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ORIGINAL ARTICLE

Phylogenetic analysis of halophyte-associated rhizobacteria and effect of halotolerant and halophilic phosphate-solubilizing biofertilizers on maize growth under salinity stress conditions

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Atriplex amnicola, halophilic plant growth-promoting rhizobacteria, maize, phosphate biofertilizers, *Salsola stocksii*, soil salinity.

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Abstract

Aims: The main objective of the present work was to evaluate plant growth-promoting abilities of bacterial strains from the rhizosphere of halophytes and their effect on maize growth under salinity stress.

Methods and Results: Halophilic bacteria were identified using 16S rRNA sequence analysis and their plant growth-promoting abilities were characterized. Phylogenetic analysis showed that bacterial strains belonging to *Bacillus*, *Halobacillus* and *Pseudomonas* were dominant in the rhizosphere of halophytes. More than 93% strains showed P-solubilization activity and IAA production. About 54% strains were able to produce ACC deaminase, 29% strains showed positive results for nitrogen fixation, 41 and 21% strains showed siderophores and HCN production ability respectively. More than 90% strains showed antifungal activity against more than two fungal pathogens and production of different hydrolytic enzymes. To study the plant growth-promoting effect on maize, five bacterial strains *Bacillus safensis* HL1HP11 and *Bacillus pumilus* HL3RS14, *Kocuria rosea* HL1RP8, *Enterobacter aerogenes* AT1HP4 and *Aeromonas veronii* AT1RP10 were used as inoculants; in the form of seed coat and enriched soil-based phosphate biofertilizers. All bacterial strains positively affected the maize growth as compared to non-inoculated control + NaCl plants. Plants inoculated with *Bacillus* HL3RS14-based soil biofertilizers showed maximum increase in dry weights of root (48–124%) and shoot (52–131%) as compared to control + NaCl (soil + rock phosphate, no inoculum). PGPR inoculations under salinity stress conditions showed high concentrations of proline, glycine betaine and malondialdehyde.

Conclusion: These results indicated that under saline soil conditions, halophilic PGPR strains combined with carrier materials are promising candidates as biofertilizers.

Introduction

Environmental factors such as soil salinity, moisture content, soil fertility and photoperiod length have significant influence on growth and yield of various crops, for example, wheat, rice, maize and sugar (Ali *et al.* 2004). Approximately 6·3 million hectares of land in Pakistan are affected due to varying degree of salinity and water logging (Ashraf 2007). It is necessary to utilize these lands by growing halophytes and xerophytes. These plants can grow under extreme environments by utilization of small organic molecules such as glycine betaine, amino acids and carbohydrates as a source of compatible solutes (Mahajan and Tuteja 2005). Halophytes can contribute to the supply of food, fuel and fibre which is the greatest demand of developing world.

Halophilic bacteria and archaea are salt-loving micro-organisms that flourish in salt affected (hypersaline)

environments. The growth of halophilic and halotolerant bacteria is affected by change in soil salinity, pH, temperature and availability of nutrients (Amoozegar *et al.* 2016). Mostly halotolerant and halophilic bacteria use small, organic compounds such as glycine proline, glutamine, betaine, potassium, glutamic acid and ectoine to survive under salt stress environments (DasSarma and DasSarma 2015).

Halophyte-associated microbes play a key role in the development and growth of plants. Plant growth-promoting rhizobacteria (PGPR) living in the rhizosphere of halophytes play an important role in plant health and soil fertility under salinity stress conditions. Halophilic PGP bacteria improve plant growth and grain yield of a variety of crops such as wheat, sugarcane, maize, cotton and rice by solubilization of minerals (P, K and Zn), production of growth-promoting hormones (indole acetic acid, cytokinins and gibberellins), siderophores and HCN (Kumar *et al.* 2011; Kumar *et al.* 2014; Gupta *et al.* 2015). A variety of plant growth-promoting bacteria related to genera *Pseudomonas, Aeromonas, Serratia, Rhizobium, Azospirillium* and *Bacillus* have been isolated and characterized from the rhizosphere of different plants (Malik *et al.* 1997; Mehnaz *et al.* 2010; Kumar *et al.* 2011; Gonzalez *et al.* 2015; Gupta *et al.* 2015).

Micro-organisms play an important role in soil P (Phosphorus) cycle by mediating the transfer of inorganic phosphate into organic forms which are readily available to plant roots (Oberson *et al.* 2001; Solangi *et al.* 2016). More than 88% soils in Pakistan are phosphorus deficient which ultimately affects plant growth (Malik 1992; Jamil *et al.* 2016). A number of bacterial genera including *Pseudomonas, Bacillus, Micrococcus, Enterobacter, Virgibacillus* and *Azospirillum* are well known for their phosphate-solubilizing ability (Malik *et al.* 1997; Mehnaz *et al.* 2010; Gupta *et al.* 2015; Mukhtar *et al.* 2017). A variety of inorganic, low-grade P-sources, such as rock phosphate can be used in combination with different phosphate-solubilizing bacteria as P biofertilizers. These biofertilizers are good alternatives to chemical fertilizers because they are cheap and environmental friendly. A mixture of carrier material peat, farmyard manure and perlite, a source of inorganic phosphate and PGPR strains has been used as biofertilizers (Mehnaz *et al.* 2001, 2010; Mukhtar *et al.* 2017).

The main objective of the present work was phylogenetic analysis of halotolerant and halophilic bacterial strains isolated from the rhizosphere of halophytes (*Salsola stocksii* and *Atriplex amnicola*) and characterization of their plant growth abilities such as IAA production, nitrogen fixation, phosphate solubilization, siderophore and HCN production and antifungal activity. Selected halophilic phosphate-solubilizing strains were used to study their effect on maize growth in pot experiments under salinity stress conditions. We also determined the presence of different osmolytes in salinity tolerance of maize plants.

Materials and methods

Selection of halotolerant and halophilic PGPR bacterial isolates

Thirty nine bacterial isolates from the rhizospheric and nonrhizospheric soils of halophytes; *Salsola stocksii* and *Atriplex amnicola* were selected from our lab collection and screened for salt tolerance by using HaP (Halophilic) medium (Tryptone 5 g l^{-1}, Yeast Extract 1 g l^{-1}, 5 g l^{-1} KCl, 10 g l^{-1} $MgSO_4$, 2 g l^{-1} K_2HPO_4 and pH 7·2) supplemented with 1·5–4 mol l^{-1} NaCl concentrations (Schneegurt 2012). The strains with the ability to grow in the presence of up to 3·5 mol l^{-1} NaCl were selected for molecular characterization and plant growth-promoting traits.

Identification of bacterial isolates on the basis of 16S rRNA sequence

Genomic DNA was isolated from each individual bacterial strain (Winnepenninckx *et al.* 1993). For PCR amplification of 16S rRNA gene, universal forward primer FD1 (AGAGTTTGATCCTGGCTCAG) and universal reverse primer (rP1) (ACGGACTTACCTTGTTACGACTT) were used. Denaturation temperature was 95°C for 5 min followed by 35 rounds of 95°C for 1 min, 54°C for 1 min and 72°C for 2 min and final extension at 72°C for 10 min. A reaction mixture of 25 μl was prepared by using Taq buffer 2·5 μl (10×), $MgCl_2$ 3 μl (25 mmol l^{-1}), Taq polymerase 1 μl, dNTPs 2 μl (2·5 mmol l^{-1}), 2 μl of forward and reverse primer (10 pmol) and the template DNA 2 μl (>50 ng μl^{-1}) (Tan *et al.* 1997). These PCR products were purified and sequenced commercially by using universal forward and reverse primers (Eurofins, Ebersberg bei München, Germany).

Phylogenetic analysis

Sequences of 16S rRNA gene were analysed by using Chromas 2.6 software (Technelysium Pty Ltd., South Brisbane, Australia). Using NCBI BLAST, these sequences were compared with related sequences that were already deposited in the GenBank database. Sequences of 16S rRNA gene were aligned by using Clustal W 2.2 software and phylogenetic tree was constructed by using MEGA7 software with neighbour joining method (Tamura *et al.* 2004; Kumar *et al.* 2016). All the parameters for

Table 1 Identification of halotolerant and halophilic bacterial strains on the basis of 16S rRNA sequence

No. of strains	Isolate code	Source of isolation	Identification based on 16S rRNA gene analysis	Sequence similarity (%)	Accession No.
1	HL1RS13	*Salsola* rhizosphere	*Bacillus licheniformis*	99·45	LT221121
2	HL1HP11	*Salsola* endosphere	*Bacillus safensis*	99·62	LT797512
3	HL1RP8	*Salsola* rhizoplane	*Kocuria rosea*	98·32	LT221125
4	HL2RS3	*Salsola* rhizosphere	*Bacillus megaterium*	99·15	LT221172
5	HL2RP7	*Salsola* rhizoplane	*Oceanobacillus oncorhynchi*	99·42	LT221138
6	HL1RS9	*Salsola* rhizosphere	*Pseudomonas monteilii*	99·72	LT221119
7	HL2RS12	*Salsola* rhizosphere	*Bacillus flexus*	99·35	LT221136
8	HL3RS14	*Salsola* rhizosphere	*Bacillus pumilus*	98·61	LT797515
9	HL3RP15	*Salsola* rhizoplane	*Planococcus rifietoensis*	99·47	LT221151
10	HL3HP5	*Salsola* endosphere	*Enterobacter aerogenes*	99·55	LT221149
11	HL3HP16	*Salsola* endosphere	*Virgibacillus* sp.	99·36	LT221155
12	HL3RP6	*Salsola* rhizoplane	*Halomonas salina*	99·18	LT221156
13	HARS26	*Salsola* rhizosphere	*Bacillus safensis*	99·92	LT797525
14	HL4RP4	*Salsola* rhizoplane	*Bacillus licheniformis*	99·12	LT221159
15	HL4HP3	*Salsola* endosphere	*Klebsiella oxytoca*	99·44	LT221158
16	AT1RP10	*Atriplex* rhizoplane	*Aeromonas veronii*	99·63	LT221167
17	AT1RP11	*Atriplex* rhizoplane	*Enterobacter aerogenes*	99·45	LT221165
18	AT1HP4	*Atriplex* endosphere	*Pseudomonas fluorescens*	99·62	LT221164
19	AT2RP4	*Atriplex* rhizoplane	*Bacillus megaterium*	99·84	LT221177
20	AT3RP10	*Atriplex* rhizoplane	*Staphylococcus equorum*	99·25	LT221191
21	AT3HP6	*Atriplex* endosphere	*Pseudomonas plecoglossicida*	99·32	LT221189
22	AT4HP10	*Atriplex* endosphere	*Halobacillus halophilus*	99·52	LT221203
23	AT3HP16	*Atriplex* endosphere	*Halomonas elongata*	99·31	LT221196
24	AT2RP3	*Atriplex* rhizoplane	*Virgibacillus halodenitrificans*	99·73	LT221174

phylogenetic tree construction were used according to Mukhtar *et al.* (2016). The sequences were submitted to NCBI GenBank data base and the accession numbers are given in Table 1.

