectrophotometrically at 560 nm by the photo reduction of nitroblue tetrazolium chloride (NBT). The reaction mixture (2.5 ml) contained 0.1 M potassium phosphate buffer (pH 7.8), 75 µm riboflavin, 2 mM NBT, 3 mM EDTA, 200 mM methionine, and 50 µl crude enzyme sample. The reaction was started under a fluorescent lamp at 25 °C and stopped after 15 min by turning off the lamp. For the blank solution, the same reaction mixture was kept under dark for the same time period. One unit of SOD was the amount of enzyme sample required for the 50 % photo reduction of the NBT (Qureshi and others 2013), and the activity was expressed as units of enzyme activity (U) g^{-1} FW.

POD Activity

POD activity was determined according to the method described by Choudhary (2011) with little modification. For estimation, 50 µl of the crude enzyme sample was added to 2.5 ml of reaction solution containing 100 mM potassium phosphate buffer (pH 7), 1 % guaiacol, and 3 % hydrogen peroxide, and the absorbance was taken at 470 nm every 30 s for 5 min. The enzyme activity was calculated using a molar extinction coefficient (e) of 26.6 mM^{-1} cm^{-1}. POD activity was expressed as µmol guaiacol oxidation min^{-1} mg $protein^{-1}$.

PPO activity

PPO activity was determined according to Weisany and others (2012). For the estimation, a total of 2.5 ml working solution contained 100 mM potassium phosphate buffer (pH 6), 1 M catechol, and 50 µl enzyme sample, and the increased absorbance was taken at 420 nm every 30 s. for 4 min. PPO activity was expressed as µmol product formed min^{-1} mg $protein^{-1}$ using a molar coefficient value of 1,300 M^{-1} cm^{-1}.

CAT Activity

The activity assay of CAT was determined by a modified method of Qureshi and others (2013). CAT activity was estimated at 240 nm by a decline in H_2O_2 concentrations due to the enzyme in the reaction mixture. Fifty µl crude enzyme sample was added to the total 2.5 ml reaction mixture containing 100 mM potassium phosphate buffer (pH 7), and 100 µl 20 mM H_2O_2. CAT activity was calculated using a 0.036 mM^{-1} cm^{-1} molar coefficient, and enzyme activity was expressed as decomposition of 1 µmol of H_2O_2 per min.

Protein estimation of crude samples was done by the Bradford (1976) method, using bovine serum albumin (BSA) as the standard. The content of soluble protein was expressed as µg g^{-1} f.w.

Native Polyacrylamide Gel Electrophoresis

Different proteins were separated by the Laemmli (1970) method in native polyacrylamide gel electrophoresis using 5 % stacking and 10 % running gels with a buffer consisting of 0.025 M Tris and 0.192 M glycine (pH 8.3) on 100 V for 4.5 h at 4 °C. A total of 10 µg crude protein sample from all treatments was loaded in every respective well of the gel. After electrophoresis, staining of the gel was done. For visualization of POD band patterns, the gel was incubated for 30 min in the same reaction mixture used in the enzyme activity assay. PPO isoforms were determined by staining the gel with 0.1 % p-phenylene diamine in the same buffer for 30 min. After incubation, the solution was discarded and gels were treated with 20 mM catechol in the same buffer (Ramamoorthy and others 2002). The gel of SOD was first stained by 0.030 mM riboflavin and 1 % *N, N, N', N'*-tetramethyl ethylenediamine (TEMED) in 100 mM potassium phosphate buffer (pH 7.8) for 30 min in the dark with gentle agitation. The gel was then washed in distilled water and treated with 2 mM NBT in the same buffer for 15 min in white light, after that the band of SOD isoforms was visualized against a purple background. Band intensity was measured by Scion Image Beta 4.02 software (Scion Corporation).

Estimation of Lipid Peroxidation and LOX Enzyme

The level of lipid peroxidation was assayed by measuring the malondialdehyde (MDA) content, according to minor modifications of the method of Hodges and others (1999). Approximately 0.50 g (fresh weight) of root and leaf tissue was separately homogenized in 1.5 ml of 5 % trichloroacetic acid (TCA, w/v). The homogenate was centrifuged at 1,500 rpm for 10 min, and then the supernatant was diluted to 10 ml. Two ml of the diluted extract was mixed with 2 ml of 0.5 % thiobarbituric acid (TBA, w/v) prepared in 15 % TCA, 1 % butyl hydroxytoluene, and 0.25 N HCl. The mixture was incubated at 100 °C in boiling water for 30 min, and then cooled at room temperature and centrifuged at 10,000 rpm for 5 min. The supernatant was then used for absorbance at 532 nm. The amount of MDA in the aqueous phase was measured by a standard curve of MDA in the range of 0.1–10 nmol.

The LOX activity of the crude enzyme samples was estimated by a slightly modified Choudhary (2011) method. The total reaction mixture for spectrophotometric absorbance at 234 nm was 2.5 ml of 0.1 M potassium phosphate buffer (pH 6.5), 10 mM linoleic acid, and 50 µl protein sample. The molar coefficient of 25,000 M^{-1} cm^{-1} of linoleic acid was used to calculate LOX activity, and the activity of the enzyme was expressed as conversion of µmol of substrate per min mg^{-1} protein.

Proline Gene Expression

To determine the gene expression pattern of *GmP5CS* in soybean, a reverse transcription polymerase chain reaction (RT-PCR) was done. For RT-PCR, total RNA was isolated from roots of SJ-5-inoculated and control plants in salt stress using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA was then reverse transcribed into cDNA. A total volume of 20 µl for RT-PCR contained, oligo $(dT)_{18}$, primer and *Avian Myeloblastosis Virus* (AMV) reverse transcriptase and was kept at room temperature for 5 min. After that RT-PCR amplification was performed in a 10 µl

reaction mixture added with 2 µl cDNA using the following specific primers for the soybean *GmP5CS* gene: 5′-GGCTGCAATGCCATGGAAACTCTT-3′ and 5′-ACTTGCCTTGGGTCCTCCATACAA-3′ designed based on accession no.U12286.1. The cycling steps included 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min, and a final extension of 72 °C for 5 min. An equal volume of PCR products was subjected to electrophoresis on 2 % (w/v) agarose gels prior to staining with ethidium bromide (Celik and Ceimen 2012). The gel was photographed under UV light, and band intensity was measured by Scion Image Beta 4.02 software (Scion Corporation).

