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Extracts from Marine Macroalgae and *Opuntia ficus-indica* Cladodes Enhance Halotolerance and Enzymatic Potential of Diazotrophic Rhizobacteria and Their Impact on Wheat Germination Under Salt Stress

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ABSTRACT

Soil salinity, which affects more than 6% of the earth's land surface and more than 20% of its irrigated areas, is a major threat to agriculture. Diazotrophic bacteria are among the functional groups of soil microbiota that are threatened by this abiotic stress, as their activity is mostly inhibited by salt stress. Seventy bacterial strains with distinct characteristics were isolated from soils by using N-free Jensen's selective medium. Based on their ability to produce metabolites of agricultural interest, four strains were selected and identified as *Flavobacterium johnsoniae*, *Pseudomonas putida*, *Achromobacter xylosoxidans*, and *Azotobacter chroococcum*. The selected strains were grown at different NaCl concentrations (0–600 mmol L^{-1} in N-free broth and 0–2 000 mmol L^{-1} in Luria-Bertani medium) in the presence and absence of glycine betaine (GB), aqueous and hydro-alcoholic extracts from marine macroalgae, *Ulva lactuca* and *Enteromorpha intestinalis*, and *Opuntia ficus-indica* cladodes. The selected bacterial strains, GB, and the aforementioned extracts were tested for their ability to promote the germination of wheat (*Triticum durum*) seeds at 0–300 mmol L^{-1} NaCl. Compared with the results obtained with the synthetic osmoprotectant GB, the extracts from *O. ficus-indica*, *U. lactuca*, and *E. intestinalis* significantly promoted bacterial growth and seed germination under salt stress.

Key Words: glycine betaine, *Ulva lactuca*, *Enteromorpha intestinalis*, plant growth-promoting rhizobacteria (PGPR), seed germination, *Triticum durum*

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INTRODUCTION

Over the last century, the rapid growth of the human population has created a global scarcity of water resources, increased salinization of soils, loss of biodiversity, and environmental degradation, and decreased nutrient availability (Shahbaz and Ashraf, 2013). Among these problems, salt stress poses an increasing number of challenges to agriculture and food security, which has led to the increased exploitation of natural resources (Ashraf *et al.*, 2012). Globally, 6% of the Earth's surface is affected by salinity, which corresponds to approximately 20% of irrigated regions, especially in the arid and semi-arid regions where rainfall is insufficient to leach salts from the upper layers of the soil. It has been estimated that more than 50% of arable land will be salinized by the year 2050 (Jamil *et al.*, 2011; Dikilitas and Karakas, 2012; Stanković *et al.*, 2015).

Soil is considered saline if the electrical conductivity (EC) of the saturation extract in the area adjacent to the roots exceeds 4 dS m^{-1} at 25 °C, and if it has 15% exchangeable sodium (Shrivastava and Kumar, 2015). High concentrations of sodium and chloride, as well as other ions (potassium, calcium, carbonate, nitrate, and sulfate), decrease the acquisition of water by plants and negatively affect many physiological processes, such as photosynthesis, protein synthesis, and lipid metabolism (Kosová *et al.*, 2011; Rojas-Tapias *et al.*, 2012).

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Salt stress also affects the microbial community in the soil, including the soil bacteria that are able to colonize the surface of the root system (and sometimes the root inner tissues) to stimulate the plant's growth and health. This group of bacteria was named in 1981 by Kloepper and Schroth as plant growth-promoting rhizobacteria (PGPR) (Barea *et al.*, 2005; Paul and Nair, 2008; Dutta and Podile, 2010; Bhattacharyya and Jha, 2012; Vacheron *et al.*, 2013). When they are exposed to solutions with increased osmolarity, the water activity in the bacterial cytoplasm decreases, consequently disturbing protein activity and inhibiting many biological processes, such as the synthesis of macromolecules or DNA replication (Nabti *et al.*, 2010; Bhattacharyya and Jha, 2012).

Plant growth enhancement by PGPR occurs through several mechanisms such as nitrogen (N) fixation, siderophore production, the solubilization of minerals essential for plant nutrition, and the synthesis of enzymes implicated directly in the degradation of organic matter and soil fertility (such as proteases, ureases, chitinases, and cellulases) (do Vale Barreto Figueiredo *et al.*, 2010; Reddy 2013). Recently, numerous studies reported the role of PGPR as an inducer of plant tolerance to abiotic stress (*e.g.*, drought and salinity) (Paul and Nair, 2008; Yang *et al.*, 2009; Lim and Kim, 2013). The term "induced systemic tolerance (IST)" has been suggested to represent the tolerance conferred to plants by PGPR against these types of stress (Bhattacharyya and Jha, 2012).

Ethylene is a plant hormone with an active role in various developmental processes, such as leaf senescence, leaf abscission, epinasty, and fruit ripening (Vogel *et al.*, 1998; Gray and Smith, 2005). However, high amounts of this hormone, produced under salt stress, lead to reductions in root and shoot growth (Shrivastava and Kumar, 2015). The enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, synthesized by a wide range of rhizospheric bacteria, contributes to the reduction of ethylene levels in plant tissues. In the presence of ACC deaminase-producing bacteria, part of the plant's ACC is sequestered and degraded to supply N and energy, contributing to the reduction of ethylene levels in the plant (Penrose and Glick, 2003; Glick *et al.*, 2007).

Under salt stress, PGPR and plants accumulate and/or synthesize compatible solutes or osmoprotectants; these metabolites are compatible with their cellular metabolism and protect their cells against plasmlysis (Cánovas *et al.*, 1997; Göller *et al.*, 1998; Wani *et al.*, 2013). The compatible solutes comprise mainly sugars and derivatives (sucrose, trehalose, sulfotrehalose, and glucosylglycerol), some amino acids and derivatives (proline, glutamic acid, glutamine, γ-aminobutyric acid, and glycine betaine (GB)), ectoin and derivatives, and polyhydric alcohols (glycerol, arabitol, and mannitol) (Courtenay *et al.*, 2000).

Marine macroalgae and *Opuntia ficus-indica* (*OFI*) are in the first line among the organisms known for their tolerance to stresses such as salinity and drought. Their adaptation to abiotic stress and chemical composition suggest that these plants might be used as efficient sources of a wide range of compatible solutes (Edwards *et al.*, 1988; Ghoul *et al.*, 1995; Kirst, 1996; Pichereau *et al.*, 1998; Nabti *et al.*, 2007; Sáenz *et al.*, 2013). Thus, the objectives of this study were: 1) to isolate, select, and identify diazotrophic bacteria with a good ability to produce "*in vitro*" metabolites of potential agricultural interest and 2) to evaluate the effect of the natural extracts from marine macroalgae, *Ulva lactuca* (*UL*) and *Enteromorpha intestinalis* (*EI*), and *OFI* on the growth of the selected bacteria and to examine the effect of these extracts and bacterial isolates on the germination of wheat (*Triticum durum*) seeds under salt stress conditions.