Assays for plant growth promotion

Phosphate solubilization assay

Phosphate solubilization ability of halotolerant and halophilic bacteria was detected by using Pikovskaya medium supplemented with 1·5 mol l^{-1} NaCl (Pikovskaya 1948). Phosphate solubilization was tested by using a plate assay as well as a colorimetric or molybdate blue colour method (Watanabe and Olsen 1965). Bacteria were grown in Pikovskaya broth supplemented with one of the three carbon sources: sucrose, glucose or maltose (10 g l^{-1}) and one of two P-sources: calcium triphosphate or rock phosphate (5 g l^{-1}).

Indole acetic acid (IAA) production assay

For qualitative assessment of IAA, bacterial cultures were aseptically inoculated in 5 ml HaP broth containing 0·1% L-tryptophan as a precursor for IAA production. The culture tubes were kept in shaking incubator at 120 rev min^{-1}, 30°C. After 7 days, 500 μl of culture was transferred to sterile Eppendorf tubes and cells were harvested at 10 000 rev min^{-1} for 10 min. The supernatant was transferred to a new Eppendorf and 1 ml Salkowski reagent was added (Gordon and Weber 1950). The tubes were kept in dark for 15–20 min. A change in colour, pink indicated a positive result. Quantitative estimation of IAA was analysed by using high-performance liquid chromatography (HPLC) according to the method described by Kamran *et al.* (2017).

Acetylene reduction assay

Nitrogenase activity was assessed by using acetylene reduction assay. Each strain was inoculated into vials containing NFM (nitrogen free malate) semi-solid medium (5 ml per vial) and incubated at 30°C. After 24 h of growth, acetylene (10% v/v) was injected and re-incubated at 30 for 24 h. Ethylene production was measured by GC-2014 System (Shimadzu Corporation, Kyoto, Japan) fitted with a column Porapak using flame ionization detection. Helium was used as a carrier gas. Nitrogenase activity was described in terms of nanomoles of ethylene per hour per milligram bacterial protein (Mehnaz and Lazarovits 2006).

ACC (1-aminocyclopropane-1-carboxylate) deaminase assay

Each bacterial strain was grown on DF-agar plates with 3 mmol l^{-1} ACC as the sole nitrogen source to

determine the ACC deaminase activity (Penrose *et al.* 2001). For the colorimetric assay, bacterial cells were grown to the mid up to the late-log phase in 15 ml of HaP broth. Bacterial cell pellets were processed for ACC deaminase activity as described by Penrose and Glick (2003).

Siderophore production assay

CAS (chrome azurol S) agar medium was used for the detection of siderophore production using the overlay method described by Pérez-Miranda *et al.* (2007). Ten millilitres of the gel was spread as an overlay on culture plates of selected strains grown at 30°C for 4 days on solid HaP medium. After 20 min, a colour change in the blue medium was observed around the colonies. The experiment was repeated 3–4 times.

Hydrogen Cyanide production assay

HCN detection is done using filter papers (soaked in 0·5% picric acid in 2% sodium carbonate) placed on the inner lid of HaP medium incubated for 3 days (Sadasivam and Manickam 1992).

Antifungal activity assay

Antifungal activity of the bacterial isolates was observed using a co-culture method with fungal pathogens. A plug of fungal culture was placed in the centre of a potato dextrose agar (PDA; Sigma, St. Louis, MO) plate. The plate was sealed with parafilm and labelled as control. Another plug from fungal culture was placed in the centre of a new plate. Using a sterile inoculating loop, single bacterial colony was transferred on the plate by streaking it 2 cm away from the plug on four sides. The plates were sealed with parafilm and placed in incubator at 30°C for 7 days. Inhibition of fungal mycelial growth near bacterial growth indicated antifungal activity. Five fungal strains were used to evaluate the antifungal activity of the bacterial isolates: *Fusarium oxysporum, Fusarium solani, Curvularia* sp., *Aspergillus niger* and *Aspergillus flavus*. These fungal strains were plant pathogens and isolated from different crops growing in Punjab, Pakistan.

Enzyme assays for bacterial isolates

Protease activity was tested on the medium described by Kumar *et al.* (2009). Amylase activity was identified by using starch hydrolysis test (Bird and Hopkins 1954). Cellulase activity was tested by using HaP medium containing 1% carboxy methyl cellulose (CMC) and 1·5 mol l^{-1} NaCl and was visualized by formation of a clear zone after staining with Gram's iodine for 3–5 min (Kasana *et al.* 2008). Lipase activity was tested by using HaP medium with 1% butyrin, Tween 80 and 1·5 mol l^{-1} NaCl as described by Sierra (1957).

Pot experiments with microbial-enriched soil and coated seeds

For preparation of biofertilizers, inoculum was prepared by growing bacterial strains in HaP medium with 1·5 mol l^{-1} NaCl at 30°C for 24 h at 150 rev min^{-1}. Optical density was measured to attain the uniform population of bacterial strains (*c.* 10^8 cells per ml). Seeds of local a salt sensitive variety of maize (Yousafwala Hybrid) were surface sterilized using 0·1% NaClO (Chlorex) for 8–10 min and washed five times with sterile dH_2O. Seeds were incubated with 10^8 bacteria per ml of individual bacterial strains and incubated with 3% PVP (Polyvinyl pyrrolidone) for 3 h. A mixture of bacterial strains (*Bacillus safensis* HL1HP11 and *Bacillus pumilus* HL3RS14, *Kocuria rosea* HL1RP8, *Pseudomonas fluorescens* AT1HP4 and *Aeromonas veronii* AT1RP10), 10^8 number of bacteria per ml was also used on the seeds to observe the effect of bacterial consortium. Seeds without bacterial inoculum served as control (with 150 mmol l^{-1} NaCl or without NaCl) for this experiment (Experiment set A).

For the second experiment (Experiment set B), phosphate biofertilizer was prepared by using soil enrichment with phosphate-solubilizing bacteria. About 500 g of unsterilized soil (S) with physicochemical properties (pH = 7·71, salinity = 1·17 EC1 : 1(dS/m), moisture = 25·53, temperature = 29·58°C, OM (g kg^{-1}) = 30·19, P (mg kg^{-1}) = 3·43, and K (mg kg^{-1}) = 0·49) was supplemented with 1% glucose + 1% RP (rock phosphate). Five bags were inoculated with 50 ml of individual phosphate-solubilizing bacteria (10^8 bacterial cells per gram of soil) and one soil bag was enriched with 25 ml mixture of phosphate-solubilizing bacteria (10^8 bacterial cells per gram of soil: same 170 consortium used in experiment A). Soil-based phosphorous biofertilizers were also analysed for available phosphorus after 1 and 2 weeks of incubation at 28°C.

Maize plants were grown in plastic pots containing 1 kg of sandy loam-unsterilized soil (EC 2·4 ds/m, pH 7·6, organic matter 0·36%, available phosphorous 2·2 mg kg^{-1} and total nitrogen 0·05%). Experiment set A was done with coated seeds (3% PVP) and about 10 g of rock phosphate was also added as inorganic P-source before sowing. Three seeds were sown in each pot and seven replicates were used for each treatment; maize seeds coated with individual bacterial strains and mixture of bacterial strains. Seeds without bacterial cultures were used as control (with 150 mmol l^{-1} NaCl or without NaCl).

In a second set of experiments, enriched soil-based phosphorus biofertilizers were applied as inoculum. Pots with 500 g soil were prepared and inoculated with 2 g of enriched soil (Experiment set B), respectively. Seven replicates for each treatment were maintained for this experiment (soil enriched with individual strains). Mixtures of strains incubated in soil were also used with same number of replicates for combined effect study. Plants with un-inoculated control soils with 150 mmol l^{-1} NaCl or without NaCl were also grown. All the plants were watered with half strength Hoagland's solution (Hoagland and Arnon, 1950) and 150 mmol l^{-1} NaCl. These pots were kept under climate control room with 12 h photoperiod and 22 ± 2°C temperature. After 5 weeks, plant growth parameters such as root and shoot dry weight and length were recorded. Plant roots and shoots were dried in an oven at 65°C for 3 days and then weighed. For measurement of available phosphorus, about 50 g of rhizosphere soil was collected by gently uprooting the plant from the soil and recovering soil attached to the root system. The available phosphorus in soil was calculated by using a colorimetric or molybdate blue colour method (Watanabe and Olsen 1965).

Proline, glycine betaine and lipid peroxidation determination in fresh leaves

Proline content from fresh leaf tissues was determined by using the Bates method (Bates *et al.* 1973). About 1 g of leaves was homogenized using 10 ml of sulfo-salicylic acid buffer. The mixture was filtered by using Whatman filter paper, then 2 ml of the filtrate was mixed with 2 ml acid ninhydrin solution and 2 ml of glacial acetic acid in a test tube. The reaction mixture was incubated at 95°C for 1 h. Toluene (4 ml) was added to the mixture and mixed vigorously for 2 min. The toluene layer was removed and absorbance of the aqueous layer was measured at 520 nm using a Specmate UV-VIS spectrophotometer (CLS-4048). The proline content was determined from standard curve and calculated by using following formula:

$$\mu\text{mol proline g}^{-1}\text{ fresh weight} = (\mu\text{g proline per ml} \times \text{ml of toluene}/115.5)/\text{g of sample}$$

For estimation of glycine betaine, oven-dried leaf tissues were ground in 10 ml of distilled H_2O. After 1 ml of this filtration was mixed with 1 ml of 2N HCl, about 2 ml of ice-cooled water and 20 ml of dichloromethane were added to this mixture and then mixed thoroughly for 2 min. The upper aqueous layer was discarded and the optical density of the organic layer was measured at 365 nm. The concentration of glycine betaine was calculated using a standard curve.

