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Halophile plant growth-promoting rhizobacteria induce salt tolerance traits in wheat seedlings (*Triticum aestivum* L.)

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ABSTRACT

Salinity is one of the most important growth-limiting factors for most crops in arid and semi-arid regions; however, the use of plant growth-promoting rhizobacteria isolated from saline soils could reduce the effects of saline stress in crops. This study aimed to evaluate the efficiency of plant growth-promoting rhizobacteria (PGPRs), isolated from the rhizosphere of halophile plants, for the growth, Na^+/K^+ balance, ethylene emission, and gene expression of wheat seedlings (*Triticum aestivum* L.) grown under saline conditions (100 mmol L^{-1} NaCl) for 14 d. A total of 118 isolates obtained from saline soils of the deserts of Iran were tested for their capacity as PGPRs. Out of the 118 isolates, 17 could solubilize phosphate ($Ca_3(PO_4)_2$), 5 could produce siderophores, and 16 could synthesize indole-3-acetic acid. Additionally, PGPRs were also evaluated for aminocyclopropane-1-carboxylate deaminase activity. A pot experiment was conducted to evaluate the ability of 28 PGPR isolates to promote growth, regulate Na^+/K^+ balance, and decrease ethylene emissions in plants. The most efficient PGPRs were *Arthrobacter aurescens*, *Bacillus atrophaeus*, *Enterobacter asburiae*, and *Pseudomonas fluorescens*. Gene expression analysis revealed the up-regulation of *H^+-PPase*, *HKT1*, *NHX7*, *CAT*, *and APX* expression in roots of *Enterobacter*-inoculated salt-stressed plants. Salt-tolerant rhizobacteria exhibiting plant growth-promoting traits can facilitate the growth of wheat plants under saline conditions. Our results indicate that the isolation of these bacteria may be useful for formulating new inoculants to improve wheat cropping systems in saline soils.

Key Words: ethylene emission, gene expression, growth-promoting trait, indole-3-acetic acid, phosphate solubility, saline soil, siderophore

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INTRODUCTION

Salinity is one of the most important growth-limiting factors for most crops in arid and semi-arid regions (Tiwari *et al*., 2010). In Iran, out of the total land area of 162.2 million hectare, 55.6 million hectaare (34% of the total) are affected by salt stress (Momeni, 2011). In these areas, infrequent rainfall, high evaporation, saline irrigation water, and inappropriate water management result in the salinization of agricultural areas. Wheat yield in saline soils is significantly decreased by soil salinity, which can promote ionic stress in plants and lead to negative physiological responses like K^+ imbalances, lower water uptake, reduced rates of photosynthesis, and dysregulation of enzyme activity and metabolism (Tester and Davenport, 2003).

Most sensitive or hypersensitive crops (glycophytes) cannot tolerate high salt levels. Consequently, saline soils are limiting for crop production (Tiwari *et al*., 2010). Wheat (*Triticum aestivum* L.) is the most important cultivated crop globally, and is a salt-sensitive glycophyte (Munns and James, 2003). The use of beneficial microbes to improve the yield and growth of wheat by reducing abiotic stresses can be achieved by the interaction between plant roots and soil microorganisms (Paul and Nair, 2008). In particular, soil rhizobacteria can colonize roots and promote plant growth through the production of phytohormones and siderophores, as well as by the solubilization of rock phosphate that then becomes available to plants (Stephen and Jisha, 2011).

Due to these beneficial effects on plant growth, these microorganisms are categorized as plant growth-promoting rhizobacteria (PGPRs). Some PGPRs also contribute to improved nutrient recycling in the rhizosphere and root colonization of the soil under salt stress (Paul and Nair, 2008). Moreover, inoculation of cereals with PGPRs to reduce salt stress has been reported (Barriuso *et al*., 2008; Marulanda *et al*., 2010). Increased crop yield in response to PGPRs has been associated with the regulation of the biosynthesis of some phytohormones, mainly indole-3-acetic acid (IAA) and ethylene (Glick, 2014; Gontia-Mishra *et al*., 2017; Ambreetha *et al*., 2018). Indeed, salinity may decrease yield by up to 65%, and is a major limiting factor for wheat production (Shafi *et al*., 2010). The PGPRs can mitigate the consequences of salt stress and increase salt tolerance in plants through several mechanisms, like the production of

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1-aminocyclopropane-1-carboxylate (ACC) deaminase, the enzyme that limits the levels of ethylene (Barriuso *et al*., 2008). Ethylene is an important phytohormone synthesized by plants under unfavorable environmental conditions; it inhibits plant shoot and root growth under abiotic or biotic stresses, causes leaf abscission, reduces leaf expansion, and promotes the onset of epinasty and chlorosis (Abeles *et al*., 1992). Glick (2014) found that bacteria in the rhizosphere can regulate ethylene levels which are positively correlated with the degree of salt stress in plants, thus alleviating salt-stress-induced deleterious effects. Indeed, under saline conditions, surplus ACC is exuded from plant roots into the soil, where bacterial ACC deaminase transforms ACC to α-ketobutyrate and ammonia (Gontia-Mishra *et al*., 2017). Moreover, PGPRs also improve plant growth under salt stress through changes in ion uptake, maintenance of the K^+/Na^+ ratio, and accumulation of osmoprotectants (Gupta and Huang, 2014). Alleviation of salinity symptoms in plants is also promoted by the PGPR-induced up-regulation of numerous genes, especially those involved in K^+/Na^+ balance like *HKT1* (encoding high-affinity K^+ transporter 1) (Zhang *et al*., 2010; Sharma *et al*., 2014), *NHX1* and *NHX7* (encoding plasma membrane Na^+/H^+ antiporter 1 and 7, respectively) (Yamaguchi *et al*., 2013; Deinlein *et al*., 2014), and *H^+-PPase* (encoding H^+-pumping pyrophosphatase), responsible for the sequestration of Na^+ into vacuoles (Schilling *et al*., 2014). Other genes, up-regulated in response to salt tolerance in plants, are related to reactive oxygen species (ROS)-scavenging enzymes, such as *APX* and *CAT* (encoding ascorbate peroxidase (APX) and catalase (CAT), respectively) (Zhang *et al*., 2010; Gururani *et al*., 2013; Suarez *et al*., 2015).