Enumeration of Introduced Bacterial Strains

Three replicates per treatment with saline and non-saline conditions were selected for the analysis of colonization of soybean plant roots by colonies of SJ-5 and AK-1 bacterial strains (Anderson and Guerra 1985). Inoculated plant roots were weighed and transferred to a sterile Petri dish, pressed with a sterile velvet pad which was subsequently replica plated onto plates of King's B (for *Pseudomonas*) and TSA (for *Bacillus*) medium containing a combination of antibiotics (ampicillin and erythromycin 30 µg/ml of each). After incubation at 28 °C for 24 h, fluorescent and whitish colonies (as same as *Pseudomonas* and *Bacillus*) were counted.

Statistical Analysis

The experimental design was randomized blocks, with three replications using a 2 × 3 factorial arrangement: two conditions (saline and non-saline) and three treatments (control: devoid of bacterial inoculation, AK-1 inoculation, and SJ-5 inoculation). Data were tested for normality using the P–P plots test ($P < 0.05$). Only leaf water content (LWC) data were significantly different from a normal distribution and then subjected to arcsine transformation. The variables obtained were tested for homogeneity of variance using Levene's Test, and then the means were subjected to analysis of variance (ANOVA) with the F test ($P < 0.05$). Comparison of treatment means was performed using the DMRT test ($P < 0.05$). All analyses described were performed in SPSS software.

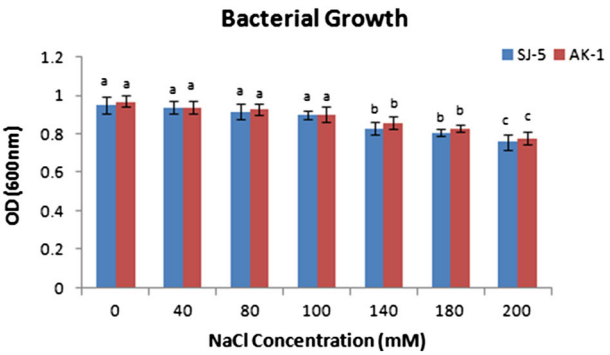

Fig. 1 Effect of different NaCl concentrations on 24-h bacterial growth. *Values* represent the mean ± SE, $n = 3$. *Different letters* on *each bar* indicate significant differences ($P = 0.05$) in bacterial growth after DMRT test

Results

Physiological Characterization of Bacterial Strains

Both the PGPR strains used in this study were isolated in our laboratory from the soybean rhizosphere and were found to positively influence plant growth, suggesting the significant role of the bacteria in plant growth promotion (Jain and Choudhary 2014). Both the bacterial strains were also screened for salinity stress tolerance as mentioned in the ''Materials and Methods'' section and showed stress tolerance in terms of better growth on the 200 mM NaCl medium (Fig. 1). Both the bacterial strains were able to produce IAA, siderophore, EPS, ACC deaminase, and phosphate solubilization activities. Bacillus strain SJ-5 produced the highest ACC deaminase, siderophore, and phosphate solubilization, whereas Pseudomonas strain AK-1 exhibited the highest IAA and EPS activities (Table 1; Fig. 2).

Effect of PGPR on Plant Growth and Chlorophyll Content

Both the bacterial strains showing tolerance to NaCl stress and exhibiting higher PGP activities on 100 mM NaCl were used to inoculate soybean plants before applying NaCl stress to the plants. The inoculation was done at the time of transferring 3-day-old geminated seeds in half MS media. After 7 days, 100 mM NaCl was added in the

Table 1 Different plant growth-promoting traits of selected bacterial isolates under salinity

Bacterial isolates	IAA (mg/L)	ACC deaminase (α-ketobutyrate nmol mg^{-1} h^{-1})	Phosphate solubilization efficiency	Siderophore production efficiency	Exopolysaccharide production (g/100 ml)
SJ-5	9.3 ± 0.5	98.0 ± 2.1	170.0 ± 8.0	280.0 ± 6.0	1.5 ± 0.3
AK-1	11.0 ± 0.3	83.5 ± 1.3	130.0 ± 6.0	247.0 ± 9.0	2.1 ± 0.2

Values represent the mean ± SE, $n = 3$

medium. This stress was observed to be toxic to soybean plants without PGPR inoculation. An early cotyledon senescence symptom was found in control salt-stressed plants (Fig. 3). However, the PGPR-inoculated plants growing under stress condition showed better growth and tolerated NaCl stress significantly better than the control plants. The inoculated plants showed better growth in terms of plant height, number of leaves, and number of lateral roots under stress conditions (Table 2; Fig. 3). Similarly, SJ-5- and AK-1-inoculated plants exhibited higher total plant fresh weight (0.70 and 0.64 g, respectively) than control plants (0.38 g) under stress. Chlorophyll content was higher (9 and 8.13 μg/g f.w.) in plants inoculated with SJ-5 and AK-1 strain, respectively, compared to control plants (3.98 μg/g f.w.) under salinity. LWC was reduced by NaCl stress in all soybean plants. However, SJ-5- and AK-1-inoculated plants had higher LWC (60 %) and (65 %), respectively, compared to control plants (30 %) under saline conditions (Table 2). These results indicate that inoculation of the two PGPR strains led to recovery from saline stress in plants.

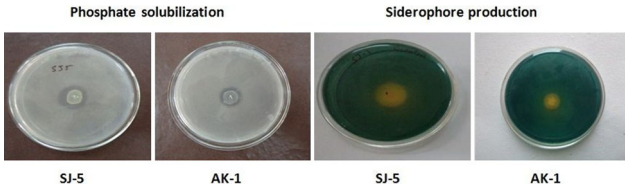

Fig. 2 Phosphate solublization and siderophore activity in SJ-5 and AK-1 bacteria on 100 mM NaCl medium

Proline Accumulation and *GmP5CS* Gene Expression

Under normal conditions, no significant difference was found in proline content between inoculated and control soybean plants. When plants were subjected to salt stress, proline content increased in all treatments, and its appearance was different in different plant tissues. In roots, SJ-5-(120 μg/g f.w.) and AK-1-(135 μg/g f.w.)inoculated plants accumulated more proline content than control plants (90 μg/g f.w.). In the same plants, shoots and leaves showed less accumulation of proline than same tissues in control plants (Fig. 4a). Proline expression was further determined using a *GmP5CS* gene-specific primer in salt stress-inoculated and control plant roots by RT-PCR technique. The result of expression analysis (Fig. 4b) shows changes in gene expression levels with a 100 bp DNA marker. We