The amount of lipid peroxidation was measured by estimating the malondialdehyde (MDA) produced by the thiobarbituric acid (TBA) reaction (Heath and Packer, 1968). Fresh leaf tissues were homogenized in a pestle and mortar using ice-cold extraction buffer (1·6% $Na_2HPO_4{\cdot}12H_2O$ and 0·7% $NaH_2PO_4.2H_2O$) and spin at 13 000 rev min^{-1} for 20 min and the supernatant was used for the estimation of MDA. About 4 ml of TBA solution (0·5% w/v) was added to 1 ml of supernatant and incubated at 95°C for 30 min. The absorbance of the mixture was measured at 534 nm and MDA concentration was estimated by its molar extinction coefficient (155 mmol l^{-1} cm^{-1}).

Statistical analysis

The pot experiment was performed in randomized complete block design with seven replicates for each treatment. Average value and standard error for each growth parameter were also calculated. Data for root and shoot dry weight and length were analyzed using ANOVA with 5% LSD test (least significant difference) by using IBM SPSS software (ver. 24).

Results

Identification of halophilic bacterial strains on the basis of 16S rRNA sequence

On the basis of salt tolerance, a total of 24 bacterial strains, 15 associated with *Salsola* roots and nine associated with *Atriplex* roots were selected for 16S rRNA sequence analysis. Identification through 16S rRNA gene analysis showed that 33·34% bacterial isolates were identified as different species of genus *Bacillus,* 12·56% related to *Pseudomonas,* 8·35% were related to *Virgibacillus,* 8·35% were related to *Enterobacter* and 8·45% were belonging to *Halomonas* (Table 1 and Fig. 1). Bacterial strains belonging to *Planococcus, Klebsiella, Aeromonas* and *Staphylococcus* were also identified in this study (Table 1 and Fig. 1).

Plant growth-promoting potential of halophilic bacterial strains

Halophilic bacterial strains were screened for various plant growth-promoting abilities such as IAA production, P solubilization, nitrogen fixation, siderophore and HCN production. Most of the strains showed more than three plant growth-promoting abilities (Table 2). All the strains showed P-solubilization activity, 23 strains showed production of IAA, seven strains showed nitrogen fixation ability, 13 strains were able to produce ACC deaminase,

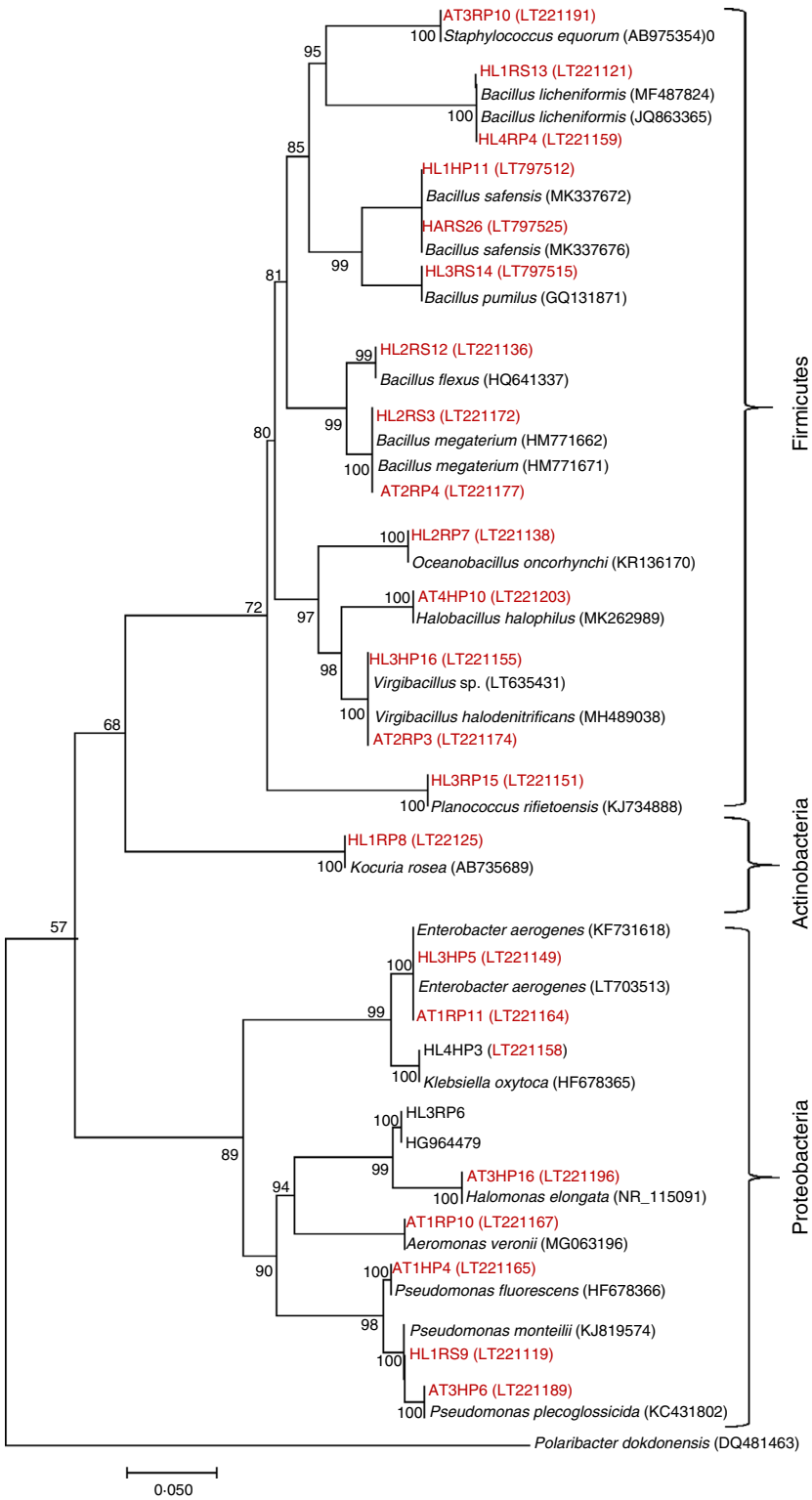

Figure 1 Phylogenetic tree based on 16S rRNA gene sequences of halophilic bacterial isolates associated with the roots of the halophytes (*Salsola stocksii* and *Atriplex amnicola*). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

Table 2 Determination of PGPR traits of halotolerant and halophilic bacterial isolates

Isolates	Phosphate solubilization ($\mu g\ ml^{-1}$)	IAA production ($\mu g\ ml^{-1}$)	Nitrogen fixation (nmol h^{-1} per $vial^{-1}$)	ACC deaminase (nmol h^{-1} mg per protein)	Siderophore production	HCN production
HL1RS13	$93{\cdot}42^{de}$	$2{\cdot}11^{a}$	0	0	–	–
HL1HP11	$83{\cdot}65^{d}$	$65{\cdot}54^{fg}$	0	397^{a}	+	–
HL1RP8	$72{\cdot}85^{d}$	$58{\cdot}84^{fg}$	256^{b}	363^{a}	++	–
HL2RS3	$55{\cdot}92^{bc}$	$13{\cdot}42^{b}$	0	421^{a}	–	–
HL2RP7	$47{\cdot}24^{bc}$	$23{\cdot}57^{c}$	0	997^{c}	–	++
HL1RS9	$49{\cdot}92^{bc}$	$56{\cdot}89^{fg}$	294^{bc}	0	++	–
HL2RS12	47.7^{bc}	$18{\cdot}54^{bc}$	0	0	–	–
HL3RS14	$128{\cdot}46^{fg}$	$42{\cdot}19^{d}$	0	325^{a}	+	–
HL3RP15	$8{\cdot}15^{a}$	$23{\cdot}23^{c}$	0	0	–	–
HL3HP5	$66{\cdot}77^{cd}$	$52{\cdot}44^{f}$	193^{a}	359^{a}	+	–
HL3HP16	$39{\cdot}22^{b}$	$39{\cdot}42^{d}$	0	0	–	+++
HL3RP6	$12{\cdot}16^{a}$	$18{\cdot}47^{b}$	0	413^{a}	–	+
HARS26	$51{\cdot}41^{bc}$	0·00	0	0	–	–
HL4RP4	$44{\cdot}84^{bc}$	11.2^{b}	0	0	++	
HL4HP3	$34{\cdot}41^{b}$	$5{\cdot}54^{a}$	0	0	–	–
AT1RP10	95.7^{de}	$105{\cdot}91^{ij}$	359^{c}	367^{a}	+	++
AT1RP11	$40{\cdot}55^{b}$	$37{\cdot}98^{d}$	249^{c}	405^{a}	–	–
AT1HP4	$94{\cdot}74^{de}$	$55{\cdot}64^{fg}$	197^{a}	753^{b}	++	–
AT2RP4	$19{\cdot}45^{a}$	$12{\cdot}54^{b}$	0	695^{b}	–	–
AT3RP10	$1{\cdot}15^{a}$	$37{\cdot}62^{d}$	0	0	++	–
AT3HP6	$6{\cdot}23^{a}$	$19{\cdot}98^{bc}$	0	677^{b}	–	–
AT4HP10	$39{\cdot}96^{b}$	$22{\cdot}15^{c}$	0	0	–	–
AT3HP16	$51{\cdot}24^{bc}$	$17{\cdot}20^{b}$	223^{b}	0	+	++
AT2RP3	$78{\cdot}58^{d}$	$6{\cdot}98^{a}$	0	441^{a}	–	–

*Letters represent statistically different values at 5% level. –, no activity; +, low activity; ++, medium activity; +++, high activity.

10 strains showed positive results for siderophore production assay and only five strains had ability to produce HCN gas (Table 2 and Figs S1 and S2).

Antifungal activity of halophilic bacterial strains

The fungi *Fusarium oxysporum, Fusarium solani, Curvularia* sp., *Aspergillus niger* and *Aspergillus flavus* were used to evaluate antifungal activity of halophilic bacterial strains (Figs 2 and S1). Six out of eight *Bacillus* strains showed positive results against *F. oxysporum*, three *Bacillus* strains showed inhibitory action against *F. solani,* seven *Bacillus* strains showed antifungal activity against *Curvularia* sp., six *Bacillus* strains showed inhibited *Aspergillus niger* growth and three *Bacillus* strains inhibited *Aspergillus flavus* growth (Table S1 and Figs 2 and S1). *Oceanobacillus* strain HL2RP7 showed antifungal activity against all fungi except *F. solani*. *Virgibacillus* strains HL3HP16 and AT2RP3 both inhibited *Curvularia* sp. and *Aspergillus niger*. *Pseudomonas* strains HL1RS9, AT1HP4 and AT3HP6 all showed antifungal activity against *Aspergillus niger,* HL1RS9 and AT1HP4 strains showed antifungal activity against *F. oxysporum* and *Curvularia* sp. and HL1RS9 and the strain AT1HP4 showed antifungal activity against *Aspergillus flavus*. *Enterobacter* strains HL3HP5 and AT1RP11 and *Halomonas* strains HL3RP6 and AT3HP16 showed positive results against *F. oxysporum* and *Curvularia* sp. (Table S1 and Figs 2 and S1). The majority of halophilic strains showed antifungal activity against more than 2 fungal pathogens.