On this basis, we hypothesized that the use of characterized PGPRs isolated from saline soils could reduce the effects of saline stress in wheat. Therefore, the aims of this study were to evaluate the plant growth-promoting (PGP) properties of isolated PGPRs and to investigate the effects of these isolates on the growth of wheat under saline conditions.

MATERIALS AND METHODS

Soil sampling and chemical analysis

Ten composite soil samples, each consisting of ten 5-cm-diameter cores pooled on site, were collected at a depth of 0–30 cm in September 2014 from the rhizosphere of wild halophile plants (*Salicornia* spp., *Echinochloa stagnina*, and *Tamarix* spp.) in the deserts of Golestan, Isfahan, Yazd, and Hormozgan provinces, Iran (Table I). This sampling method, based on various composite soil samples rather than single soil samples, was used to minimize spatial variability as the experimental setup did not apply to single plots (Greenway and Munns, 1980). Soon after collection, the soil samples were immediately placed in sterilized plastic bags, stored at 4 °C, and transported to the laboratory, where visible crop residues and coarse bulk soil were removed under a laminar flow hood using sterile gloves. The soil samples were then collected in open plastic bags, once in the laboratory, transferred to microbiological flasks capped with a cotton wool stopper, and stored at 4 °C for 3–4 d before being analyzed. Before each subsequent analysis, soils were acclimated at room temperature for 24 h. Rhizosphere soil was isolated after sonication for 2 min, using the protocol of White *et al*. (2015). The physicochemical properties of the soil samples, such as the exchangeable Na^+ content, exchangeable Mg^{2+}/Ca^{2+} ratio, exchangeable sodium percentage (ESP), sodium adsorption ratio (SAR), soil organic carbon (SOC) by wet oxidation, carbonate (CO_3^{2-}) content, pH (in water), and electrical conductivity (EC) of soil water extract, were determined according to Pansu and Gautheyrou (2006).

Isolation of rhizobacteria

The collected soil samples (50 g) were suspended in 450 mL sterile NaCl solution (0.85%, weight/volume) and shaken on an orbital shaker at 100 × *g* and room tempera-

TABLE I

Geographical coordinates of the sampling sites and chemical properties of the composite soil samples in four provinces of Iran

Province	Coordinate		Na^+	Mg^{2+}/Ca^{2+}	ESP[a)]	SAR[b)]	SOC[c)]	CO_3^{2-}	pH	EC[d)]
	Longitude	Latitude								
			mg kg^{-1} DW[e)]		%		g kg^{-1} DW	mg kg^{-1} DW		dS m^{-1}
Golestan	54°20′01.1″ E	37°12′53.8″ N	36.1 ± 2.1[f)]	0.98 ± 0.32	37 ± 5	32 ± 3	8.4 ± 2.5	13.4 ± 1.5	7.9 ± 0.5	8.3 ± 2.0
Isfahan	52°33′43.6″ E	33°38′42.7″ N	48.4 ± 5.4	0.80 ± 0.45	42 ± 6	45 ± 4	9.8 ± 3.4	15.6 ± 1.3	8.1 ± 0.4	14.4 ± 3.2
Hormozgan	56°27′20.4″ E	28°03′54.6″ N	32.3 ± 2.6	0.89 ± 0.26	33 ± 4	30 ± 2	3.1 ± 2.7	16.6 ± 1.3	7.2 ± 0.5	9.9 ± 1.5
Yazd	55°12′33.4″ E	32°02′26.8″ N	40.6 ± 4.9	0.74 ± 0.11	31 ± 3	29 ± 5	2.8 ± 6.5	12.3 ± 1.0	7.5 ± 0.3	11.7 ± 1.9
Mean			39.4	0.68	35.8	34.0	8.1	14.5	7.7	11.1

[a)] Exchangeable sodium percentage.
[b)] Sodium adsorption ratio.
[c)] Soil organic carbon.
[d)] Electrical conductivity.
[e)] Dry weight.
[f)] Mean ± standard error ($n = 10$).

ture for 20 min. The soil suspensions were serially diluted with NaCl solution (0.85%, weight/volume), and 100 µL of each suspension was plated on different media to maximize bacterial variation. Bacterial isolation was conducted on ten media (Tables SI and SII, see Supplementary Material for Tables SI and SII): yeast extract mannitol agar (YEMA), King's B agar (KBA), water yeast extract agar (WYEA), glycerol yeast extract agar (GYA), Luria Bertani agar (LBA), triptic soy agar (TSA), eosin methylene blue agar (EMBA), and two synthetic saline culture media (A and B) (Tables SI and SII). All the plates were supplemented with 3% NaCl and incubated at 30 ± 2 °C for 3 d. A representative of each colony was picked according to colony morphology and transferred to liquid, non-specific, nutrient agar (NA) medium (Table SI) to establish pure cultures. The bacterial isolates were kept in 50% (volume/volume) glycerol at −80 °C.

Screening of salt-tolerant bacterial isolates for plant growth-promoting traits

Salt-tolerant bacterial isolates were screened for plant growth-promoting traits, such as the ability to solubilize phosphate, produce siderophores and IAA, and show ACC deaminase activity.