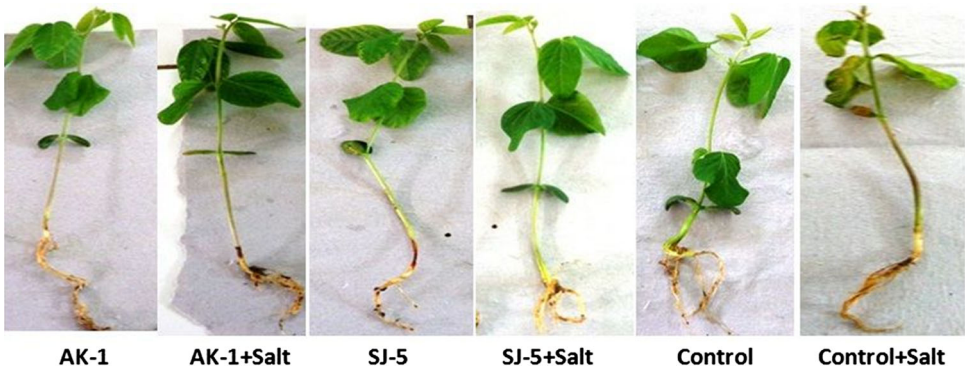

Fig. 3 Phenotypic alteration in different treatments of soybean plants. AK-1 (AK-1-inoculated plants), AK-1 + Salt (AK-1 salt-stressed plants), SJ-5 (SJ-5-inoculated plants), SJ-5 + Salt (SJ-5 salt-stressed plants), Control plants (devoid of bacterial inoculation), Control + salt (Control salt-stressed plants)

Table 2 Effect of bacterization with selected strains on different plant growth parameters in saline (100 mM) and non-saline conditions

Experimental groups	Shoot length (cm)	Root length (cm)	Leaves (no.)	Total plant fresh weight (gm)	Lateral roots (No.)	Chlorophyll content (μg/g f.w.)	Leaf water content (%)
Control							
Non-stressed	$15^{a} \pm 0.6$	$6^{b} \pm 0.2$	$8^{a} \pm 0.3$	$0.74^{a} \pm 0.08$	$12^{b} \pm 1.1$	$13.0^{a} \pm 1.3$	$87^{a} \pm 3.2$
Salt stressed	$8^{c} \pm 0.4$	$3^{c} \pm 0.1$	$2^{c} \pm 0.1$	$0.38^{c} \pm 0.06$	$8^{c} \pm 0.4$	$3.9^{c} \pm 1.1$	$30^{c} \pm 4.1$
SJ-5 inoculated							
Non-stressed	$18^{a} \pm 0.3$	$12^{a} \pm 0.4$	$8^{a} \pm 0.3$	$0.79^{a} \pm 0.06$	$24^{a} \pm 1.4$	$14.0^{a} \pm 1.4$	$93^{a} \pm 2.5$
Salt stressed	$12^{b} \pm 0.7$	$7^{b} \pm 0.3$	$5^{b} \pm 0.2$	$0.70^{b} \pm 0.08$	$14^{b} \pm 1.4$	$9.0^{b} \pm 1.2$	$60^{b} \pm 3.3$
AK-1 inoculated							
Non-stressed	$16^{a} \pm 0.4$	$10^{a} \pm 0.4$	$8^{a} \pm 0.4$	$0.76^{a} \pm 0.05$	$28^{a} \pm 1.6$	$12.2^{a} \pm 1.2$	$90^{a} \pm 4.6$
Salt stressed	$13^{b} \pm 0.6$	$8^{b} \pm 0.2$	$5^{b} \pm 0.2$	$0.64^{b} \pm 0.03$	$16^{b} \pm 1.4$	$8.1^{b} \pm 0.8$	$65^{b} \pm 5.1$

Superscripted letters indicate values within the same column that are either significantly different (when the letters are different) or not (when the letters are the same) using DMRT test at $P = 0.05$, $n = 3$

Values represent the mean ± SE, $n = 3$

found that the *GmP5CS* gene was more expressed in bacterial-inoculated plant roots than control plant roots (Fig. 4c).

MDA Content

MDA content was measured as an indicator of lipid peroxidation in soybean plants under salt stress. Results showed that salt stress caused a significant increase in MDA levels in root tissue in all treatments. Bacterial-inoculated plant roots SJ-5 (520 nmol/g f.w.) and AK-1 (480 nmol/g f.w.) had increased MDA levels during salt stress but it was lower than control plant roots (730 nmol/g f.w.). However, in shoots, bacterial-inoculated plants showed non-significant increased MDA levels under salt stress. Control plant shoots exhibited higher MDA content (930 nmol/g f.w.) than SJ-5-(250 nmol/g f.w.) and AK-1-(310 nmol/g f.w.)inoculated plant shoots under salt stress. Results indicate that both PGPR prevent plants from oxidative damage caused by salt stress (Fig. 5).

LOX Activity

Our data showed that root tissue exhibited higher LOX activity compared to shoots in all treated plants. Bacterial-inoculated plants had the highest LOX activity under saline and non-saline conditions. In salinity, SJ-5-inoculated plant roots (0.414 U/mg protein) and AK-1-inoculated plant roots (0.403 U/mg protein) showed higher LOX activity than control plant roots (0.366 U/mg protein) (Fig. 6).