Production of halophilic extracellular enzymes

All halophilic bacterial strains produced at least for two different hydrolytic enzymes. Four out of eight *Bacillus* strains showed cellulase activity, six strains showed positive results for lipase, three strains showed chitinase activity, four strains had the ability to degrade amylose and seven strains showed proteolytic activity (Table S2 and Fig. 3). *Oceanobacillus* strain HL2RP7 showed positive results for lipase, chitinase and protease. *Virgibacillus* strain HL3HP16 showed hydrolytic activity for all enzymes except amylase. *Pseudomonas* strains HL1RS9 and AT1HP4 showed positive results for lipase, chitinase and protease. *Enterobacter* strains AT1RP11 showed hydrolytic activity for cellulase, lipase, chitinase and protease and HL3HP5 strain showed positive results for

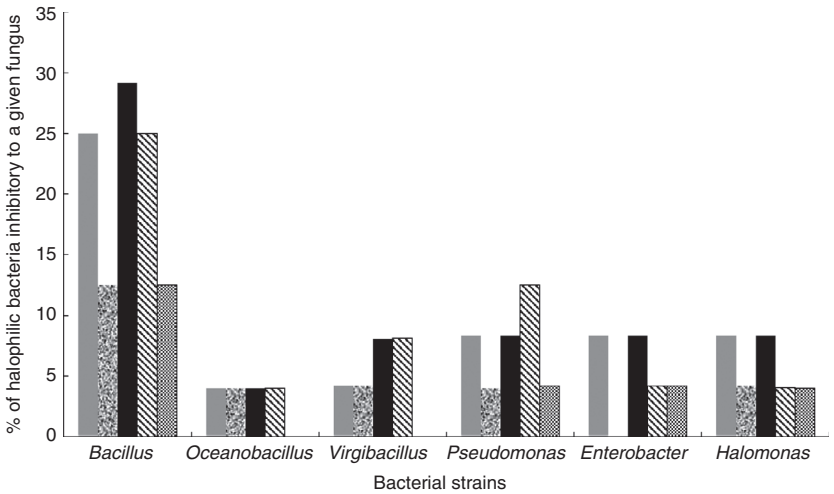

Figure 2 Antifungal assays of bacterial isolates with: (▩) *Fusarium oxysporum,* (▩) *Fusarium solani,* (■) *Curvularia* sp., (▩) *Aspergillus niger* and (▩) *Aspergillus flavus.*

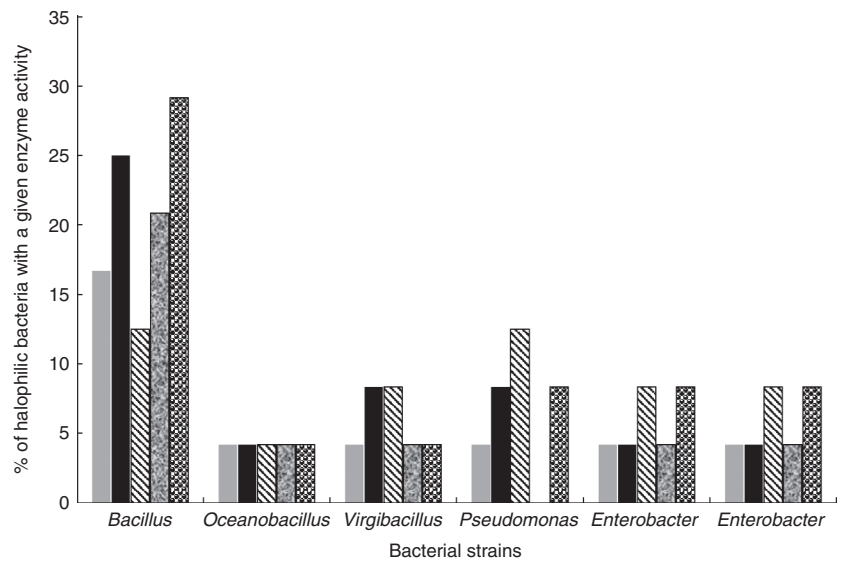

Figure 3 Agar plate-based enzyme assays for bacterial strains isolated from the rhizosphere of halophytes *Salsola* and *Atriplex*; (▩) Cellulase (■) Lipase (▩) Chitinase, (▩) Amylase and (▩) Protease.

chitinase, amylase and protease. *Halomonas* strains AT3HP16 showed positive results for cellulase, chitinase, amylase and protease and HL3RP6 strain showed hydrolytic activity for cellulase, lipase, chitinase and protease (Table S2 and Fig. 3).

Quantification of P solubilization by halophilic bacterial strains using different carbon and inorganic P-sources

Five bacterial strains (HL1HP11, HL3RS14, HL1RP8, AT1RP10 and AT1HP4) with highest P-solubilizing ability were selected for further analysis. These strains were grown in Pikovskaya broth supplemented with three carbon sources: sucrose, glucose and maltose and 262 two P-sources: calcium triphosphate and rock phosphate. *Bacillus* strains HL1HP11 and HL3RS14 showed maximum P solubilization (85·2 and 127·5 μg ml^{-1}) by using sucrose as a carbon source and calcium triphosphate as P-source while *Aeromonas* AT1RP10 and *Pseudomonas* AT1HP4 showed maximum P solubilization (82·4 and 100·98 μg ml^{-1}) by using glucose as a carbon source and calcium triphosphate as P-source (Fig. 4A).

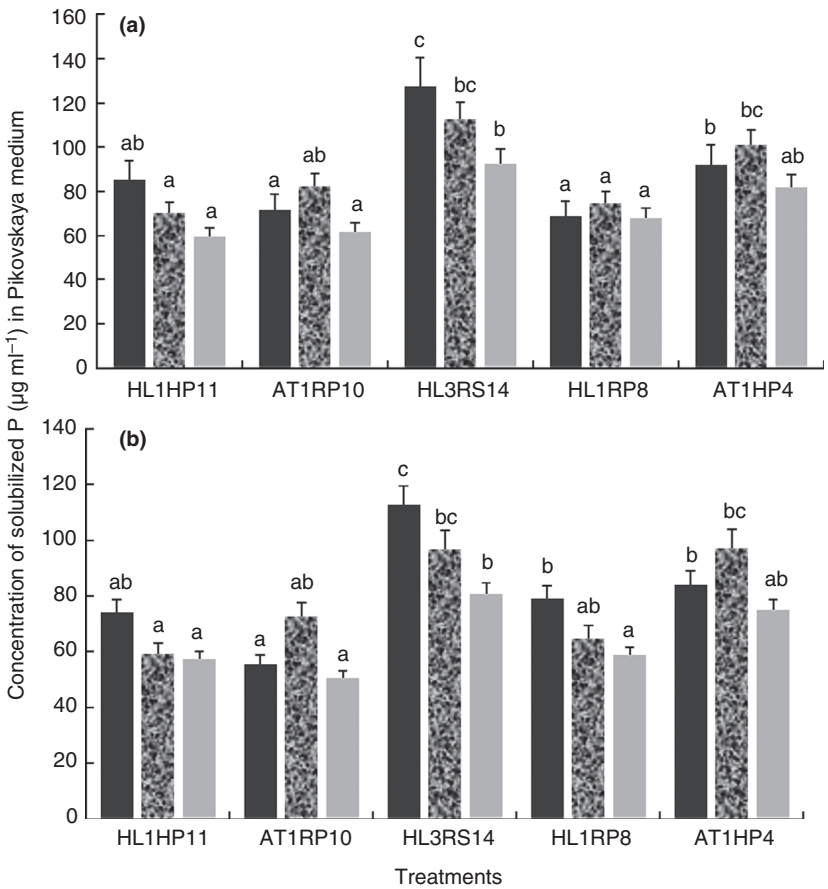

Figure 4 Phosphate solubilization (μg ml^{-1}) after incubation of 14 days by bacterial in Pikovskaya broth medium supplemented with three carbon sources; sucrose, glucose and maltose and two P-sources, (A) tricalcium phosphate and (B) rock phosphate after incubation of 14 days. Significance between means was tested using Duncan's Multiple Range test at $P < 0{\cdot}05$ after a variance analysis was performed. Letters on graph bars represent statistically different values at the 5% level. (■) P-estimation using sucrose; (▩) P-estimation using glucose and (▒) P-estimation using maltose.

When rock phosphate used as a P-source, *Bacillus* HL3RS14 showed highest P solubilization (112·52 μg ml^{-1}) with sucrose as a carbon source and *Pseudomonas* AT1HP4 showed highest P solubilization (96·92 μg ml^{-1}) with glucose (Fig. 4B). There is a little difference in P-solubilization values when Pikovskaya broth supplemented with both calcium triphosphate and rock phosphate as a P-source. Because rock phosphate is comparatively a cheap P-source, so we used it for plant experiment at pot scale.

Quantification of available phosphorus in enriched soil-based biofertilizers

By using colorimetric method, available P was quantified in soil-based biofertilizers after incubation of 7 and 14 days. *Pseudomonas* AT1HP4 showed maximum P solubilization (7·83 mg kg^{-1}) as compared to other soil biofertilizers and control soil samples after 7 days while *Bacillus* HL3RS14 and *Pseudomonas* AT1HP4 showed highest P solubilization (13·67 and 12·33 mg kg^{-1}) after 14 days (Fig. 5).

