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PLANT GROWTH-PROMOTING BACTERIA FACILITATE THE GROWTH OF BARLEY AND OATS IN SALT-IMPACTED SOIL: IMPLICATIONS FOR PHYTOREMEDIATION OF SALINE SOILS

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Plant growth-promoting bacteria (PGPB) strains that contain the enzyme 1-amino-cyclopropane-1-carboxylate (ACC) deaminase can lower stress ethylene levels and improve plant growth. In this study, ACC deaminase-producing bacteria were isolated from a salt-impacted (∼50 dS/m) farm field, and their ability to promote plant growth of barley and oats in saline soil was investigated in pouch assays (1% NaCl), greenhouse trials (9.4 dS/m), and field trials (6–24 dS/m). A mix of previously isolated PGPB strains UW3* (Pseudomonas sp.) *and UW4* (P. *sp.) was also tested for comparison. Rhizobacterial isolate CMH3* (P. corrugata) *and UW3+UW4 partially alleviated plant salt stress in growth pouch assays. In greenhouse trials, CMH3 enhanced root biomass of barley and oats by 200% and 50%, respectively. UW3+UW4, CMH3 and isolate CMH2 also enhanced barley and oat shoot growth by 100%–150%. In field tests, shoot biomass of oats tripled when treated with UW3+UW4 and doubled with CHM3 compared with that of untreated plants. PGPB treatment did not affect salt uptake on a per mass basis; higher plant biomass led to greater salt uptake, resulting in decreased soil salinity. This study demonstrates a method for improving plant growth in marginal saline soils. Associated implications for salt remediation are discussed.

KEY WORDS: ACC deaminase, PGPB/PGPR, salt remediation

INTRODUCTION

The amount of salt-affected land (i.e., soils with elevated salt levels; generally electrical conductivity (EC) >4 dS/m) is estimated to be approximately 900 million hectares worldwide, and comprises 6% of the total global land area, representing about 20% of the world's cultivated land (Flowers 2004). In Canada, approximately 1 million hectares of

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surface soil within the agricultural regions of the prairies are affected by moderate (EC_e 8–16 dS/m) to severe (EC_e > 16 dS/m) soil salinity (Wiebe *et al*. 2006). Elevated soil salinity poses an enormous threat to the global food supply because soil salinity inhibits plant growth and decreases crop yields. Because of the global need for good quality agricultural land, it is important to find ways to improve plant growth in marginal soils, and to remediate salt from impacted soils (Qadir *et al*. 2002). Phytoremediation is a strategy that uses plants to degrade, stabilize, and/or remove soil contaminants (Gerhardt *et al*. 2009). Phytoremediation of salt involves removal of soil salt into plant tissue, and requires high amounts of plant biomass to attain acceptable rates of remediation. This is often difficult to achieve in salt-impacted soils (Qadir *et al*. 2003).

Soil salinity affects plants in a variety of ways: plants grown in salt-impacted soils can exhibit drought stress symptoms, decreased germination rates, inhibition of photosynthesis, loss of membrane integrity and increased generation of reactive oxygen species (Qadir *et al*. 2003; Greenberg *et al*. 2008). Some of these responses are the result of elevated stress ethylene production, which triggers a cascade of plant defence and senescence responses (O'Donnell *et al*. 1996; Cuartero and Fernandez-Munoz 1999; Blumwald 2000; Petruzzelli, Coraggio, and Leubner-Metzger 2000; Hall *et al*. 2001; Parida and Das 2005; Shibli *et al*. 2007). However, some soil bacteria can lower plant ethylene levels and thereby ameliorate some inhibitory effects of salt on plant growth and development (Glick, Penrose, and Li 1998; Glick *et al*. 1999; Mayak, Tirosh, and Glick 2004; Cheng, Park, and Glick 2007). One group of plant growth-promoting bacteria (PGPB) (also referred to as PGPR, plant growth-promoting rhizobacteria) is comprised of bacterial strains that can lower plant ethylene levels and promote plant growth via the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Glick *et al*. 1998; Gerhardt, Greenberg, and Glick 2006). ACC deaminase-producing PGPB have been investigated for their ability to promote crop growth in the presence of high salinity. These studies, which have used soils with EC_e as high as 30 dS/m, have involved tomatoes (Mayak *et al*. 2004), peppers (Mayak *et al*. 2004), groundnuts (Saravanakumar and Samiyappan 2007), maize (Nadeem *et al*. 2007; Principe *et al*. 2007), cotton (Yue *et al*. 2007), canola (Cheng *et al*. 2007), and grasses (Greenberg *et al*. 2008). Of particular relevance to this study, Cheng *et al*. (2007) demonstrated that *Pseudomonas putida* UW4, a bacterial strain that produces high levels of ACC deaminase, protected canola plants against growth inhibition caused by salt stress.

Although lowering ethylene levels via ACC deaminase is a prevalent mechanism for alleviating salt stress on plant growth, some bacteria utilize other mechanisms to protect plants; for example, a number of researchers have reported that some *Azospirillum* spp. strains were able to partially mitigate the inhibitory effects of salt stress on wheat, maize, beans, lettuce and sweet pepper (Bacilio *et al*. 2004; Hamdia, Shaddad, and Doaa 2004; Rabie and Almadini 2005; Barassi *et al*. 2006; del Amor and Cuadra-Crespo 2012), but not all of these beneficial strains had high ACC deaminase activities (Holguin and Glick 2001). It is postulated that indoleacetic acid (IAA, an auxin) synthesized by *A*. spp. strains also plays an important role in plant growth promotion under salt stress. Patten and Glick (2002) also reported that primary roots of canola seedlings treated with wild-type *P. putida* GR12-2 were 35% to 50% longer than those treated with an IAA-deficient mutant of this strain. Other mechanisms used to ameliorate salt stress include altering mineral uptake, which results in an increase in the K^+/Na^+ ratio (Yildrim, Donmez, and Turan 2008), and elevating the production of quorum sensing molecules, which have an effect on bacterial biochemical pathways (Barriuso *et al*. 2008).

The objective of the research was to determine if PGPB would enhance plant growth on salt-impacted soils and if salt remediation would be observed. To achieve this, plant root associated bacteria (rhizobacteria) were isolated from field soils. Rhizobacterial strains that produce high levels of IAA and ACC deaminase were tested for their ability to promote the growth of plants (barley and oats) grown in saline soil. The experiments included newly isolated PGPB, and previously reported PGPB (UW3 (*P.* sp.) and UW4 (*P.* sp.)) for comparison. Finally, the phytoremediation potential of the PGPB-treated plants was tested. To the best of our knowledge, this paper is the first to report the use of PGPB to ameliorate the inhibitory effects of excess salt on barley and oats in the field, and to use PGPB-treated plants to remediate salt.