MATERIAL AND METHODS

Soil samples

Soil samples were aseptically collected from seven agricultural sites used for wheat cultivation in Algeria (Table I). Soil samples (500 g) from each site were collected at depths from 10 to 30 cm by using sterile metallic corers. The soil surface was disinfected with ethanol (70%, volume:volume) before sampling. It is important to mention that the seven sites are situated at a range of different distances from the Mediterranean (approximately from 500 m to 200 km) and encompass a range of temperatures, to ensure the high diversity of the isolated bacteria.

Macroalgae and OFI cladodes

Marine macroalgae (Chlorophyta), *UL* and *EI*, and the cladodes of *OFI*, were harvested from Bejaïa Province, Algeria (Table I), and transported at 4 °C to the laboratory for the preparation of the extracts.

Soil physicochemical properties

Effective and titratable acidity (pH (water), pH (KCl)), EC, gravimetric moisture content (θ_g), and soil organic matter (SOM) were determined according to Rouiller *et al.* (1994), Hardie and Doyle (2012), Pepper and Gerba (2004), and Huang *et al.* (2009), respectively.

TABLE I

Date and location of soil, *Ulva lactuca* (*UL*), *Enteromorpha intestinalis* (*EI*), and *Opuntia ficus-indica* (*OFI*) sampling

Sample	Location	Latitude, longitude	Date
Soil			
BEA	Bejaïa	36°42′11.60″ N, 5°5′14.47″ E	Jan. 21, 2014
BEB	Bejaïa	36°41′30.19″ N, 4°55′46.13″ E	Jan. 2, 2014
BEC	Bejaïa	36°40′44.54″ N, 4°53′35.55″ E	Jan. 29, 2014
BOA	Bouira	36°7′41.29″ N, 3°32′55.89″ E	Jan. 29, 2014
MEA	Medea	36°15′38.8″ N, 3°23′29.25″ E	Jan. 31, 2014
SEA	Setif	36°12′3.81″ N, 5°22′1.44″ E	Feb. 24, 2014
SEB	Setif	36°11′30.55″ N, 5°22′16.79″ E	Feb. 24, 2014
UL	Bejaïa	36°48′54.5″ N, 4°59′11.1″ E	May 13, 2014
EI	Bejaïa	36°48′54.5″ N, 4°59′11.1″ E	May 13, 2014
OFI	Bejaïa	36°48′05.5″ N, 5°00′15.7″ E	May 15, 2014

Isolation of diazotrophic bacteria from soil samples

Free-living N-fixing bacteria were isolated by using the N-free Jensen's selective medium containing 20 g mannitol, 1.0 g K_2HPO_4, 2.0 g $CaCO_3$, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.5 g NaCl, 0.1 g $FeSO_4$, 0.005 g Na_2MoO_4, and 17 g agar in 1 L distilled water, with pH adjusted at 7.4 ± 0.02 (Thompson, 1989). Ten grams of each sample, homogenized in a sterile manner, was thoroughly mixed with 90 mL of sterile distilled water to form the first dilution (10^{-1}), from which successive dilutions from 10^{-2} to 10^{-7} were prepared. Each dilution was inoculated to three Petri dishes (100 µL each) containing N-free Jensen's medium. The Petri dishes were incubated for 7 d at 30 °C. At the end of the incubation period, 10 isolates with different appearances were purified from each soil sample, tested for their motility and oxidase and catalase activity, subjected to Gram staining, and stored for further studies.

Plant growth-promoting traits

Enzymatic activities. The seventy purified strains were screened for their potential hydrolytic enzyme activities using the agar disk method. Cellulolytic (Carder, 1986), esterasic (Sierra, 1957), lipolytic (Sierra, 1957), chitinolytic (Kopečný *et al.*, 1996), proteolytic (Kavitha *et al.*, 2013), amylolytic (Raj *et al.*, 2009), and ureasic (Christensen, 1946) activities were tested. Twenty-eight isolates with the best enzymatic scores (the sum of the positive results for each isolate) were chosen for further studies. To reveal any possible influence of the environmental parameters (pH, EC, and SOM) on the enzyme expression by the selected isolates, the correlations between the physicochemical properties of each soil sample and the total score of the enzymatic activities of their corresponding strains together (the sum of the positive results for all the strains purified from the same sample) were determined.

Indole 3-acetic acid (IAA) detection and quantification. Indole 3-acetic acid was quantified on Luria-Bertani (LB) medium supplemented with 1 g L^{-1} tryptophan (Bric *et al.*, 1991). After incubation, 1.5 mL of the culture was transferred to microtubes and centrifuged under approximately 25 °C (room temperature) at 9 500 *g* for 2 min. One milliliter of the supernatant, together with 1 mL of Salkowski's reagent (1 mL of 0.5 mol L^{-1} $FeCl_3$ and 49 mL of 35% $HClO_4$), was transferred to the tubes. After 30 min, the absorbance at 350 nm was measured. A standard curve of IAA (Sigma-Aldrich®) was prepared. The development of a pink color after the addition of Salkowski's reagent indicated the production of auxin by the isolate; 14 auxin-producing isolates were selected and tested for their ability to produce siderophores.

Siderophore production. The ability of the 14 selected strains to produce siderophores was tested by using chrome azurol S (CAS) agar medium prepared in accordance with the protocol originally proposed by Schwyn and Neilands (1986) and modified by Alexander and Zaberer (1991). The Fe-CAS indicator solution (Solution 1) was prepared by mixing 10 mL of 1 mmol L^{-1} $FeCl_3{\cdot}6H_2O$ (in 10 mmol L^{-1} HCl) with 50 mL of CAS solution (1.21 mg mL^{-1}). The mixture was added slowly to 40 mL of an aqueous solution of hexadecyltrimethylammonium bromide (HDTMA) (1.82 mg mL^{-1}). The buffer solution (Solution 2) was prepared by dissolving 30.24 g of 1,4-piperazinediethanesulfonic acid (PIPES) in 750 mL of a salt solution: 0.3 g KH_2PO_4, 0.5 g NaCl, 1.0 g NH_4Cl, and 15 g agar. The pH was adjusted to 6.8 with 50% (weight:weight) KOH, and water was added to bring the volume to 800 mL. The composition of Solution 3, per 70 mL water, was: 2 g glucose, 2 g mannitol, 493 mg $MgSO_4{\cdot}7H_2O$, 11 mg $CaCl_2$, 1.17 mg $MnSO_4{\cdot}H_2O$, 1.4 mg H_3BO_3, 0.04 mg $CuSO_4{\cdot}5H_2O$, 1.2 mg $ZnSO_4{\cdot}7H_2O$, and 1.0 mg $Na_2MoO_4{\cdot}2H_2O$. The three obtained solutions were

autoclaved separately and cooled to 50 °C. An aliquot of 30 mL of the filter-sterilized solution (Solution 4) containing 10% (weight:volume) casamino acids was mixed with Solutions 3 and 2. The indicator solution was added last, with sufficient stirring to mix the ingredients without forming bubbles. Based on this test, eight siderophore-producing isolates were selected for further study.

