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Effect of inoculation with plant growth-promoting bacteria (PGPB) on amelioration of saline stress in maize (Zea mays)

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ABSTRACT

Our objective was to evaluate the role of *Azotobacter* strains to protect maize plants against salt damage. Four candidate *Azotobacter* strains were evaluated, and the two most tolerant to salinity (C5 and C9) were selected for further studies. They were phylogenetically related to *Azotobacter chroococcum* based on their 16S rDNA sequences. Strains were inoculated on maize roots growing in sterilized soil under different salinity conditions (0, 2.93 and 5.85 g NaCl/kg soil). After 4 weeks plant biomass (length and weight), ion uptake (Na^+, K^+, Ca^{2+}, Mg^{2+}), chlorophyll content, and accumulation of proline and polyphenols were evaluated. Strains C5 and C9 fixed nitrogen and solubilized phosphate regardless of NaCl concentration in most cases, while auxins were synthesized by C5 only under conditions of salinity. In pot experiments, plant growth was promoted by bacterial inoculation only at 2.93 and 5.85 g NaCl/kg soil ($P < 0.05$). Bacteria improved Na^+ exclusion and K^+ uptake in maize, thereby increasing their K^+/Na^+ ratio. Content of polyphenol and chlorophyll was enhanced by inoculation with strains C5 and C9. The concentration of proline in leaves was increased by salinity, but was decreased when C5 and C9 were used as inoculants. The present observations showed that strains C5 and C9 partially alleviated the saline stress in maize, likely through the integration of several mechanisms that improve the plant response. Hence, the use of nitrogen-fixing plant growth-promoting bacteria may represent an important biotechnological approach to decrease the impact of salinity in crops.

1. Introduction

Salinity is one major limiting factor to plant growth and crop productivity (Allakhverdiev et al., 2000). In most saline soils, sodium chloride is the predominant salt species, and its effect can be observed by decreased productivity or plant death (Munns and Tester, 2008). Soil salinity causes plant stress in two ways: (1) making water uptake by the roots more difficult, and (2) causing plant toxicity via accumulation of high salt concentrations in the plant (Munns and Tester, 2008). Several biochemical processes can be affected by salinity, including protein synthesis, photosynthesis, and lipid metabolism (Parida and Das, 2005). However, most plants possess several mechanisms to decrease the negative effects of salinity including regulation and compartmentalization of ions, synthesis of compatible solutes, induction of antioxidative enzymes, induction of plant hormones, and changes in photosynthetic pathways (Cheeseman, 1988; Parida and Das, 2005).

Currently, more than 800 million hectares of land throughout the world are affected by levels of salt that could substantially reduce crop productivity (Munns and Tester, 2008). Suboptimal irrigation can result in further damage, caused by salinity in irrigation waters, on several important agricultural crops. For instance, maize is considered to be a moderately salt-sensitive plant (Zörb et al., 2004), and under irrigation, it can be subjected to salt toxicity (Fu et al., 2010). Several strategies have been developed in order to decrease the toxic effects caused by high salinity on plant growth, including plant genetic engineering (Wang et al., 2003), and recently the use of plant growth-promoting bacteria (PGPB) (Dimkpa et al., 2009).

PGPB are usually defined as microorganisms that can grow in, on, or around plant tissues, stimulating plant growth by a variety of mechanisms (Vessey, 2003). These mechanisms and their effects can be classified as direct or indirect. The direct mechanisms are associated with an increase in availability of nutrients and include biological nitrogen fixation (BNF) (Graham and Vance, 2000), phosphate solubilization and mineralization (Rodríguez et al., 2007), siderophore production (Neilands, 1993), and synthesis of plant hormones such as indole, cytokinins, or gibberellins (Costacurta and Vanderleyden, 1995). Utilization of PGPB has become a

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promising alternative to alleviate plant stress caused by salinity (Fu et al., 2010; Mayak et al., 2004; Shilev et al., 2010; Yao et al., 2010). *Pseudomonas fluorescens* biotype F and *P. fluorescens* CECT 378^T increased fresh weight of sunflowers by more than 10% under saline conditions (100 mM NaCl), and similarly improved the K^+/Na^+ ratio (Shilev et al., 2010). Studies showed that inoculation with *Azospirillum* spp. increased plant growth and the K^+/Na^+ ratio of two maize cultivars *cv*. 323 and *cv*. 324 (Hamdia et al., 2004). Moreover, Yao et al. (2010) reported that inoculation with *Pseudomonas putida* Rs-198 promoted cotton growth and germination under conditions of salt stress.

Hence, the main purposes of this research were: (1) to focus on the identification of the *Azotobacter* sp. strains C5 and C9 with known plant protection against salt stress, (2) to study the effect of PGPB on plant growth in the presence and absence of salt stress, and (3) to evaluate the influence of bacteria on uptake of ions (Na^+, K^+, Ca^{2+}, Mg^{2+}) and accumulation of proline, total polyphenols, and chlorophyll in maize.