MATERIALS AND METHODS

Isolation, Analysis and Identification of Bacterial Strains

Pseudomonas sp. UW3 (GenBank Accession Number KF145175) was originally isolated from the rhizosphere of corn plants and classified as *P.* sp. (Glick, Karaturovic, and Newell 1995). *P.* sp. UW4 (GenBank Accession Number CP003880) was originally isolated from the rhizosphere of common reeds growing on the campus of the University of Waterloo (Waterloo, Ontario, Canada) and was classified as *P.* sp. (Glick *et al*. 1995). It was later reclassified as *Enterobacter cloacae* based on fatty acid profiles (Shah *et al*. 1998), and then as *P. putida* based on 16S rDNA sequence analysis and metabolic profiling (Hontzeas *et al*. 2005). Recently, based on the complete genomic sequence (obtained by pyrosequencing) and phylogenetics, strain UW4 was redesignated *Pseudomonas* sp. (Duan *et al*. 2013). These previously reported PGPB were maintained in laboratory cultures and were re-tested for ACC deaminse activity prior to use in this study. These PGPB strains will subsequently be referred to as UW3 and UW4.

The method used to isolate UW3 and UW4 was used to isolate indigenous strains from the salt-impacted sites in this study. The detailed isolation procedure is described in Shah *et al*. (1998). Briefly, soil sampled from field sites was transferred into sterile tryptic soy broth (TSB) rich medium and incubated for 16 h. Bacterial cells were washed and cultured with sterile DF minimum salt medium (Dworkin and Foster 1958). After rinsing cells with DF salt medium that contained no nitrogen, this culture was streaked onto 1.5% agar DF minimum salt agar medium with 300 mM 1-aminocyclopropane-1-carboxylate (ACC) as the sole nitrogen source. The ACC deaminase activity of each isolate was then determined by the ACC deaminase activity assay (Penrose and Glick 2003).

Each strain was named after its site of origin, Cannington Manor (CM), and the salinity level of soil from which it was isolated. For example, bacterial strains CMH1 and CML1 were isolated from soil of high salinity (average $EC_e = 50.4$ dS/m) and from soil of lower salinity (average $EC_e = 9.4$ dS/m), respectively. CMH2 (GenBank Accession Number KF041155) and CMH3 (GenBank Accession Number KF041156) were isolated as described (Glick *et al*. 1995) from the rhizosphere of monocot grasses grown on one target field site (Cannington Manor, Saskatchewan, Canada) that had high soil salinity ($EC_e =$ 50.4 dS/m). These strains were sent for identification based on 16S rDNA sequence analyses ($\sim$500 bp) (Accugenix Inc., Newark, DE, USA) and characterization using the Biolog^{TM} system. For a negative control, *P. putida* UW4/AcdS$^-$ was used; it is an ACC deaminase gene knockout mutant of strain *P. putida* UW4 (Li *et al*. 2000).

ACC Deaminase and IAA Assays

ACC deaminase activity was measured as the production of α-ketobutyrate, an end product derived from ACC (Honma and Shimomura 1978; Penrose and Glick 2003). Bacterial cells of each isolate were cultured with ACC as the only nitrogen source to induce the expression of ACC deaminase, followed by cell lysis with toluene. ACC was added into the cell lysate and reacted with ACC deaminase, producing α-ketobutyrate. The enzyme activity was determined by the amount of α-ketobutyrate produced, normalized to the total protein in the lysate. IAA production was quantified using Salkowski's reagent as described elsewhere (Gordon and Weber 1951). Cells were incubated with a DF minimal medium (Dworkin and Foster 1958) containing 500 μg/mL L-tryptophan. For both assays, each experiment was repeated three times.

Bacterial Inoculation of Seeds

Hordeum vulgare cv. Ranger (barley) and *Avena sativa* cv. CDC Baler (oats) seeds were purchased from Cribit Seeds (West Montrose, ON, Canada) and Wagon Wheel Seed (Churchbridge, SK, Canada), respectively. The bacterial cultures for inoculation were prepared as follows: After overnight (18 h) growth at 25°C in 50 ml of tryptone soy broth (TSB, 30 g/L) *(Difco, Detroit, MI, USA*), bacterial cells were centrifuged at 800 $\times$ *g* for 10 min before being suspended in 250 ml of 1% sodium pyrophosphate. The cells were centrifuged again and resuspended in 0.25% methylcellulose (adhesive reagent; Sigma-Aldrich, St. Louis, MO, USA). Bacterial density was 10^9–10^{10} cfu/ml. The methylcellulose solution was autoclaved for 30 min at 121°C prior to use. A blue colorant (Color Coat Blue; Becker Underwood, Saskatoon, SK, Canada) was added to the methylcellulose-bacteria suspension for a ratio of 1.75 ml of colorant per 100 ml suspension. The presence of the colorant meets safety regulations of the Seed Act of Canada, which requires coloring of treated seeds to avoid their use for animal consumption. A 20 ml aliquot of the blue bacterial slurry was mixed with 600 ml of seeds using a seed treater (HEGE 11, Wintersteiger Inc., Ried, Austria) that generates air flow in the chamber, resulting in an even seed coating with the bacteria slurry. These air-dried seeds were transferred into sealed plastic bags and stored at 4°C for up to four weeks prior to use. For seeds treated with two bacterial strains, a 10 ml aliquot of each bacterial slurry was applied. Bacterial density was approximately 10^8–10^9 cfu/seed for barley and oats, measured as described elsewhere (Germida and Walley 1996).

Growth Pouch Assays

The root elongation growth pouch tests were conducted as previously described (Penrose and Glick 2003). Six seeds were placed evenly along the seed line of each growth pouch (Mega International, Minneapolis, MN, USA) that was drenched with an aseptic solution of 1% NaCl. Ten pouches were set up for each bacterial treatment. Primary root length was measured five days after the onset of germination. The entire experiment was repeated three times.