Phosphate solubilization. The eight selected strains were tested for their ability to solubilize inorganic rock phosphate ($Ca_3(PO_4)_2$). Pikovskaya's medium (PKV) was used, which contained 10 g glucose, 5 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)_2SO_4$, 0.2 g NaCl, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 0.5 g yeast extract, 0.002 g $MnSO_4 \cdot H_2O$, 0.002 g $FeSO_4 \cdot 7H_2O$, and 15 g agar in 1 L of distilled water. The capacity of an isolate to solubilize inorganic phosphate was indicated by a clear halo around the agar disk (Sagervanshi *et al.*, 2012). Four strains considered to be phosphate solubilizers were molecularly identified and subsequently used in the second part of this work.

Use of ACC as a sole N source. The bacteria were cultivated on a rich medium containing 5 g tryptone, 2.5 g yeast extract, and 2 g glucose in 1 L of distilled water and incubated with shaking (100 r min^{-1}) for 48 h at 30 °C. The obtained biomass was washed three times with a sterile solution of 0.1 mol L^{-1} $MgSO_4$ and finally resuspended in the same solution. In a 96-well plate, 120 µL of Dworkin and Foster (DF) salt medium, which contained (in 1 L) 4.0 g KH_2PO_4, 6.0 g Na_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 2.0 g glucose, 2.0 g gluconic acid, 2.0 g citric acid, 1 mg $FeSO_4 \cdot 7H_2O$, 10 mg H_3BO_3, 11.19 mg $MnSO_4 \cdot H_2O$, 124.6 mg $ZnSO_4 \cdot 7H_2O$, 78.22 mg $CuSO_4 \cdot 5H_2O$, and 10 mg MoO_3, at pH 7.2 (Penrose and Glick, 2003), was added to each well. Aliquots of 15 µL of 0.1 mol L^{-1} $MgSO_4$ or 15 µL of 0.1 mol L^{-1} $(NH_4)_2SO_4$ were added to the first and the second thirds of the microplates, respectively. The ACC solution (3 mmol L^{-1}) was filter-sterilized (0.2 µm) and stored at −20 °C until the assay. The ACC was thawed and 15 µL was placed in the final third of the microplates. Each well was inoculated with 15 µL of the washed bacterial culture. A control without bacteria was used (Shahzad *et al.*, 2010). After incubation (48 h at 28 °C), the bacterial growth was determined by the measurement of optical density at 600 nm (OD_{600}).

Molecular identification

The selected strains were identified based on their coding DNA fragments for 16S rRNA sequences. The total DNA extraction was performed, and the 16S rRNA gene was amplified by using the universal primers 5′-S-D-Bact-0008-a-S-20-3′ and 5′-S-D-Bact-1495-a-S-20-3′, with the sequences 5′-AGAGTTTGATCCTGGCTCAG-3′ and 5′-AAGGAGGTGATCCAGCCGCA-3′, respectively (Daffonchio *et al.*, 2000).

The amplification reaction mixture consisted of 2.5 µL PCR reaction buffer, 2.5 µL $MgCl_2$, 0.2 µL deoxynucleoside triphosphate, 0.3 µL of each primer, 0.2 µL *Taq* DNA polymerase, and 1 µL of total DNA. The PCR program consisted of an initial step of 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 1 min, and elongation at 72 °C for 2 min, followed by a final elongation step at 72 °C for 8 min.

An agarose gel (1.5%, weight:volume) was used for the separation of the PCR products in 0.5× Tris-borate-EDTA buffer, stained for 30 min in 0.5 mg L^{-1} ethidium bromide solution, and developed by exposure to ultra-violet light. An enzymatic sequencing of the amplified DNA fragments was conducted in accordance with the method of Sanger (Sanger and Coulson, 1975). The obtained sequences were deposited in GenBank and compared with sequences available from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) using the BLAST algorithm. A phylogenetic dendrogram was conducted by using the neighbor joining method, and tree topology was evaluated by performing bootstrap analysis of 1 000 data sets using MEGA4.1 (Kumar *et al.*, 2004).

Determination of halotolerance

The halotolerance of the selected strains was studied using an N-free broth medium (N-Fb) containing 6 g K_2HPO_4, 4 g KH_2PO_4, 0.2 g $MgSO_4$, 0.1 g NaCl, 0.02 g $CaCl_2$, 0.01 g $FeCl_3$, 0.002 g Na_2MoO_4, 0.05 g yeast extract, and 5 g glucose (added after autoclaving) in 1 L of distilled water. This medium was supplemented with various concentrations of NaCl (0, 250, 300, 350, 400, 450, 500, 550, 600, and 650 mmol L^{-1}) (Nabti *et al.*, 2007).

Marine macroalgae and *OFI* cladodes were cut into fine pieces to facilitate the improved release of intracellular substances. The finely cut pieces (2.5 g fresh weight) were immersed in vials containing 50 mL of N-Fb medium with the aforementioned NaCl concentrations. The vials were then autoclaved at 110 °C for 30 min. After decantation, 10 mL of the supernatant was distributed in sterile tubes to be loaded into microplates for the study of bacterial halotolerance (Ghoul *et al.*, 1995).

Luria-Bertani broth (5 mL) was inoculated with a fresh culture (18 h) of the tested strains (BEA4, BEC9,

BOA4, and SEB9). After incubation at 28 °C for 24 h, the culture was centrifuged at 3 000 r min^{-1} for 10 min. The pellet was washed three times with 5 mL of normal saline solution (8.5 g L^{-1} NaCl) and finally resuspended in 5 mL of the same solution. The obtained bacterial suspensions were inoculated to the culture medium already distributed in microplates. After incubation (28 °C for 4 d), the bacterial growth was estimated through the measurement of the absorbance of the culture at 600 nm.

The above procedure was then repeated after the replacement of N-Fb with LB medium (0, 300, 500, 800, 1 000, 1 300, 1 500, 1 800, and 2 000 mmol L^{-1} of NaCl) supplemented, or not, with GB (1 mmol L^{-1}) or hydro-alcoholic extracts (10^{-2}, volume:volume) from *OFI*, *UL*, and *EI*. The hydro-alcoholic extracts were prepared through the immersion of 5 g (dry weight) of *UL*, *EI*, and *OFI* cladodes in vials containing 150 mL of 70% ethanol with shaking for 5 to 10 min. The obtained extract was filtered through a filter paper to eliminate solids, evaporated to dryness, and resuspended in 10 mL distilled water (Ghoul *et al.*, 1995).

