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ORIGINAL PAPER

Plant growth promoting bacteria confer salt tolerance in *Vigna radiata* by up-regulating antioxidant defense and biological soil fertility

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Abstract Salinity, a frequently occurring abiotic stress, is a major constraint for crop productivity worldwide. The present study was conducted to evaluate the ability of plant growth promoting rhizobacteria (PGPR) *Bacillus cereus* Pb25, isolated from soil irrigated with saline water, to promote *Vigna radiate* (mungbean) growth in the absence and presence of salt stress (9 dS m^{-1}). Results demonstrated that *B. cereus* promoted *V. radiate* plant growth significantly even in the presence of salt. Inoculations with PGPR improved the plant growth, and increased the root, shoot fresh and dry biomass and yield as compared to plants with no bacterial treatment (control). Results showed that both chlorophyll content and plant growth were inhibited by saline stress and the salt-induced oxidative damage (measured by MDA, H_2O_2) was alleviated by PGPR inoculation. Furthermore, PGPR inoculation significantly increased the antioxidant enzymes (POD, SOD and CAT) activities and enhanced the accumulation of proline, potassium, nitrogen and phosphorus as well as decreased sodium accumulation in saline stressed plants. Regarding the soil biological activity, inoculated PGPR enhanced the activity of dehydrogenase, alkaline phosphatase, microbial biomass carbon, available phosphorus and total organic carbon under saline stress as compared to saline treatment alone. These results suggest that *B. cereus* can be used in salinized agricultural lands as bio-inoculant to increase crop productivity.

Keywords Alkaline phosphatase · *Bacillus cereus* Pb25 · NaCl · Dehydrogenase · Mungbean (*Vigna radiata* (L.) Wilczek) · Yield

Introduction

For agriculture, saline stress is the most serious threat because salt deposition on arable land turns cultivatable land into unproductive area. Almost 3.6 billion hectares (Mha) of agriculture land (5.2 billion ha) is affected from soil degradation, erosion and salinity (Riadh et al. 2010). The 10 % of the land surface (950 Mha) and 50 % of arable land (230 Mha) are salt affected globally (Ruan et al. 2010). Soil salinization is reducing the area that can be used for agriculture by 1–2 % every year ultimately, reducing food production that result in the negative ecological, socio-economic outcomes. For example, the accumulation of salt in tsunami affected areas of Maldives damaged approximately 70 % of agriculture land, destroyed approximately 370,000 fruit trees and affected around 15,000 farmers (FAO 2005). Ghassemi et al. (1995) reported loss of $12 billion per year based on crop yield loss due to salanization. The inflation-adjusted cost of salt-induced land degradation in 2013 was $441 ha^{-1}. The cost of salt-induced land degradation in 2013 was estimated at

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US $441 per hectare, yielding an estimate of global economic losses at US $27.3 billion per year (Qadir et al. 2014).

Salinization consists of an accumulation of water soluble salts in the soil that include the ions of potassium (K^+), magnesium (Mg^{2+}), calcium (Ca^{2+}), chloride (Cl^-), sulfate (SO_4^{2-}), carbonate (CO_3^{2-}), bicarbonate (HCO_3^-) and sodium (Na^+). Depending on the soil, the extract solution differs in the content of dissolved salts. Extract solution of soil with electrical conductivity (EC), exceeding 20 mM ($\sim$2 dS m^{-1}) can be categorized as salt affected soil (Silva and Fay 2012). Saline stress has a negative impact on plant growth and metabolism, mainly associated with enhanced uptake of Na^+ in plant tissue, resulting in restricted photosynthesis and increase production of reactive oxygen species (ROS) which accelerate toxic reactions like membrane damages, protein degradation and DNA mutation (Islam et al. 2015; Sudhir and Murthy 2004). To alleviate these oxidative stresses, plants upregulate different kinds of enzymatic and non-enzymatic antioxidants like superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and proline to scavenge overproducing ROS (Noreen et al. 2010).

Remediation of salt affected areas with efficient, low cost and adaptable method is a challenging goal for scientists (Munns and Tester 2008). However, the use of beneficial microorganisms has great potential in ecofriendly and sustainable agriculture. Recent studies have shown that local adaptation of plants to their environment is driven by genetic differentiation in closely associated microbes (Lau and Lennon 2011). Rhizospheric microorganisms have shown potential to enhance the growth of different crops grown in a wide range of root-zone salinization, this approach may succeed where it has proved difficult to develop salt-tolerant germplasm (Dodd and Perez-Alfocea 2012). The potential application of PGPR in agriculture is based on their ability to increase crop growth and stress amelioration without harming the environment. The complex and dynamic interaction between microorganisms and plant roots under abiotic stresses, affects not only the plant growth, but also the soil.

On the other hand, the identification, selection and application of suitable beneficial microorganism can increase the option to deal with the growing problem (Kilian et al. 2000) and also it is environmental friendly (Woitke et al. 2004). PGPR inoculation mediates physiological and anatomical changes in plants, which plays a vital role during environmental stresses. Owing to obvious evidence of the adverse effect of saline stress on plant growth, it was hypothesized that the selected PGPR used in this study can overcome the injurious effects of NaCl stress on mungbean plants. The aims of this research were (1) to determine the influence of PGPR inoculation on the growth of *Vigna radiata* (mungbean) plants under saline stress; (2) to evaluate the effects of PGPR on physiological responses of mungbean; (3) to examine the changes in mineral (N, P, Na^+ and K^+) contents of the plant as well as the activities of soil enzymes.

Materials and methods

Sampling and bacterial isolation

The soil samples were taken from soil irrigated with saline waste water near Chakera (31°46N, 73°00W), Faisalabad, Pakistan. The bacterial isolation was carried out at same day through the pore plate method (Islam et al. 2014). Briefly, 100 µL of 10^6 times serially diluted soil solution (prepared in physiological saline) was spread on Luria–Bertani (LB) media plates and were incubated for 24 h at 30 °C (McLellan et al. 2009). Purified bacterial strains were evaluated for their tolerance to NaCl in term of maximum inhibitory concentration (MIC) by gradually increasing NaCl concentration in LB plates until the bacterial isolates failed to grow. This concentration was considered as MIC of respective isolate.

Evaluation for PGPR characters

Auxin production assay

For indole acetic acid production, NaCl resistant bacterial isolates were grown in LB broth with the addition of 0.5 mg L^{-1} tryptophan. A 1 mL of overnight grown culture (10^8 CFU mL^{-1}) was added in 20 mL of LB broth and incubated at 150 rpm for 96 h. The absorbance of pink color was read at 530 nm on spectrophotometer (Halo DB-20 UV–VIS Double beam spectrophotometer) (Gordon and Weber 1951). The indole acetic acid (IAA) concentration in the culture was determined using a calibration curve of pure IAA as a standard following linear regression analysis (Sheng et al. 2008).