Greenhouse Trials

Soil used in the greenhouse trials was taken from the top 30 cm of a salt-impacted site in Cannington Manor, Saskatchewan, Canada. The soil was sieved through a 2 mm mesh and

homogenized prior to potting and analysis. Soil analysis (ALS Laboratory, Waterloo, ON, Canada) indicated a cation exchange capacity (CEC) of 36.8 meq/100 g, electroconductivity (EC_e) of 9.4 dS/m, and a sodium adsorption ratio (SAR) of 11. SAR is a measurement of sodicity (optimal SAR values are less than 4.0), which is the amount of Na^+ relative to Ca^{2+} and Mg^{2+} in soil solution according to:

$$\mathrm{SAR} = \frac{[\mathrm{Na}]}{\sqrt{\frac{[\mathrm{Ca}]+[\mathrm{Mg}]}{2}}}$$

This clay loam soil had a pH of 8.2, contained 12.7% organic material, and was composed of 34% sand, 36% silt and 30% clay (Agri-Food Laboratories, Guelph, ON, Canada). Pro-Mix 'BX' (General Horticulture, Inc., Red Hill, Penn., U.S.A.) was used as a growth medium for controls to indicate healthy plant growth at regular salinity (<2 dS/m) in a greenhouse environment. The Pro-Mix used was composed of sphagnum peat moss (75–85% by volume), perlite, vermiculite, macronutrients (calcium, magnesium, nitrogen, phosphorus, potassium, sulfur), micronutrients (boron, copper, iron, manganese, molybdenum, zinc), dolomitic limestone, calcite limestone, and a wetting agent. The pH was approximately 6.0.

Barley and oat seeds inoculated with rhizobacterial isolates were sown in salt-impacted soil ($EC_e = 9.4$ dS/m) from the CM field site. Ten seeds were sown per 5.7 cm pot containing $\sim$80 cm^3 of soil. Each bacterial treatment included four replicates. Plants were watered with reverse osmosis-purified water at an average daytime temperature of 25°C without supplemental lighting. After a growth period of 45 days, plants grown in one pot were gathered and soil was carefully washed away with tap water to avoid breaking off any root tissue. The total biomass collected from one pot was oven dried at 60°C for 72 h prior to being divided for root and shoot dry weight measurements. Two independent experiments were carried out.

Field Trials

High soil salinity of the three salt-impacted sites was a result of upstream gas and oil production (i.e., exploration, drilling, extraction and recovery of petroleum) in Saskatchewan, Canada. Two sites are located in the Cannington Manor region and are referred to as the north ('CMN', 38 ha) and the south ('CMS, 16 ha) site; the third site is located in Alameda ('AL', 21 ha). All three sites were tilled and mixed with a 10 cm thick layer of manure prior to seeding. The manure came from a feedlot in High River, Alberta, Canada that was piled and allowed to heat to over 60°C. The field was divided into 5 m-wide plots with a length of 15 m to 50 m, depending on the size of the site. Plots were planted with untreated or PGPB-treated seeds of barley or oats, at a density of 6,000–8,000 seeds/m^2.

The ± PGPB effects were tested using a paired block design so that the plots with/without PGPB would have the most comparable soil quality and salinity possible. Parameters from the experiments such as plant biomass were directly compared between the paired blocks, after a five month growing season. The average soil salinity for 0.25 m^2 sampling areas was determined by using the salinity values of three random soil samples taken from surface soils (to a depth of 30 cm) within these areas. The field experiment was replicated at three different sites (i.e., each independent site was a replicate) using ± PGPB. The purpose of this preliminary trial was to determine if the same PGPB effects observed in the greenhouse studies would also occur in the field.

Analyses for Na and Cl in Plants

For one greenhouse trial, the total biomass of oven-dried shoots and roots of each treatment was homogenized prior to analyses. For field trials, the total aboveground biomass collected from each 0.25 m^2 area, including the grain portion of the plants, was analyzed as a composite sample. One exception was the untreated barley sampled from the CMN site: one half of the biomass was homogenized for a composite sample; and the other half was used in a separate related study (Chang, Gerhardt and Greenberg, unpublished results). All Na and Cl analyses were conducted by ALS Laboratory (Waterloo, ON, Canada). For Na concentrations, tissues were completely decomposed with concentrated nitric acid (US EPA method #6020 (U.S. EPA 1996)) prior to inductively coupled plasma mass spectroscopy (ICP-MS) measurements. Chloride was analyzed by ion chromatography according to the American Public Health Association method #4110B (APHA 1995).

Statistical Analysis

Root length and greenhouse biomass data were analyzed by one-way analysis of variance (ANOVA) and *post-hoc* Dunnett's tests ($^*P < 0.1$ versus Control) with random sampling. The software package STATISTICA (StatSoft, Inc., Tulsa, OK, USA) was used for all statistical analyses.

RESULTS

ACC Deaminase Activity and IAA Production of Isolated Rhizobacteria

Eighteen strains of potential PGPB were isolated from salt-impacted soils from Saskatchewan. The ACC deaminase activities and IAA levels of these strains were determined (Figure 1). The ACC deaminase activity of UW3 was the highest of bacterial strains assayed (Figure 1). We also compared ACC deaminase enzyme activity of the knockout mutant UW4/AcdS$^-$ and it was found to be nearly undetectable (Figure 1). Not all isolates

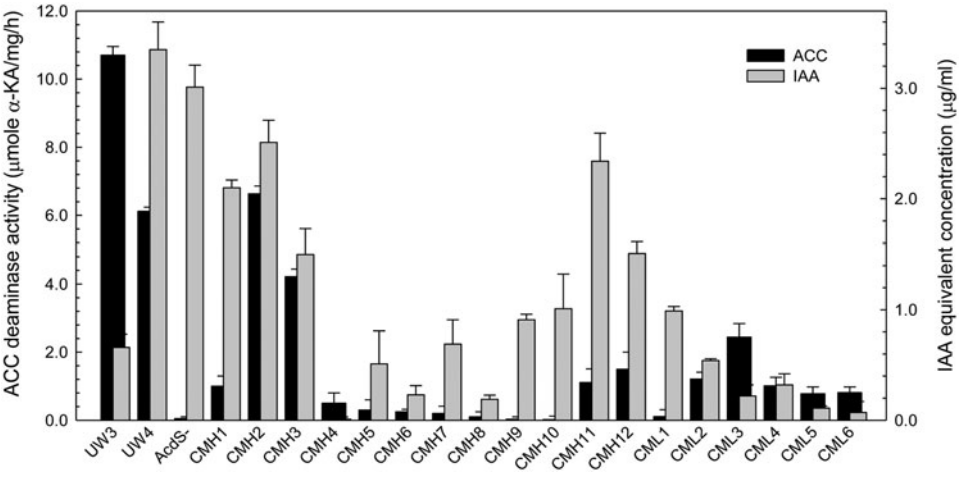

Figure 1 ACC deaminase activity (black) and IAA production (grey) of newly isolated bacterial strains. PGPB UW3 and UW4 have been tested previously and were used here as positive controls. Strain UW4/AcdS-, an ACC deaminase gene knock-out mutant, was used as a negative control. Results are expressed as means ± SEM. KA, α-ketobutyric acid; ACC, 1-aminocyclopropane-1-carboxylate; IAA, indole-3-acetic acid.

that were capable of using ACC as the sole nitrogen source produced high levels of ACC deaminase. Of the newly isolated strains, only CMH2 and CMH3 expressed ACC deaminase activity at a level similar to that of strain UW4 (Figure 1). In addition to expressing ACC deaminase activity, CMH2 and CMH3 also produced high levels of IAA (Figure 1). Given that bacterial IAA and ACC deaminase are beneficial to plant growth, and that CMH2 and CMH3 produced high levels of both ACC deaminase and IAA, these potential PGPB were selected for further testing.

