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ORIGINAL PAPER

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Mitigation of salt stress in wheat seedlings by a *gfp*-tagged *Azospirillum lipoferum*

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Abstract Root colonization and mitigation of NaCl stress on wheat seedlings were studied by inoculating seeds with *Azospirillum lipoferum* JA4::*ngfp*15 tagged with the green fluorescent protein gene (*gfp*). Colonization of wheat roots under 80 and 160 mM NaCl stress was similar to root colonization with this bacterial species under non-saline conditions, that is, single cells and small aggregates were mainly located in the root hair zone. These salt concentrations had significant inhibitory effects on development of seedlings, but not on growth in culture of *gfp-A. lipoferum* JA4::*ngfp*15. Reduced plant growth (height and dry weight of leaves and roots) under continuous irrigation with 160 mM NaCl was ameliorated by bacterial inoculation with *gfp-A. lipoferum* JA4::*ngfp*15. Inoculation of plants subjected to continuous irrigation with 80 mM NaCl or to a single application of either NaCl concentration (80 or 160 mM NaCl) did not mitigate salt stress. This study indicates that, under high NaCl concentration, inoculation with modified *A. lipoferum* reduced the deleterious effects of NaCl; colonization patterns on roots were unaffected and the genetic marker did not induce undesirable effects on the interaction between the bacterium and the plants.

Keywords *Azospirillum* · *gfp* · Plant growth-promoting bacteria · PGPR · Salt stress mitigation

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Introduction

Cultivated soils worldwide are becoming more saline from marginal irrigation water, excessive fertilization, and desertification processes. Impacted soils are a major limiting production factor worldwide for every major crop (Shannon and Grieve 1999). Strategies for alleviation of salt stress involve developing salt-resistant cultivars, leaching excess soluble salts from upper to lower soil depths, flushing soils that contain salt crusts at the surface, reducing salt by harvesting salt-accumulating aerial plant parts in areas with negligible irrigation water or rainfall for leaching, and amelioration of saline soils under cropping and leaching (most successful; Qadir et al. 2000). An experimental alternative is to alleviate salt stress by inoculating crops seeds and seedlings with various plant growth-promoting bacteria (PGPB), such as *Rhizobium* and *Azospirillum* spp. and also with mycorrhizal fungi.

Apart from *Azospirillum halopraeferens*, which tolerates high salinity (Reinhold et al. 1987), colonizes mangrove roots in seawater (Puente et al. 1999), and enhances the growth of halophytes irrigated with seawater (Bashan et al. 2000), most strains of *Azospirillum* sp. can tolerate only limited levels of salt (NaCl). *A. brasilense* Cd (the wild-type strain for this species) can tolerate 2% NaCl when co-cultured with *Staphylococcus* sp. (Holguin and Bashan 1996). The common cellular mechanism of osmotic-stress adaptation is intracellular accumulation of organic solutes (osmolytes; Choi and Gal 1998; Tripathi et al. 1998). However, decreasing diversity of native azospirilla is apparently a consequence of increased salinity of soils (Saleena et al. 2002). Salt tolerance in *Azospirillum* has been ranked *A. halopraeferens* > *A. brasilense* > *A. lipoferum* > *A. amazonense* (Hartmann et al. 1991).

Relative to the tolerance of these bacteria to saline conditions, none of the most important crops tolerates high levels of salts, and NaCl is the most destructive salt affecting plant growth. Inoculation of crops with PGPB may assist growth by alleviating negative effects of salt. Inoculation with *Rhizobium* has shown effectiveness under

saline conditions (Cordovilla et al. 1996, 1999), and cultivation of chickpeas and fava beans inoculated with *Azospirillum* and irrigated with saline water performed better than plants that were not inoculated (Hamaoui et al. 2001). To verify that the effects on plant growth resulted from inoculation, there is need to demonstrate that seeds inoculated with bacterial strains yield colonized roots. In recent years, methods for identifying colonization of *Azospirillum* on roots have been developed and among them is the molecular insertion of marker genes (Vande Broek et al. 1993; Assmus et al. 1995; Jacoud et al. 1998; Fischer et al. 2000). One approach is the insertion of the green fluorescent protein (*gfp*) marker into *Azospirillum* (Xi et al. 1999; Ramos et al. 2002) and other bacterial chromosomes and plasmids (Errampalli et al. 1999; Unge and Jansson 2001).