Siderophore production and ACC deaminase assay

The siderophore production of isolated bacteria was determined by chrome azurol-S method (CAS) following the method of Schwyn and Neilands (1987) by the production of orange halo zone around the bacterial isolates on blue ager. The production of α-ketobutyrate (a-KB) through the enzymatic cleavage of 1-aminocyclopropane-1-carboxylate (ACC) was determined by measuring its absorbance in bacterial culture at 540 nm and compared with the absorbance of known concentration of pure α-ketobutyrate (a-KB). Protein concentration in bacterial

culture was estimated by the method of Bradford (1976) and the enzymatic activity was expressed as 1 M a-KB mg^{-1} h^{-1} (Huaidong et al. 2012; Jalili et al. 2009).

Phosphate (P) solubilization assay

For the quantitative measurement of P solubilization, the freshly prepared bacterial culture (10^8 CFU mL^{-1}) was inoculated in Pikovskaia's broth containing 2.5 g of TCP (Tri-Calcium Phosphate). The culture was incubated at constant shaking of 200 rpm for 7 days at 30 °C. Supernatant of the culture was obtained by centrifugation at 2817*g* and was used to determine P-solubilization through the calorimetric method of Olsen et al. (1954).

Bacterial characterization and phylogenetic analysis

Among the isolated salt resistant bacteria Pb25 was found highly salt resistant (MIC = 60 g L^{-1} NaCl) and efficient among other isolates in studied plant growth promoting activities under stress condition. So, the bacterial strain Pb25 was further characterized and evaluated in pot experiment.

Molecular identification

For molecular identification, bacterial genomic DNA was extracted using QIAGEN genomic DNA isolation kit according to the manufacturer's recommendations. The 16S rRNA gene was amplified using universal primers 27 F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′). The amplification reaction was carried out in a final volume of 25 µL containing 2.5 µL of *Taq* polymerase buffer, 200 µM of each dNTP, 1.5 mM of $MgCl_2$, 0.5 µM of each primer, 0.625 U of Taq polymerase and 1 µL of template solution. PCR was performed in a thermocycler (PTC 200 Gradient Cycler, MJ Research, Waltham, Massachusetts) according to the following program: one cycle of 4 min at 94 °C; 39 cycles of 1 min at 94 °C, 1 min at 55 °C, 1.5 min at 72 °C and one final cycle of 5 min at 72 °C. The amplified 16S rRNA gene products were purified by Mini Elute PCR Purification Kit following manufacturer's instructions (QIAGEN). Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied Bio-Systems, USA). The obtained 16S rRNA gene sequence of bacterial isolate Pb25 was compared with the known nucleotide sequences in the GenBank database using BlastN (http://www.ncbi.nlm.nih.gov/ BLAST). Pb25 showed 100 % resemblance with *Bacillus cereus* strains of the database. On the basis of resemblance with specific group of bacteria, we conclude isolate Pb25 as *B. cereus*. The sequence was deposited in the GenBank database under the Accession number KF471506. Biochemical characterization of the bacterial isolates was carried out according to Bergey's Manual of Determinative Bacteriology (Holt et al. 1994) (Supplementary Table 1). A phylogenetic tree was constructed by carrying out the multiple alignments among different selected bacterial 16S rRNA gene sequences of database and Pb25 using Clustal X version 2.0.11 (Thompson et al. 1997) and neighbour-joining method of Saitou and Nei (1987). Nucleotides substitution rate (Kimura 1980) and 1000 times of bootstrap analysis for evaluation of phylogenetic tree topology (Felsenstein 1993) were also calculated. The data of aligned and processed sequences was visualized using NJ Plot version 2.3 (Perrière and Gouy 1996; Fig. 1).

Pot experiment

Inoculum preparation

Bacterial culture was prepared by inoculating pure single colony of *B. cereus* Pb25 to LB broth and incubated at 30 ± 1 °C for overnight (12 h). The bacterial cells were harvested by centrifugation at 2012*g* for 5 min. The pellet obtained was re-suspended in sterilized distilled water. The optical density of the bacterial culture was maintained at 10^7–10^8 CFU mL^{-1} using a UV visible spectrophotometer by following the method of Mortensen et al. (1992). This bacterial suspension of *B. cereus* Pb25 was used to inoculate mungbean (*Vigna radiata* (L.) Wilczek) seeds.

Plant material and growth conditions

For the pot experiment, soil was collected from the botanical garden of the university. The soil was sieved (2 mm) and sterilized by autoclaving at 121 °C for 15 min at 15 psi. Analytical characteristics of the soil, including pH (7.4), electrical conductivity (1.3 dS m^{-1}) and total nitrogen (97.34 kg ha^{-1}) were recorded. Soil enzyme activities were measured in fresh moist soil. Dehydrogenase activity was measured according to Pepper et al. (1995) through the reduction of 2,3,3 tri-phenyltetrazolium chloride and expressed in µg triphenyl formazan g^{-1} soil h^{-1}. Alkaline phosphatase activity was estimated by the method of Eivazi and Tabatabai (1977). Total organic carbon was analyzed by using Walkley and Black (1934) method. Available phosphorus was analyzed by the method of Olsen et al. (1954). The microbial biomass carbon was determined using the chloroform fumigation extraction method of Vance et al. (1987).

For seed inoculation, seeds of mungbean (NIAB-2006) were obtained from National institute of agriculture and biology (NIAB), Faisalabad, Pakistan. The seeds were