Effect of PGPR strains on maize growth

PGPR strains exerted a significant influence on growth characteristics of maize shoot and root dry weight and shoot and root lengths under salinity stress conditions. Plants with coated seeds (bacterial strains with 3% Polyvinylpyrrolidone: exp. set A) showed 49–117% increase in dry weight of root and 56–116% increase in dry weight of shoots as compared to un-inoculated control + NaCl (3% PVP, no inoculum) (Figs 6 and 7A,B). Similarly, plants treated with enriched soil-based phosphorus biofertilizers (exp. set B) showed significant increase in root and shoot dry weight as compared to

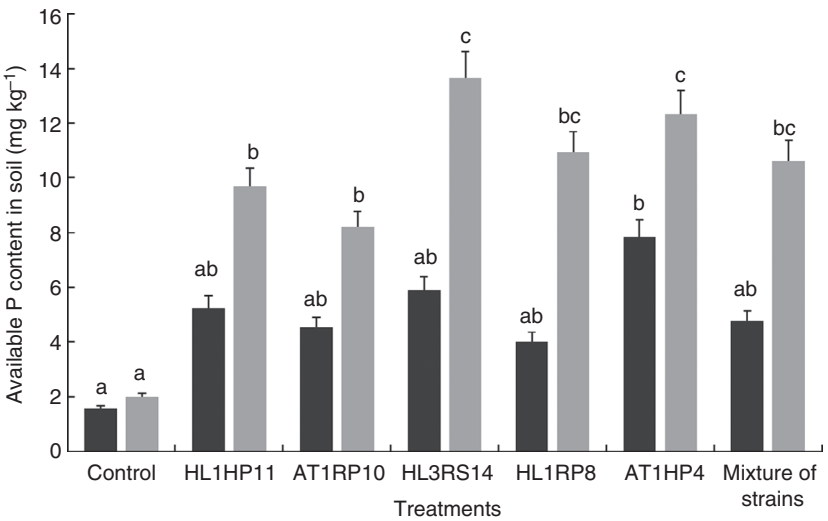

Figure 5 Quantification of available phosphorous (mg/kg) in enriched soil after incubation of 7 and 14 days. Significance between means was tested using Duncan's Multiple Range test at $P < 0{\cdot}05$ after a variance analysis was performed. Letters on graph bars represent statistically different values at the 5% level. (■) P-estimation after 7 days and (■) P-estimation after 14 days.

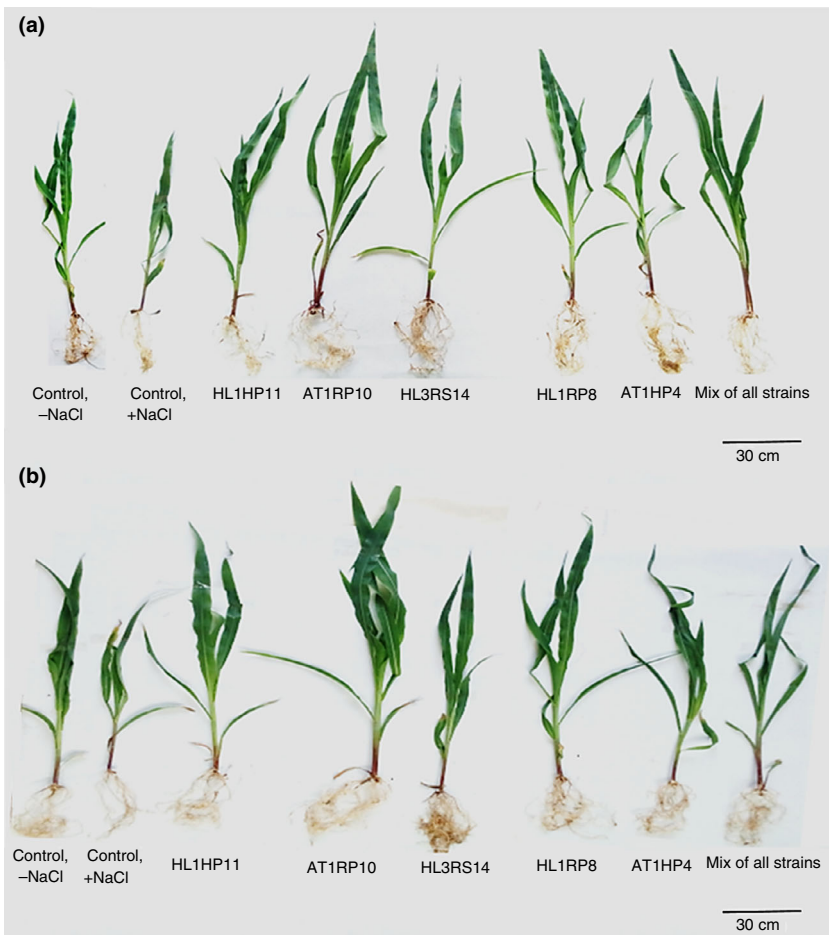

Figure 6 Effect of phosphate-solubilizing bacteria on maize growth after 5 weeks (a) Seed coated with bacterial strains, 3% PVP and rock phosphate (b) Soil-based phosphate biofertilizers.

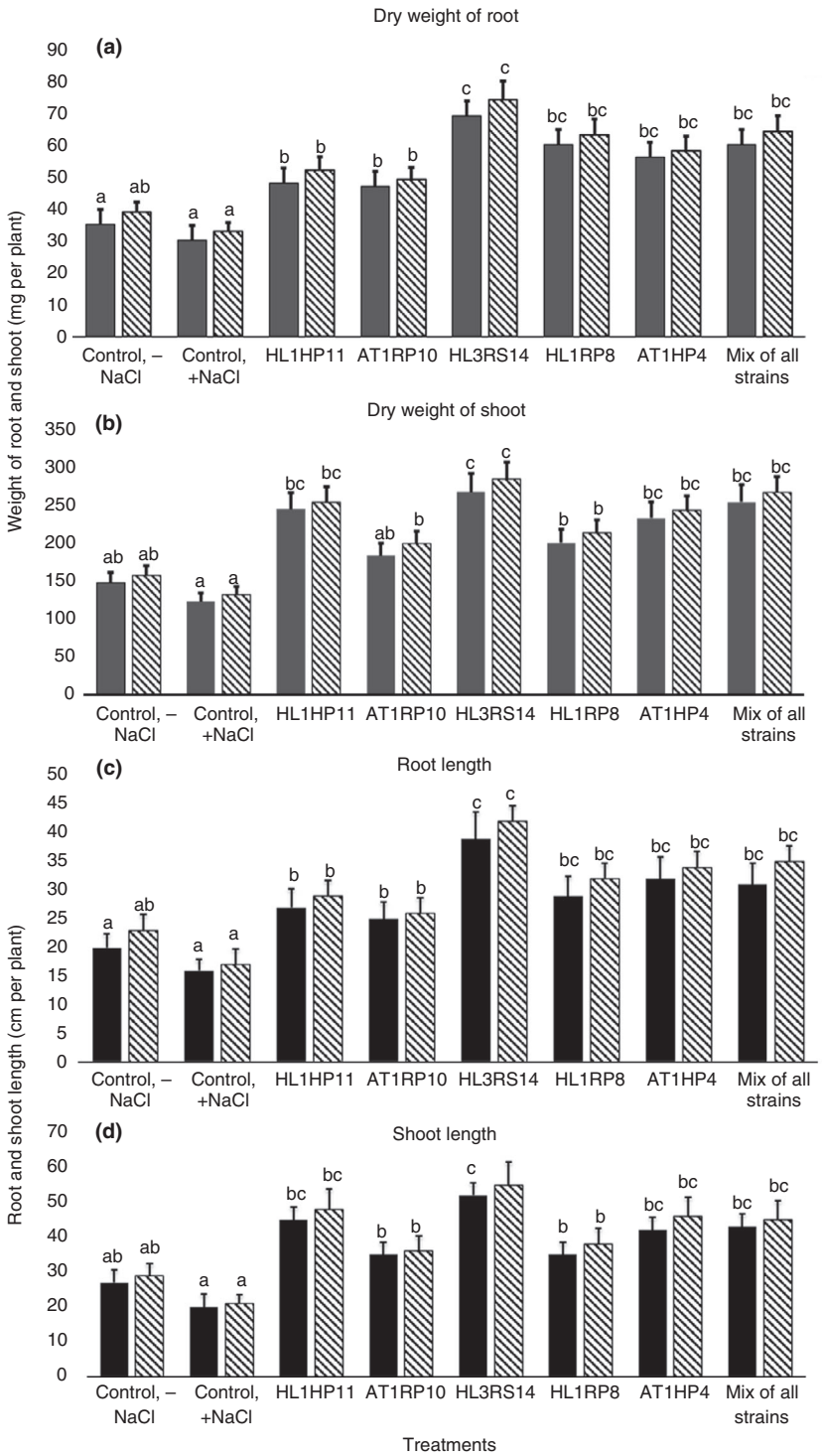

Figure 7 Effect of coated bacterial inoculum and enriched soil on dry weight and length of roots and shoots of maize plants. Significance between means was tested using Duncan's Multiple Range test at $P < 0{\cdot}05$ after a variance analysis was performed. Letters on graph bars represent statistically different values at the 5% level. (■) seed-coated bacterial strains and rock phosphate and (▨) Bacterial strains with soil and rock phosphate.

control + NaCl (soil + rock phosphate, no inoculum). Plants treated with bacterial consortium showed a significant increase in shoot and root dry weights as compared to un-inoculated control + NaCl plants and plants treated with individual bacterial strains except *Bacillus* strain HL3RS14 (Figs 6 and 7A,B).

The inoculated plants showed increased root growth as compared to un-inoculated control + NaCl plants under salinity stress conditions. The relative increase in length varied between 61–137% for roots and 76–141% for shoots in plants with coated seeds (bacterial strains with 3% PVP) over the control + NaCl (3% PVP, no inoculum) (Figs 6 and 7C,D). Plants inoculated with enriched soil-based P biofertilizers showed significant increase in root length 65–149% and 70–152% shoot length as compared to control + NaCl (soil + rock phosphate, no inoculum). Root and shoot length were longer in plants treated with bacterial consortium showed a pattern similar to shoot and root dry weight (Figs 6 and 7C,D). The overall results indicated that plants treated with enriched soil-based P biofertilizers showed better results as compared to non-inoculated control plants seeds coated with bacterial strains.

The inoculated plants showed better root structure and growth as compared to un-inoculated control + NaCl plants under salinity stress conditions (Fig. S4). The relative increase in length varied between 61–137% for roots and 76–141% for shoots in plants with coated seeds (bacterial strains with 3% PVP) over the control + NaCl (3% PVP, no inoculum) (Figs 6 and 7C,D). Plants inoculated with enriched soil-based P biofertilizers showed significant increase in root length 65–149% and 70–152% shoot length as compared to control + NaCl (soil + rock phosphate, no inoculum) (Figs 6 and 7C,D). The overall results indicated that plants treated with enriched soil-based P biofertilizers showed better results as compared to non-inoculated control plants seeds coated with bacterial strains.