Growth Pouch Tests

Growth pouch experiments were conducted to examine the effect of strains UW3+UW4, CMH2, and CMH3 on root elongation in a gnotobiotic environment. When seeds were germinated in the absence of salt, these rhizobacteria did not have an effect on seedling root length (Figure 2A), whereas some of these strains promoted root growth in the presence of 1% salt (Figure 2B). In particular, the UW3+UW4 treatment significantly improved the growth of both barley and oat roots. Also, the newly isolated strain CMH3 had a beneficial effect on early root development of barley germinated in a saline solution.

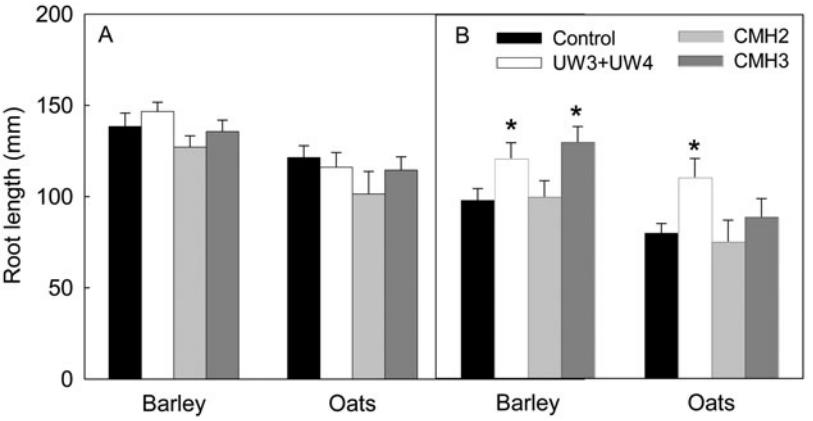

Figure 2 Plant growth pouch root elongation assays. PGPB-treated and untreated seeds of barley and oats were germinated in seed pouches. Seeds were watered with H_2O (Panel A) or H_2O + 1.0% NaCl (w/v) (Panel B). Root length was measured 5 days after germination to determine root growth. The results are expressed as means ± SEM of ten replicates. Data were analyzed by one-way analysis of variance (ANOVA) and Dunnett's test. * indicate statistical differences ($P < 0.1$) between root growth for untreated and PGPB-treated seeds.

Greenhouse Trials

The shoot and root dry weights of 45-day-old plants are shown in Figure 3. All three bacterial inoculations partially lowered the inhibitory effects of soil salinity on barley and oats. UW3+UW4 increased growth of barley roots and oat shoots by ∼50%. Shoot biomass of both barley and oats more than doubled, relative to untreated plants (Control) when the new isolates CMH2 and CMH3 were used. In particular, CMH2 tripled the shoot biomass of barley. As well, root biomass increased relative to the controls for plants treated with CMH2 and CMH3.

The Na and Cl concentrations in barley and oat shoots are listed in Table 1. Regardless of plant species or bacterial treatment, plants grown in saline soil had elevated levels of Na and Cl in shoot tissue relative to plants grown in Promix. Moreover, the increase in

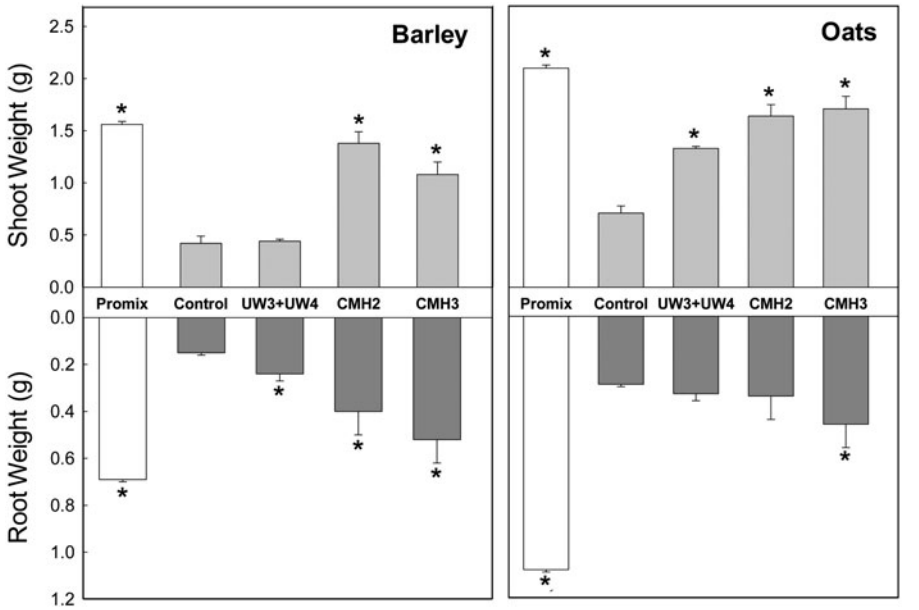

Figure 3 Plant growth in field soil. The dry weight (g) of shoot and root biomass of barley and oat plants grown in the greenhouse in saline field soil (ECe = 9.4 dS/m). "Promix"(white) represents the baseline normal plant growth in ProMix^{TM} (ECe < 2 dS/m) growth medium. "Control" stands for plants grown in saline soil without a bacterial treatment. The results are expressed as means ± SEM of four replicates in each of two independent trials (n = 8). Data were analyzed by one-way analysis of variance (ANOVA) and Dunnett's test. * indicate statistical differences (P < 0.1) between biomass for untreated and PGPB-treated seeds.

Table 1 Concentrations of Na and Cl in oat and barley shoots in greenhouse trials.