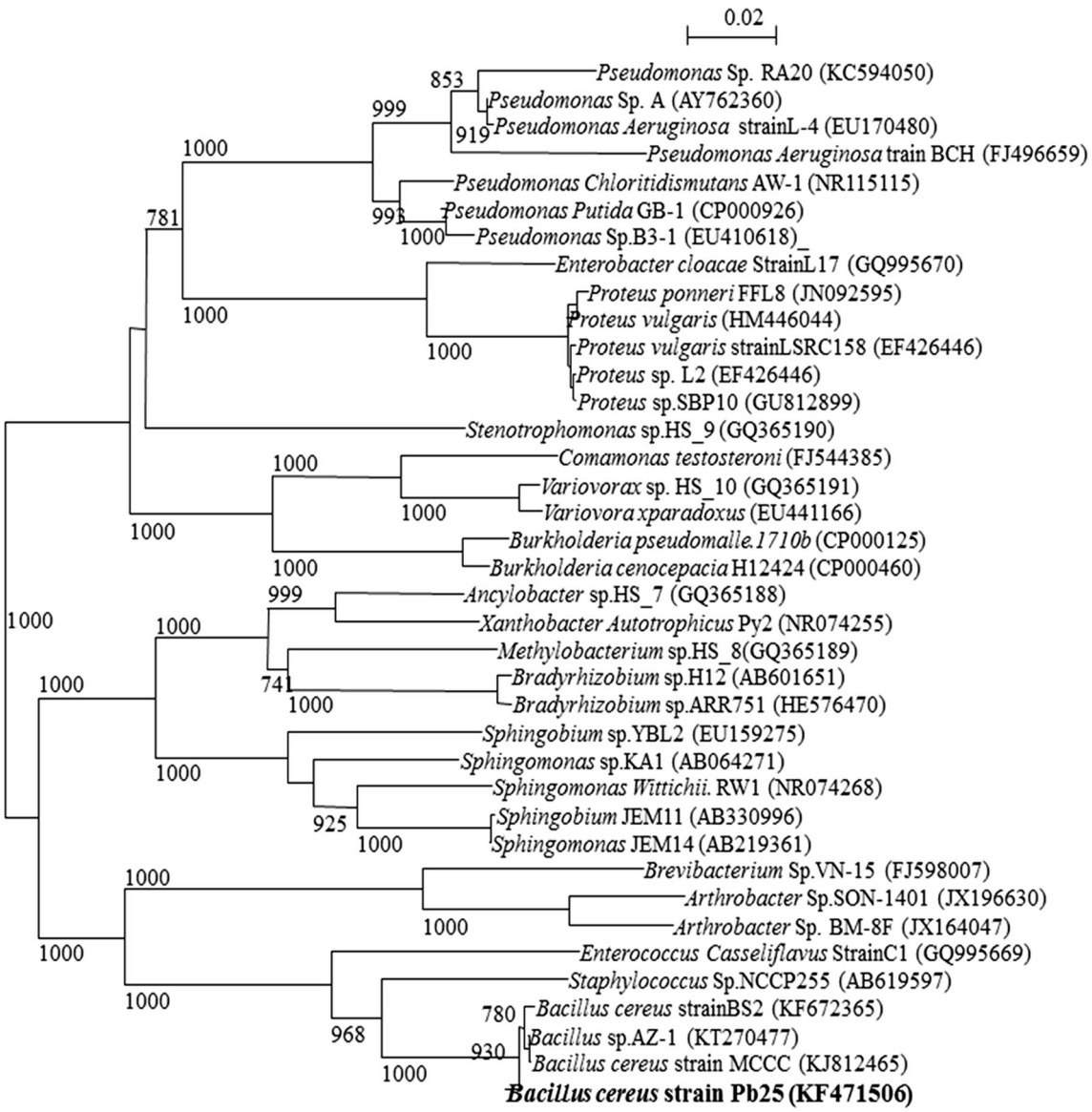

Fig. 1 A phylogenetic relationship of bacterial isolates Pb25 identified on the basis of 16S rRNA gene sequences as *B. cereus* with accession number KF471506. The branching pattern was generated by the neighbor-joining method. *Numbers* indicate bootstrap values greater than 700 Bar, 0.02 *K*nuc unit

surface sterilized with 70 % ethanol for 5–10 min and washed thoroughly with double distilled water. Mungbean seeds were inoculated by seed dressing with *B. cereus* Pb25 culture of approximately 10^8 CFU mL^{-1}. The seeds were sown in earthen pots lined with polythene layer containing 1.5 kg of autoclaved soil. All the pots were kept under natural light (sunlight) with average 39/27 °C day and night temperature and humidity 30 % during the study period. The pot experiment was laid out in complete randomized design (CRD) in triplicate with four treatments as T_0 (control containing Non-saline soil), T_1 (Saline soil), T_2 (Non-saline soil + *B. cereus* Pb25), T_3 (Saline soil + *B. cereus* Pb25*)*. Two salinity levels, 1.41 (i.e. original), 9 dS m^{-1}, by adding NaCl in each pot and mixed it thoroughly. Levels of salinity were determined according to Rhoades et al. (1989). To maintain the salinity stress throughout the experiment, drainage water was collected from each pot was put back into the same pot as per requirement. For treatment, T_0 and T_1 sterilized seeds were sown while for treatment T_2 and T_3, sterilized seeds were inoculated with bacterial culture by seed dressing. To maintain a sufficient community of bacterial isolates 2 mL of bacterial inoculum was poured along sides of emerging seedlings.

Plant growth and yield attributes

At maturity, three replicates from each treatment were harvested to measure the fresh weight of root and shoot; while, dry weight was recorded after drying at 70 °C for 5 days. Yield and yield contributing parameters were also taken from the remaining plants of each treatment.

Biochemical studies

Fresh leaves (0.5 g) were homogenized in 10 mL of ice cold potassium buffer (pH 7.0) in an ice bath by grinding with a mortar pestle. The mixture was centrifuged at 4 °C for 20 min at 12,000 rpm. The supernatants were stored at 4 °C and used for the determination of various antioxidant enzymes (Islam et al. 2014). Superoxide dismutase (SOD) activity was measured through the photoreduction of nitro blue tetrazolium chloride (NBT) (Dhindsa et al. 1981). The reaction mixture was placed under lamp below 15 W for 25 min then stopped by switching off the light. Non illuminated and illuminated reactions without supernatant served as calibration standard. The absorbance was measured at 560 nm. One unit of SOD activity was defined as the quantity of SOD required to produce a 50 % inhibition of NBT, and the specific enzyme activity was expressed as Units mg^{-1} protein. Modified method of Aebi (1984) was followed for the measurement of catalase. The absorbance of mixture was measured at 240 nm by the addition of 6 mM H_2O_2 in a reaction mixture for 2 min. A 0.1 unit min^{-1} change in absorbance was defined as 1 unit of CAT activity, and CAT activity was expressed as U mg^{-1} protein.

The activity of POD was determined by guaiacol oxidation method (Maehly and Chance 1954). Changes in absorbance of the reaction solution at 470 nm were determined every 20 s. One unit of POD activity was defined as an absorbance change of 0.01 unit min^{-1} and POD activity was expressed as U mg^{-1} protein. Total chlorophyll contents were determined according to the method of Arnon (1949).

MDA, H_2O_2 and proline contents

To find out the extent of NaCl-induced oxidative stress, lipid peroxidation was evaluated by measuring the malondialdehyde (MDA) formation in the leaves of mungbean plant. Briefly, 1 g of fresh weight of leaves was homogenized in 20 mL of 0.1 % TCA solution and centrifuged for 10 min at 12,000 rpm. The 1 mL of supernatant was mixed with 4 mL of TCA containing 5 % TBA, heated for 30 min at 95 °C and cooled on ice. The mixer was centrifuged at 12,000 rpm for 10 min and absorbance of the supernatant was taken at 532 and 660 nm (Demiral and Türkan 2005). Concentration of H_2O_2 was determined by following Velikova et al. (2000). Fresh leaf tissues (0.5 g) were homogenized with 5 mL of 0.1 % (w/v) trichloroacetic acid (TCA) in a pre-chilled pestle and mortar and the homogenate was then centrifuged at 12,000 rpm for 15 min. To 0.5 mL of the supernatant, 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of potassium iodide (1 M) were added. The mixture was vortexed and its absorbance was read at 390 nm. The H_2O_2 concentrations were calculated using a standard curve prepared with known concentrations of H_2O_2. Moreover, proline content was measured according to Bates et al. (1973) method.