This study evaluated whether a common *A. lipoferum* strain, tagged with the *gfp* gene inserted into its chromosome, was capable of mitigating negative effects of salt on plant growth and colonizing wheat roots grown in sand containing salt.

Materials and methods

Organisms and growth conditions

*Azospirillum lipoferum*strain JA4::*ngfp*15 served as a model bacterium. This strain was obtained by tagging the wild-type strain *A. lipoferum* JA4 with the *gfpmut2* gene that codifies the green fluorescent protein molecular marker, under the constitutive *npt2* promoter, by transposon mutagenesis (Rodríguez and Bashan 2002). Bacteria were cultivated in 250-ml Erlenmeyer flasks containing 50 ml TYG medium (Bashan et al. 2002) at 30±2°C, 120 rpm for 18 h (incubator shaker series 25; New Brunswick, Edison, N.J.). Bacteria were harvested by centrifugation at 4,000*g*for 20 min and rinsed three times in 0.06 M, pH 7.0 phosphate buffer solution supplemented with 0.15 M NaCl (PBS; Bashan et al. 1993). A wheat cultivar (*Triticum aestivum* cv. Rayon), whose growth is promoted by inoculation with *Azospirillum* spp. (Bashan et al. 2002; Bacilio et al. 2003), served as a model. Seeds were disinfected with 2% Tween-20 (Sigma) for 10 min, rinsed, soaked in 3% commercial NaOCl for 5 min, and then thoroughly washed in sterile tap water for 10 min. The disinfected seeds were immersed in a bacterial suspension (10^6 cfu ml^{-1} in 0.85% saline solution) and subjected to a vacuum of 600 mm Hg for 5 min. The vacuum was released abruptly to allow penetration of bacteria into the seeds' cavities (Bashan 1986; Puente and Bashan 1993). Tests for colonization were performed on seedlings grown from inoculated seeds at the level of 10^7 cfu ml^{-1}.

Plant growth conditions

Dry riverbed sand, sorted to 10-mesh particles, was collected in the CIB desert conservation area, 20 km northwest of La Paz, Baja California Sur, Mexico. Sand was washed with 20-l bottled drinking water to remove debris, then sterilized in an oven at 160°C for 48 h. Seeds were sown in 200-ml white, round plastic pots containing 180 g sand. Each pot contained five seedlings grown from seeds sown at an even distance from each other at 5 mm depth. The control treatment was sand saturated with 50% standard Hoagland's nutrient solution prior to the start of the experiment. Other treatments were potted sand irrigated once with 50% Hoagland's nutrient solution supplemented with 80 or 160 mM NaCl. Two modes of supplying NaCl were used. Sand was irrigated once with 80 or 160 mM NaCl (final concentrations) before sowing and the seedlings were irrigated later with distilled water every 2–4 days until the sand substrate was saturated, depending on the size of the seedlings. Alternatively, pots were irrigated at the same intervals, but with distilled water supplemented with 80 or 160 mM NaCl. Plants were raised in a growth chamber (Conviron, Winnipeg, Canada) at 26±1°C, 200 μmol m^{-2} s^{-1} light intensity, and 70% relative humidity. Treatments were carried out for 21 days, with weekly samplings of the seedlings (described later) and measurements of soil conductivity and Na^+ ion concentration (conductivity meter CO150, Hach, Loveland, Co.; atomic absorption model Avanta, GBC Atomic Absorption, Australia, respectively).

To verify that fluorescence observed on roots was from the *gfp*-tagged strain and not from native soil bacteria, an additional experiment was carried out in large 70-ml test tubes filled with sterile and non-sterile sand (identical to the pot treatment and growth medium) with a cotton plug seal to avoid external contamination. Five seedlings were grown inside the tubes for 21 days under identical growth conditions as the pot treatments. During cultivation, 5 ml sterile water was added twice. At extraction time, roots were prepared for immediate observation under fluorescent microscopy and counting of bacteria colonizing the roots.

Extraction of plants and dry weight determination

After 7, 14, and 21 days, the height of each plant shoot was measured. Plants were carefully removed from the sand. The roots in each pot were excised and gently washed with tap water to eliminate sand particles. Plants from each pot were dried with a paper towel to eliminate excess water. Roots and shoots were dried separately for 2 days in a forced air oven at 70°C. After drying, plants were placed in hermetically sealed desiccators to avoid absorption of humidity from the air. Each sample group was weighed on an analytical balance; each measurement was the weight of all plants in one pot.