Wheat germination assay

The bacterial cultures of the four strains finally selected were washed three times with sterile distilled water (OD_{600}: 0.8) and used for the germination assays. The seeds of wheat (variety Boussalem) were surface sterilized (Götz *et al.*, 2006) and imbibed in washed bacterial suspension for 30 min. The imbibed seeds were placed in Petri dishes layered with sterile filter paper and soaked (5 mL per Petri dish) in various NaCl solutions (100, 200, and 300 mmol L^{-1}). Hydro-alcoholic extracts from *OFI* and *UL* (1%, weight:volume) and GB (1 mmol L^{-1}) were added to the salt solutions (Ramadoss *et al.*, 2013). All tests were repeated in triplicate (12 seeds per Petri dish). Controls without bacterial treatment, without GB, and without extracts were used. The experiment was conducted in darkness at 20 °C. Readings were recorded every 2 d for 12 d, and the final germination percentage was determined at the end of the experiment.

Statistical analysis

Statistical analyses were computed by using GraphPad Prism software (version 6). The data from the halotolerance and germination assays were analyzed using two-way analysis of variance (ANOVA) subjected to a 95% confidence limit. The correlations between the enzymatic scores and physicochemical properties of the soil samples were analyzed by the calculation of Pearson's coefficient of correlation (r) and linear regression ($P < 0.05$).

RESULTS

Soil physicochemical properties

All soil samples were slightly alkaline. One sample from Bejaïa (BEA) was identified as a saline soil (EC > 4 dS m^{-1}); for the other samples, the EC was between 3.8 dS m^{-1} for the samplefrom Bouira (BOA) and 2.1 dS m^{-1} for for the samplefrom Setif (SEA). The samples collected from Medea (MEA) and Setif (SEA and SEB) were richer in SOM (11, 13, and 15 g kg^{-1}, respectively) than those collected from Bejaïa (BEA, BEB, and BEC) and Bouira (BOA). Other physicochemical properties were similar for the seven samples (Table II).

TABLE II

Selected physicochemical properties[a)] of the collected soil samples

Sample	pH (H_2O)	pH (KCl)	θ_g	EC	SOM
			g kg^{-1}	dS m^{-1}	g kg^{-1}
BEA	8.05	7.16	1.37	4.2	8
BEB	8.54	7.63	2.00	3.5	5
BEC	8.20	7.29	2.40	2.4	9
BOA	8.29	7.60	1.86	3.8	7
MEA	8.12	7.34	2.11	2.9	11
SEA	8.20	7.15	2.87	2.1	15
SEB	8.19	7.41	2.22	2.4	13

[a)] θ_g = gravimetric moisture content; EC = electrical conductivity; SOM = soil organic matter.

Enzymatic activities

Sixty-three strains (90% of the selected bacteria) were amylase positive. Ureasic activity was detected in 44.29% of the isolates, with less protease activity detected (37.14%). Lipolytic, esterasic, chitinolytic, and cellulolytic activities were detected in 45.71%, 47.14%, 70%, and 50% of the 70 tested strains, respectively (data not shown). The intensity of the enzymatic activities of the selected isolates from each soil sample and their respective pH (KCl) values were positively correlated; for a sample with a basic pH, the bacteria produced more hydrolytic enzymes (Fig. 1a). The SOM of all samples, except BEA, and their θ_g, except BEA and SEB, were negatively correlated with the total enzymatic activities in each group of isolates, whereas the EC of all samples, except BEA, was positively correlated with the enzymatic activities of the isolates (Fig. 1b, c). In all samples, both SOM and θ_g were negatively correlated with EC (Fig. 1d).

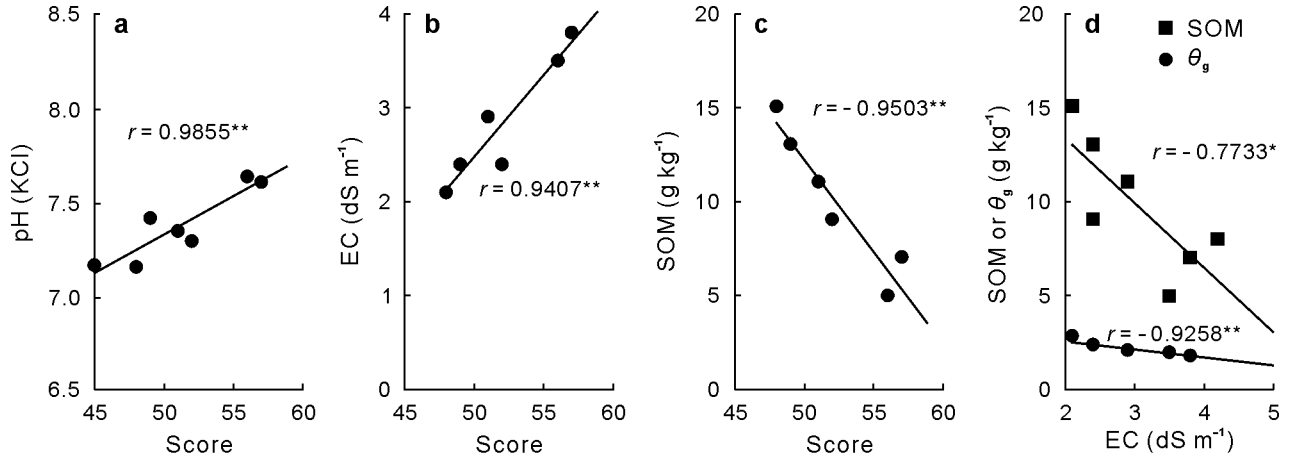

Fig. 1 Pearson's coefficient of correlation (r) and linear regression between the total enzymatic activities (score) of isolates selected from each soil sample and soil pH (KCl) (a), electrical conductivity (EC) (b), and soil organic matter (SOM) (c) and EC and SOM and gravimetric moisture content (θ_g) of each soil sample (d). *, **Significant at $P < 0.05$ and $P < 0.01$, respectively.

IAA quantification

The phytohormone IAA was produced by all the tested strains, ranging from 76.31 μg mL^{-1} for the isolate BEA4 to 3.35 μg mL^{-1} for the isolate BOA2. The isolates BEC9, BOA4, and SEB9 produced 25.7, 47.4, and 32.2 μg IAA mL^{-1}, respectively (Table III).

Detection of siderophores

All the tested isolates were able to produce siderophores, except the strains BEB4, BOA5, and MEA4. The isolates BEB6 and SEA3 showed a weak ability to produce siderophores (Table III).

Phosphate solubilization

Only two isolates, BEA3 and SEA3, were unable to solubilize inorganic phosphate ($Ca_3(PO_4)_2$). The solubilization index of the isolates BEA4, BEC9, BOA4, and SEB9 was 0.79, 0.40, 0.36, and 0.65, respectively (Table III).

Use of ACC as a sole N source

Four strains (BEA4, BEC9, BOA4, and SEB9) were able to grow with ACC as the sole N source. The growth of the two isolates BEA4 and BEC9 was much faster in the presence of ACC and $(NH_4)_2SO_4$ than in the absence of N. However, no significant difference was detected in the growth of the two strains BOA4 and BEC9 in the presence and absence of ACC as a N source (Fig. 2).