Sodium, potassium, phosphorus and nitrogen contents estimation

For the determination of sodium and potassium ions, the dried ground leaf of mungbean (0.1 g) was digested with H_2SO_4 and H_2O_2 and their concentration were determined by using a flame photometer. Phosphorus was determined spectro-photometrically following method of Jakson (1967). Nitrogen was determined following the Kjeldahl method as described by Bremner (1965).

Statistical analysis

All data were presented as mean values of three replicates. The data were analyzed using a statistical package, SPSS (Version 19.0). One-way ANOVA (analysis of variance) was employed followed by Duncan's multiple range tests to determine the significant difference among means of the treatment at $P \leq 0.05$.

Results

Isolation, identification and growth conditions of *Bacillus cereus* Pb25

During the initial screening process, five bacterial isolates showed high resistance to salt stress. However, the bacterial isolate Pb25 which was identified as *B. cereus* (KF471506) through 16S rRNA gene sequencing, was able to grow at 60 g L^{-1} of NaCl. *B. cereus* Pb25 also showed tolerance to 50 mg L^{-1} of Pb, 100 mg $L^{-1}$of Zn, 25 mg L^{-1} of Cr and 80 mg L^{-1} of Cu; while, other bacterial isolates were unable to grow at an above mention concentration of heavy metals. The growth curve of the isolate showed extended lag phase of 60 h in the presences of 20–32 g L^{-1} of NaCl with 3.2×10^7 CFU mL^{-1} while, lag phase increased from 60 to 144 h when the

concentration of NaCl was increased from 33 to 55 g L^{-1} with 1.2 × 10^3 to 1.7 × 10^2 CUF mL^{-1}.

Plant growth promoting (PGP) characteristics

To identify PGP activities of *B. cereus* Pb25, quantitative estimation of IAA production in LB broth supplemented with tryptophan, utilization of ACC as the sole N source, phosphorous solubilization and siderophore production was evaluated under salt stress (9 dS m^{-1}) condition (Table 1). Among the initially screened five isolates, *B. cereus* Pb25 produced 19 mg L^{-1} of IAA and solubilized 39 mg L^{-1} of phosphorus from TCP. Moreover, the ACC deaminase and siderophores production were only observed in *B. cereus* Pb25 inoculation under salt stress (Table 1).

Effect on microbial biomass and soil enzymes

The data regarding effect of bacterial inoculation and salt stress on microbial biomass and soil enzymes is presented in Table 2. All the studied parameters exhibited remarkable difference between bacterial inoculation and saline stress. *B. cereus* Pb25 inoculation not only increased microbial biomass carbon (20.86 μg g^{-1}) but also increased the contents of available phosphorus (0.51 μg g^{-1}) in saline stress soil. Statistically significant difference among soil enzymes (dehydrogenase and phosphatase) could also be observed (Table 2). The maximum value of dehydrogenase enzymes was found in the sole inoculation of PGPR inoculated soil (0.41 μg g^{-1}) followed by the PGPR inoculated saline stress soil (0.28 μg g^{-1}). However, salinity alone decreased the dehydrogenase activity as compared to control soil. A similar trend was found in the case of phosphatase enzyme; where the minimum activity of enzymes was observed in saline treatment soil; while, significant increase in phosphatase activity was found in the sole inoculated PGPR soil (32.39 μg g^{-1}) followed by the PGPR inoculated saline soil (20.40 μg g^{-1}). The increase in phosphatase enzyme activity is correlated with the increase in availability of phosphorous in soil. The salinity decreases the availability of phosphorous in the soil while the addition of bacteria along with salinity enhanced the availability of phosphorous in soil (Table 2).

Table 1 Plant growth promoting characters of *B. cereus* Pb25 under 9 dS m^{-1} NaCl

Sr. no.	PGP character	Value
1	ACCDA (1 Mα-KB mg^{-1} h^{-1})	29.0 ± 1.65
2	IAA (mg L^{-1})	19.0 ± 1.20
3	Siderophores (cm)	2.7 ± 0.95
4	P solubilization (mg L^{-1})	39.0 ± 3.12

Average ± standard error from three separate replicates

Bacillus cereus Pb25 increased the plant growth and yield parameters under NaCl stress

The response of plant growth parameters such as fresh and dry weight of shoot and root, nodules fresh and dry weight per plant has been presented in Table 3. The addition of NaCl alone caused a significant reduction in plant growth parameters as compared to control. Inoculation of *B. cereus* Pb25 significantly increased fresh and dry weight of shoots and roots as well as nodules fresh and dry weight per plant under NaCl treated conditions. The PGPR inoculations increased the root and shoot fresh weight by 57 and 43 % respectively under saline stress; while, enhanced more than 6 % in leaf area and 65 % in nodules fresh weight per plant as compared to saline stress plants. Effect of bacterial inoculation and salt stress on different yield parameters (numbers of pods $plant^{-1}$, number of seeds pod^{-1}, seeds yields $plant^{-1}$, 1000-seed weight) are summarized in Table 4. Yield attributes were significantly influenced by salinity and bacterial inoculation. Statistical analysis showed that *B. cereus* Pb25 inoculation under saline stress increased the number of green pods per plant by 45 % as compared to salinity treatment. Saline stress significantly reduced the number of seeds per plant; while, *B. cereus* Pb25 inoculation increased number of seeds per plant by 38 % under saline stress. The highest increase in 1000-seed weight was found in the sole inoculation of *B. cereus* Pb25 followed by *B. cereus* Pb25 inoculation under saline stress; while, minimum weight was found in plants treated with saline stress alone.

Bacillus cereus Pb25 inoculation improved the mineral contents

The plants subjected to salinity stress demonstrated a significant increase in the sodium level in comparison to control plants. Under saline stress, significant decrease in K^+, P and N was also observed in mungbean plants (Fig. 2a, b). However, inoculation of *B. cereus* Pb25 significantly decreased the Na^+ accumulation by 22 % in mungbean under saline stress as compared to un-inoculated salt stressed plants. In addition to this, a significant increase in phosphorus (48 %), nitrogen (33 %) and potassium (50 %) contents were measured in inoculated stress plants as compared to the un-inoculated salt stress plants (Fig. 2a, b).