Antioxidant Activity

All antioxidant enzymes showed increasing activity under salt stress. In salinity, bacterial-inoculated plants exhibited lower activities of CAT, SOD, and PPO, whereas POD showed higher activity in roots than control plants. SJ-5- and AK-1-inoculated plant roots, (14.4 U/mg protein) and (14.1 U/mg protein), respectively, had higher POD activity than control plant roots (12.5 U/mg protein), whereas shoots showed (7.2 and 7.4 U/mg protein), respectively, less POD activity than control plant shoots (9.5 U/mg protein) under salt stress. In non-saline conditions, no significant difference was found in POD activity between inoculated and control plants (Fig. 7). CAT activity was notably higher in shoots than roots in all treatments. In salinity, SJ-5-inoculated shoots (376.11 µmol H_2O_2/mg protein) and AK-1-inoculated shoots (393.49 µmol H_2O_2/mg protein) had less activity than control plant shoots (520.93 µmol H_2O_2/mg protein) (Fig. 7). SOD activity was higher for all times in shoots during salinity, whereas in non-saline conditions SOD activity was elevated in roots in every treatment. Bacterial-inoculated plants exhibited lower SOD activity than control plants in salt stress. SJ-5-inoculated plant shoots and roots (112 and 94 U/mg

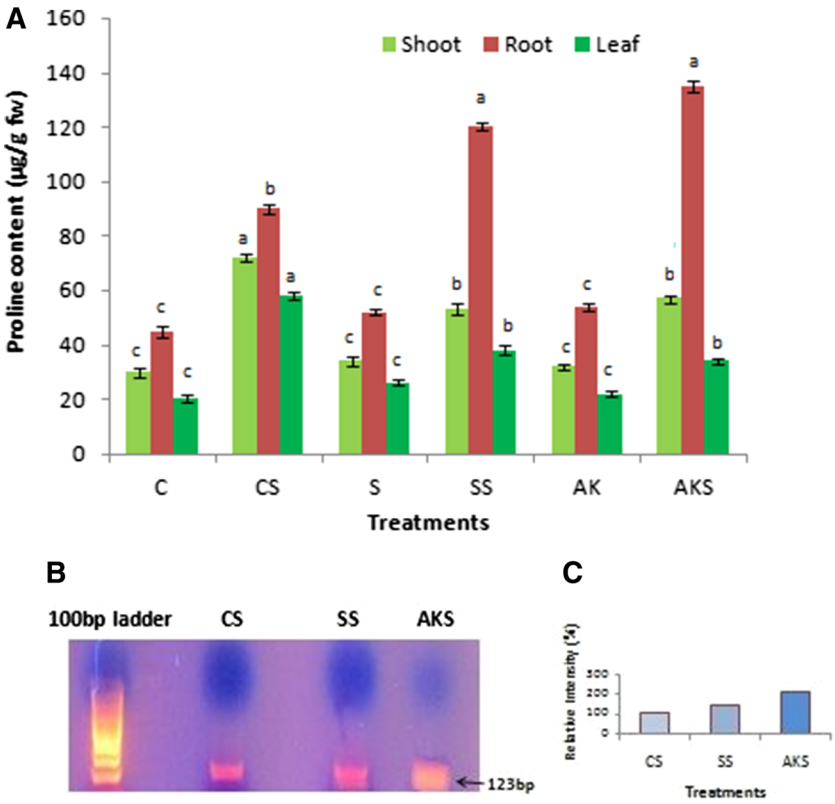

Fig. 4 Impact of salinity and PGPRs inoculation on **a** proline content (µg/g f.w) in shoot, root, and leaf, **b** *GmP5CS* gene expression in roots of different treatments of soybean plants and **c** band intensity of *GmP5CS* gene expression in different treatments. *C* control plants without any treatment, *CS* control-stressed, *S* SJ-5-inoculated plants, *SS* SJ-5-stressed plants, *AK* AK-1-inoculated plants, and *AKS* AK-1-stressed plants. In **a** *values* represent the mean ± SE, $n = 3$. *Different letters* on *each bar* indicate significant differences ($P = 0.05$) in bacterial growth after DMRT test

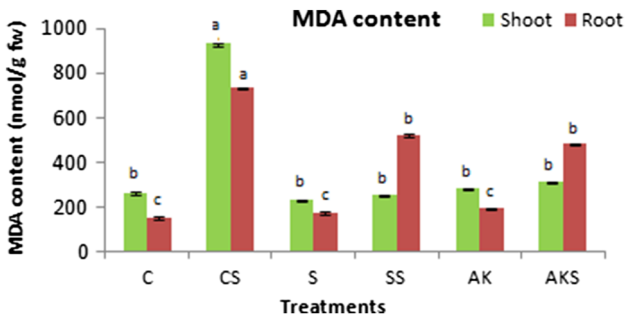

Fig. 5 Impact of salinity and PGPRs inoculation on MDA content (nmol/g fw) in shoots and roots of different treatments of soybean plants. C control plants without any treatment, *CS* control-stressed, *S* SJ-5-inoculated plants, *SS* SJ-5-stressed plants, *AK* AK-1-inoculated plants, and *AKS* AK-1-stressed plants. *Values* represent the mean ± SE, $n = 3$. *Different letters* on *each bar* indicate significant differences ($P = 0.05$) in bacterial growth after DMRT test

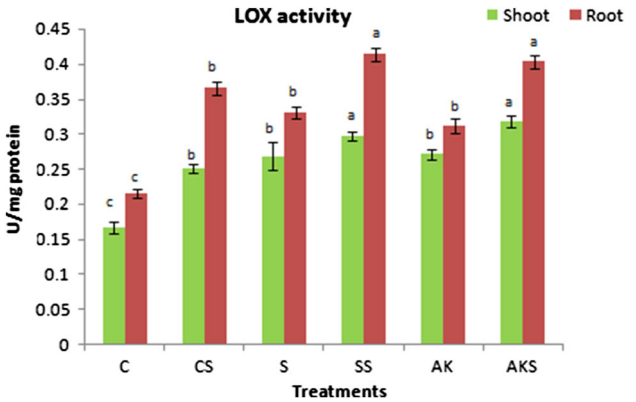

Fig. 6 Impact of salinity and PGPRs inoculation on LOX activity (U/mg protein) in shoots and roots of different treatments of soybean plants. *C* control plants without any treatment, *CS* control-stressed, *S* SJ-5-inoculated plants, *SS* SJ-5-stressed plants, *AK* AK-1-inoculated plants, and *AKS* AK-1-stressed plants. *Values* represent the mean ± SE, $n = 3$. *Different letters* on *each bar* indicate significant differences ($P = 0.05$) in bacterial growth after DMRT test

protein, respectively) and AK-1-inoculated shoots and roots (123 and 108 U/mg protein, respectively) showed lower SOD activity in contrast to control plants shoot and root (140 and 122 U/mg protein, respectively) during saline conditions (Fig. 7). PPO activity was always higher in roots of different treatment plants, except control salt-stressed plants, which had higher activity in shoots. In salinity, control plants exhibited the highest activity in both roots and shoots, whereas bacterial-inoculated plants showed slightly increased PPO activity in contrast to their corresponding non-stressed plants (Fig. 7).