Quantification of available phosphorus in soil samples before sowing and after harvesting

Available phosphorus was quantified from soil samples of all the PGPR inoculated as well as un-inoculated control plants before sowing and after harvesting. Overall P solubilization was higher in soil samples from all the treated plants as compared to un-inoculated control plants (Table 3). Soil samples from plants inoculated with *Bacillus* strain HL3RS14-based soil biofertilizers showed a maximum increase (108%) in available P as compared to other treated and control plants (Table 3).

Analysis of osmolytes from maize leaves

Salinity stress caused a significant increase in proline content of leaf tissues in the inoculated plants. Leaf tissues of plants with seeds (bacterial strains with 3% Polyvinylpyrrolidone) showed 44–122% increase in proline content as compared to un-inoculated control + NaCl (3% PVP, no inoculum) while plants treated with enriched soil-based phosphorus biofertilizers showed significant increase in proline content (50–106%) as compared to control + NaCl (soil + rock phosphate, no inoculum). Plants treated with bacterial consortium showed a significant increase in proline content as compared to un-inoculated control + NaCl plants and plants treated with individual bacterial strains with the exception of *Bacillus* strain HL3RS14 (Fig. 8A).

Similarly, a significant increase in glycine betaine content was observed in treated plants due to salinity stress. The relative increase in glycine betaine varied between 46–105% in plants with coated seeds (bacterial strains with 3% PVP) over the control + NaCl (3% PVP, no inoculum) (Fig. 8B). Similarly, plants treated with enriched soil-based phosphorus biofertilizers showed significant increase in glycine betaine content of leaf tissues (39–94%) as compared to control + NaCl (soil + rock phosphate, no inoculum). Glycine betaine levels in plants treated with bacterial consortium showed a pattern similar to the content of proline (Fig. 8B).

There was a significant increase in the malondialdehyde (MDA) contents among treated plants under salinity stress conditions. Plants with coated seeds (bacterial strains with 3% Polyvinylpyrrolidone) showed 70–161% increase in MDA contents as compared to un-inoculated control-NaCl (3% PVP, no inoculum) (Fig. 8C). Similarly, plants treated with enriched soil-based phosphorus biofertilizers showed significant decrease in MDA contents (50–166% decrease) as compared to control + NaCl (soil + rock phosphate, no inoculum). Plants treated with bacterial consortium showed a significant decrease in MDA contents as compared to un-inoculated control + NaCl plants and plants treated with *Bacillus* strain HL3RS14 (Fig. 8C).

Discussion

The rhizosphere of halophytes hosts a considerable diversity of plant growth-promoting halotolerant and halophilic bacteria. These organisms have a unique ability to survive and grow at high salt concentrations. The main objective of the present work was to study microbial diversity and plant growth-promoting potential of bacterial strains from the rhizosphere of halophytes (*Salsola stocksii* and *Atriplex amnicola*). Twenty four halophilic bacterial strains from the rhizosphere of halophytes were phylogenetically analysed on the basis of 16S rRNA sequence. Bacterial genera *Bacillus, Pseudomonas, Enterobacter* and *Halomonas* were found to be abundant in the rhizosphere of all the plants. *Bacillus* plays an important role in plant growth promotion and biodegradation of hemicelluloses and lipids (Piñar *et al.* 2014; Gupta *et al.*

Table 3 Quantification of available phosphorous (mg kg^{-1}) in soil samples before sowing and after harvesting

Treatment	Before seeds sowing	After plant harvesting	
		Bacterial strains and rock phosphate	Soil-based phosphorous biofertilizers
Control, −NaCl	2·14[a]	2·21[a]	2·44[ab]
Control, +NaCl	2·12[a]	2·25[a]	2·41[a]
Bacillus safensis HL1HP11	2·16[a]	4·04[c]	4·34[c]
Aeromonas veronii AT1RP10	2·21[a]	3·11[ab]	3·58[b]
Bacillus pumilus HL3RS14	2·17[a]	4·19[c]	4·52[c]
Kocuria rosea HL1RP8	2·13[a]	3·43[b]	3·62[b]
Pseudomonas fluorescens AT1HP4	2·16[a]	4·14[c]	4·35[c]
Mixture of strains	2·15[a]	3·74[b]	4·19[c]

*Letters represent statistically different values at 5% level.

2015). They play important role in plant growth promotion and are a good source of halophilic and thermophilic enzymes such as proteases, amylases, cullulases and lipases (Saharan and Nehra 2011). Members of Proteobacteria (*Pseudomonas, Enterobacter* and *Halomonas*) were dominant in the rhizosphere of wheat and halophytes (Kumar *et al.* 2014; Goswami *et al.* 2016).

Most of the halophilic strains showed more than three plant growth-promoting abilities. More than 95% strains showed P-solubilization activity and IAA production. About 54% strains were able to produce ACC deaminase, 29% strains showed positive results for nitrogen fixation, 41% strains had ability to produce siderophores and only 21% strains showed HCN production ability. A large number of PGPR strains such as *Enterobacter, Pseudomonas, Bacillus, Aeromonas* and *Azospirillum* have been isolated from the rhizosphere of various crops like wheat, rice, maize and sugarcane (Mehnaz *et al.* 2001, 2010; Mukhtar *et al.* 2017). Bacterial strains related to genus *Bacillus* colonize the halophyte rhizosphere and promote plant growth by adopting different mechanisms such as mineral solubilization (P, Zn), production of phytohormones (Indole acetic acid) and biocontrol agents (HCN and siderophores) under salt stress conditions (Kumar *et al.* 2011; Zhang *et al.* 2014). *Enterobacter aerogenes* strains HL3HP5 and AT1RP11 showed the presence of four plant growth-promoting traits and were positive for phosphate solubilization, IAA production, nitrogen fixation and HCN production (Mehnaz *et al.* 2010). Many halophilic and drought tolerant PGPR strains produce ACC deaminase to stimulate plant growth under abiotic stress such as drought and salinity. Plants treated with ACC deaminase producing halo- and drought tolerant PGPR have ability to reduce the deleterious effects of ethylene (Glick *et al.* 1998; Zahir *et al.* 2009).

Halophilic *Bacillus, Oceanobacillus, Virgibacillus, Pseudomonas* and *Enterobacter* strains identified in this study showed antifungal activity against *F. oxysporum, F. solani, Curvularia* sp., *A. niger,* and *A. flavus.* More than 60% halophilic strains showed positive results for antifungal assay against *F. oxysporum, Curvularia* sp., and *A. niger.* Many PGPR genera such as *Bacillus, Pseudomonas* and *Enterobacter* have been used as growth inhibitors for different fungal pathogens and provide protection against a number of plant diseases (Mehnaz *et al.* 2010; Ali *et al.* 2015a; Khan *et al.* 2018). Mostly halophilic bacterial strains showed hydrolytic activity for at least two enzymes. About 66·67% bacterial strains showed protease activity, 54·17% strains showed positive results for lipase, 41·66% strains showed cellulase activity and 33·33% strains had ability to degrade starch and other carbohydrates. A number of PGPR strains such as *Bacillus*, *Enterobacter,* and *Pseudomonas* promote plant growth and suppress plant diseases by producing a variety of siderophores, hydrolytic enzymes (chitinase, cellulase, amylase and protease) and HCN (Mehnaz *et al.* 2010; Chen *et al.* 2013; Mukhtar *et al.* 2019).

Bacillus strains HL1HP11, HL3RS14 showed maximum P solubilization using sucrose as a carbon source with both calcium triphosphate (85·2 and 127·5 $\mu g\ ml^{-1}$) and rock phosphate (79·95 and 112·52 $\mu g\ ml^{-1}$) as P-source. Using glucose as carbon source, *Pseudomonas* AT1HP4 showed maximum P-solubilization calcium triphosphate (100·98 $\mu g\ ml^{-1}$) and rock phosphate (96·92 $\mu g\ ml^{-1}$) as P-source. A number of studies have previously reported P solubilization by PGPR strains using different carbon sources with calcium triphosphate as the main P-source (Nautiyala *et al.* 1999; Karpagam and Nagalakshmi 2014). All soil-based biofertilizers showed increase in P solubilization as compared to control soil samples. Overall biofertilizer inoculated with *Bacillus* strain HL3RS14 showed highest P solubilization as compared to other treated as well as control samples. Different PGPR strains such as *Pseudomonas, Bacillus, Azospirillum* and *Enterobacter* have been used as biofertilizers with the aim of improving nutrient availability and plant growth