Table 2 Effect of *B. cereus* Pb25 inoculation on microbial biomass carbon (μg g^{-1}), available phosphorous (μg g^{-1}), total organic carbon (%), dehydrogenase and phosphatase enzymes

Treatment	DHE (μg g^{-1})	PE (μg g^{-1})	MBC (μg g^{-1})	AP (μg g^{-1})	TOC (%)
Control	0.14 ± 0.02c	11.19 ± 1.19c	11.23 ± 1.86c	0.21 ± 0.02c	0.27 ± 0.02c
NaCl	0.09 ± 0.01c	5.65 ± 1.64c	7.33 ± 0.65c	0.20 ± 0.03c	0.23 ± 0.02c
B. cereus	0.41 ± 0.04a	32.39 ± 3.38a	31.33 ± 3.28a	0.67 ± 0.03a	0.79 ± 0.05a
B. cereus + NaCl	0.28 ± 0.02b	20.40 ± 2.05b	20.86 ± 2.02b	0.51 ± 0.03b	0.61 ± 0.03b

Average ± standard error from three separate replicates. Values with different letters are significantly different at $P \leq 0.05$ as determined by Duncan's test

NaCl, 9 dS m^{-1}; MBC, microbial biomass carbon; AP, available phosphorous; TOC, total organic carbon (%); DHE, dehydrogenase enzyme; PE, phosphatase enzyme

Table 3 Effect of *B. cereus* Pb25 inoculation on shoot fresh weight (g), shoot dry weight (g), root fresh weight (g), root dry weight (g), nodules fresh weight (mg) and nodule dry weight (mg) of mungbean $plants^{-1}$

Treatment	Shoot Fw (g)	Shoot Dw (g)	Leaf area (cm^2 $plant^{-1}$)	Root Fw (g)	Root Dw (g)	Nodules Fw (mg)	Nodules Dw (mg)
Control	15.00 ± 3.06ab	6.07 ± 0.58b	534.70 ± 14.65a	9.63 ± 1.30ab	3.67 ± 0.30a	339.45 ± 5.08b	104.60 ± 1.07b
NaCl	9.01 ± 1.00c	3.13 ± 0.54c	447.67 ± 21.53a	5.30 ± 0.44c	2.30 ± 0.31b	170.00 ± 2.21c	58.50 ± 3.25d
B. cereus	18.07 ± 1.52a	8.43 ± 0.68a	565.53 ± 15.45a	11.88 ± 0.93a	4.50 ± 0.35a	489.47 ± 3.78a	143.34 ± 2.70a
B. cereus + NaCl	14.13 ± 1.17ab	7.23 ± 0.58ab	488.43 ± 12.54a	8.33 ± 0.72b	3.13 ± 0.56a	290.53 ± 1.64b	87.15 ± 4.88c

Average ± standard error from three separate replicates. Values with different letters are significantly different at $P \leq 0.05$ as determined by Duncan's test

NaCl, 9 dS m^{-1}; Fw, fresh weight; Dw, dry weight

Table 4 Yield attributing parameters of mungbean as influenced by *B. cereus* Pb25 inoculation

Treatment	Numbers of pods $plant^{-1}$	Number of seeds pod^{-1}	Seed yields $plant^{-1}$ (g)	1000-seed weight (g)
Control	18.00 ± 1.73b	8.03 ± 0.69a	3.53 ± 0.54ab	27.33 ± 3.28b
NaCl	7.00 ± 0.67c	5.07 ± 0.50b	2.13 ± 0.23b	11.67 ± 1.45c
B. cereus	26.00 ± 1.53a	11.03 ± 1.52a	4.20 ± 0.38a	35.33 ± 2.96a
B. cereus + NaCl	16.00 ± 1.73b	6.83 ± 0.41b	2.90 ± 0.53ab	21.67 ± 1.45b

Average ± standard error from three separate replicates. Values with different letters are significantly different at $P \leq 0.05$ as determined by Duncan's test

NaCl, 9 dS m^{-1}

Bacillus cereus Pb25 lowered the NaCl-induced oxidative stress

Analysis of the rhizospheric soil showed that *B. cereus* Pb25 successfully colonized in the roots of mungbean plants with an average of 10^6 and 10^3 CUF mL^{-1} in the sole inoculation of *B. cereus* Pb25 and *B. cereus* Pb25 + saline stress treatment, respectively. The effect of inoculation on the accumulation of MDA as well as H_2O_2 under salt stress is presented in Fig. 3. Approximately one fold in MDA and 1.5 fold increase in H_2O_2 contents was observed in salt stressed plants as compared to control. While, MDA and H_2O_2 contents were found significantly lower in bacterially inoculated salt stress plants as compared to un-inoculated salt stress plants. Salt stress significantly decreased the total chlorophyll content of mungbean plants (Fig. 3). However, *B. cereus* Pb25 inoculated salt stress plants maintained higher contents of chlorophyll (1.63 mg g^{-1} FW) as compared to un-inoculated salt stress plants (1.03 mg g^{-1} FW).

Bacillus cereus Pb25 enhanced the antioxidant activity

Data showed that CAT activity was significantly modulated in inoculated salt stress plants (Fig. 4a) compared to the un-inoculated salt stress plants, while no change in the activity of CAT was noted between sole inoculation of bacterial inoculation and control plants. SOD activity was significantly higher in un-inoculated salt stress plants as compared to bacterially inoculated stress plants (Fig. 4b). The increase in SOD activity under salt stress and a relative

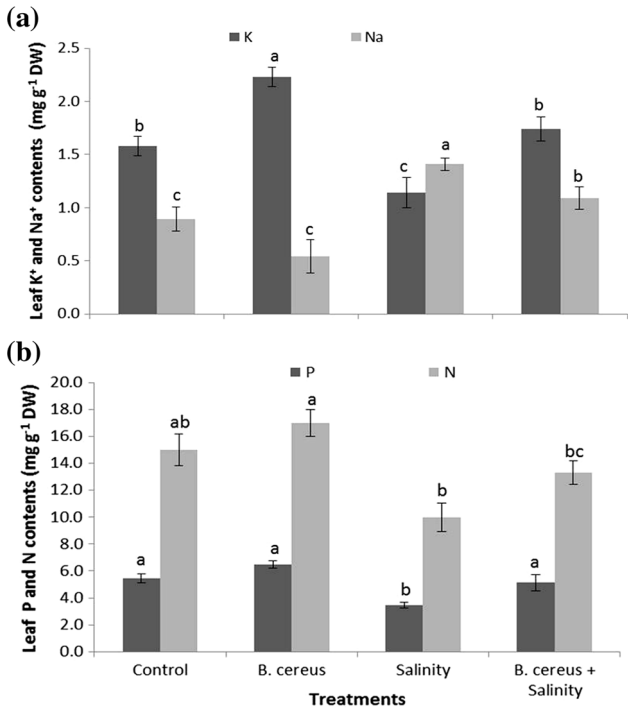

Fig. 2 Effect of *B. cereus* Pb25 inoculation and salinity (NaCl, 9 dS m^{-1}) on (**a**) K^+ (mg g^{-1} DW) and Na^+ (mg g^{-1} DW), (**b**) P (mg g^{-1} DW) and N (mg g^{-1} DW) contents of mungbean plants. Average ± standard error from three separate replicates. Values with *different letters* are significantly different at $P \leq 0.05$ as determined by Duncan's test