Bacterial counts and location on roots

Chromosomally, *gfp*-labeled *A. lipoferum* JA4::*ngfp*15 was detected on roots using a fluorescent microscope (Olympus BX41 equipped with fluorescence accessories series 2B, with excitation at 450–480 nm, a blue filter U-MW 640, and a digital camera—CoolSnap-Pro Color, Japan) attached to an image analyzer (Image ProPlus 4.5, Media Cybernetics, Japan). The pale yellow autofluorescence of roots was distinguishable from the green fluorescence created by the *gfp*-tagged *A. lipoferum*.

Bacteria counts from roots were made by the standard plate count method described earlier (Puente et al. 1999). After homogenization of the roots in 0.85% NaCl solution by a root disruptor (Polytron PT1200, Kinematica AG, Switzerland), serial dilutions of root homogenates were plated for counting bacteria. As the *gfp* insertion transposon also included resistance to tetracycline (Dandie et al. 2001), the nutrient agar (Sigma) used for plating was supplemented with 15 μg/ml tetracycline (Sigma). This limited bacterial morphotypes on the petri dishes to two. As the developed colonies are non-fluorescent to the naked eye, samples (ten colonies from each plate) from the two developed morphotypes were evaluated under a fluorescent microscope to verify which morphotype corresponds to the *gfp-A. lipoferum* JA-4 strain. Colonization of roots was expressed as colony forming units per milligram dry weight of roots. Resistance of the bacterium to salt was determined by growing the bacterium in nutrient broth supplemented with 80 or 160 mM NaCl and counting the developing bacterial colonies after incubation for 24 h at 30°C.

Experimental design and statistical analysis

All experiments were repeated three times. Each plant growth experiment had four or five replicates per treatment; five seedlings served as a single replicate. The pots were placed in the growth chamber in a random block design. Data were analyzed by one-way ANOVA and post hoc analysis (Tukey HSD) at $P \leq 0.05$. All analyses were made with Statistica software (StatSoft, Tulsa, Okla.). Data are accompanied by standard error bars.

Results

Root colonization by *gfp*-tagged *A. lipoferum* and level of colonization under saline stress

When *gfp*-tagged *A. lipoferum* JA4::*ngfp*15 (Fig. 1a) colonized wheat roots under normal growth conditions (data not shown) and saline conditions of 80 or 160 mM NaCl (Fig. 1c), the mode of colonization was single-cell and small-aggregate colonization. Cells were located mainly in the root hair zone. Fluorescence was not