Native-PAGE Analysis

Isoform expressions of SOD, POD, and PPO were analyzed in salt-stressed root and shoot extracts subjected to native-PAGE. In the peroxidase activity gel, five isoforms were detected in roots and four in shoots. In roots, POD II isoform band intensity was higher in both the bacterial stressed plants, and POD I intensity was more elevated in SJ5-inoculated plants. In shoot, the POD IV isoform showed higher activity in control-stressed plants (Fig. 8).

Four isoforms were found in the SOD activity gel. SOD I and II isoforms were not detected in roots of any treatment, whereas in shoots these isoforms showed higher intensity in control-stressed plants. SOD III and IV bands were found with higher intensity in shoots than roots in all treatments. The SOD III isoform was similar in salt-stressed root of all treated plants, and the SOD IV isoform was similar in control and SJ-5-inoculated plant shoots during salt stress (Fig. 9). The PPO activity gel showed five isoforms. PPO I and II isoforms were higher in both roots and shoots, whereas the PPO V isoform was higher only in shoots of control salt-stressed plants. PPO III was higher in both bacterial-inoculated plant shoots, whereas in roots it was negligible. PPO IV was higher in SJ-5 salt-stressed plant shoots and in roots, it was higher in control plants without salt stress (Fig. 10).

Colonization of Soybean Roots by SJ-5 and AK-1 Strain

Colonies of *Pseudomonas* sp. strain AK-1 and *Bacillus* sp. strain SJ-5 were identified by morphological and antibiotic susceptible assay. The result showed that the fluorescent and whitish-like colonies were identical and resistant to ampicillin and erythromycin antibiotics to the strains of AK-1 and SJ-5, respectively. The colonies of SJ-5 and AK-1 were present at 6×10^5 and 6×10^3 cfu per gram fresh weight of roots, respectively, under control conditions. During salinity, populations declined to 5×10^8 and 5×10^1 for SJ-5 and AK-1, respectively (Table 3).

Discussion

This study investigated physiological, biochemical, and molecular aspects related to salt tolerance in bacterial-inoculated and control soybean plants. Two plant growth-promoting strains, SJ-5-*Bacillus* spp. and AK-1-*Pseudomonas* spp. (isolated from soybean rhizosphere), were used in the present study. Both bacterial strains exhibited in vitro PGP characteristics that include Pi solubilization, ACC deaminase activity, and production of siderophores, EPS and IAA, on medium containing 100 mM NaCl. Salinity causes the depletion and precipitation of available phosphorus, so our bacterial Pi solubilization activity in MS medium may have a tendency to solubilize precipitated forms of phosphorus and therefore play an important role in providing phosphorus to the plant system (Gyaneshwar

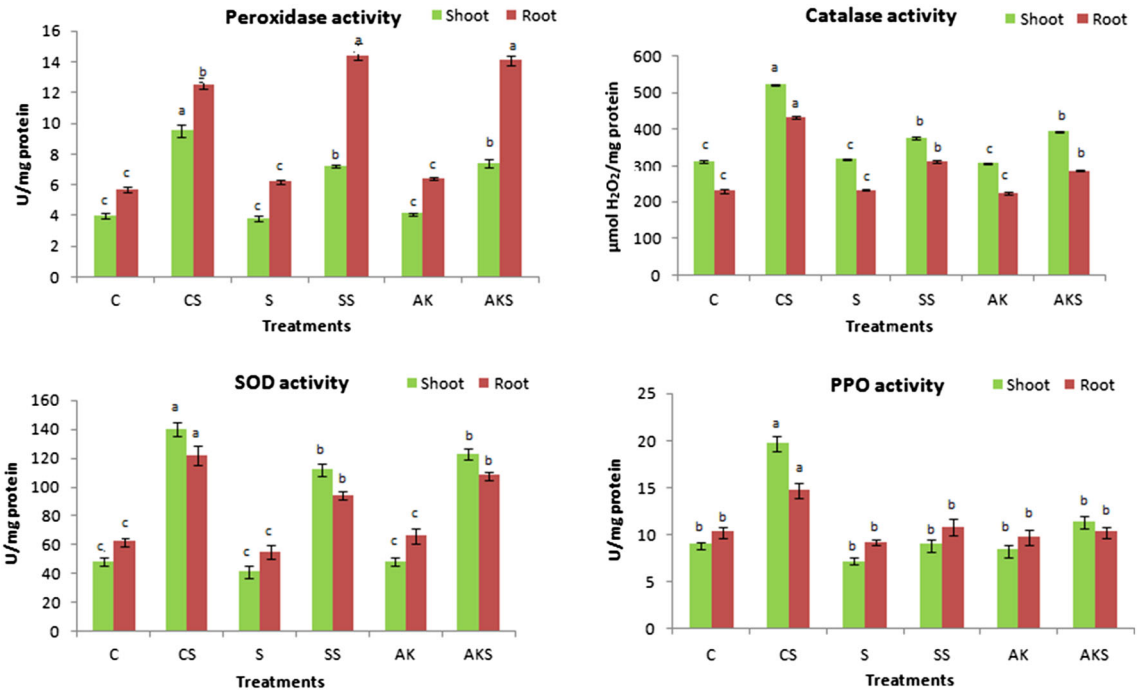

Fig. 7 Impact of salinity and PGPRs inoculation on different antioxidant enzyme activities in shoots and roots of different treatments of soybean plants. *C* control plants without any treatment, *CS* control-stressed, *S* SJ-5-inoculated plants, *SS* SJ-5-stressed plants, *AK* AK-1-inoculated plants, and *AKS* AK-1-stressed plants. *Values* represent the mean ± SE, $n = 3$. *Different letters* on *each bar* indicate significant differences ($P = 0.05$) in bacterial growth after DMRT test