The capacity for phosphate solubilization was determined by observing a clear zone around each isolate after a 5-d incubation at 30 ± 2 °C, according to Oteino *et al*. (2015). Phosphate solubilization was quantified through a reduction in pH. A loop of each rhizobacterial isolate was cultured on Pikovskaya's agar plates containing bromophenol blue, according to Illmer *et al*. (1995). Phosphate solubilization activity was calculated by the method developed by Pikovskaya (1948); tri-calcium phosphate ($Ca_3(PO_4)_2$) solubilization by the isolates, as well as the patterns of pH decrease, was also recorded. The quantity of soluble phosphate was measured by the stannous chloride method (Watanabe and Olsen, 1965) after 5 d of incubation at 28 °C.

Siderophores were quantified using the chrome azurol sulphonate (CAS)-shuttle assay, with cultures grown in deferated Fiss minimal medium (Payne, 1994). In brief, samples were collected and centrifuged at 2 700 × g for 15 min, and equal proportions of culture supernatant and CAS assay solution were mixed and allowed to stand for 20 min. Siderophores removed iron from the dye complex, causing a blue-to-orange color change, which was recorded at 630 nm. Percentage production by bacteria was calculated as: siderophores (%) $= [(A_r - A_s)/A_r] \times 100$, where A_r is the absorbance of reference (minimal medium + CAS assay solution) and A_s is absorbance of the sample.

Determination of IAA was performed according to Bric *et al*. (1991) with some modifications. Briefly, the bacteria were cultured in NA medium containing 500 g L^{-1} L-tryptophan at 30 ± 2 °C for 72 h. The supernatant was mixed with Salkowski reagent (50 mL 35% perchloric acid, 1 mL 0.5 mol L^{-1} $FeCl_3$ solution) at 1:1 (volume/volume), and 20 min later, absorbance at 530 nm was determined. The IAA produced was estimated against an IAA standard curve in the range of 10–100 mg mL^{-1}.

To estimate the bacterial ACC deaminase activity, the method developed by Penrose and Glick (2003) was used. Briefly, the isolates were grown in 20 mL half-strength Marine Broth (Roth), centrifuged at 3 500 × g for 10 min, re-suspended in Dworking and Foster (DF) minimal salt medium filled with ACC (Penrose and Glick, 2003) to a final concentration of 1%, 2%, and 3% (weight/volume) NaCl and incubated for 48 h at 28 °C. The quantity of α-ketobutyrate produced by ACC deaminase activity was estimated at 540 nm by comparing the absorbance values of the samples to a standard curve of α-ketobutyrate between 0.1 to 1 mmol L^{-1}. The activity of ACC deaminase was expressed as µmol α-ketobutyrate mg^{-1} protein h^{-1}. Protein concentration was determined by the Bradford assay (Bradford, 1976).

Molecular characterization of bacterial isolates

The isolates with the most effective PGP ability were characterized by partial sequence analysis of the 16S rRNA gene. The AccuPrep® Genomic DNA extraction kit (Bioneer, the Republic of Korea) was used to extract the DNA, which was dissolved in 20 µL *Tris*-EDTA buffer. Polymerase chain reaction (PCR) amplification was performed using the universal forward primer 8f (5′-AGAGTTTGATCCTGGCTCAG-3′) and the 1492r reverse primer (5′-GG(C/T)TACCTTGTTACGACTT-3′). The PCR reactions were performed in a total volume of 25 µL, including 25 ng of template DNA, 2.5 mmol L^{-1} dNTP mixture, 10 pmol L^{-1} of each primer, and Taq DNA polymerase (1 U), with the following conditions: 95 °C for 2 min, followed by 35 cycles of 95 °C for 1 min, 56 °C for 1 min, and 72 °C for 1.5 min, with a final extension of 7 min at 72 °C (Qin *et al*., 2011). The PCR products were verified on 1% agarose gel and purified using the AccuPrep® PCR purification kit (Bioneer). The 16S rRNA gene sequences of selected isolates were obtained using an automated sequencer at the Seq Lab Laboratory (Göttingen, Germany). The sequences of the isolates were compared with related 16S rRNA gene sequences in the National Center for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov) *via* a BLAST search.

Pot trial

Pots (0.016 m^3) were filled with sterilized soil (121 °C for 20 min) composed of a mixture of loam, peat, and sand (1:1:1) and placed in a greenhouse at a temperature ranging 20–35 °C and 30% mean average humidity. The two treatments were: a) un-inoculated control and b) with

bacteria inoculation. The pot experiments were performed as a randomized complete block design with five replicates. Wheat seeds (*Triticum aestivum* L. cv. drya) were sterilized in 70% ethanol for 2 min and 1.2% (volume/volume) sodium hypochlorite for 10 min, and then washed ten times in sterile tap water. Seeds for the inoculation treatments were then rinsed in the bacterial suspensions for 30 min under sterile conditions. Only 28 bacterial isolates showing at least one of the PGP traits described above were used for seed inoculation. To obtain the bacterial suspensions, isolates were grown in nutrient broth medium at 30 °C and diluted to a final density of approximately 10^8 colony-forming units (CFU) mL^{-1} in sterile distilled water containing 0.025% (volume/volume) Tween 20.