Molecular identification

The selected strains BEA4, BEC9, BOA4, and SEB9 were molecularly similar to the species *Flavobacterium johnsoniae*, *Pseudomonas putida*, *Achromobacter xylosoxidans*, and *Azotobacter chroococcum*, respectively (Fig. 3).

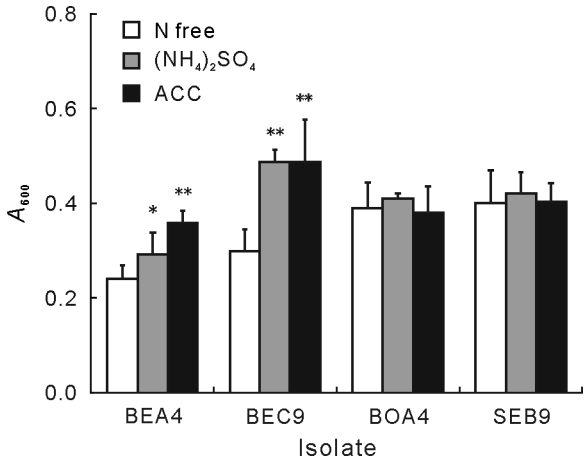

Fig. 2 Utilization of 1-aminocyclopropane-1-carboxylate (ACC) and $(NH_4)_2SO_4$ as the sole N source by isolates BEA4, BEC9, BOA4, and SEB9. Readings were performed in four replicates. Error bars represent the standard deviations of means. A_{600} = absorbance of the culture at 600 nm. *, **Significant at $P < 0.05$ and $P < 0.01$, respectively.

Halotolerance of bacteria

The LB medium conferred greater protection to bacteria against salt stress than the N-Fb medium in the absence of GB and extracts. At high salt concentrations, the *OFI* extract conferred better protection than the GB and *UL* and *EI* extracts. The isolate BEA4 seemed to be the most tolerant to salt stress. The isolate SEB9 was the most sensitive to salinity on the LB medium, whereas BEC9 expressed the weakest tolerance to NaCl on the N-Fb medium (Figs. 4 and 5).

Compared with the control, in the absence of salt stress, *OFI* extract enhanced the growth of the isolates BEA4 and SEB9 on the N-Fb medium by 115.342% and 124.51%, respectively, and that of the isolates

TABLE III

Hierarchical strain selection based on the production of indole 3-acetic acid (IAA) and siderophores and phosphate solubilization

Strain code	IAA	Siderophore production[a)]	Phosphate solubilization		
			Colony zone diameter	Halo zone diameter	Solubilization index
	$\mu g\ mL^{-1}$		cm		
BEA1	12.3 ± 2.8				
BEA3	38.0 ± 2.7	+++	1.1	1.1	1
BEA4	76.3 ± 7.7	+++	1.1	1.4	0.79
BEA6	12.3 ± 4.2				
BEB3	15.4 ± 2.8				
BEB4	23.1 ± 4.1				
BEB6	30.3 ± 8.4	+			
BEB9	05.3 ± 1.3				
BEC1	10.2 ± 4.2				
BEC4	34.2 ± 2.8	+++	0.6	1.5	0.40
BEC5	34.4 ± 5.9				
BEC9	25.7 ± 2.6	+++	0.2	2	0.40
BOA2	03.3 ± 2.8				
BOA4	47.4 ± 5.6	+++	0.9	2.5	0.36
BOA5	40.2 ± 5.5	−			
BOA8	20.3 ± 2.7				
MEA1	11.3 ± 2.6				
MEA2	05.3 ± 2.5	++			
MEA4	31.4 ± 1.4	−			
MEA6	07.4 ± 0.0				
SEA3	26.9 ± 1.4	+	1.2	1.2	1
SEA4	24.3 ± 5.9	+++			
SEA6	03.4 ± 0.8				
SEA8	23.3 ± 2.7				
SEB1	12.7 ± 0.7				
SEB4	08.4 ± 1.2				
SEB7	32.6 ± 5.0	+++	1	1.2	0.83
SEB9	32.2 ± 1.4	+++	1.3	2	0.65

[a)] − = negative; + = weakly positive; ++ = moderately positive; +++ = highly positive.

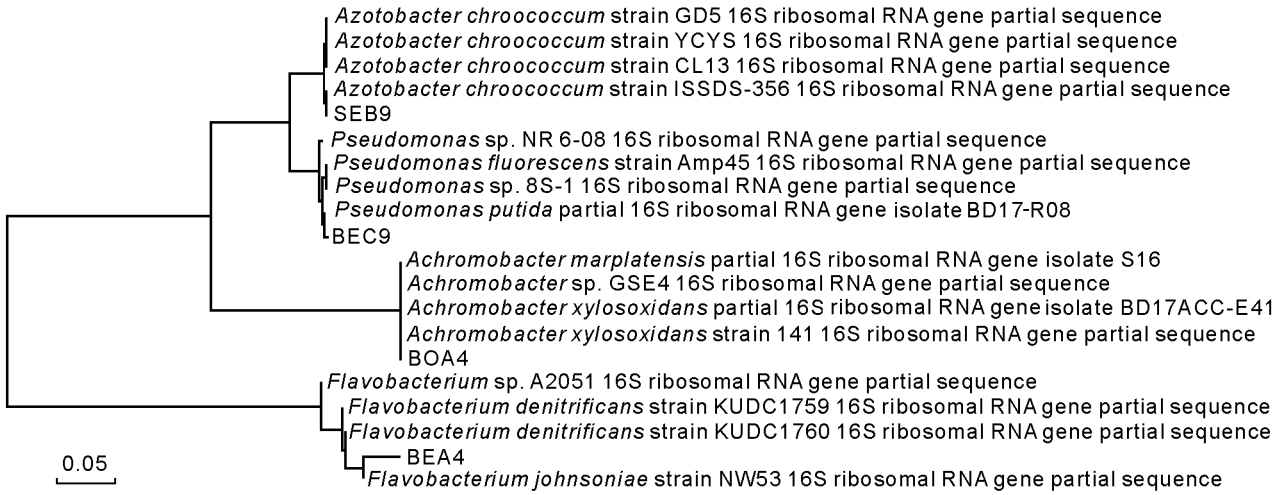

Fig. 3 Neighbor-joining tree revealing the phylogenetic relationship of the analyzed isolates BEA4, BEC9, BOA4, and SEB9. The bar indicates 5% sequence divergence.