and others 2002, Shukla and others 2012). PGPR strains showing Pi solubilization activity could help plants growth by increasing nutrient uptake (Bano and Fatima 2009). When strains were introduced into MS medium, populations of both strains increased rapidly around the soybean root. However, the bacterial population was slightly decreased in salt-supplemented MS medium. The difference in population increases around the root area suggests that *Bacillus* strain SJ-5 might multiply more rapidly than *Pseudomonas* stain AK-1. These results confirm the previous findings that *Bacillus* strains were the most predominant colonizer on the root surface (Yan and others 2003). Our statistical analysis results showed no significant difference between different plant growth parameters affected by *Bacillus* and *Pseudomonas* bacterial strains. Both the bacterial-treated plants showed a higher tolerance to salt stress that was significantly different from non-treated plants, as shown by their enhanced plant length, shoot and root biomass production, higher LWC, higher photosynthesis activity, and lower osmotic stress injury (MDA content). These results clearly suggest that the selected microorganisms can alleviate some of the deleterious effects of salt stress. Plants inoculated with SJ-5 and AK-1 bacteria showed higher shoot and root lengths and maximum number of lateral roots under salt stress, whereas in controls plant lengths decreased in salinity. The higher root length of inoculated plants is probably due to the availability of IAA hormone in the medium secreted by both the bacterial inoculi. Several other researchers also reported that PGPR strains show IAA activity, and the interaction of IAA with plants could help in increased root growth under salinity, which may be an adaptive response to stresses, and also can contribute to maintaining leaf growth which is considered as a primary response of plant productivity under conditions of salinity (Albacete and others 2008). IAA-producing PGPR strain colonization with plant roots seems to be responsible for the higher root length and nutrient uptake under hydroponic conditions (Shukla and others 2012; Baset Mia and others 2010). *Bacillus* and *Pseudomonas* bacterial strains are known to increase root length in hydroponic conditions (Yan and others 2003; Nadeem and others 2007; Maziah and others 2010).

The shoot and root fresh weights of control plants were decreased under saline conditions, whereas bacterial-inoculated plants had higher fresh weights of both tissues. *Bacillus* and *Pseudomonas* bacterial strains can alter the morphology of the root system by enhancing root length and number of lateral roots, resulting in enhanced water uptake and increased shoot and root biomass under hydroponic conditions (Maziah and others 2010; Ryu and others 2005; Baset Mia and others 2010), which may be considered as a mechanism of salinity tolerance (Nadeem

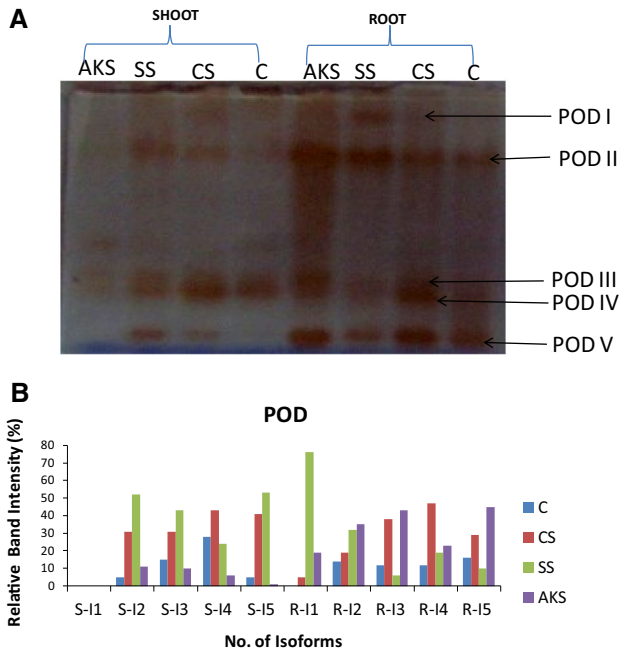

Fig. 8 Impact of salinity and PGPRs inoculation on **a** native-PAGE analysis of POD isoforms in shoots and roots of different treatments of soybean plants, **b** band intensity of POD isoforms. *C* control plants without any treatment, *CS* control-stressed, *S* SJ-5-inoculated plants, *SS* SJ-5-stressed plants, *AK* AK-1-inoculated plants, and *AKS* AK-1-stressed plants. On horizontal axis, S-I1 to S-I5 represent isoforms of POD1 to POD5 in shoot, and R-I1 to R-I5 represent isoforms of POD1 to POD5 in root

and others 2007; Shukla and others 2012). However, another explanation is also possible, that of lowering of plant ethylene concentrations by the action of bacterial ACC deaminase activity, thereby decreasing the ethylene inhibition of seedling root biomass (Saravana and Samiyappan 2007). A *Pseudomonas* strain containing ACC deaminase activity has been reported to increase root and shoot biomass in maize plants under hydroponic conditions with salt stress (Nadeem and others 2007). LWC is the best indicator of water stress, which is reduced during stress conditions (Mayak and others 2004b). Bacterial-inoculated plants leaves were more hydrated than the control plant leaves under saline conditions. A high level of salinity had adversely affected the sink function and decreased the ions and energy stored in cotyledons, resulting in poor growth of seedlings (Yan and others 2007). In this study, an early cotyledon senescence symptom was found only in non-inoculated salt-stressed plants. During propagule development, cotyledons may act as a sink for ions and nutrients that support the seedlings in their early growth. However, another explanation is also possible, that the induction of endogenous nitric oxide due to salt stress triggered early cotyledon senescence (David and others 2010). Salinity and other environmental stresses are reported to produce nitric oxide gas that has been suggested to be a signaling component that mediates senescence (Neill and others 2008; Corpas and others 2009; Xuan and others 2010).

Maintaining water homeostasis and the functioning of photosynthetic structures are essential for alleviating the impact of salinity on plant growth and crop yield (Munns and Tester 2008). In this study, bacterial-inoculated plants showed higher levels of proline and chlorophyll contents than control plants, which enables the plants to maintain an osmotic balance and increase photosynthesis activity under salt stress. Greener leaves of bacterial-inoculated plants under salinity suggest that SJ-5 and AK-1 bacteria relieve negative effects of salt. It is reported that salt tolerance in glycophyte species is mostly related to the exclusion of Na^+ ions from the leaves thereby avoiding inhibition of photosynthesis machinery (Munns and Tester 2008). There are several reports on the contribution of PGPR for maintaining the homeostasis of toxic ions in plants under salinity. Ashraf and others (2004) explained that a *Bacillus* strain secreting EPSs helps bind cations (especially Na^+) in