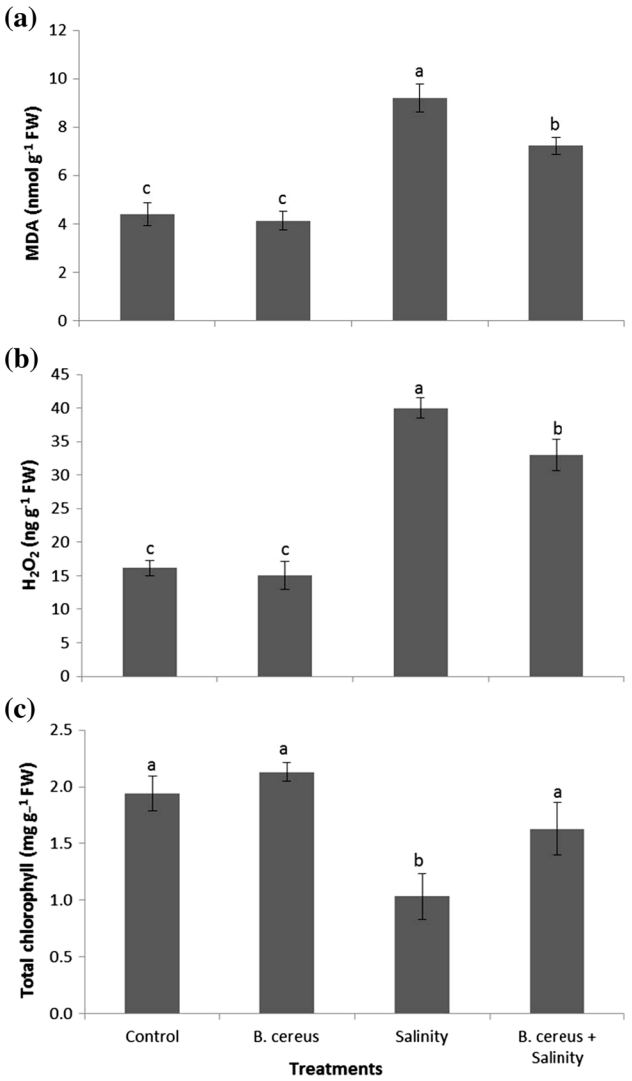

Fig. 3 Effect of *B. cereus* Pb25 inoculation and salinity (NaCl, 9 dS m^{-1}) on H_2O_2 (ng g^{-1} FW), MDA (nmol g^{-1} FW) and chlorophyll (mg g^{-1} FW) contents of mungbean plants. Average ± standard error from three separate replicates. Values with *different letters* are significantly different at $P \leq 0.05$ as determined by Duncan's test

decrease in SOD activity in inoculated stress plants are correlated with the production of H_2O_2 in mungbean plants (Fig. 3). The POD activity increased non-significantly under sole inoculation of bacteria as compared to control. The highest increase in POD activity was found under inoculated salt stress plants (Fig. 4c). Similarly, a significant increase in the proline content was recorded in inoculated plants grown under stress conditions as compared with that in un-inoculated salt stress plants (Fig. 4d), while significant increase of proline contents were also observed in inoculated non stress plants. Thus, data obtained from the present investigation suggest that the accumulation of proline itself confers salt tolerance in mungbean plants.

Discussion

Strains with plant growth-promoting activity have been identified from various genera of which Pseudomonas and *Bacillus* are the most extensively studied (Islam et al. 2014; Kumari et al. 2015). In this study, we have reported *Bacillus* strain possesses ACC deaminase, IAA, phosphate solubilization, and siderophore production activity that stimulated the growth of *V. radiata* under saline stress soil. *Bacillus* spp. reportedly has favorable effects on plant growth, higher yield, and tolerance (Compant et al. 2005; Wahyudi et al. 2011). The selected *Bacillus* strain was not only resistant to the saline stress, but also maintained higher growth rate under lead stress (unpublished results from author's lab). Due to multiple stress tolerance (salinity and Pb), chelation, production of organic acid, siderophores, capable of enhancing nutrient bioavailability and improving soil aggregation make this isolate superior than other reported *Bacillus* stains.

The intrinsic ability of bacteria to produce plant growth promoting (PGP) substances in the rhizosphere is influenced by different factors. Solubilization of phosphorus correlates with the production of organic acid in