Wheat seeds were planted at the same depth (approximately 2.5 cm below the soil surface) in all the pots. The pots were covered with plastic film and aluminum foil to avoid evaporation from the soil surface and to minimize temperature increases inside the containers. Plants were irrigated with water for 7 d after germination. All the pots were weighed every evening to calculate the daily transpiration rates of the plants, and to maintain soil water content at a constant value of approximately 70%–75% of soil water-holding capacity to keep wheat roots well aerated. Seven days after germination, the plants were irrigated with Hoagland nutrient solution supplemented with up to 100 mmol L^{-1} NaCl for 14 d. The solution was applied in increments (30 mmol L^{-1} NaCl in days 1 and 2, 50 mmol L^{-1} in day 3, 70 mmol L^{-1} in day 4, and 100 mmol L^{-1} in the remaining 10 d) to not shock the seedlings with the added saline solution. The 100 mmol L^{-1} NaCl concentration was chosen based on the fact that it induces a moderate, rather than severe, degree of salt stress, and was similar to the NaCl levels commonly found in saline soils cultivated with wheat (Munns and James, 2003; Tester and Davenport, 2003; Tiwari *et al*., 2010). Seedlings without NaCl supplementation in the Hoagland solution were used as controls. An image of the pot trial with PGPR-inoculated and un-inoculated wheat seedlings, after 14 d of exposure to NaCl, is shown in Fig. 1.

At the end of experiment, seedlings under both non-saline (0 mmol L^{-1} NaCl) and saline (100 mmol L^{-1} NaCl) conditions were harvested and shoot dry biomass, root dry biomass, and total dry biomass were measured after oven-dried at 80 °C for 48 h and cooled in a desiccator. Leaves were dry-ashed at 550 °C and digested with an acid mixture (HNO_3:H_2SO_4:$HClO_4$ = 10:1:3) before Na^+ and K^+ contents were determined.

Ethylene production in plants

Ethylene production was measured with a gas chromatograph (HP5890, Hewlett-Packard, USA) according to Siddikee *et al*. (2011), using a flame ionization detector (FID) and a stainless-steel column (150 × 0.4 cm internal diameter packed with Hysep T) at temperatures of 70 and 350 °C, respectively, with N_2 as the carrier gas at a flow rate of 30 mL min^{-1}. Ethylene emission levels were determined in seedlings incubated for 24 h.

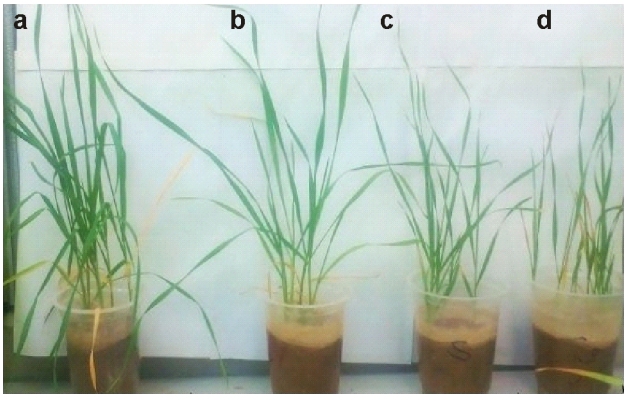

Fig. 1 A photo showing wheat seedlings inoculated (a and b) or un-inoculated (c and d) with *Enterobacter asburiae*, a plant growth-promoting rhizobacterium, under non-saline (0 mmol L^{-1} NaCl) (a and c) and saline (100 mmol L^{-1} NaCl) (b and d) conditions for 14 d.

Quantitative reverse transcription (qRT)-PCR

TRIzol reagent (Sigma-Aldrich, USA) was used to extract total RNA from the roots of wheat seedlings subjected to salts stress for 14 d. Active, fine roots (with a diameter < 1 mm) were chosen and carefully washed before being immediately analyzed. Root tissues were selected for gene expression analysis due to the importance of ion uptake and transport, and regulation of water status and signal transduction processes in the root system. To remove genomic DNA contamination, RNase-free DNase I (Sigma-Aldrich) was utilized during RNA preparation. Total RNA (3 μg) was reverse-transcribed using a first-strand cDNA synthesis kit (Thermo Fisher Scientific, USA). Gene-specific primers (Table SIII, see Supplementary Material for Table SIII) and SYBR Green dye were applied to 1 μL of the 10-fold diluted cDNA for qRT-PCR. The following conditions were used: 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 1 min, and PCR was performed using an Applied Biosystems StepOne PlusTM real-time PCR system (Applied Biosystems, USA). Furthermore, the $2^{-\Delta\Delta Ct}$ method was applied using actin (*ACT2*) as an endogenous control to calculate relative gene expression levels, according to Remans *et al*. (2008).

Statistical analysis

All the data obtained in this study were tested for statistical significance using one-way analysis of variance (ANOVA) in the SAS statistical software package version 9.1 (SAS Inc., USA). The means were compared by the least significant difference (LSD) test at $P < 0.05$.

RESULTS

Soil chemical properties

The geographical coordinates of the sampling sites and the measured chemical properties of the collected soil samples are presented in Table I. The results indicated that the EC of the soil samples ranged from −8.3 to 14.4 dS m^{-1}, with an average value of 11.1 dS m^{-1}. Soils showed a mean pH of 7.7 but a low SOC content (mean = 8.1 g kg^{-1}).

Isolation and purification of salt-tolerant bacteria

Screening on the selective media used allowed the morphological distinction of 118 isolates, which were purified by successive sub-culturing in the same media. Among the 118 isolates, 21 were obtained on KBA, 24 on YEMA, 26 on GYA, 9 on LBA, 10 on medium A, 4 on EMBA, 4 on TSA, and 22 on NA. No bacteria grew in medium B.