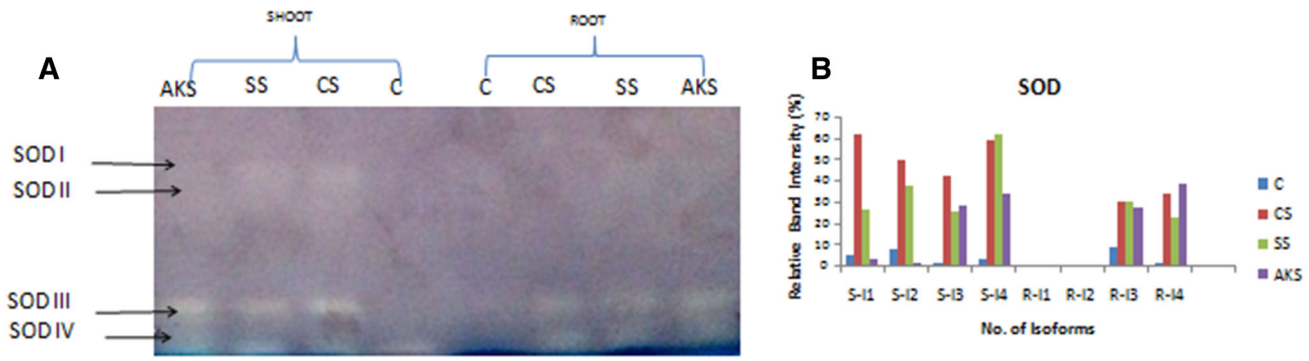

Fig. 9 Impact of salinity and PGPRs inoculation on **a** native-PAGE analysis of SOD isoforms in shoots and roots of different treatments of soybean plants and **b** band intensity of SOD isoforms. *C* control plants without any treatment, *CS* control-stressed, *S* SJ-5-inoculated plants, *SS* SJ-5-stressed plants, *AK* AK-1-inoculated plants, and *AKS* AK-1-stressed plants. On horizontal axis, S-I1 to S-I4 represent isoforms of SOD1 to SOD4 in shoots, and R-I1 to R-I4 represent isoforms of SOD1 to SOD4 in roots

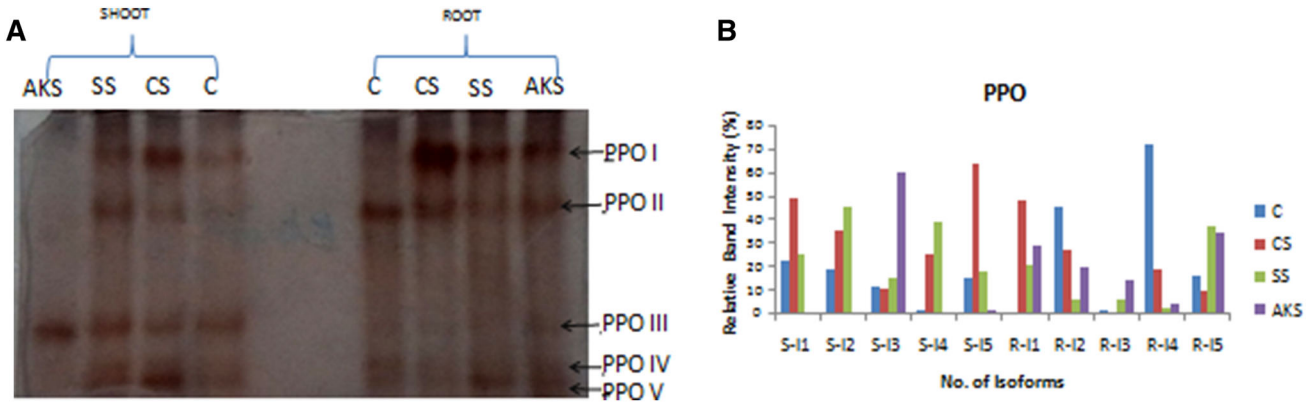

Fig. 10 Impact of salinity and PGPRs inoculation on **a** native-PAGE analysis of PPO isoforms in shoots and roots of different treatments of soybean plants and **b** band intensity of PPO isoforms. *C* control plants without any treatment, *CS* control-stressed, *S* SJ-5-inoculated plants, *SS* SJ-5-stressed plants, *AK* AK-1-inoculated plants, and *AKS* AK-1-stressed plants. On horizontal axis S-I1 to S-I5 represent isoforms of PPO1 to PPO5 in shoots, and R-I1 to R-I5 represent isoforms of PPO1 to PPO5 in roots

Table 3 Bacterial colonization on soybean plant roots under control and salt conditions

Treatment	Bacteria	
	Control (CFU/g root)	Salt (CFU/g root)
SJ-5	$6 \times 10^5 \pm 9.1$	$5 \times 10^8 \pm 11.6$
AK-1	$6 \times 10^3 \pm 10.3$	$5 \times 10^1 \pm 10.7$

Values represent the mean ± SE, $n = 3$

roots, thus preventing their transfer to leaves and enhancing the photosynthesis mechanism, which alleviates salt stress in plants. Furthermore, PGPR containing ACC deaminase activity increased ion uptake efficiency (Mayak and others 2004a), resulting in higher K^+/Na^+ ratios in salt-stressed plants under hydroponic conditions (Nadeem and others 2007; Shukla and others 2012). Our result also showed that SJ-5 and AK-1 bacterial strains had EPS and ACC deaminase production activity suggesting their role in increased chlorophyll content in inoculated soybean plants. A high level of proline protects plants against osmotic stresses, not only by adjusting osmotic pressure, but also by stabilizing membrane proteins and enzymes, scavenging of ROS, and maintaining redox homeostasis (Banu and others 2009). Enhanced proline synthesis in abiotically stressed plants has been reported in the presence of beneficial bacteria such as *Pseudomonas* (Kohler and others 2008), *Burkholderia* (Barka and others 2006), *Arthrobacter* (Sziderics and others 2007), and *Bacillus* (Vardharajula and others 2011; Gururani and others 2013). In our study, bacterial-inoculated plant roots accumulated higher proline content compared to control plant roots under salt stress, which may be due to higher uptake of nutrients, resulting in a high biosynthesis rate (Maziah and others 2010; Vardharajula and others 2011). The accumulation of proline in roots suggests an osmotic mechanism to keep a positive water potential for water entrance into the roots, leading to lower stress damage in plants (Porcel and others 2004). In another study in hydroponic conditions, Nadeem and others (2007) and Shukla and others (2012) reported that proline contents increased by salinity stress, but decreased by inoculation with PGPR. These authors suggested that the lower accumulation of proline indicates successful alleviation of salt stress. In a similar way, bacterial-inoculated plant shoots and leaves showed less proline contents than control plants under salinity, suggesting that bacterial-inoculated plant shoots and leaves were less strained by salinity. In addition, we determined the relation between proline accumulation and *GmP5CS* gene expression level. *P5CS* has been reported as the main gene responsible for proline biosynthesis and accumulated in plants under salt stress (Yamada and others 2005; Celik and Ceimen 2012). Our RT-PCR results showed that the *GmP5CS* gene was more expressed in SJ-5-inoculated plant roots compared to control plant roots under salinity. Proline accumulation in these plants also showed the same behavior. This reveals a clear correlation between proline accumulation and GmP5CS gene expression, indicating that proline accumulation in bacterial-treated plant roots is caused by overexpression of the *GmP5CS* gene (Hien and others 2003; Yamada and others 2005; Kim and others 2007).