BEA4, BEC9, BOA4, and SEB9 by 109.231%, 105.5%, 103.115%, and 144.887% on the LB medium. The same extract increased the bacterial growth of the isolates BEA4, BEC9, BOA4, and SEB9 on LB medium with 1 000 mmol L^{-1} of NaCl by 141.085%, 230.805%, 215%, and 221%, respectively, whereas their growth was restored under 350 mmol L^{-1} of NaCl on N-Fb medium to 187.877%, 414.398%, 183.321%, and 414.267%

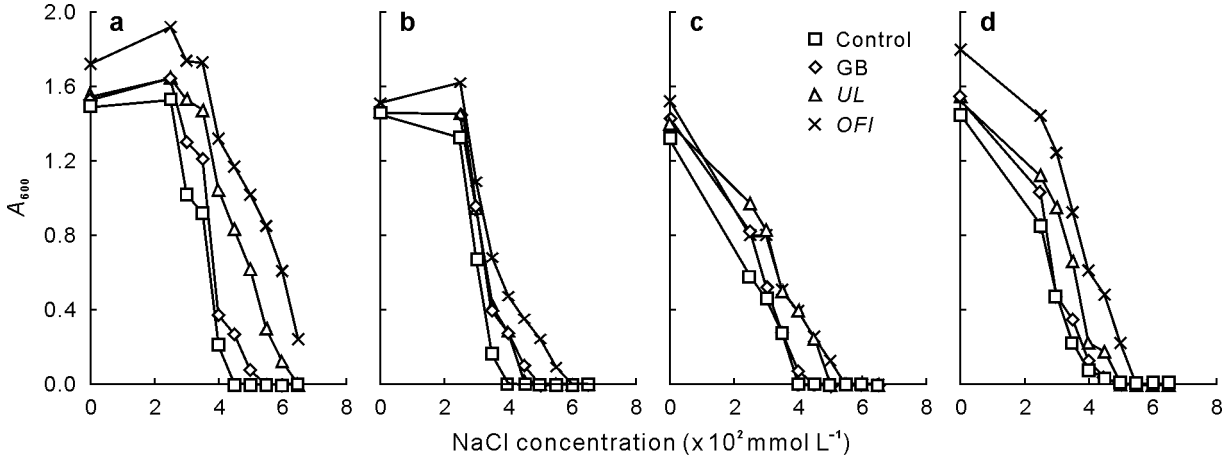

Fig. 4 Maximum growth of isolates BEA4 (a), BEC9 (b), BOA4 (c), and SEB9 (d) achieved on N-free broth medium with different NaCl concentrations in the the absence (control) and presence of glycine betaine (GB) (1 mmol L^{-1}) and hydro-alcoholic extracts from *Ulva lactuca* (*UL*) and *Opuntia ficus-indica* (*OFI*) (1%, weight:volume). The readings were performed in duplicate. A_{600} = absorbance of the culture at 600 nm.

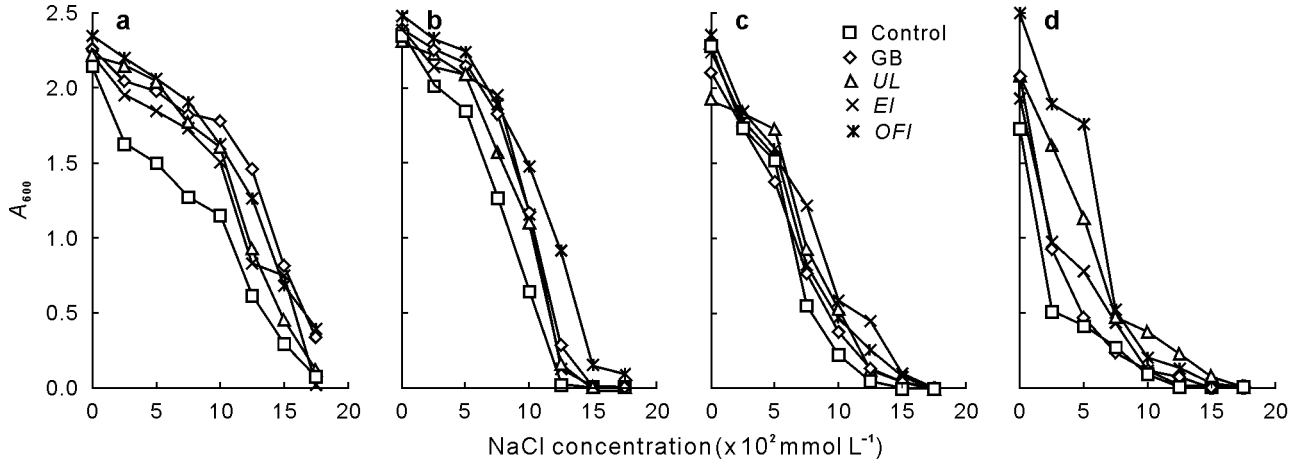

Fig. 5 Maximum growth of isolates BEA4 (a), BEC9 (b), BOA4 (c), and SEB9 (d) achieved on Luria-Bertani medium with different NaCl concentrations in the absence (control) and presence of glycine betaine (GB) (1 mmol L^{-1}) and aqueous extracts from *Ulva lactuca* (*UL*), *Enteromorpha intestinalis* (*EI*), and *Opuntia ficus-indica* (*OFI*). The readings were performed in duplicate. A_{600} = absorbance of the culture at 600 nm.

of the control (without extract). *EI*-extract ameliorated the growth of the isolates BEA4, BEC9, and BOA4 on LB medium with 1 300 mmol L^{-1} of NaCl by 135.529%, 641.538%, and 949.895%, respectively. Glycine betaine stimulated the growth of the isolates BEA4, BEC9, BOA4, and SEB9 on N-Fb medium with 300 mmol L^{-1} of NaCl by 127.286%, 142.22%, 113.269%, and 101.834%, respectively (Figs. 4 and 5).

Wheat germination

The presence of GB and *UL* and *OFI* extracts markedly promoted seed germination under salt stress. The bacterial treatment, especially with BOA4 and SEB9, improved seed germination success. The germination percentage was restored from 2.77% to 22.23%, 38.89%, and 30.57% under 300 mmol L^{-1} of NaCl and from 22.2% to 47.23%, 44.45%, and 47.23% under 200 mmol L^{-1} NaCl in the presence of GB and *OFI* and *UL* extracts, respectively. Bacterial inoculation with the strains BEA4, BEC9, BOA4, and SEB9 enhanced the germination success from 22.2% to 44.43%, 41.66%, 61.1%, and 63.86% under 200 mmol L^{-1} NaCl and from 2.77% to 25%, 22.23%, 50%, and 44.43% under 300 mmol L^{-1} NaCl, respectively (Fig. 6).