2. Materials and methods

2.1. Strains and culture conditions

In this study, four presumptive strains of *Azotobacter* sp.: C5, C7, C8, and C9, were studied. These were previously selected by their potential as biofertilizers (data not shown), and were provided by Laboratorio de Microbiología de Suelos of Corpoica, Colombia. Strains were isolated in Provincia de Ricaurte, Boyacá, Colombia (5°38′02.69″N 73°31′24.02″O, elevation 2142 m). The standard culture conditions for incubation were 28 ± 2 °C and 150 rpm. Bacterial maintenance utilized Ashby medium (in g/L: mannitol 10, K_2HPO_4 0.2, $MgSO_4{\cdot}7H_2O$ 0.2, NaCl 0.2, $CaSO_4$ 0.1, $CaCO_3$ 10.0, agar 15.0, pH 7.5). Morphological characteristics, such as gram reaction, cell shape, and cyst formation, were examined after incubation in nitrogen-free Ashby culture medium for 3 days using an optic microscope (Olympus, Japan). Pigmentation was also observed after 3 days of incubation in the same culture media. For catalase assay, one bacterial colony and one drop of 3% hydrogen peroxide (Merck, USA) were used, the appearance of bubbles was considered as positive. The API20NE kit (bioMérieux, France) was used for the evaluation of hydrolysis of urea, esculine, and gelatine hydrolysis; utilization of glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine, maltose, caprate, malate, citrate, and phenyl acetate; and for nitrate reduction to nitrite. All tests were performed in duplicate.

2.2. Effect of NaCl on bacterial growth

Tolerance of strains to NaCl was evaluated on Ashby modified broth (in g/L: mannitol 10, K_2HPO_4 0.2, $MgSO_4{\cdot}7H_2O$ 0.2, NaCl 0.2, $CaSO_4$ 0.1), supplemented with increasing NaCl concentrations ranging between 0 and 58.5 g/L. Flasks (125 mL) were incubated for 72 h at standard conditions. In addition, we studied the effect of NaCl (0, 2.93, and 5.85 g/L) on growth kinetics of the C5 and C9 strains employing culture flask (250 mL) containing 50 mL of Ashby modified broth. Flasks were inoculated with 500 μL of an overnight culture adjusted to OD_{600} = 0.500, and incubated for 72 h at standard conditions. The bacterial growth was monitored by measuring optical density at 600 nm.

2.3. Genetic characterization of strains

Bacterial DNA was extracted by using DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. Genomic DNA extracted was diluted in sterile Milli-Q water before conducting PCR analysis under the following conditions: a 25 μL PCR mixture contained 1× Taq DNA polymerase buffer (Invitrogen, USA), 2.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 25 pmol of each forward and reverse primers, 1 U of DNA polymerase (Invitrogen, USA), and 50 ng of genomic DNA as template. Nearly complete 16S rDNA genes were amplified with the forward primer 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and the reverse primer 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) (Gauri et al., 2009). The DNA was amplified with an iCycler thermocycler (BioRad, USA) with the following program: 2 min of pre-heating at 95 °C, 35 cycles of 30 s of denaturation at 95 °C, 30 s of primer annealing at 57 °C, 2 min of elongation at 72 °C, and 10 min of extension step at 72 °C. Amplicon size was confirmed to be as expected by using agarose gel electrophoresis (1.5% w/v) in TAE buffer (0.04 M Tris acetate, 0.001 M EDTA) containing 1 μg mL^{-1} ethidium bromide. The amplified DNA was purified using the PureLink™ Quick Gel Extraction Kit (Invitrogen, USA) according to the manufacturer's instructions. Automated sequencing of the purified PCR products was performed using the BigDye terminator cycle sequencing kit and the ABI 310 DNA Sequencer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Partial sequences obtained were matched against nucleotide sequences present in GenBank using the BLASTn program and deposited in the EMBL-EBI/GenBank database.

2.4. Plant growth-promoting (PGP) features

Measurement of plant growth-promoting features was carried out at 0, 2.93, and 5.85 g NaCl/L. Each experiment was performed in triplicate at two different times.

2.4.1. Biological nitrogen fixation

Flasks (250 mL) containing 50 mL free-nitrogen Ashby medium were inoculated with 25 μL of bacterial suspension adjusted to OD_{600} = 0.500 and incubated for 24 h at 30 °C. Biological nitrogen fixation was measured by using a gas chromatograph (Perkin Elmer, USA) with flame ionization detector and a Poropak column N 200/300 Mesh of 6.0 ft and 3.0 mm diameter, according to Eckert et al. (2001). A calibration curve was determined by using ethylene (chromatographic grade) as standard. Confirmation of nitrogen fixation capability was evaluated by amplifying the *nifH* gene according to the method described by Widmer et al. (1999).

2.4.2. Bacterial phosphate solubilization

For the evaluation of phosphate solubilization, the quantitative phosphomolybdate method was employed. Each strain was grown on Pikovskaya broth (in g/L: glucose 10; $(NH_4)_2SO_4$ 0.5; $MgSO_4$ 0.1, KCl 0.2, yeast extract 0.05, $Ca_3(PO_4)_2$ 5.0); and incubated for 120 h at 150 rpm (Pikovskaya, 1948). Five-hundred microliters of supernatant from each culture, including controls, were used for analysis (Fiske and Subbarow, 1925). A calibration curve was determined by using K_2HPO_4 (Merck, USA).