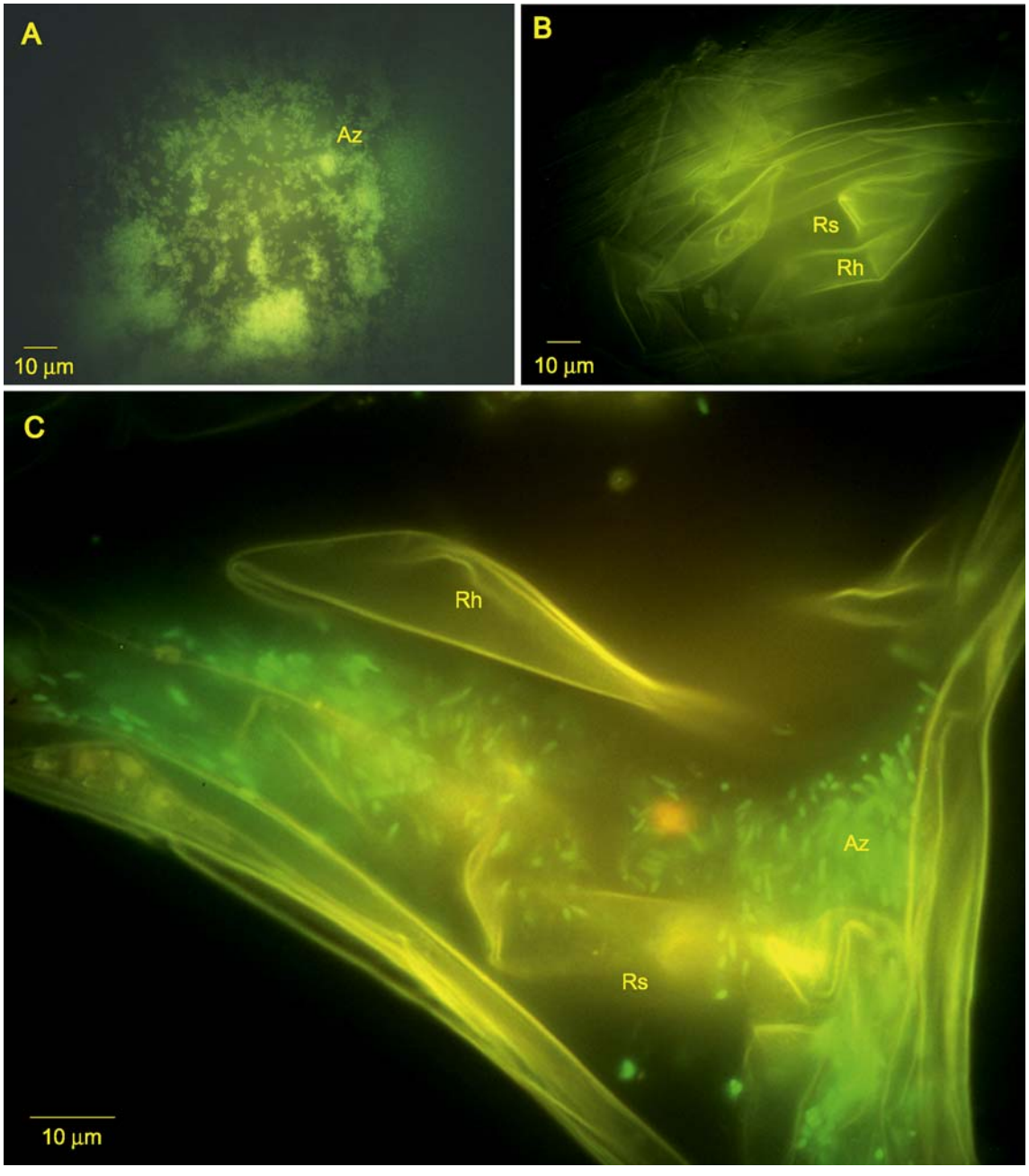

Fig. 1a–c Pattern of wheat root colonization with *Azospirillum lipoferum* JA4::*ngfp*15 tagged with the green fluorescent protein gene. **a** Inoculum (similar: verification of fluorescent colony isolated from inoculated roots); **b** roots of control growing in soil without inoculation (similar: inoculated plants with unmodified *A. lipoferum* JA-4); **c** root colonization of the root hair zone under saline conditions (*Az Azospirillum lipoferum* JA4::*ngfp*15, *Rs* root surface, *Rh* root hair, *bars* represent 10 µm)

detected in the sand where plants grew (data not shown), on roots of controls without inoculation in the same sand (Fig. 1b), or on roots of plants inoculated with the unmodified strain of *A. lipoferum* JA4 (data not shown). Colonies isolated from inoculated roots and grown on growth medium showed similar fluorescence to the inoculant used (data not shown). Root colonization under saline conditions was $1\pm0.1\times10^{7}$ cfu g^{-1} dw after 11 days and $1\pm0.1\times10^{5}$ cfu g^{-1} dw after 21 days. NaCl level (80 and 160 mM) did not affect the growth of bacteria in liquid medium, reaching 5×10^{9} cfu ml^{-1} after 24 h.

Inoculation of plants with *Azospirillum* under continuous saltwater irrigation

Plant height and leaf and root dry weights were measured in seedlings inoculated with *Azospirillum* and in seedlings that were not inoculated. Plants were irrigated with either 80 or 160 mM NaCl every 4 days during the first week and every 2 days during the last two weeks. Inoculation did not affect the three growth parameters in the absence of NaCl (Fig. 2a, d, g). In plants that were not inoculated, under either concentration of NaCl, there was a significant decline in plant growth parameters. In the presence of continuously irrigated saline water (80 and 160 mM NaCl), plant height was significantly greater in inoculated plants than the ones without inoculation, and about equal to the height of plants grown without salt (Fig. 2b, c). After 21 days, leaf and root dry weight of inoculated plants significantly increased in the higher-level NaCl treatment compared to controls without inoculation growing under similar saline conditions. Growth values were lower than those of controls that were not treated with salt (Fig. 2f, I). As a result of continuous irrigation with NaCl, Na^{+} and electrical conductivity of the sand substrate gradually increased (Table 1).