DISCUSSION

Morrissey *et al.* (2014) indicated that salinity increased the microbial decomposition rates in low-salinity wetlands, which was consistent with the positive correlation between the EC of soil samples and the enzymatic activities of their corresponding strains found in this work. The isolates from the saline soil BEA (EC > 4) showed a high enzymatic score when

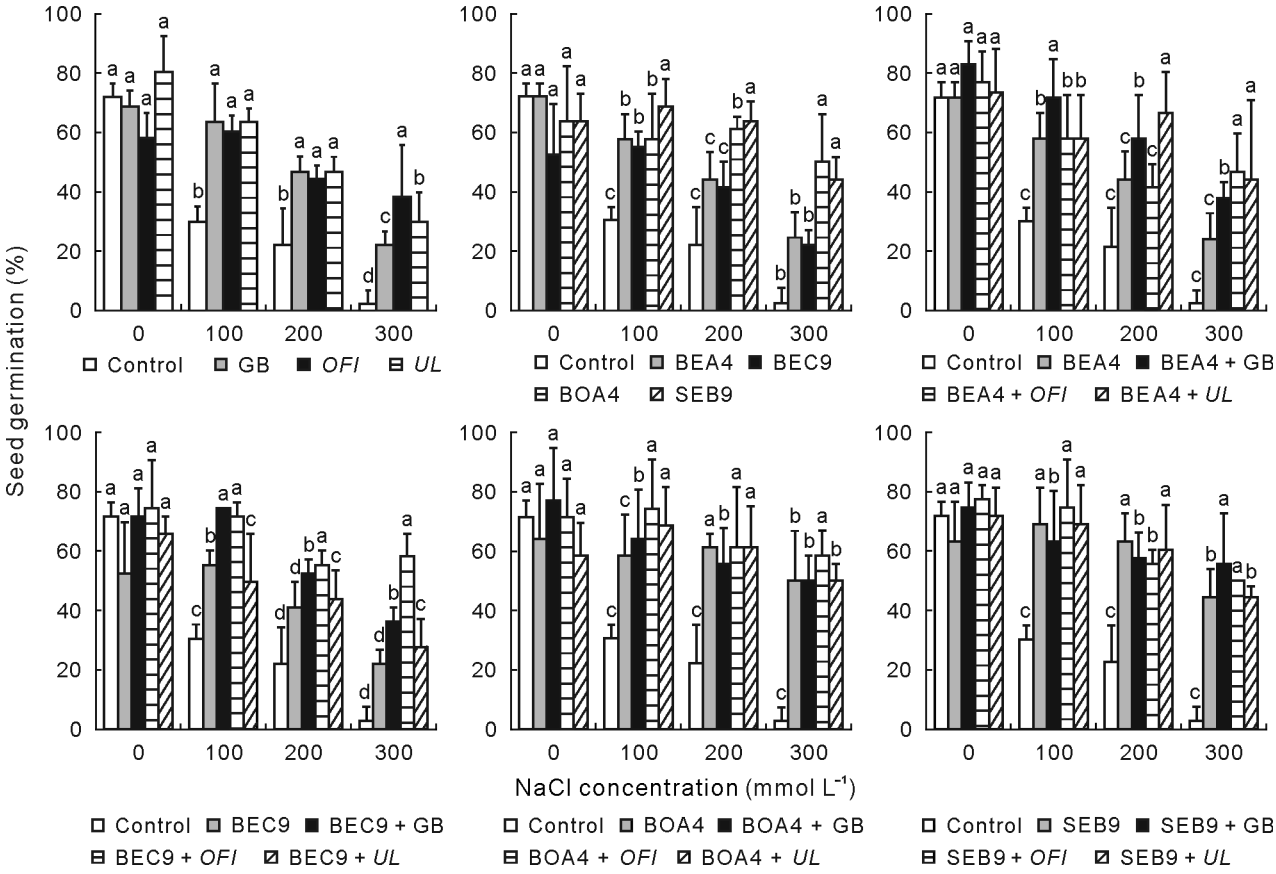

Fig. 6 Seed germination success (percentage) of wheat in the presence and absence of glycine betaine (GB) (1 mmol L^{-1}) and hydro-alcoholic extracts from *Opuntia ficus-indica* (*OFI*) and *Ulva lactuca* (*UL*) (1%, weight:volume). Seeds other than controls were treated with isolate BEA4, BEC9, BOA4, or SEB9. Controls without bacterial, without GB, and without extracts were used. Error bars are the standard deviations of means. Different letters above the bars indicate significant differences at $P < 0.05$ within each NaCl concentration.

tested in non-saline medium. Despite this, BEA contained high levels of SOM, probably due to inhibition of the lytic enzymes under salt stress. Eivazi and Tabatabai (1988), García *et al.* (1994), and Batra and Manna (1997) showed that the activities of the soil enzymes were seriously reduced by salinity, which affected nutrient recycling and release for use by plants. pH (KCl) in soil assesses the exchangeable hydrogen and affects the N-fixing efficiency of diazotrophic bacteria (Niklińska *et al.*, 2005; Lapinskas and Piaulokaitė-Motuzienė, 2006; Rousk *et al.*, 2009). Generally, bacterial extracellular enzymes in soil are alkaline, in contrast to those released by fungi and plants (Caldwell, 2005), which could explain the positive correlation observed between the pH of soil samples and the total detected enzymes.

Plant growth-promoting rhizobacteria may influence plant growth through several mechanisms, such as the increased availability of other nutrients and the production of phytohormones. In the first part of this work, we established a hierarchy of potential properties in order to isolate diazotrophic bacteria with high plant growth-promoting potential: N fixation; catabolic activity (urease, protease, lipase, chitinase, esterase, amylase, and cellulase); IAA and siderophore production; and phosphate solubilization. Through this method, we selected four bacterial strains (*Flavobacterium johnsoniae* BEA4, *Pseudomonas putida* BEC9, *Achromobacter xylosoxidans* BOA4, and *Azotobacter chroococcum* SEB9) with a high *in vitro* potential to promote plant growth.

One of the mechanisms by which rhizobacteria ameliorate plant growth is N fixation (James *et al.*, 1994; Glick *et al.*, 1997; Vessey 2003; Gray and Smith 2005). Several strains belonging to the species *F. johnsoniae*, *P. putida*, *A. xylosoxidans*, and *A. chroococcum*, are known to be plant growth promoters; they are diazotrophic bacteria in the soil (Altman and Waisel, 1997; Malik *et al.*, 1997; Kader *et al.*, 2002; Jha and Kumar, 2009; Ma *et al.*, 2009; Sgroy *et al.*, 2009; Laskar *et al.*, 2013; Vacheron *et al.*, 2013; Pathani *et al.*, 2014).

The degradation of organic matter through catabolic activity is one of the most important means through which bacteria increase plant growth. Mi-

crobial enzymes, such as ureases, esterases, lipases, proteases, chitinases, amylases, and cellulases, play key roles in biological transformation processes (Glick, 1995; Martínez-Viveros *et al.*, 2010; Rana *et al.*, 2012; Xun *et al.*, 2015). Enzymes such as chitinase and cellulose play important roles as biocontrol agents through the degradation of fungal cell walls (Mitchell and Alexander, 1963; Sindhu and Dadarwal 2001). Kathiresan *et al.* (2011) reported that a bacterium of the genus *Azotobacter* produces important quantities of amylases, cellulases, lipases, chitinases, and proteases. Ahmad *et al.* (2013) revealed that several strains of the genus *Pseudomonas* produce proteases, cellulases, chitinases, and lipases, that are implicated in SOM degradation and the biocontrol of plant pathogens.