Treatment	Concentration (g/kg)			Molar Ratio Cl/Na
	Na	Cl	NaCl	
Barley				
Promix	3.2	21.1	24.3	4.3
Control	15.9	33.9	49.8	1.4
UW3+UW4	21.8	41.1	62.9	1.2
CMH2	15.8	33.7	49.5	1.4
CMH3	18.4	43.2	61.6	1.5
Oats				
Promix	5.3	20.8	26.1	2.5
Control	18.3	44.4	62.7	1.6
UW3+UW4	26.1	57.0	83.1	1.4
CMH2	17.7	38.4	56.1	1.4
CMH3	16.5	36.3	52.8	1.4

No differences in plant tissue NaCl concentrations were observed between different PGPR treatments. The total biomass of four replicates of each treatment was homogenized as one composite sample for analyses. "Promix" represents normal plant growth in ProMix^{TM} (ECe < 2 dS/m). "Control" indicates plants grown in saline soil without a bacterial treatment.

Na was greater than that of Cl; Na concentrations increased by 3–7 times, while those of Cl increased about two fold. As a consequence, the Cl/Na molar ratio decreased. PGPB treatment did not affect NaCl uptake on a per mass basis (i.e., there was no increase in NaCl concentration per unit of plant biomass).

Identification of PGPB Strains

Isolates that demonstrated the best growth promotion effects in greenhouse trials were deemed PGPB, and were selected for identification. The classification of strain CMH2 was based on 16S rDNA sequence analysis (99.9% identity; Accugenix Inc., Newark, DE, USA). It was identified as *Acinetobacter* sp. We had multiple concerns regarding the use of this genus for further testing because there is evidence that *Acinetobacter* spp. have the ability to rapidly develop resistance to antimicrobials, they have rapid transformation potential, and they persist in the environment for an extremely long period of time (see Doughari *et al*. (2011) for a review of this topic). Strain CMH3 was identified as *Pseudomonas corrugata* based on both 16S rDNA sequence analysis (99.0% identity) and extensive characterization using the Biolog^{TM} system (100% probability; Biolog Inc., Hayward, CA, USA). *P. corrugata* has been shown previously to be a PGPB (Antoun and Prevost 2005). It is categorized as a biosafety level 1 organism, which is the safest category, posing little risk to the environment or human health (http://www.atcc.org/). It was deemed to be safe for the field trials reported below.

Field Trials

Three PGPB (UW3+UW4 and CMH3) were selected for field trials, based on the laboratory and greenhouse experiments (Figures 1–3, Table 1) and previously reported research (Huang *et al*. 2005; Cheng *et al*. 2007; Pandey and Palni 1998; Guo *et al*. 2007). The PGPB tested in the field enhanced the growth of barley and oat plants (Table 2, Figure 4C). Strain CMH3 increased the foliage biomass of oats by one-third on the CMS site ($EC_e \approx 6$ dS/m) (Table 2). The UW3+UW4 treatment doubled the shoot biomass of barley on the CMN site ($EC_e \approx 4$ dS/m) (Table 2) and almost tripled that of oats on the AL site ($EC_e \approx 24$ dS/m).

Table 2 PGPB effects on dry biomass (g) and NaCl concentrations (g/kg) of the above ground tissue of plants.

Site	Plant	Treatment	Salinity (dS/m)	Dry Weight (g/0.25 m^2)	Increase (%)	NaCl (g/kg)	Cl/Na (molar ratio)
AL	oats	−PGPB	23	160		96.7	2.8
	oats	UW3+UW4	24	440	175	61.6	3
CMN	barley	−PGPB	4.2	600		55.4	1.3
	barley	UW3+UW4	4.5	1400	133	50.3	1.2
CMS	oats	−PGPB	6.5	360		57.4	2
	oats	CMH3	6.1	460	28	48.9	1.8
Average ± SEM		− PGPB		373 ± 127	112 ± 44	70 ± 13	2 ± 0.4
		+ PGPB (UW3+UW4, CMH3)		767 ± 317		54 ± 4	2 ± 0.5

−PGPB indicates no PGPB treatment. Only a subset of plant tissue samples was used in the analyses to obtain plant biomass and salt uptake. Paired samples for ± PGPB were selected from plots with similar salinity levels in the rhizosphere soil. No differences were observed in plant tissue NaCl concentrations between UW3+UW4 and CMH3 treatments.

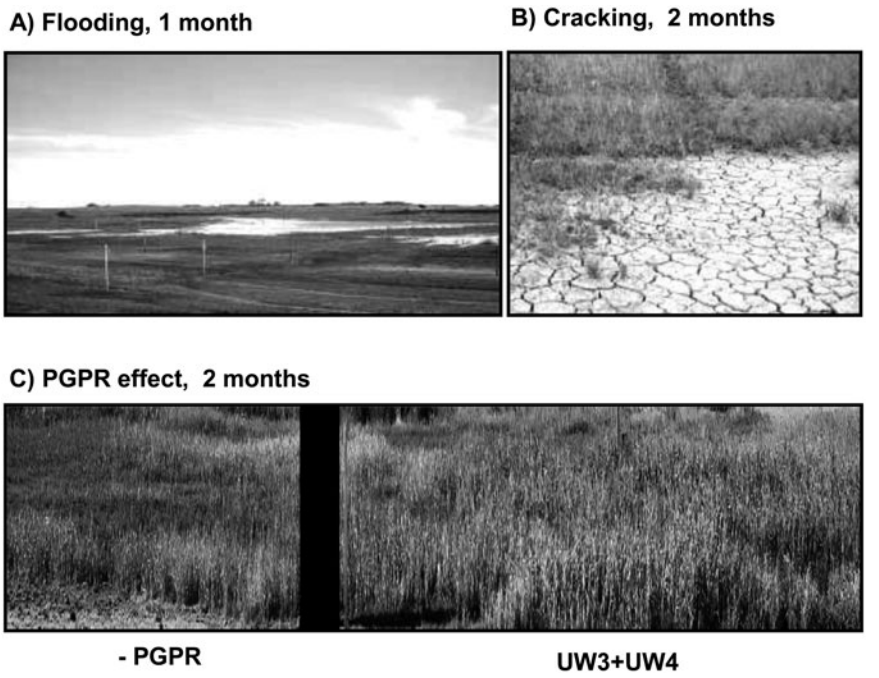

Figure 4 Photographs of the CMS and CMN field sites. At the CMS site, flooding occurred one month after seeding (A), followed by a period of drought that led to soil cracking (B). On the CMN site, the PGPB effect was observable after 2 months of barley growth (C).

The Na and Cl concentrations in the foliage of barley and oats following a five-month growing season are given on a per mass basis in Table 2. The average aboveground plant NaCl concentrations ranged from 50 g to 97 g per kg of plant dry weight; that is, 5% to 10% of the total foliar biomass (DW) is NaCl. Concurrent with NaCl accumulation in plant foliage, a marked decrease in soil salinity was observed at the CMN and CMS field sites (Figure 5).