Contrary to treatments with continuous salt irrigation, when salt (80 and 160 mM NaCl) was applied in a single irrigation prior to sowing inoculated seeds and then followed by irrigation with distilled water for 21 days, no growth enhancement in these inoculated plants occurred, and no recovery from mild salt stress was induced (data not shown). As yet, we have no additional data to explain this.

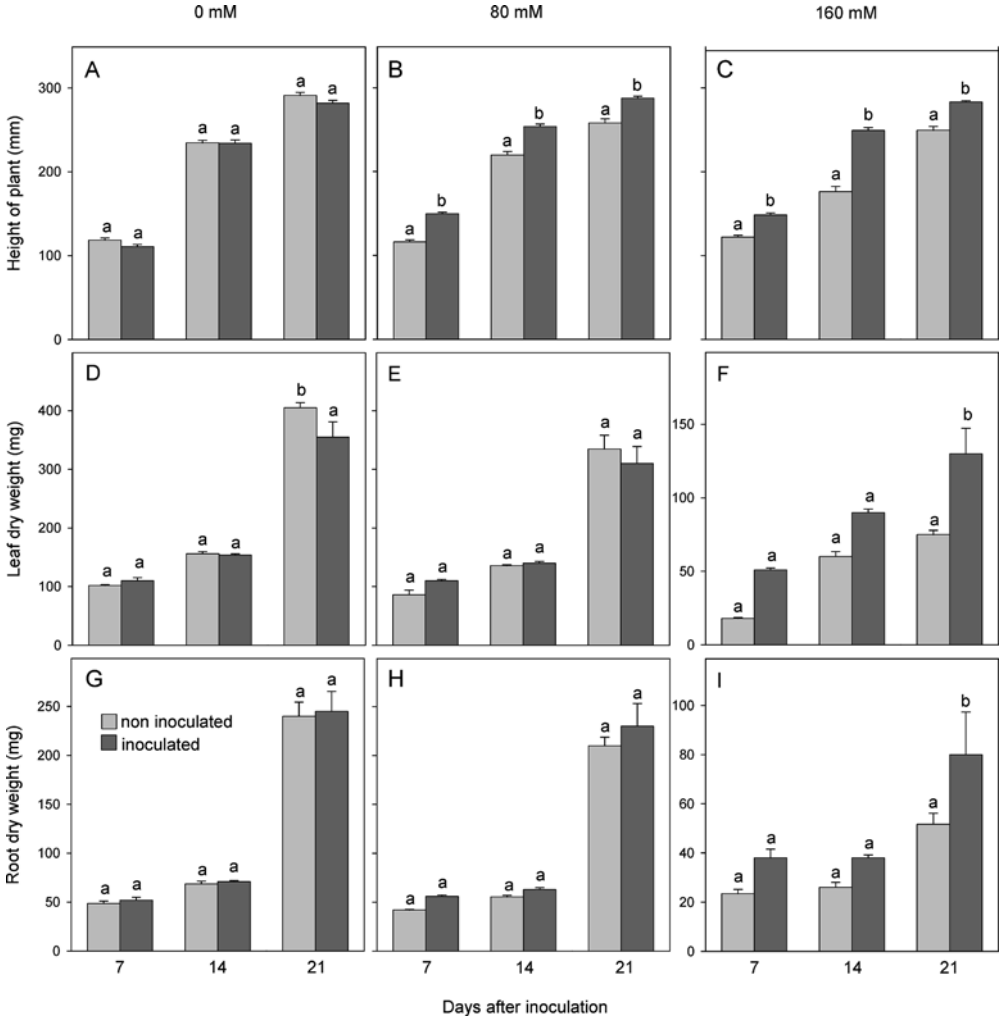

Fig. 2A–I Effect of wheat plants' inoculation with *Azospirillum lipoferum* JA4::*ngfp*15 under continuous irrigation with either of two concentrations of NaCl on plant growth parameters over 21 days. **A**–**C** Height; **D**–**F** leaf dry weight; and **G**–**I** root dry weight. *Columns denoted by a different letter* in each subfigure differ significantly at $P \leq 0.05$ by one-way ANOVA. *Bars* represent SE

Table 1 Na^+ ion concentration and electrical conductivity of sand used in experiments under continuous irrigation with either of two concentrations of NaCl. Values for each day after inoculation, separately, *denoted by a different letter* differ significantly at $P \leq 0.05$ by one-way ANOVA ± SE