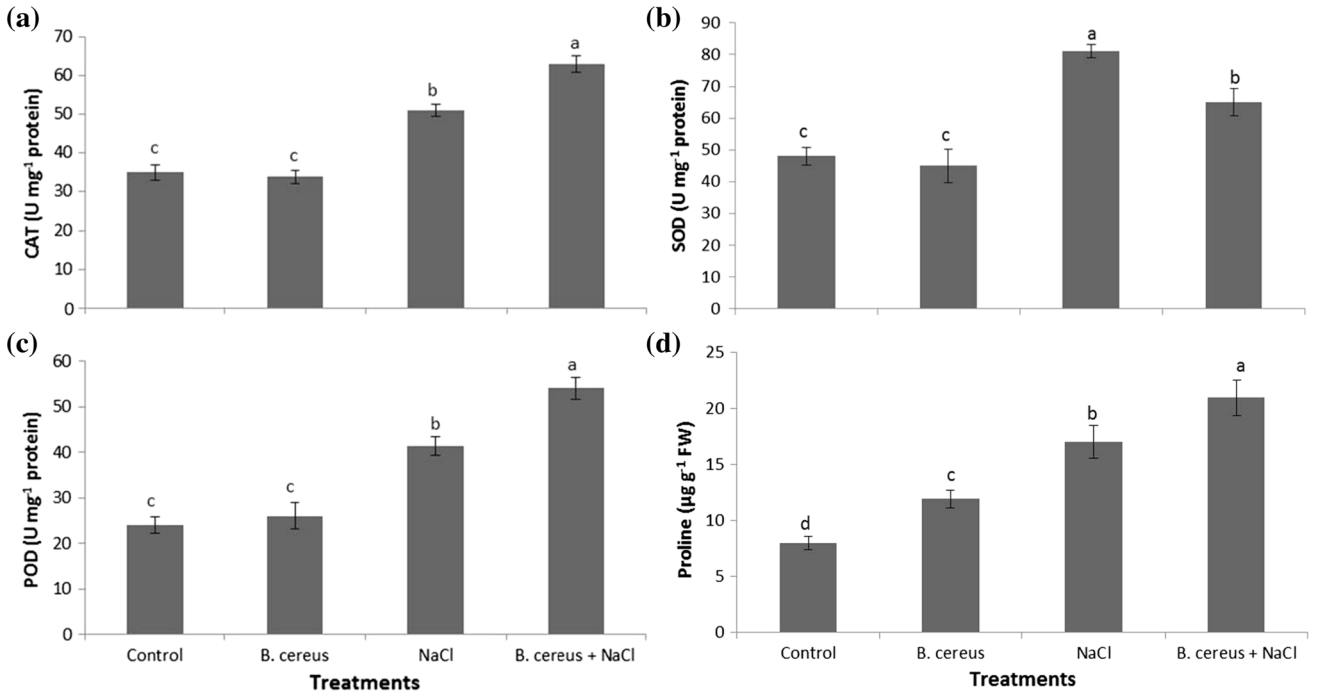

Fig. 4 Effect of *B. cereus* Pb25 inoculation and salinity (NaCl, 9 dS m^{-1}) on antioxidant enzymes activity and proline content of mungbean. Average ± standard error from three separate replicates. Values with *different letters* are significantly different at $P \leq 0.05$ as determined by Duncan's test

rhizosphere. The PGP bacteria are major contributors of solubilization of phosphorus in soil that ultimately help in the promotion of plant growth (Rajkumar et al. 2009). Strains of *Bacillus* genus have also been reported for production of plant growth regulators (like IAA), extracellular phytase, chitinase, antifungal peptides and phosphorus solublization (Yang et al. 2009; Nautiyal et al. 2013). Siderophore production is another important character of PGP bacteria that increases the uptake of iron under saline stress. The role of ACC deaminase in decreasing ethylene level by the enzymatic hydrolysis of ACC into α-KB and ammonia has been presented as one of the critical mechanism of PGP bacteria in promoting plant growth. ACC deaminase activity as measured in *B. cereus* Pb25 reflects its ability to utilize ACC as a sole source of nitrogen which can enhance root length and plant growth under salt stress. The ACC producing bacteria not only lowered the stress ethylene level in plants, but also prevent chlorophyll degradation (Glick et al. 2007; Fig. 3). In accordance with this, a reduction in chlorophyll due to salt stress and its relative recovery in the presence of *B. cereus* Pb25 has also been observed in the present study (Fig. 3). It might be due that the presence of ACC, deaminase in bacteria mitigated the damaging effect of salt on chlorophyll by suppressing the synthesis of ethylene, thus reduced the salinity-induced ethylene effect on chlorophyll decay.

The increase in chlorophyll content may also be due to the increased photosynthetic leaf area of *B. cereus* Pb25 inoculated plants compared to salt stress plants where the leaf area (Table 3) was reduced due to salinity stress (Nadeem et al. 2007). The reduction in growth attributes such as fresh and dry weights, and shoot and root lengths of mungbean under saline stress is also observed under salinity (Mohammed 2007). The adverse effects of salinity on plant growth in term of root, shoot dry and fresh weights could overcome by inoculation with the bacterium *B. cereus* (Chakraborty et al. 2011). Shukla et al. (2012) observed similar responses of growth in *Arachis hypogaea* inoculated with different bacterial isolates. It has been suggested that apart from bacterial ACC deaminase activity, elevated phosphorous availability (Upadhyaya et al. 2011) potassium uptake and phytohormones secretion might play a critical role in plant growth under saline stress (Mayak et al. 2004). Consistent with this, several PGP bacteria alter root development by the secretion of phytohormones such as IAA that results in increase root surface area and number of root tips, enabling the plant to uptake more nutrient and also can contribute to maintain leaf growth, which is considered as a primary response of plant productivity under the condition of salinity (Mantelin and Touraine 2004; Albacete et al. 2008). Therefore, PGPR can have multiple impacts on the phytohormone status, modifying root-to-shoot signalling and shoot hormone concentrations, which may improve growth, development, and physiological processes of plants under salt stress (Dodd et al. 2010).

Soil enzyme activities are the indicator of the ecosystem health and sustainability (Bergstrom et al. 1998; Cao et al. 2014). Previous studies have shown that saline conditions severely inhibit soil enzyme activities (Rietz and Haynes 2003). The enzymes produced by tolerant or resistant bacteria are also adapted to saline environment and perform well than those enzymes produced by non-tolerant or resistant bacteria (Cao et al. 2014). The reduced soil enzyme activities under salinity may be influenced by decrease enzyme synthesis due to the decrease in abundance and activity of soil microorganisms and/or to the "salting-out" effects on enzyme proteins (Zhang et al. 2011). Under microbial inoculation, enhanced neutral phosphatase, alkaline phosphatase, urease, and catalase activities (Table 3) in both bulk and rhizosphere soils were observed (Zhang et al. 2011). Dehydrogenase usually exists in every viable microbial cell (Singh and Kumar 2008). In this investigation, the subsequent and tangible activity of dehydrogenase was measured in *B. cereus* Pb25 inoculated soil. The high correlation of this enzyme with soil microbial biomass has widely been reported (Garcıa-Gil et al. 2000; Taylor 2002). Recently, Cao et al. (2014) also claimed higher dehydrogenase enzyme activity under bacterial inoculation and suggested that the soil microbial biomass is correlated with dehydrogenase enzyme because it is an intracellular enzyme which may be involved in microbial oxidoreductase metabolism. Previously, Eivazi and Tabatabai (1977) reported a predominance of acid phosphatase in acidic soils and alkaline phosphatase in alkaline soils. Therefore, in the present study only alkaline phosphatase activity has been determined in the soil as soil pH was 7.2. Alkaline phosphatase activity of inoculated soil was higher than un-inoculated soil (Table 2).

The findings of this study are in line with the study of Dodd et al. (1987) and Ezawa and Yoshida (1994) who conclude that inoculated bacteria may alleviate the negative effect of salinity on enzyme activities, probably due to increased soil microbial biomass (Zhang et al. 2011). A significant effect of the interaction between available P and phosphatase activity {($r^2 = .974$** (data not shown)} was observed because available P is a secondary dominant factor affecting phosphatase activity (Cao et al. 2014). Significantly higher available P in soil, even under salt stress with *B. cereus* Pb25 inoculation was observed (Table 2) which indicated the presence of organic acid in the rhizospheric soil released by plant roots or microbes (Amos and Walters 2006; Nguyen 2003). Many rhizosphere microorganisms are heterotrophs and might use root exudates to produce organic acids, which would increase P concentration in soil (Hameeda et al. 2006; Reyes et al. 2007). Probably, *B. cereus* Pb25 secreted organic acid in rhizospheric soil that solubilize insoluble inorganic or organic phosphate, responsible for this increase in available P as *B. cereus* Pb25 showed phosphorous solubilization in vitro (Table 1).