Bacterial phosphate solubilization, siderophore and IAA biosynthesis, and ACC deaminase activity

Of the 118 isolates, 17 showed a zone of phosphate solubilization on Pikovskaya's agar supplemented with bromophenol blue (Table II). Ten isolates presented phosphate solubilization levels greater than 1 000 μg mL^{-1} (Table II). Only five isolates produced siderophores, and 16 synthesized IAA (Table II). Isolates 53 and 84 showed the highest levels of IAA (121 and 78 μg mL^{-1}, respectively) (Table II). The highest ACC deaminase activity was observed in isolates 53 and 87 (33.25 and 25.22 μmol α-ketobutyrate mg^{-1} protein h^{-1}, respectively) (Table II). The isolates not reported in Table II did not show any phosphate solubilization, IAA production, or ACC deaminase activity.

TABLE II

Phosphate solubility, production of siderophores and indole-3-acetic acid (IAA), and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity of rhizobacterial isolates from the rhizosphere of wild halophile plants (*Salicornia* spp., *Echinochloa stagnina*, and *Tamarix* spp.) in the deserts of Golestan, Isfahan, Yazd, and Hormozgan provinces, Iran ($n = 10$)

Isolate No.	Phosphate solubility	Siderophores	IAA	ACC deaminase activity
	μg mL^{-1}	%	μg mL^{-1}	μmol α-ketobutyrate mg^{-1} protein h^{-1}
2	966.30	< LOD[a)]	< LOD	13.32
3	147.20	< LOD	< LOD	19.82
7	1 480.53	< LOD	< LOD	1.89
8	146.21	< LOD	< LOD	23.23
21	73.23	< LOD	9.25	12.89
22	< LOD	< LOD	17.90	9.22
23	< LOD	< LOD	15.45	7.62
32	73.23	< LOD	< LOD	5.22
38	< LOD	14.43	18.80	17.09
39	< LOD	5.11	< LOD	12.29
40	1 850.10	< LOD	10.37	11.09
41	1 202.32	< LOD	12.52	11.48
42	< LOD	17.21	< LOD	19.99
43	< LOD	< LOD	7.35	4.89
45	< LOD	< LOD	10.15	3.34
47	1 008.44	< LOD	< LOD	5.52
48	2 012.12	< LOD	< LOD	3.52
53	1 599.10	7.23	121.32	33.25
54	< LOD	< LOD	16.41	22.90
55	< LOD	< LOD	25.96	10.89
57	219.00	< LOD	5.76	18.85
65	1 480.41	< LOD	17.74	6.33
69	1 377.88	< LOD	< LOD	2.33
76	388.69	< LOD	< LOD	7.88
84	1 797.31	< LOD	77.92	12.30
87	188.22	11.12	< LOD	25.22
96	1 667.81	< LOD	17.65	21.25
97	< LOD	< LOD	17.04	22.89
LSD[b)] ($P < 0.05$)	21.13	4.48	5.20	0.26

[a)] Limit of detection.
[b)] Least significant difference.

Plant growth parameters, Na^+ and K^+ levels, and ethylene production

The highest values for total plant biomass were found in the plants inoculated with isolates 53 and 84 under both non-saline conditions and NaCl stress (Table III). The Na^+ and K^+ contents in the shoots were influenced by isolate inoculation under both saline and non-saline conditions, with significantly reduced Na^+ levels in the leaves compared to the un-inoculated control (Table IV). At 100 mmol L^{-1} NaCl, the percentage of Na^+ in the wheat seedlings was the lowest in those inoculated with isolates 38, 53, 84, and 96 (Table IV). Overall, the foliar levels of K^+ increased with bacterial isolate inoculation, in both saline and non-saline conditions (Table IV). The lowest Na^+/K^+ ratios were observed in the leaves of seedlings inoculated with isolates 38, 53, 84, and 96 grown under salt stress (Table IV). The highest ethylene emissions were found in the un-inoculated plants treated with 100 mmol L^{-1} NaCl, while inoculation with PGPRs had no significant effect on ethylene production in non-stressed plants (Table V). Ethylene production increased in non-stressed plants, whereas the values were significantly lower compared to salt-stressed plants (Table V). The lowest values for ethylene emission were obtained in salt-stressed plants inoculated with isolate 53 (Table V).

Molecular characterization and phylogenetic analysis of salt-tolerant bacterial isolates

Four isolates (38, 53, 84, and 96) were selected for their capacity to promote the accumulation of total biomass. Through 16S rRNA gene sequence homology, isolate 38 was identified as *Pseudomonas fluorescens*, isolate 53 as *Enterobacter asburiae*, isolate 84 as *Arthrobacter aurescens* TC1, and isolate 96 as *Bacillus atrophaeus* (Table VI). The values for DNA sequence identity are reported in Table VI.

Effect of Enterobacter sp. inoculation on plant gene expression

Isolate 53 (*Enterobacter asburiae*) showed the best PGP

TABLE III

Shoot, root, and total dry weights of wheat seedlings grown under non-saline (0 mmol L^{-1} NaCl) and saline (100 mmol L^{-1} NaCl) conditions for 14 d ($n = 10$)