Days after inoculation	Hoagland's nutrient solution + NaCl concentration (mM)	Na^+ concentration (μg/mg)	Electrical conductivity (μS/cm)
7	0	24±3 a	102±9 a
	80	52±8 b	172±27 b
	160	54±5 b	209±14 b
14	0	39±8 a	133±20 a
	80	102±13 b	322±46 b
	160	167±27 b	457±49 c
21	0	47±7 a	155±18 a
	80	86±18 b	265±42 b
	160	160±32 c	538±134 c

Discussion

A. brasilense and *A. lipoferum* are known to have remarkable salinity tolerance (Rivarola et al. 1998), exceeding the tolerance of wheat seedlings. These bacteria can colonize roots under saline conditions. Inoculation of plants with *Azospirillum* spp. may alleviate external stresses imposed on the plant by water scarcity, excessive fertilization, and salinity. Subjecting inoculated sorghum plants to potential osmotic stress in hydroponic systems diminished some of the adverse effects (reduction in leaf senescence) caused by osmotic stress (Sarig et al. 1990). Inoculation with *Azospirillum* spp. promotes germination of wheat grown in soil-compost mixtures that otherwise inhibit seed germination, and inoculated plants develop better than untreated plants (Bacilio et al. 2003). Maize plants inoculated with *Azospirillum* sp. then cultured at high NaCl concentrations showed significantly increased chlorophyll, K, Ca, soluble saccharides, and protein content compared with controls growing without NaCl (Hamdia and El-Komy 1997).

Interactions between *Azospirillum* sp. and many plant species under saline stress have yielded contradictory evidence; some interactions were beneficial and some were somewhat negative. Saline stress was shown to alter the interaction between *A. brasilense* Cd and maize and wheat—normal colonization patterns changed to inadequate colonization and N_2-fixation was affected by an alteration of the expression of *A. brasilense nif* gene promoters. While *nifA* expression increased in stressed bacteria, *nifH* transcription was diminished (Jofré et al. 1998a, b). In these studies, the attachment of *A. brasilense* Cd to maize and wheat roots was altered when the bacteria were grown under saline stress conditions. Alteration in the adsorption phase of attachment appeared to be related to the disappearance of a 100-kDa external membrane protein in the bacterium. Gene expression in *A. brasilense* was influenced by plant root exudates; wheat root exudates induced the reappearance of the 100-kDa protein (Fischer et al. 1999). Unstressed bacteria grown under standard conditions were distributed along the entire root system of wheat except the elongation zone. Bacteria subjected to saline stress were mainly located on root tips and lateral roots, but salt treatment reduced surface colonization (Fischer et al. 2000). However, *A. halopraeferens* and *A. brasilense* were capable of colonizing roots of mangrove trees at very high concentrations of seawater, similar to colonization of crop plants (Puente et al. 1999). The colonization pattern of wheat roots in this study was not different from colonization of wheat roots that were unaffected by salt, both in appearance (single cells and small aggregates) and population levels. Colonization under NaCl stress resembled numerous colonization studies of cereal roots without NaCl (for review, Bashan and Holguin 1997). These contradictions may originate from the numerous variables employed in the previous studies (different plant genotypes, technical aspects of the experiments, and different plant parameters). Therefore, this study does not necessarily reject, as artifacts, the negative effects of salt on root colonization observed in previous studies.

As support for our study, Creus et al. (1997) showed that wheat seedlings inoculation with wild-type strain *A. brasilense* and exposed to severe salt (NaCl) or osmotic (polyethylene glycol) stresses significantly reversed part of the negative effects both stresses produced on the relative elongation rate of shoots.

An explanation for the ameliorating phenomenon induced by *Azospirillum* spp. in wheat seedlings under NaCl stress might be that the bacterium enhanced water uptake. Creus et al. (1998) found that turgor pressure at low water potential was higher in inoculated seedlings in two wheat cultivars under osmotic stress. This could be the result of better water uptake induced by inoculation that, in turn, is reflected in faster shoot growth in inoculated seedlings exposed to these stresses.

In summary, a genetically tagged strain of *A. lipoferum* was capable of colonizing the roots of wheat seedlings that were under NaCl stress, similar to unmodified bacteria. Chromosomal insertion of the marker gene in the bacterium did not change the basic characteristics of plant-bacteria interaction, and inoculation with the modified bacteria alleviated NaCl stress in wheat seedlings.

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