The results showed that saline stress induced oxidative stress in mungbean plants via increased lipid peroxidation and hydrogen peroxide accumulation. However, we noted that inoculated plants, both in stress and un-stress conditions exhibits lower a level of H_2O_2 and MDA contents than saline stress plants, indicating a lower accumulation of ROS and membrane damage in inoculated plants (Fig. 3). This ameliorated effect on MDA and H_2O_2 accumulation can be linked with the higher accumulation of nitrogen containing compounds such as proline (Fig. 4d), which is involved in the stabilization of sub-cellular structures (e.g., membrane and proteins), scavenge free radicals, and buffer cellular redox potential under stress conditions (Kavi-Kishor et al. 2005; Yang et al. 2008). The reduction of MDA and H_2O_2 contents in inoculated stress plants may be an efficient mechanism to attenuate the activation of plant defenses. During salinity-induced oxidative stress, availability of atmospheric CO_2 is reduced because of stomatal closure and consumption of NADPH by the Calvin Cycle is decreased, When ferrodoxine is over-reduced during photosynthetic electron transfer, electrons may be transferred from PS-I to oxygen to form superoxide radicals ($O_2^{\cdot-}$) by the process called Mehler Reaction (Türkan and Demiral 2009).

The plants have efficient mechanisms to compensate the possible oxidative damage to cellular components by inducing specific ROS-scavenging antioxidative enzymes (Türkan and Demiral 2009). In the present study, a significant increase in antioxidant enzyme activities (SOD, POD and CAT) was recorded in salt stressed mungbean plants as compared to control (Fig. 4). Significantly reduced SOD activity in *B. cereus* Pb25 inoculated mungbean plants under salt stress accompanied by enhanced H_2O_2 scavenging enzymes like CAT and POD activates, were of great importance to cope with oxidative stress during salt stress conditions (Kohler et al. 2008). This indicate the effectiveness of enzymatic activities in controlling the possible oxidative damage under bacterial inoculation (Pérez Rodríguez et al. 2013). The increase in the activity of H_2O_2 scavenging enzymes such as CAT and POD allows us to speculate that H_2O_2 homeostasis was altered in inoculated plants, what led to increased plant growth under stress. The increase in the activities of ROS scavenging enzymes under inoculated plants may be due to the triggering effect of PGPRs on genes encoding for antioxidative enzymes (Gururani et al. 2012). Similar results of modulation in antioxidant enzymes were reported for lettuce inoculated with *Pseudomonas mendocin* (Kohler et al. 2010) and for *Solanum tuberosum* inoculated with two *Bacillus* strains (Gururani et al. 2012).

One of the main consequences of NaCl stress is the loss of intracellular water. Plants accumulate many metabolites that are also known as "compatible (organic) solutes" in the cytoplasm to increase their hyperosmotic tolerance against salt stress-induced water loss from the cells. These compatible solutes accumulation help to balance the osmotic potential of Na^+ and Cl^- being sequestered into the vacuole. Proline contents were enhanced in the leaves of inoculated mungbean plants under salinity stress (Fig. 4d). Previously, studies on *Jatropha curcas* and *Triticum aestivum* showed an increase in proline in inoculated plants (Patel and Saraf 2013; Upadhyay et al. 2011). The higher proline accumulation in PGPR inoculated plants may be due to the higher uptake of nutrients, resulting in a high biosynthesis rate (Maziah et al. 2010; Vardharajula et al. 2011). The accumulation of proline in inoculated plants suggests a protective mechanism to keep positive water potential for water availability from the soil, leading to lower stress damage (Porcel and Ruiz-Lozano 2004). However, Kumari et al. (2015) demonstrated that proline accumulation in PGPR inoculated plants was caused by over expression of the P5CS gene. This may suggest that bacterial inoculation triggers the biosynthesis of proline in inoculated plants due to which enhanced accumulation of proline was measured in present study. Thus, proline accumulation at suitably high concentrations to create osmotic pressure is related to improving adaptation to salt stress by acting as a protein-compatible hydrotropic and radical scavenger.

A decrease in Na^+ uptake under bacterial inoculation (Fig. 2 a) might be due to the higher proportion of bacteria in rhizosphere of inoculated seedlings which caused a reduced apoplastic flow of Na^+ into the stele (Ashraf and Harris 2004) or may be the dilution effect due to growth enhancement (Table 3) and could be related with enhanced availability of P in rhizosphere that reduced the Na^+ uptake under saline conditions as observed by Talaat et al. (2015). Under saline stress, phosphorous nutrition is a limiting factor for plant growth and its uptake is also reduced due to the precipitation with other ions in soil. This may explain the reduced contents of P in saline treated soil and plants. The plants inoculated with PGPR have higher contents of P as compared to salt stress plants (Fig. 2b). The results showed that the inoculation of *B. cereus* Pb25, with the ability to solubilize P (Table 1), enhanced the phosphorous availability (Table 2) and increased the plant growth under saline stress (Table 3). In addition to this, we also observed the significant increase in N contents of inoculated plants. We did not measure the N assimilation ability of *B. cereus* Pb25 stain, but this increase might be due to the activity of ACC deaminase that increased the nitrogen contents in inoculated plants through the degradation of ethylene (role of ACC deaminase as we discussed earlier). The salinity decreases the N content in plant tissue due to direct competition of Cl^- with nitrate at membrane level or change plasma lemma integrity (Kohler and Raschke 2000). Inoculation of PGPR also elevated the level of K^+ in the plants and enhanced level of K^+ may be involved in the maintenance of the turgor pressure and the mitigation of oxidative stress impose by higher salinity (Upadhyaya et al. 2011). Possibly, the increased K^+ accumulation in salt stress plants may be related to the impact of PGPR inoculation on the stability of membranes that facilitate compartmentalization within vacuoles and selective ion uptake (Ramoliya et al. 2004). The increased concentration of Na^+ and decreased K^+ in this study under saline stress may be due to maintain turgor pressure in salt stress plants (Fig. 2a). Decrease uptake of Na^+ is considered as mechanism of salt tolerance by limiting the translocation of Na^+ ions to the aerial part to protect the photosynthetic apparatus from salt damage (Esechie et al. 2002). The results of present investigation proposed that PGPR inoculation had a remarkable impact on nutrient acquisition. PGPR inoculation reduced the sodium uptake and enhanced the phosphorous, potassium and nitrogen contents in mungbean leaves, especially under saline conditions.

Conclusions

From the findings of the present study, it is concluded that *B. cereus* strain Pb25 exhibited resistance to high concentration of NaCl and protected the plants against the inhibitory effects of NaCl through its plant growth promoting activity (IAA, ACC, siderophore and solubilizing the phosphate). The bacterial inoculation successfully enhanced the nodulation and yield of mungbean plants under saline condition by increasing plant antioxidative capacity and by decreasing oxidative stress. Moreover, inoculation of *B. cereus* also modulated the soil enzymatic activities under salinity. Hence, use of PGP bacteria may be important input to decrease the deleterious effect of saline soils.

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