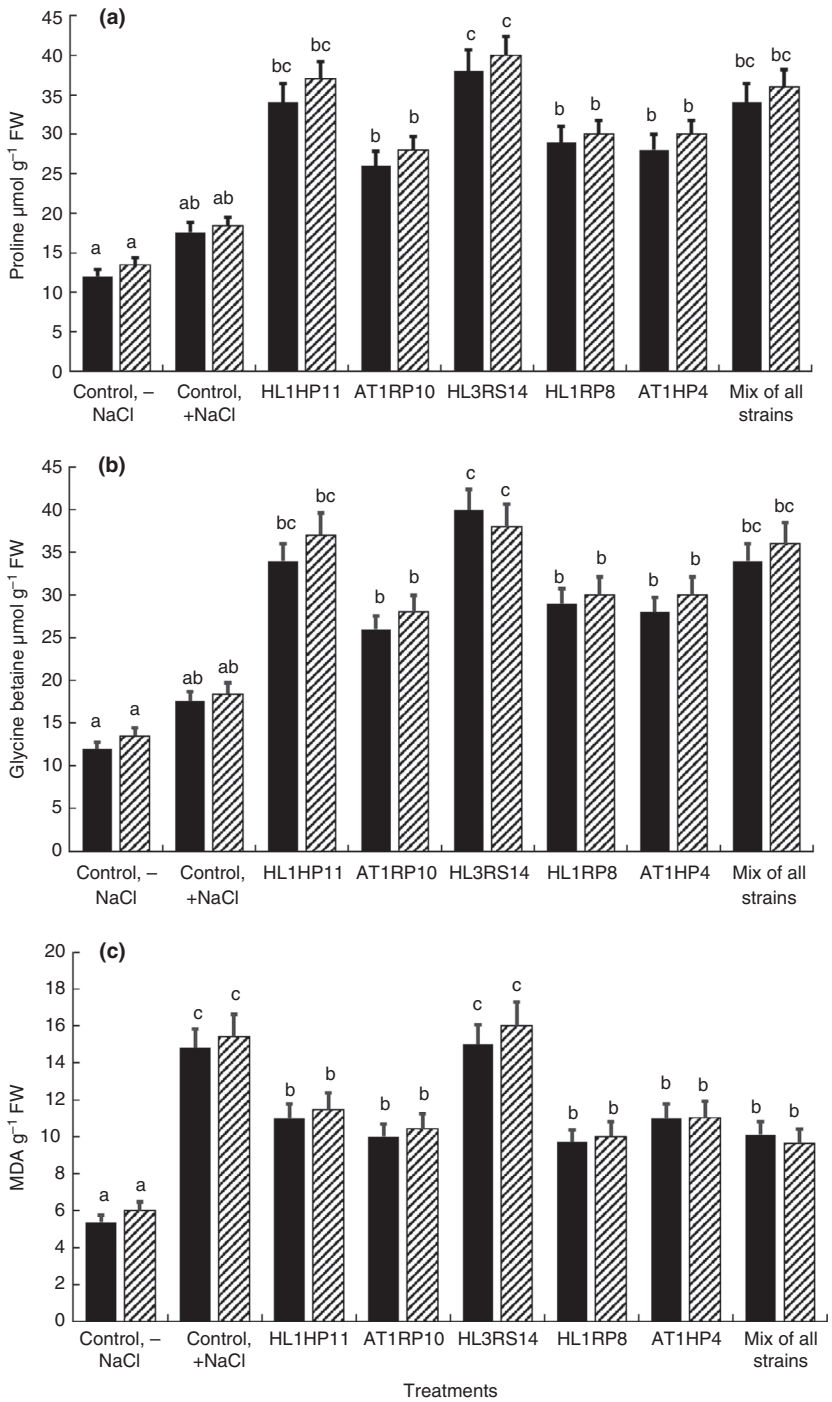

Figure 8 Effect of salt stress on (A) proline, (B) glycine betaine and (C) MDA content of halophilic PGPR-inoculated and un-inoculated control maize leaf tissues. Significance between means was tested using Duncan's Multiple Range test at $P < 0{\cdot}05$ after a variance analysis was performed. Letters on graph bars represent statistically different values at the 5% level. (■) seed-coated bacterial strains and rock phosphate and (▨) Bacterial strains with soil and rock phosphate.

promotion (Mehnaz *et al.* 2010; Paul and Lade 2014; Mukhtar *et al.* 2017). A mixture of different carrier materials such as farmyard manure and peat, source of inorganic phosphate and PGPR strains has been used as biofertilizers. The main advantage of using sterile soil as a carrier material is that it is not only provides a good

medium for bacterial survival but also contains several macro and micronutrients necessary for plant growth (El Husseini *et al.* 2012).

Five bacterial strains; two *Bacillus* (HL1HP11 and HL3RS14) strains, one *Kocuria rosea* (HL1RP8) strain one *Pseudomonas fluorescens* (AT1HP4) strain and one *Aeromonas veronii* (AT1RP10) were applied as inoculants in the form of coated seeds and soil-based phosphate biofertilizers. Plants inoculated with halophilic PGPR strains showed better growth as compared to control plants (with or without salt). The maximum increase in plant biomass or dry weights of root (114%) and shoot (97%) was observed in plants treated with *Bacillus* strain HL3RS14-based soil biofertilizers as compared to other treated and un-inoculated control plants (with or without salt). Inoculation of wheat, rice, and sugarcane varieties with *Pseudomonas, Enterobacter* and *Bacillus* strains resulted in an increase in plant biomass and grain yield (Mirza *et al.* 2007; Mehnaz *et al.* 2009). Plant growth-promoting abilities of halophilic *Bacillus, Enterobacter* and *Pseudomonas* strains promote plant growth and enhance grain yield under saline conditions (Li *et al.* 2017). They can also be used for reclamation of saline soils (Saikia *et al.* 2012; Orhan 2016; Mukhtar *et al.* 2017).

Under salt stress conditions, plants use small organic molecules or osmolytes such as proline, glycine betaine, polyamines and certain carbohydrates to maintain their internal osmotic balance. Leaf tissues of plants treated with halophilic PGPR strains showed a significant difference in proline and glycine betaine content as compared to un-inoculated control plants with and without salt. Plants inoculated with *Bacillus* strain HL3RS14-based enriched soil biofertilizers showed maximum level of proline (40 μmol g^{-1} FW) and glycine betaine (3·67 μmol g^{-1} FW). A number of previous studies also showed that plant experiments on different crops such as wheat, maize and sugarcane under salinity stress conditions showed increased production of glycine betaine and proline (Cha-Um and Kirdmanee 2009; Luo *et al.* 2018). There was a significant increase in the malondialdehyde (MDA) content among treated plants and un-inoculated control-NaCl plants under salinity stress conditions. Control + NaCl un-inoculated plants and *Bacillus* strain HL3RS14 treated plants showed maximum production of MDA as compared to other treated plants and control-NaCl un-inoculated plants. A recent study on wheat growth under salt stress environment showed a significant increase in lipid peroxidation MDA and proline contents of leaf tissues (Bharti *et al.* 2016).

In summary, our results suggested bacterial strains belonging to *Bacillus, Halobacillus, Halomonas* and *Pseudomonas* were dominant in the rhizosphere of halophytes. More than 70% bacterial strains showed positive results for P solubilization, nitrogen fixation, IAA and ACC deaminase production. Selected PGPR strains were used as bio-inoculants for growth promotion of maize. The present study is the first report of its kind that deals with the comparative inoculation effect of both seeds coated with halophilic bacterial culture and enriched soil-based phosphate biofertilizers on maize plant growth and physiology. Salinity stress caused a significant increase in proline, glycine betaine and MDA contents of leaf tissues in the PGPR-inoculated plants. These results suggest that halophilic PGPR strains can be used as biofertilizers for crops growth under salinity affected land.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

Ali, Y., Aslam, Z., Hussain, F. and Shakur, A. (2004) Genotype and environmental interaction in xerophytes for yield and disease resistance. *Int J Environ Sci Technol* **1**, 119–123.

Ali, G.S., Norman, D. and El-Sayed, A.S. (2015a) Soluble and volatile metabolites of plant growth-promoting rhizobacteria (PGPRs): role and practical applications in inhibiting pathogens and activating induced systemic resistance (ISR). *Adv Bot Res Acad Press* **75**, 241–284.

Amoozegar, M.A., Bagheri, M., Makhdoumi, A., Nikou, M.M., Fazeli, S.A., Schumann, P., Spröer, C., Sanchez-Porro, C. *et al.* (2016) *Oceanobacillus halophilus* sp. nov., a novel moderately halophilic bacterium from a hypersaline lake. *Int J Syst Evol Microbiol* **66**, 1317–1322.

Ashraf, M.Y. (2007) Variation in nutritional composition and growth performance of some salt tolerant plants growing under saline environments. *Afr J Rang Forage Sci* **24**, 19–23.

Bates, L.S., Waldren, R.P. and Teare, I.D. (1973) Rapid determination of proline for water stress studies. *Plant Soil* **39**, 205–207.

Bharti, N., Pandey, S.S., Barnawal, D., Patel, V.K. and Kalra, A. (2016) Plant growth promoting rhizobacteria *Dietzia*

natronolimnaea modulates the expression of stress responsive genes providing protection of wheat from salinity stress. *Sci Rep* **6**, 34768.

Bird, R. and Hopkins, R.H. (1954) The action of some alpha-amylases on amylase. *Biochem J* **56**, 86–99.

Cha-Um, S. and Kirdmanee, C. (2009) Effect of salt stress on proline accumulation, photosynthetic ability and growth characters in two maize cultivars. *Pak J Bot* **41**, 87–98.

Chen, L.H., Lin, C.H. and Chung, K.R. (2013) A non-ribosomal peptide synthetase mediates siderophore production and virulence in the citrus fungal pathogen *Alternaria alternata*. *Mol Plant Patholog* **14**, 497–505.

DasSarma, S. and DasSarma, P. (2015) Halophiles and their enzymes: negativity put to good use. *Curr Opin Microbiol* **25**, 120–126.

El Husseini, M.M., Helmut, B. and Helmut, J. (2012) The biofertilising effect of seed dressing with PGPR *Bacillus amyloliquefaciens* FZB 42 combined with two levels of mineral fertilizing in African cotton production. *Arch Phytopathol Plant Prot* **45**, 2261–2271.

Glick, B.R., Penrose, D.M. and Li, J. (1998) A model for the lowering of plant ethylene concentration by plant growth promoting bacteria. *J Theor Biol* **190**, 63–68.

Gonzalez, A.J., Larraburu, E.E. and Llorente, B.E. (2015) *Azospirillum brasilense* increased salt tolerance of jojoba during in vitro rooting. *Ind Crop Prod* **76**, 41–48.

Gordon, S.A. and Weber, R.P. (1950) Colorimetric estimation of indole acetic acid. *Plant Physiol* **26**, 192–195.

Goswami, D., Thakker, J.N. and Dhandhukia, P.C. (2016) Portraying mechanics of plant growth promoting rhizobacteria (PGPR): a review. *Cog Food Agri* **2**, 11275.

Gupta, G., Parihar, S.S., Ahirwar, N.K., Snehi, S.K. and Singh, V. (2015) Plant growth promoting rhizobacteria (PGPR): current and future prospects for development of sustainable agriculture. *J Microb Biochem Technol* **7**, 96–102.

Heath, R.L. and Packer, L. (1968) Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch Biochem Biophys* **125**, 189–198.

Hoagland, D.R. and Arnon, D.I. (1950) *The Water-Culture Method for Growing Plants Without Soil*. Berkley, CA: California Agricultural Experimental Station Publications, College of Agriculture, University of California.

Jamil, S., Mehmood, A., Akhtar, M.S., Memon, M. *et al.* (2016) Changes in soil phosphorus fractions across a toposequence in the estuary plains of Pakistan. *Arch Agron Soil Sci* **62**, 1567–1577.

Kamran, S., Shahid, I., Baig, N.D., Rizwan, M., Malik, A.K. and Mehnaz, S. (2017) Contribution of zinc solubilizing bacteria in growth promotion and zinc content of wheat. *Front Microbiol* **8**, 2593.