Indole 3-acetic acid is the most commonly produced phytohormone whose effect on plant growth depends on the sensitivity of the plant and the amount of IAA produced (Pant and Agrawal, 2014). Our results with respect to IAA production are in agreement with previous works (Torres-Rubio *et al.*, 2000; Tsavkelova *et al.*, 2006; Jha and Kumar, 2009; Soltani *et al.*, 2010; Kumar *et al.*, 2014), which reported that bacteria of the genera *Flavobacterium*, *Pseudomonas*, *Achromobacter*, and *Azotobacter* can produce large quantities of IAA and promote plant growth.

Siderophores are iron-chelating compounds whose biosynthesis is regulated by the availability of iron in the surrounding medium (Simões *et al.*, 2007). In soil, they play a crucial role in plant growth improvement, enhancing iron uptake by roots. Recent work has demonstrated that iron-chelating siderophores suppress soil-borne fungal pathogens (Beneduzi *et al.*, 2012). Others have shown that the bacterial species *F. johnsoniae*, *P. putida*, *A. chroococcum*, and *A. xylosoxidans* produce siderophores (Suneja *et al.*, 1996; Hynes *et al.*, 2008; Moretti *et al.*, 2008; Sgroy *et al.*, 2009; Tian *et al.*, 2009; Ahemad and Kibret, 2014).

After N, phosphorus is the most limiting element for plant nutrition. Phosphate-solubilizing microorganisms (PSM) in soil are considered the most ecofriendly option to provide inexpensive phosphorus to plants (Kumar *et al.*, 2000; Chen *et al.*, 2006; Hussain *et al.*, 2013; Sharma *et al.*, 2013; Khan *et al.*, 2014; Nosrati *et al.*, 2014). Sashidhar and Podile (2010) highlighted the phosphate solubilization capacity of the bacteria *A. xylosoxidans*, *A. chroococcum*, *P. putida*, and *F. johnsoniae*.

All the discussed traits (N fixation, hydrolytic enzymes, IAA synthesis, solubilization of inorganic insoluble phosphorus, ACC degradation, and siderophore production) are known to be involved in the enhancement of plant tolerance to salt stress (Egamberdieva, 2009; Walpola and Arunakumara, 2010; Kizildag *et al.*, 2012; Shrivastava and Kumar, 2015).

The use of PGPR is becoming a widespread practice. However, they may be even more beneficial under stress conditions. It is therefore important to ensure that the tested inoculant is able to survive in the particular conditions under which its use is proposed. Microbial tolerance to salt stress may be improved using compatible solutes synthesized by the microorganism and/or taken up from the medium (Madkour *et al.*, 1990); the latter mechanism is preferred when the appropriate substances are present in the environment (Lamosa *et al.*, 1998; Paul and Nair 2008).

In the second part of this work, we demonstrated that *OFI* extract was, in most cases, better at promoting bacterial growth (BEA4, BEC9, BOA4, and SEB9) and wheat seed germination under high salinity than GB. The LB medium most likely conferred greater protection to bacterial cultures than N-Fb medium in the absence of other sources of compatible solutes owing to the presence of proline, betaines, and other molecules known for their osmoprotectant role, especially in the yeast extract (Nagata *et al.*, 1996; Wood, 2007). It is also important to report the positive results obtained by the addition of the extracts of *UL* and *EI*. Both marine macroalgae have been reported to synthesize and accumulate a wide variety of osmoprotectants under salt stress (Dickson *et al.*, 1982; Edwards *et al.*, 1987; Plettner *et al.*, 2005). Marine macroalgal extracts have already been used to improve the halotolerance of bacteria such as *Sinorhizobium meliloti*, *Azospirillum brasilense*, and *Escherichia coli* (Ghoul *et al.*, 1995; Pichereau *et al.*, 1998; Nabti *et al.*, 2007). Dimethylsulfoniopropionate and other tertiary sulfoniums, sugars, polyols, and N-organic compounds are the most abundant compatible solutes accumulated under salt stress conditions by marine macroalgae, including *UL* and *EI* (Edwards *et al.*, 1988; Kirst, 1996).

It is pertinent to note that this is the first recorded use of *OFI* as a source of natural osmoprotectants. No prior reports have detailed the chemical nature of these compounds, nor the conditions of their synthesis by this plant, which is known for its resistance to environmental stress. However, the total content of free amino acids (257.24 mg per 100 g) was higher than the average for other fruits. *Opuntia ficus-indica* has relatively high levels of serine, g-amino butyric acid, glutamine, proline, arginine, and histidine, and contains methionine (Sáenz *et al.*, 2013), suggesting that the amino acids may constitute the majority of the

natural osmoprotectants provided by the extract of this plant. In addition, *OFI* cladodes are rich in minerals such as potassium, calcium, phosphorus, and iron (Hadj Sadok *et al.*, 2008), which are involved in the enrichment of the culture media, and thus promote bacterial growth. Tryptophan, the direct precursor of IAA, is an abundant compound in *OFI* cladodes (1.04 g per 100 g) (El-Mostafa *et al.*, 2014). The production of such elements plays a key role in the promotion of plant growth and the amelioration of salt stress (Egamberdieva 2009; Kaya *et al.*, 2009; Liu *et al.*, 2013).

Nabti *et al.* (2007) reported the use of PGPR and natural compatible solutes from *UL* to promote wheat seed germination and growth under salt stress. However, our report on the use of extracts of *OFI* and *EI* to promote plant growth under high salinity is new. Basavaraju *et al.* (2002), Kaymak *et al.* (2009), Tam and Diep (2014), and Rathi *et al.* (2014) reported the role of *A. xylosoxidans*, *Flavobacterium* sp., *P. putida*, and *A. chroococcum* in the amelioration of seed germination and plant growth under salt stress conditions. The extract from *OFI* cladodes appears to be an economically viable, natural alleviator of salt stress in agriculture. These plants are distributed worldwide in many countries with arid and semi-arid zones, the most extensive dry lands on the planet, where there is a need for plant species that can adapt to provide food and materials under abiotic stress conditions (Sáenz *et al.*, 2013).

CONCLUSIONS

The bacterial strains *F. johnsoniae* BEA4, *P. putida* BEC9, *A. xylosoxidans* BOA4, and *A. chroococcum* SEB9 were able to fix atmospheric N, produce a wide range of hydrolytic enzymes, solubilize inorganic insoluble phosphorus, produce siderophores, synthesize useful amounts of IAA, and use ACC as their sole N source. Aqueous and hydro-alcoholic extracts from *OFI*, *UL*, and *EI* improved the halotolerance of the aforementioned strains. The germination of wheat seeds was markedly enhanced by bacterial inoculation and the application of the *OFI* and *UL* extracts. *In vivo* studies are required to determine the effect of the extracts and the strains on extended stages of the plant growth, and to determine the nature of the compounds implicated in the enhancement of bacterial and plant halotolerance.

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