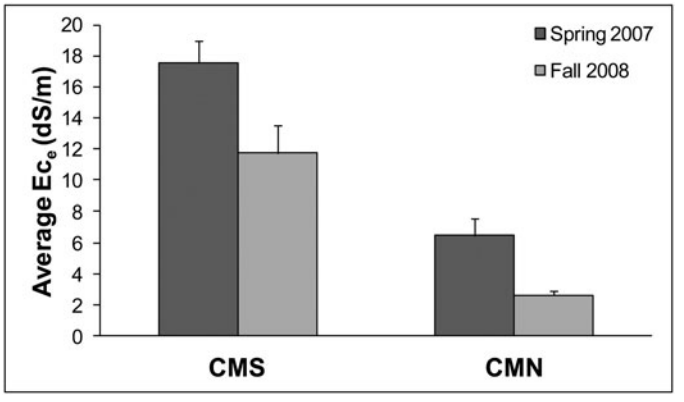

Figure 5 Electrical conductivity (ECe) values from the CMS and CMN sites for Fall 2007 and Fall 2008. Error bars represent SEM (n = 48 for CMS 2007, n = 21 for CMN 2007, n = 36 for 2008).

DISCUSSION

The first goal of this project was to isolate PGPB from saline soils and determine if these PGPB promoted plant growth on saline soils. The second goal was to determine if the plants could grow well enough to effect salt remediation from saline soils. We found that PGPB isolated from saline soils do indeed promote plant growth on saline soils and that PGPB-enhanced phytoremediation of saline soils is a real possibility.

The growth pouch assays showed that strains CMH3 and UW3+UW4 promoted root growth of barley and/or oats that germinated in a 1% salt solution. No significant increase in root length was observed for seeds grown with distilled water (Figure 2). This lack of PGPB-induced growth promotion on monocotyledonous plants in the absence of salt was observed previously; for example, Hall *et al*. (1996) observed that, without additional salt in the growth media, strain *P. putida* GR12-2, an ACC deaminase-containing PGPB, did not increase the root length of barley or oats, both of which are monocots. In contrast, strain GR12-2 enhanced the root length of dicotyledonous plants such as tomatoes, lettuce, canola, and wheat. Holguin and Glick (2001) postulated that this difference in the response to PGPB between dicotyledons and monocotyledons is a result of lower sensitivity of monocotyledons to ethylene stress, and this sensitivity can be induced by high salinity. It also shows that when there is a plant stress, PGPB effects are more readily observed. For a review of the topic, see Bashan and de-Bashan (2010).

The growth pouch tests were conducted to examine the effects of rhizobacteria on root growth in a controlled environment as a preliminary indicator for growth promotion effects of isolates. In general, the rhizobacterial strains that were beneficial to root growth in the pouch tests had positive effects on plant growth in the greenhouse trials. In that regard, pouch tests served as a good pre-screening method in this instance. Nonetheless, we found that strains which did not promote root growth in the pouch tests could still promote plant growth in another test environment; for instance, strain CMH2, which did not promote root growth in pouch tests (Figure 2), enhanced shoot and root biomass of barley, and shoot biomass of oats, by more than one fold in the greenhouse trials relative to controls (Figure 3). Thus, pouch tests may be a cost-efficient means to pre-select beneficial strains for further testing, but may not always reflect potential plant growth effects in soil. Factors such as soil pH, texture, root exudates, and the indigenous microbial community can alter the proliferation or functioning of inoculated bacteria and, subsequently, their effects on plant growth.

PGPB strains UW3 and UW4 were previously characterized as having ACC deaminase activity. These PGPB strains also produce IAA. Both UW3 and UW4 have previously been shown to promote plant growth in greenhouse and field trials (Huang *et al*. 2004; Huang *et al*. 2004; Huang *et al*. 2005; Cheng *et al*. 2007; Gurska *et al*. 2009). Greenhouse trials indicated that barley and oats accumulated more shoot biomass when inoculated with the indigenous strains CMH2 and CMH3 than with the exogenous PGPB UW3+UW4 (Figure 3). A similar result was observed in the field trials of Bhattarai and Hess (1993); they found that the maximum increase in local spring wheat yield occurred when locally isolated *Azospirillum* spp. (i.e., indigenous rhizobacteria) were used. However, our preliminary field tests at the AL site suggested that using indigenous strains is not necessary for significant increases in plant growth, as treatment with exogenous strains UW3 and UW4 resulted in almost a two-fold increase in oat biomass (Table 2). It is of note that the ability of the indigenous strain CMH3 to promote plant growth might have been compromised because of poor soil properties. Soil at the CMS site had high sodium absorption ratios

(SAR), thus clay particles easily disperse and plug soil pores, especially after flooding; it often results in a hard crust or soil cracking on the surface. This cycle of flooding (Figure 4A) and soil cracking (Figure 4B) could have inhibited seedling emergence and plant growth to such an extent that the PGPB CMH3 was ineffective at alleviating the environmental stress. Overall, the field tests indicated varying degrees of effectiveness for both indigenous and exogenous PGPB, which may be attributable to a host of environmental factors.

Greenhouse trials indicated that Cl/Na molar ratios of plants grown in saline soil ($EC_e = 9.4$ dS/m) were lower than those of plants grown in Pro-Mix ($EC_e < 2$ dS/m). This indicates that barley and oats might readily take up Na^+ than Cl^- under saline conditions similar to those tested in these experiments. In the greenhouse and the field, plants assimilated large amounts of NaCl to their foliar tissue. This tissue can be removed from salt-impacted sites, effecting remediation.

Notably, NaCl concentrations in barley and oats did not change proportionally with the increase in soil salinity from 6 to 24 dS/m (Table 2). Between 5%–10% of total foliar biomass (DW) may be the maximum amount of NaCl these plant species can take up, regardless of soil salt levels. The Cl/Na molar ratio results are consistent with those in the greenhouse trials (Table 1). It is also important to note that greenhouse trials indicated that PGPB did not alter plant tissue NaCl concentrations (i.e., there was no increase in NaCl uptake per unit biomass) (Table 1) which has pronounced implications for phytoremediation: Without increases in foliar NaCl concentrations (per unit biomass), phytoremediation efficiency will be proportional to the total accumulation of plant biomass at any given salt-impacted site. Because PGPB treatment increases the total aboveground biomass on saline soils, the total amount of NaCl that would be removed by harvesting the plants would also increase. As in the greenhouse experiments, although PGPB treatment resulted in increases in plant biomass in the field, it did not affect NaCl uptake on a per mass basis (Table 2). Thus, marked decreases in soil salinity (Figure 5) were a result of more Na and Cl being removed from the soil by the extra biomass that resulted from PGPB treatment.