Treatment[a)]	Shoot dry weight		Root dry weight		Total dry weight	
	Non-saline	Saline	Non-saline	Saline	Non-saline	Saline
	g $plant^{-1}$					
Un-inoculated	0.099	0.060	0.077	0.085	0.167	0.154
2	0.209	0.183	0.177	0.139	0.386	0.322
3	0.168	0.148	0.182	0.145	0.350	0.293
7	0.203	0.170	0.107	0.117	0.310	0.288
8	0.154	0.136	0.144	0.100	0.298	0.236
21	0.185	0.147	0.184	0.148	0.369	0.295
22	0.179	0.127	0.160	0.113	0.339	0.240
23	0.181	0.161	0.187	0.162	0.368	0.323
32	0.187	0.182	0.200	0.201	0.387	0.383
38	0.158	0.234	0.167	0.225	0.325	0.409
39	0.170	0.210	0.189	0.221	0.359	0.331
40	0.165	0.128	0.154	0.129	0.319	0.257
41	0.186	0.151	0.208	0.171	0.394	0.322
42	0.257	0.173	0.281	0.144	0.337	0.317
43	0.205	0.187	0.210	0.191	0.414	0.378
45	0.174	0.242	0.242	0.237	0.416	0.379
47	0.173	0.131	0.175	0.117	0.347	0.248
48	0.129	0.126	0.214	0.133	0.343	0.259
53	0.207	0.245	0.256	0.250	0.462	0.495
54	0.222	0.181	0.197	0.113	0.410	0.294
55	0.158	0.156	0.143	0.167	0.301	0.323
57	0.191	0.175	0.207	0.162	0.398	0.336
65	0.175	0.138	0.161	0.138	0.336	0.276
69	0.154	0.167	0.158	0.153	0.312	0.321
76	0.239	0.184	0.316	0.147	0.555	0.332
84	0.182	0.169	0.134	0.152	0.416	0.421
87	0.181	0.137	0.166	0.130	0.347	0.267
96	0.189	0.154	0.197	0.253	0.386	0.407
97	0.178	0.153	0.193	0.114	0.371	0.267
LSD[b)] ($P < 0.05$)	0.08	0.05	0.04	0.39	0.12	0.37

[a)] For the treatments named with a number, the number refers to the No. of the isolate (a plant growth-promoting rhizobacterium) used to inoculate the wheat seedlings.
[b)] Least significant difference.

TABLE IV

Contents of Na^+ and K^+ and Na^+/K^+ ratio in wheat seedlings grown under non-saline (0 mmol L^{-1} NaCl) and saline (100 mmol L^{-1} NaCl) conditions for 14 d ($n = 10$)

Treatment[a)]	Na^+		K^+		Na^+/K^+ ratio	
	Non-saline	Saline	Non-saline	Saline	Non-saline	Saline
	g kg^{-1} dry weight					
Un-inoculated	11.53	10.51	27.25	05.23	0.423	2.010
2	5.96	39.22	41.65	17.02	0.143	2.304
3	5.42	47.25	32.25	25.32	0.168	1.866
7	5.13	53.37	39.52	21.00	0.130	2.541
8	3.35	54.13	33.74	15.65	0.099	3.459
21	3.88	41.24	28.36	18.15	0.137	2.272
22	5.72	35.14	21.63	39.32	0.264	0.894
23	4.23	26.35	36.32	24.01	0.116	1.097
32	3.91	45.51	43.62	19.51	0.090	2.333
38	3.02	18.46	45.51	26.55	0.066	0.695
39	4.43	28.63	42.24	27.66	0.105	1.035
40	5.98	54.26	38.39	20.11	0.156	2.698
41	4.29	29.21	36.41	21.24	0.118	1.375
42	4.95	46.74	41.28	19.32	0.120	2.419
43	3.46	35.20	42.31	23.28	0.082	1.512
45	7.98	52.42	37.41	12.03	0.213	4.357
47	3.46	42.31	35.51	15.11	0.097	2.800
48	5.43	43.62	39.23	21.42	0.138	2.036
53	3.96	18.22	38.20	26.35	0.104	0.691
54	3.95	51.23	43.42	20.20	0.091	2.536
55	4.51	38.22	35.36	22.01	0.128	1.736
57	4.71	28.21	36.21	18.33	0.130	1.539
65	4.43	44.62	36.44	21.62	0.122	2.064
69	4.14	52.51	39.33	12.55	0.105	4.184
76	3.82	25.22	26.62	41.11	0.144	0.613
84	4.95	23.13	52.56	45.55	0.094	0.508
87	3.42	32.35	46.24	36.33	0.074	0.890
96	4.64	23.42	32.20	38.28	0.144	0.612
97	4.93	48.33	38.56	09.23	0.128	5.236
LSD[b)] ($P < 0.05$)	0.29	0.32	0.56	0.13	0.02	0.28

[a)] For the treatments named with a number, the number refers to the No. of the isolate (a plant growth-promoting rhizobacterium) used to inoculate the wheat seedlings.
[b)] Least significant difference.

traits and highest ACC deaminase activity (Table II) in the screen and was chosen for plant gene expression analysis. The results of differential gene expression of wheat roots inoculated with isolate 53 showed that the expression of *H^+-PPase*, *HKT1*, and *NHX7* in wheat seedlings was significantly upregulated after inoculation with *Enterobacter asburiae* by factors of 12, 8, and 7, respectively, compared to non-stressed control plants (Fig. 2). Under salt stress, isolate 53 significantly increased CAT and APX activity by factors of 14 and 10, respectively, compared to non-stressed controls (Fig. 2).

DISCUSSION

In this study, PGPRs were isolated from saline soils of Isfahan, Golestan, Yazd, and Hormozgan provinces (Iran) (Table I). Bacterial isolates under conditions of salinity and high temperatures can increase the growth and yield of plants in soils with high Na^+ and low water reserves (Chang *et al.*, 2014). These bacteria evolved to cope with environments with elevated osmolarity by accumulating a limited range of low molecular mass molecules, called compatible solutes (Sleator and Hill, 2001). Phosphate solubilization is one of the most important PGP traits, by which insoluble phosphate is solubilized and become available to plants (Suarez *et al.*, 2015). In this study, among the isolates capable of solubilizing $Ca_3(PO_4)_2$, only isolates 53 and 87 also produced siderophores (Table II). Typical phosphate solubilization values in phosphate solubilizing bacteria (PSBs) range 10–2 000 mg L^{-1} (Stephen and Jisha, 2011; Oteino *et al.*, 2015). El-Azeem *et al.* (2007) reported that several PSB isolates could solubilize $Ca_3(PO_4)_2$ in both solid and liquid media and produce organic acids, thus reducing pH. As shown in Table II, 25% of the isolated bacteria produced IAA, with *Enterobacter asburiae* (isolate 53) producing the highest amount. This result is particularly important since