Karpagam, T. and Nagalakshmi, P.K. (2014) Isolation and characterization of phosphate solubilizing microbes from agricultural soil. *Int J Curr Microbiol App Sci* **3**, 601–614.

Kasana, R.C., Salwan, R., Dhar, H., Dutt, S. and Gulati, A. (2008) A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. *Curr Microbiol* **57**, 503–507.

Khan, N., Martínez-Hidalgo, P., Ice, T.A., Maymon, M., Humm, E.A., Nejat, N., Sanders, E.R., Kaplan, D. *et al.* (2018) Antifungal activity of *Bacillus* species against *Fusarium* and analysis of the potential mechanisms used in biocontrol. *Front Microbiol* **9**, 2363.

Kumar, K.V., Srivastava, S., Singh, N. and Behl, H.M. (2009) Role of metal resistant plant growth promoting bacteria in ameliorating fly ash to the growth of *Brassica juncea*. *J Haz Mat* **170**, 51–57.

Kumar, A., Prakash, A. and Johri, B. (2011) *Bacillus* as PGPR in crop ecosystem. In *Bacteria in Agrobiology: Crop Ecosystems* ed. Maheshwari, D.K. pp 37–59. Berlin, Heidelberg: Springer-Verlag.

Kumar, A., Maurya, B.R. and Raghuwanshi, R. (2014) Isolation and characterization of PGPR and their effect on growth, yield and nutrient content in wheat (*Triticum aestivum* L.). *Biocatal Agric Biotechnol* **3**, 121–128.

Kumar, S., Stecher, G. and Tamura, K. (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* **33**, 1870–1874.

Li, Y., Liu, X., Hao, T. and Chen, S. (2017) Colonization and maize growth promotion induced by phosphate solubilizing bacterial isolates. *Int J Mol Sci* **18**, E1253.

Luo, M., Zhao, Y., Wang, Y., Shi, Z., Zhang, P., Zhang, Y., Song, W. and Zhao, J. (2018) Comparative proteomics of contrasting maize genotypes provides insights into salt-stress tolerance mechanisms. *J Proteome Res* **17**, 141–153.

Mahajan, S. and Tuteja, N. (2005) Cold, salinity and drought stresses: an overview. *Arch Biochem Biophys* **444**, 139–158.

Malik, D.M. (1992) Phosphorus management for wheat production in Punjab. In *Proceeding Symposium on the Role of Phosphorus in Crop Production* ed. AbdulRashid pp 175–196. Islamabad: NFDC.

Malik, K.A., Bilal, R., Mehnaz, S., Rasool, G., Mirza, M.S. and Ali, S. (1997) Association of nitrogen-fixing, plant growth promoting rhizobacteria (PGPR) with kallar grass and rice. *Plant Soil* **194**, 37–44.

Mehnaz, S. and Lazarovits, G. (2006) Inoculation effects of *Pseudomonas putida, Gluconaacetobacter azotocaptans* and *Azospirillum lipoferum* on corn plant growth under greenhouse conditions. *Microb Ecol* **51**, 326–335.

Mehnaz, S., Mirza, M.S., Haurat, J., Bally, R., Normand, P., Bano, A. and Malik, K.A. (2001) Isolation and 16S rRNA sequence analysis of the beneficial bacteria from the rhizosphere of rice. *Can J Microbiol* **47**, 110–117.

Mehnaz, S., Weselowski, B., Aftab, F., Zahid, S., Lazarovits, G. and Iqbal, J. (2009) Isolation, characterization and effect of fluorescent *Pseudomonads* on micropropagated sugarcane. *Can J Microbiol* **55**, 1007–1011.

Mehnaz, S., Baig, D.N. and Lazarovits, G. (2010) Genetic and phenotypic diversity of plant growth promoting

rhizobacteria isolated from sugarcane plants growing in Pakistan. *J Microbiol Biotechnol* **20**, 1614–1623.
Mirza, B.S., Mirza, M.S., Bano, A. and Malik, K.A. (2007) Inoculation of chickpea with Rhizobium isolates from roots and nodules and phytohormone producing *Enterobacter* strains. *Aust J Exp Agric* **47**, 1008–1015.
Mukhtar, S., Mirza, M.S., Awan, H.A., Maqbool, A., Mehnaz, S. and Malik, K.A. (2016) Microbial diversity and metagenomic analysis of the rhizosphere of Para Grass (*Urochloa mutica*) growing under saline conditions. *Pak J Bot* **48**, 779–791.
Mukhtar, S., Shahid, I., Mehnaz, S. and Malik, K.A. (2017) Assessment of two carrier materials for phosphate solubilizing biofertilizers and their effect on growth of wheat (*Triticum aestivum*). *Microbiol Res* **205**, 107–117.
Mukhtar, S., Mehnaz, S., Mirza, M.S. and Malik, K.A. (2019) Isolation and characterization of halophilic bacteria from the rhizosphere of halophytes and non-rhizospheric soil samples. *Braz J Microbiol* **50**, 85–97.
Nautiyala, C.S., Bhadauria, P., Kumar, H.L. and Mondal, R. (1999) Stress induced phosphate solubilization in bacteria isolated alkaline soils. *FEMS Microbiol Lett* **182**, 291–296.
Oberson, A., Friesen, D.K., Rao, I.M., Bühler, S. and Frossard, E. (2001) Phosphorus transformations in an oxisol under contrasting landuse system: the role of the microbial biomass. *Plant Soil* **237**, 197–210.
Orhan, F. (2016) Alleviation of salt stress by halotolerant and halophilic plant growth-promoting bacteria in wheat (*Triticum aestivum*). *Braz J Microbiol* **47**, 621–627.
Paul, D. and Lade, H. (2014) Plant-growth-promoting rhizobacteria to improve crop growth in saline soils: a review. *Agron Sustain Dev* **34**, 737–752.
Penrose, D.M. and Glick, B.R. (2003) Methods for isolating and characterizing ACC deaminase containing plant growth promoting rhizobacteria. *Physiol Plant* **118**, 10–15.
Penrose, D.M., Moffat, B.A. and Glick, B.R. (2001) Determination of 1-aminocyclopropane-1-carboxylic acid (ACC) to assess the effects of ACC deaminase-containing bacteria on roots of canola seedlings. *Can J Microbiol* **47**, 77–80.
Pérez-Miranda, S., Cabirol, N., George-Téllez, R., Zamudio-Rivera, L.S. and Fernández, F.J. (2007) O-CAS, a fast and universal method for siderophore detection. *J Mircobial Meth* **90**, 127–131.
Pikovskaya, R. (1948) Mobilization of phosphorus in soil in connection with vital activity of some microbial species. *Mikrobiologiya* **17**, 362–370.
Piñar, G., Kraková, L., Pangallo, D., Piombino-Mascali, D., Maixner, F., Zink, A. and Sterflinger, K. (2014) Halophilic bacteria are colonizing the exhibition areas of the Capuchin Catacombs in Palermo, Italy. *Extremophiles* **18**, 677–691.
Sadasivam, S. and Manickam, A. (1992) *Biochemical Methods for Agricultural Sciences*. pp 246–257. New Delhi: Wiley Eastern Ltd.
Saharan, B. and Nehra, V. (2011) Plant growth promoting rhizobacteria: a critical review. *Life Sci Med Res* **21**, 1–30.
Saikia, S.P., Bora, D., Goswami, A. and Mudoi, K.D. (2012) A review on the role of *Azospirillum* in the yield improvement of non-leguminous crops. *Afr J Microbiol Res* **6**, 1085–1102.
Schneegurt, M.A. (2012) Media and conditions for the growth of halophilic and halotolerant bacteria and archaea. In *Advances in understanding the biology of halophilic microorganisms* ed. Vreeland, R.H. pp 35–58. Dordrecht: Springer.
Sierra, G. (1957) A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty acid substrates. *Antonie van Leeuw J Microbiol* **23**, 15–22.
Solangi, M.K., Solangi, S.K. and Solangi, N.K. (2016) Grain yield, phosphorus content and uptake of wheat (*Triticum aestivum* L.) as affected by phosphorus fertigation. *Pak J Biotechnol* **13**, 205–209.
Tamura, K., Nei, M. and Kumar, S. (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc Natl Acad Sci USA* **101**, 11030–11035.
Tan, Z.Y., Xu, X.D., Wang, E.T., Gao, J.L., Martinez-Romero, E. and Chen, W.X. (1997) Phylogenetic and genetic relationships of *Mesorhizobium tianshanense* and related *Rhizobia*. *Int J Sys Bacteriol* **47**, 874–879.
Watanabe, F. and Olsen, S. (1965) Test of an ascorbic acid method for determining phosphorus in water and $NaHCO_3$ extracts from soil 1. *Soil Sci* **29**, 677–678.
Winnepenninckx, B., Backeljau, T. and de Wachter, R. (1993) Extraction of high molecular weight DNA from molluscs. *Trends Genet* **9**, 407–412.
Zahir, Z.A., Ghani, U., Naveed, M., Nadeem, S.M. *et al.* (2009) Comparative effectiveness of *Pseudomonas* and *Serratia* sp. containing ACC-deaminase for improving growth and yield of wheat (*Triticum aestivum* L.) under salt-stressed conditions. *Arch Microbiol* **191**, 415–424.
Zhang, H., Chen, L., Zhao, S., Ren, J. and Cao, X. (2014) Knockout of ituD Gene of *Bacillus subtilis* S44 strain and impact of its biocontrol effect to cotton rhizoctoniosis. *Plant Pathol J* **13**, 125–132.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Antifungal activity of halophilic bacterial strains.

Table S2. Hydrolytic enzymes produced by halophilic bacterial strains.

Figure S1. Siderophore production by halotolerant and halophilic bacterial strains with: (A) −, no activity; (B) +, low activity; (C) ++, medium activity; and (D) +++, high activity.

Figure S2. HCN production by halotolerant and halophilic bacterial strains with: (A) −, no activity; (B) +, low activity; (C) ++, medium activity; and (D) +++, high activity.

Figure S3. Antifungal assays of bacterial isolates with: (i) *Fusarium oxysporum,* (ii) *Aspergillus niger*, (iii) *Curvularia* sp., and (iv) *Aspergillus flavus*.

Figure S4. Effect of halophilic phosphate-solubilizing bacteria on maize root growth under salinity stress conditions (A) Seed coated with bacterial strains, 3% PVP and rock phosphate (B) Soil-based phosphate biofertilizers.