Salt-impacted soils are an unavoidable problem in Western Canada resulting from upstream oil and gas production. This is because brine and hydrocarbons often occur together geologically (Alberta Environment 2001). Brine water contains sulphates, bicarbonates, and chlorides of Na, Ca, and Mg; NaCl is the most prevalent brine salt (Alberta Environment 2001). Remediation of salt-impacted soils has proven difficult and costly due to the absence of a versatile *in situ* technology (Glass 2000, Alberta Environment 2001). Often the impacted soil must be removed to landfill and replaced with clean soil. Results from the field experiments reported above showed that rhizobacterial strains selected for their superior ACC deaminase activity and IAA production can promote plant growth in salt-impacted soil with salinity up to 24 dS/m. Strains isolated from one site can be effective on another site although the magnitude of the positive effects on plant growth can vary with bacterial strains, plant species, and soil properties. PGPB-related increases in plant biomass were concomitant with accumulation of NaCl in foliar tissue and decreases in soil EC_e levels, which indicates the potential for *in situ* phytoremediation of saline soils using the system reported here.

CONCLUSIONS

This research was undertaken to test the ability of PGPB that produce high levels of ACC deaminase and IAA to promote plant growth under salt stress conditions. Two

indigenous PGPB isolated for this study (CMH2 and CMH3) and two exogenous PGPB previously isolated (UW3 and UW4) improved shoot and/or root growth of barley and oats in saline soils under greenhouse and/or field conditions. This work suggests the potential for using PGPB-treated plants for farming on naturally salinized land, and for phytoremediation of salt-impacted soils that are the result of oil and gas exploration and processing.

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REFERENCES

Alberta Environment, Environmental Sciences Division, Environmental Service. 2001. Salt contamination assessment & remediation guidelines. Pub. No. T/606.

Antoun H, Prévost D. 2005. Ecology of plant growth promoting rhizobacteria. In: Siddiqui ZA, ed. PGPR: Biocontrol and biofertilization, Dordrecht, The Netherlands: Springer. p. 1–38.

APHA. 1995. Standard methods for the examination of water and waste water. 19th ed. Washington, DC: American Public Health Association.

Bacilio M, Rodriguez H, Moreno M, Hernandez JP, Bashan Y. 2004. Mitigation of salt stress in wheat seedlings by a gfp-tagged *Azospirillum lipoferum*. Biol Fertil Soils. 40:188–193.

Barassi CA, Ayrault G, Creus CM, Sueldo RJ, Sobrero MT. 2006. Seed inoculation with *Azospirillum* mitigates NaCl effects on lettuce. Sci Hortic. 109:8–14.

Barriuso J, Solano BR, Fray RG, Camara M, Hartmann A, Manero FJG. 2008. Transgenic tomato plants alter quorum sensing in plant growth-promoting rhizobacteria. Plant Biotechnol J. 6:442–452.

Bashan Y, de-Bashan LE. 2010. How the plant growth-promoting bacterium *Azospirillum* promotes plant growth - A critical assessment. Adv Agron. 108:77–136.

Bhattarai T, Hess D. 1993. Yield Responses of Nepalese Spring Wheat (*Triticum aestivum* L) Cultivars to Inoculation with *Azospirillum* spp. of Nepalese Origin. Plant Soil. 151:67–76.

Blumwald E. 2000. Sodium transport and salt tolerance in plants. Curr Opin Cell Biol. 12:431–434.

Cheng Z, Park E, Glick BR. 2007. 1-Aminocyclopropane-1-carboxylate deaminase from *Pseudomonas putida* UW4 facilitates the growth of canola in the presence of salt. Can J Microbiol. 53:912–918.

Cuartero J, Fernandez-Munoz, R. 1999. Tomato and salinity. Sci Hortic 78(1–4): 83–125.

de-Bashan LE, Hernandez J-P, Bashan Y. 2012. The potential contribution of plant growth-promoting bacteria to reduce environmental degradation - A comprehensive evaluation. Appl Soil Ecol. 61:171–189.

del Amor FM, Cuadra-Crespo P. 2012. Plant growth-promoting bacteria as a tool to improve salinity tolerance in sweet pepper. Funct Plant Biol. 39:82–90.

Doughari, HJ, Ndakidemi PA, Human IS, Benade S. 2011. The ecology, biology and pathogenesis of *Acinetobacter* spp.: an overview. Microbes Environ. 26:101–112.

Duan J, Jiang W, Cheng Z, Heikkila JJ, Glick BR. 2013. The complete genome sequence of the plant growth-promoting bacterium *Pseudomonas sp*. UW4. PLoS ONE. 8(3): e58640. doi:10.1371/journal.pone.0058640.

Dworkin M, Foster JW. 1958. Experiments with some microorganisms which utilize ethane and hydrogen. J Bacteriol. 75:592–603.

Flowers T. 2004. Improving crop salt tolerance. J Exp Bot. 55:307–319.

Gerhardt KE, Greenberg BM, Glick BR. 2006. The role of ACC deaminase in facilitating the phytoremediation of organics, metals and salt. Curr Trends Microbiol. 2:61–72.

Gerhardt KE, Huang X-D, Glick BR, Greenberg BM. 2009. Phytoremediation of organic soil contaminants: Potential and challenges. Plant Sci. 176:20–30.

Germida JJ, Walley FL. 1996. Plant growth-promoting rhizobacteria alter rooting patterns and arbuscular mycorrhizal fungi colonization of field-grown spring wheat. Biol Fertil Soils. 23:113–120.

Glass D. 1999. U.S. and International Markets for Phytoremediation, *1999–2000*. Needham, (MA): D. Glass Associates, Inc.

Glick BR, Karaturovic DM, Newell PC. 1995. A novel procedure for rapid isolation of plant growth promoting pseudomonads. Can J Microbiol. 41:533–536.

Glick BR, Liu CP, Ghosh S, Dumbroff EB. 1997. Early development of canola seedlings in the presence of the plant growth-promoting rhizobacterium *Pseudomonas putida* GR12–2. Soil Biol Biochem. 29:1233–1239.

Glick BR, Penrose DM, Li J. 1998. A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. J Theor Biol. 190:63–68.

Gordon SA, Weber RP. 1951. Colorimetrie estimation of indoleacetic acid. Plant Physiol. 26:192–195.