TABLE V

Ethylene emission in wheat seedlings grown under non-saline (0 mmol L^{-1} NaCl) and saline (100 mmol L^{-1} NaCl) conditions for 14 d ($n = 10$)

Treatment[a)]	Ethylene emission	
	Non-saline	Saline
	nmol g^{-1} fresh weight h^{-1}	
Un-inoculated	8.03	11.56
2	7.63	8.91
3	6.97	7.52
7	8.22	9.83
8	7.46	9.53
21	7.34	8.61
22	6.88	7.32
23	7.71	9.34
32	7.81	8.89
38	7.63	8.40
39	8.02	8.76
40	7.90	8.24
41	6.92	8.68
42	7.83	7.42
43	7.52	8.19
45	8.09	9.26
47	7.46	8.13
48	7.98	8.73
53	7.01	7.26
54	8.24	9.15
55	7.85	8.69
57	8.55	9.46
65	7.36	7.75
69	8.23	8.73
76	7.64	8.48
84	7.93	7.45
87	6.99	7.48
96	7.85	8.53
97	7.92	10.12
LSD[b)] ($P < 0.05$)	2.55	1.33

[a)] For the treatments named with a number, the number refers to the No. of the isolate (a plant growth-promoting rhizobacterium) used to inoculate the wheat seedlings.
[b)] Least significant difference.

IAA production by bacteria can facilitate IAA uptake in plants, promoting their growth and tolerance against stressors (Barazani and Friedman, 2000).

Wheat is known to have moderate resistance to salinity. All the selected 28 bacterial isolates affected shoot, root, and total dry weights of wheat seedlings grown under both saline and non-saline conditions (Table III). Shoot and root dry weights decreased in plants under salt stress compared to non-saline conditions, but PGPR-inoculated plants also showed increased shoot and root weights under saline stress (Table III). Vivas *et al*. (2003) reported a rise in the shoot and root weights of lettuce inoculated with *Bacillus* sp. under drought stress. In the present study, isolates 53 and 84 strongly increased the dry weight of salt-stressed plants (Table III); additionally, isolates 53 and 84 produced the highest amount of IAA (Table II), were efficient at solubilizing phosphate (Table II), and elicited low Na^+ concentration in shoots (Table IV).

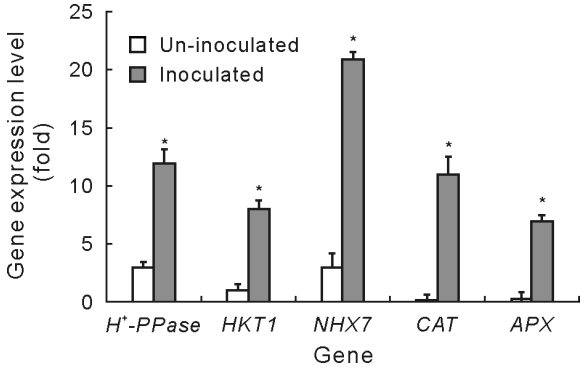

Fig. 2 Quantitative reverse transcription polymerase chain reaction analysis of H^+-*PPase*, *HKT1*, *NHX7*, *CAT*, and *APX* in roots of un-inoculated and *Enterobacter asburiae*-inoculated wheat seedlings subjected to salt stress (100 mmol L^{-1} NaCl) for 14 d. Vertical bars indicate standard errors of the means ($n = 10$), and * indicates significant difference at $P < 0.05$ between inoculated and un-inoculated plants (Duncan's multiple range test). The actin gene *ACT2* was used as the endogenous control to calculate relative gene expression levels.

Treatment with PGPR isolates can improve plant growth under saline conditions by decreasing the effects of potentially toxic ions (Zhang *et al*., 2010; Goswami *et al*., 2014), and avoiding nutrient imbalance in plants (Greenway and Munns, 1980). Egamberdiyeva and Höflich (2003) reported that PGPRs increased the uptake of N, P, and K in wheat. In particular, Vivas *et al*. (2003) found that *Bacillus* sp. increased the levels of N, P, and K by 5%, 70%, and 50%, respectively, in the leaves of lettuce plants under drought stress, compared to non-saline controls. In this study, the Na^+/K^+ values were lower in the leaves of PGPR-inoculated plants (Table IV), suggesting that Na^+ uptake by roots and translocation to the shoots was inhibited in the presence of these bacteria. A low Na^+/K^+ ratio is positive for plant growth and crop yield, and Na^+ and K^+ transport and ac-

TABLE VI

Molecular characterization of the selected plant growth-promoting rhizobacterium isolates for their capacity to promote total biomass of wheat seedlings under saline condition of 100 mmol L^{-1} NaCl

Isolate No.	Closest relative	NCBI[a)] accession No.	16S rRNA fragment length	Sequence identity
			bp	%
38	*Pseudomonas fluorescens*	KU374975	1 348	99.6
53	*Enterobacter asburiae*	KU374974	1 366	99.4
84	*Arthrobacter aurescence* TC1	KU374976	1 338	97.3
96	*Bacillus atrophaeus*	KU374973	1 127	100.0

[a)] National Center for Biotechnology Information.

cumulation are of basic importance for salt tolerance (Liu *et al*., 2014). In particular, the Na^+/K^+ ratio in wheat decreases in response to treatment with *Pseudomonas putida*, *Enterobacter cloacae*, *Serratia ficaria*, *and Pseudomonas fluorescens*, and this is linked to salinity tolerance in this species (Nadeem *et al*., 2013).