The peroxidation of membrane lipids severely affects its permeability, integrity and can cause irreversible damage to the cell function (Noctor and Foyer 1998). Lipid peroxidation can be produced by both enzymatic and non-enzymatic processes. The non-enzymatic process starts by the action of free ROS and MDA is a secondary product of lipid peroxidation in this process. Therefore, MDA is used as an indication of the extent of stress induced by ROS (Jain and others 2001). Decreased MDA content has been reported in

the presence of PGPR under stress conditions (Porcel and others 2003, 2004; Han and Lee 2005; Shukla and others 2012). In the present investigation, an increased MDA level was found in salt-stressed roots of soybean plants. It indicates that NaCl causes oxidative damage to plants. Bacterial-inoculated plants were less affected by lipid peroxidation as shown by lower MDA content than control plants during salt stress conditions. Compatible solutes such as proline protect cellular components from oxidative stress. Bacterial-inoculated plant roots showed higher proline content that may protect plants against salt stress. Under salt stress, proline has been reported to decrease lipid peroxidation (Banu and others 2009; Islam and others 2009; Okuma and others 2004).

Enzymatic lipid peroxidation is initiated by LOX enzyme (Halliwell and Chirico 1993). LOX-generated oxylipins are reported to protect roots from oxidative damage and are involved in root-to-shoot signaling during environmental stress (Hasegawa and others 2011; Grebner and others 2013). In our results, bacterial-inoculated plants showed higher LOX activity in saline as well as non-saline conditions, which suggests its role in regulation of interactions between roots and bacteria (Hause and Schaarschmidt 2009).). In addition, LOX is also reported in the adaptation of plants to adverse growth conditions (Yang and others 2012; Rodrigues and others 2013; Grebner and others 2013) and in the defense reactions against bacterial and fungal pathogens (Choudhary 2011; Jain and Choudhary 2014). In this study, MDA content in bacterial-inoculated plants was not paralleled with LOX activity, which explains that tissue damage occurred due to non-enzymatic lipid peroxidation. Our results show that higher LOX activity in bacterial-inoculated plant roots was involved in salt tolerance by reducing oxidative damage. Lower MDA content in these plant shoots also supports this explanation.

In response to environmental stress such as salinity, ROS may be generated in excess, which is extremely harmful to living organisms. The excess production of ROS can cause cell death by various pathways such as peroxidation of lipids, oxidation of proteins, damage to nucleic acids, and finally leading to programmed cell death. The efficient destruction of ROS requires the action of several antioxidant enzymes acting simultaneously. The induction of antioxidant enzymes such as CAT, peroxidase (POD), PPO, and SOD can be considered as one mechanism of salt tolerance in plants. CAT and POD enzymes are involved in elimination of H_2O_2, whereas SOD catalyzes the dismutation of O_2^- redicals (Sharma and others 2012). PPO oxidizes some phenols to quinones. This enzyme has been studied in wounding and browning in plants during biotic and abiotic stress conditions (Bilková and others 2005).

Khedr and others (2003) suggested that proline acts as a free radical scavenger and its presence enhances POD activity to alleviate salt stress. A similar result was found in our study, POD enzyme activity was higher in the roots of bacterial-inoculated plants, which also contained higher proline levels than control plant roots under salt stress. In the same plants, shoots had less POD activity along with less proline content. Many reports suggested that proline enhanced antioxidant enzymes to improve salt tolerance (Banu and others 2009; Molinari and others 2007). Sharma and others (2012) reported that increased activities of antioxidant enzymes are an indication of high oxidative stress in a plant. CAT, SOD, and PPO activity results support the above explanation. In our results, CAT, SOD, and PPO showed less activity in bacterial-inoculated plants than control plants under salt stress suggesting that these plants were under lower oxidative stress under saline conditions (Porcel and others 2004; Kohler and others 2008, 2009; Vardharajula and others 2011).

In native-PAGE analysis, salt stress caused the enhancement of enzyme isoforms. We analyzed three antioxidant enzyme activities (SOD, POD, PPO) in salt-stressed root and shoot extracts of different treatments. The band intensity on native-PAGE gel was measured in relative band intensity percentage by Scion Image Beta 4.02 software. Our results indicated that SJ-5 and AK-1 bacteria induced more activity of POD I and POD II isoforms in roots than control plant roots under salt stress, which is probably responsible for the enhancement of POD activity in bacterial-inoculated plant roots. Furthermore, the higher expression of SOD and PPO activity in control plants than bacterial-inoculated plants may be induced by the induction of SOD I, II, and III and PPO I, II, IV, and V isoform activity. Lee and Lee (2000) also performed native-PAGE analysis of antioxidant enzymes and observed high expression of enzyme isoforms in chilling stress. These authors proposed that plants can synthesize new isoforms of antioxidant enzymes with altered kinetic properties during severe osmotic stress. This could explain why the SOD and PPO isoforms were more induced in control plants in salt stress, whereas the bacterial-inoculated plants showed suppression of both the enzyme isoforms. As indicated above, decreased isoforms of SOD and PPO antioxidant enzymes in bacterial-inoculated plants could be explained by the fact that these plants may have experienced lower oxidative stress under saline conditions.

The overall data show that both root and shoot tissues are influenced by PGPR inoculation by means of salt stress tolerance and avoidance mechanisms. PGPR inoculation first enhances LOX activity in soybean plants under normal conditions, which helps plants survive better under salt stress. In addition, proline content increased in roots to

maintain favorable water potential to the water passing from soil into the roots. This enables higher water content in shoots and leaves during stress and keeps the plants protected against oxidative stress. Hence, use of selected PGPRs may be important for alleviating salinity stress in glycophyte plants.

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