Greenberg BM, Huang X-D, Gerwing P, Yu X-M, Chang P-C, Wu SS, Gerhardt K, Nykamp J, Lu X, Glick B. 2008. Phytoremediation of salt-impacted soils: greenhouse and the field trials of plant growth promoting rhizobacteria (PGPR) to improve plant growth and salt phytoaccumulation. In: Proceedings of the 33rd AMOP Technical Seminar on Environmental Contamination and Response. Environment Canada, Ottawa, ON. 2: 627–637.

Guo Y, Zheng H, Yang Y, Wang H. 2007. Characterization of *Pseudomonas corrugata* Strain P94 isolated from soil in Beijing as a potential biocontrol agent. Curr Microbiol. 55:247–253.

Gurska J, Wang W, Gerhardt KE, Khalid AM, Isherwood DM, Huang X-D, Glick BR, Greenberg BM. 2009. Three year field test of a plant growth promoting rhizobacteria enhanced phytoremediation system at a land farm for treatment of hydrocarbon waste. Environ Sci Tech. 43:4472–4479.

Hall JA, Peirson D, Ghosh S, Glick BR. 1996. Root elongation in various agronomic crops by the plant growth promoting rhizobacterium *Pseudomonas putida* GR12–2. Isr J Plant Sci. 44:37–42.

Hall MA, Moshkov IE, Novikova GV, Mur LA, Smith AR. 2001. Ethylene signal perception and transduction: multiple paradigms? Biol Rev Cambridge Philos Soc. 76:103–128.

Hamdia ABE, Shaddad MAK, Doaa MM. 2004. Mechanisms of salt tolerance and interactive effects of *Azospirillum brasilense* inoculation on maize cultivars grown under salt stress conditions. Plant Growth Regul. 44:165–174.

Holguin G, Glick BR. 2001. Expression of the ACC deaminase gene from *Enterobacter cloacae* UW4 in *Azospirillum brasilense*. Microb Ecol. 41:281–288.

Honma M, Shimomura T. 1978. Metabolism of 1-aminocyclopropane-1-carboxylic acid. Agricult Biol Chem. 42:1825–1831.

Hontzeas N, Richardson AO, Belimov A, Safronova V, Abu-Omar MM, Glick BR. 2005. Evidence for horizontal transfer of 1-aminocyclopropane-1-carboxylate deaminase genes. Appl Environ Microbiol. 71:7556–7558.

Huang X-D, El-Alawi Y, Gurska J, Glick BR, Greenberg BM. 2005. A multi-process phytoremediation system for decontamination of persistent total petroleum hydrocarbons (TPHs) from soils. Microchem J. 81:139–147.

Huang XD, El-Alawi Y, Penrose DM, Glick BR, Greenberg BM. 2004. A multi-process phytoremediation system for removal of polycyclic aromatic hydrocarbons from contaminated soils. Environ Pollut. 130:465–476.

Huang XD, El-Alawi Y, Penrose DM, Glick BR, Greenberg BM. 2004. Responses of three grass species to creosote during phytoremediation. Environ Pollut. 130:453–463.

Li JP, Ovakim DH, Charles TC, Glick BR. 2000. An ACC deaminase minus mutant of *Enterobacter cloacae* UW4 no longer promotes root elongation. Curr Microbiol. 41:101–105.

Mayak S, Tirosh T, Glick BR. 2004. Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. Plant Physiol Biochem. 42:565–572.

Nadeem SM, Zahir ZA, Naveed M, Arshad M. 2007. Preliminary investigations on inducing salt tolerance in maize through inoculation with rhizobacteria containing ACC deaminase activity. Can J Microbiol. 53:1141–1149.

O'Donnell PJ, Calvert C, Atzorn R, Wasternack C, Leyser HMO, Bowles DJ. 1996. Ethylene as a signal mediating the wound response of tomato plants. Science. 274:1914–1917.

Pandey A, Palni L. 1998. Isolation of *Pseudomonas corrugata* from Sikkim Himalaya. World J Microbiol Biotechnol. 14:411–413.

Parida, AK, Das AB. 2005. Salt tolerance and salinity effects on plants: a review. Ecotoxicol Environ Safety. 60:324–349.

Patten CL, Glick BR. 2002. Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. Appl Environ Microbiol. 68:3795–3801.

Penrose DM, Glick BR. 2003. Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. Physiol Plant. 118:10–15.

Petruzzelli L, Coraggio I, Leubner-Metzger G. 2000. Ethylene promotes ethylene biosynthesis during pea seed germination by positive feedback regulation of 1-aminocyclo-propane-1-carboxylic acid oxidase. Planta. 211:144–149.

Principe A, Alvarez F, Castro MG, Zachi L, Fischer SE, Mori GB, Jofre E. 2007. Biocontrol and PGPR features in native strains isolated from saline soils of Argentina. Curr Microbiol. 55:314–322.

Qadir M, Steffen, D, Yan F, Schubert S. 2003. Sodium removal from a calcareous saline-sodic soil through leaching and plant uptake during phytoremediation. Land Degrad Develop. 14:301.

Qadir M, Oster JD. 2002. Vegetative bioremediation of calcareous sodic soils: history, mechanisms, and evaluation. Irrigation Sci. 21:91–101.

Rabie GH, Almadini AM. 2005. Role of bioinoculants in development of salt-tolerance of *Vicia faba* plants under salinity stress. Afr J Biotechnol. 4:210–222.

Saravanakumar D, Samiyappan R. 2007. ACC deaminase from *Pseudomonas fluorescens* mediated saline resistance in groundnut (*Arachis hypogea*) plants. J Appl Microbiol. 102:1283–1292.

Shah S, Li JP, Moffatt BA, Glick BR. 1998. Isolation and characterization of ACC deaminase genes from two different plant growth-promoting rhizobacteria. Can J Microbiol. 44:833–843.

Shibli RA, Kushad M, Yousef GG, Lila MA. 2007. Physiological and biochemical responses of tomato microshoots to induced salinity stress with associated ethylene accumulation. Plant Growth Regulation. 51:159–169.

U.S. EPA. 1996. Method 6020A, Inductively coupled plasma-mass spectrometry. Washington (DC). Test methods for Evaluating Solid Waste: Physical/Chemical Methods, SW-846.

Wiebe BH, Eilers RG, Eilers WD, Brierley JA. 2006. The presence and extent of moderate to severe soil salinity on the Canadian Prairies. In: Proceedings of the Manitoba Soil Science Society Annual Meetings, Winnipeg, MB.

Yildrim E, Donmez MF, Turan M. 2008. Use of bioinoculants in ameliorative effects on radish plants under salinity stress. J Plant Nutr. 31:2059–2074.

Yue HT, Mo WP, Li C, Zheng YY, Li H. 2007. The salt stress relief and growth promotion effect of Rs-5 on cotton. Plant Soil. 297:139–145.