In addition, PGPRs can mobilize and/or solubilize nutrients, increase water use efficiency, and stimulate root and shoot growth through the production of phytohormones and the enzymatic reduction of plant ethylene concentrations (Suarez *et al*., 2015). Several physiological, enzymatic, and biochemical changes in plants after inoculation with PGPRs have been suggested to help plants alleviate salt or drought stress (Barriuso *et al*., 2008; Suarez *et al*., 2015). The results of the ethylene emissions study collectively suggest that salt stress enhanced ethylene production, and that ethylene production was reduced in PGPR-inoculated wheat seedlings under conditions of salt stress (Table V). Reduced ethylene levels suggest the existence of crosstalk between IAA and ethylene biosynthesis (Glick, 2014). The activity of ACC deaminase lowers ethylene levels locally, facilitating the stimulation of plant growth by IAA (Gontia-Mishra *et al*., 2017). In this study, isolate 53 had the highest ACC deaminase activity, which was associated with the lowest ethylene emissions (Tables II and V), likely contributing to the mitigation of the effects of salt stress in wheat seedlings.

Previous findings (Paul and Nair, 2008; Nadeem *et al*., 2013; Suarez *et al*., 2015) suggest that inoculation of plants with PGPRs increases resistance and resilience to salinity stress. The reasons for this have not been fully elucidated. Enhanced plant shoot and root weights in wheat by inoculation with *Bacillus simplex* (KBS1F-3) and *Bacillus cereus* (KFP9-F) isolated from the rhizosphere of grasses from soils with high salinity were also observed by Hassen and Labuschagne (2010). Zhang *et al*. (2010) reported that *Bacillus subtilis* confers salt resistance through the up-regulation of the high-affinity K^+ transporter *HKT1* in *Arabidopsis thaliana*. The transcriptional up-regulation of *HKT1* was also observed by Sharma *et al*. (2014) in salt-stressed plants inoculated with *Enterobacter* (Fig. 2). Moreover, *Arabidopsis* treated with *B. subtilis* GB03 showed up-regulated *HKT1* expression levels in the shoots and roots, increased Na^+ recirculation from shoots to roots, and limited Na^+ uptake in the roots (Zhang *et al*., 2010). The observed up-regulation of *HKT1* in wheat roots may also explain the high K^+ and consequent low Na^+/K^+ ratio in inoculated plants (Table IV). The levels of Na^+ export and sequestration are controlled by Na^+/H^+ antiporters. In *Arabidopsis*, these are located in the tonoplast (*NHX1*) and plasma membrane (*NHX7*), where vacuolar intake and cellular export of Na^+ occur (Yamaguchi *et al*., 2013; Deinlein *et al*., 2014). In this study, *NHX7* was significantly up-regulated by *Enterobacter asburiae* (isolate 53) in the roots of salt-stressed plants (Fig. 2), showing that PGPRs can promote Na^+ sequestration in the vacuoles, as suggested by the results reported in Table IV. In *Arabidopsis*, salt-stress tolerance is coupled to the up-regulation of *H^+-PPase* through the sequestration of Na^+ in the vacuoles (Schilling *et al*., 2014). In this study, the expression levels of *H^+-PPase* were up-regulated in *Enterobacter*-inoculated plants under saline conditions compared to the un-inoculated control (Fig. 2).

Both osmotic stress and ROS accumulation are frequently observed in plants subjected to salt stress (Mittova *et al*., 2004). In particular, lipid membrane peroxidation results from increased cellular levels of ROS (Yang *et al*., 2009). In plants, ROS levels and oxidative stress can be reduced by the activity of various antioxidant molecules and enzymes (Mittova *et al*., 2004). In this study, the *APX* and *CAT* transcript levels were higher in the roots of *Enterobacter*-inoculated plants grown under salt stress compared to the un-inoculated controls (Fig. 2). Previous reports (Zhang *et al*., 2010; Suarez *et al*., 2015) have shown that reduced lipid peroxidation and increased APX and CAT activity resulting from the activity of soil microorganisms can increase salt tolerance in plants under saline stress. Finally, Gururani *et al*. (2013) found that salt-stress tolerance can be mediated by PGPRs through modulation of the expression of ROS-scavenging enzymes.

CONCLUSIONS

The results of this study highlighted that some of the bacteria isolated from highly saline soils could solubilize phosphate and/or produce IAA. Other PGP traits included siderophore production and relevant ACC deaminase activity. In the pot experiment conducted to test the plant growth-promoting activity of the isolated bacteria on wheat seedlings under saline conditions, four of the screened isolates were the most effective as PGPRs (isolates 38, 53, 84, and 96), with each contributing at least two PGP traits, and reducing Na^+ accumulation in plants. *Enterobacter asburiae*, exhibiting all three PGP traits, showed the most relevant plant growth-promoting activity, protecting wheat seedlings from salt stress-induced injury. This isolate likely conferred tolerance to salt stress in wheat seedlings by reducing Na^+ content and ethylene production and by up-regulating the expression of *HKT1*, *H^+-PPase*, *NHX7*, *CAT*, and *APX* in the roots. Salt-tolerant rhizobacteria exhibiting plant growth-promoting traits can facilitate the growth of wheat plants under saline conditions, and mitigate the deleterious effects of salt stress. Considering the urgency of cultivating in saline soils due to increasing food demand and decreasing soil availability and quality/fertility, the isolation of halophile PGPRs can be useful for the formulation of new inoculants to improve the cropping systems of wheat grown under saline conditions.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article can be found in the online version.

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