2.4.3. Indolic compound synthesis

Indolic compounds were estimated using the colorimetric assay based on the Salkowsky reagent using the PC reagent (12 g/L $FeCl_3$ in 7.9 M H_2SO_4) (Glickmann and Dessaux, 1995). The culture medium used was K-lactate (Carreno-Lopez et al., 2000), and the incubation was carried out for 72 h at 150 rpm in the dark. The reaction between the PC reagent and culture supernatant was performed in a 1:1 ratio for 30 min in the dark. Indolic compounds were spectrophotometrically determined at 540 nm. A calibration curve was determined using indol-3-acetic acid pure (Merck, USA).

Table 1
Characteristics of soil used for pot experiments.

Parameter	Value	Parameter	Value
pH	5.95 ± 0.07	Sodium (cmol/kg)	0.39 ± 0.15
Organic matter (%)	15.15 ± 0.21	Effective cationic interchange capacity	8.26 ± 1.81
Phosphorus (ppm)	13.6 ± 0.70	Electric conductivity (dS/m)	0.69 ± 0.2
Sulphur (ppm)	12.6 ± 0.70	Minor elements	
Interchangeable cations		Iron (ppm)	134.5 ± 16.2
Calcium (cmol/kg)	4.72 ± 0.69	Copper (ppm)	1.85 ± 0.07
Magnesium (cmol/kg)	1.11 ± 0.35	Manganese (ppm)	17.30 ± 0.01
Potassium (cmol/kg)	1.95 ± 0.75	Zinc (ppm)	3.45 ± 0.9

± shows standard deviation.

2.5. Effects of NaCl and bacterial inoculation on maize growth

Pot experiments were conducted in order to evaluate the effect of NaCl and bacterial inoculation on growth of *Zea mays*. The experimental design was a full factorial design with six replicates per treatment. Pots containing 400 g of dry-sterilized soil were supplemented to reach 0, 2.93 and 5.85 g NaCl/kg soil, which was prepared by adding 0, 1.078 and 2.248 g NaCl dissolved in 100 mL water. The treatment without exogenous addition of NaCl was considered as 0 g NaCl/kg soil concentration. Characteristics of the soil without added salt are listed in Table 1. For measuring electrical conductivity, 30 g dry soil was mixed with 20 mL deionized water and shaken for 1 h. Then, the soil extract was filtered, and conductivity was measured using a conductivimeter (Thermo Corporation, USA). Maize seeds Var. ICA-508 were disinfected by soaking in 30% hydrogen peroxide and 70% ethanol (1:1) for 10 min, and followed by rinsing several times in sterilized distilled water. The seeds were then pre-germinated in sterilized peat at room conditions for five days. For inoculum preparation, bacteria were growth in nutrient broth (Merck, USA) for 24 h at 28 °C, rinsed twice, and finally resuspended to the same initial volume using 0.03 M $MgSO_4$. Roots of seedlings, with the same size, were submerged three times in bacterial suspension adjusted to OD_{600} = 1.000 and planted in each pot supplemented or not with NaCl. Seedlings submerged in sterilized water were used as a control. Plants were grown in a growth chamber at a day/night temperature of 22/18 °C with 120 μmol m^{-2} s^{-1} of light supplied for 16 h during daytime for 4-weeks. Finally, vegetative tissue was employed for the respective analyses described below. To determine the dry weight, shoots and roots were oven-dried separately at 60 °C for 48 h (at constant weight). In addition, lengths of both shoots and roots were recorded.

2.6. Determination of chlorophyll

Photosynthetic pigment content of *Zea mays* leaves was estimated by the method of Hiscox and Israelstam (1979), employing the equations described by Wellburn (1994).

2.7. Determination of Na^+, K^+, Ca^{2+} and Mg^{2+} in plant tissues

Roots and shoots were washed several times with deionized water. These were oven-dried at 60 °C for 48 h and afterward ground. Samples of 200 mg of each plant tissue were digested at 150 °C for 2.0 h in a microwave digester in a mixture of 30% H_2O_2, 65% HNO_3 (Merck, USA), and deionized water in a ratio of 1:1:1 to final volume of 12.0 mL; after digestion, volume was adjusted to 20 mL. An Absorption Atomic Spectrophotometer (AAS 2380, Perkin Elmer, USA) was employed for measuring the concentration of Na^+, K^+, Ca^{2+}, and Mg^{2+}. Reagent blank and analytical duplicates were used where appropriate to ensure accuracy and precision of the analysis.

2.8. Estimation of proline in plant

Proline content was estimated according to Bates et al. (1973). Briefly, 0.5 g fresh leaves were frozen in liquid nitrogen, homogenized with 3% sulfosalicylic acid (Fisher Scientific, USA), and immediately centrifuged at 10,000 × *g* for 5 min. One milliliter of supernatant was taken for analysis. Absorbance was measured at 520 nm and the calibration curve was determined using pure L-proline (Sigma–Aldrich, USA) as standard.

2.9. Estimation of total polyphenol content

Total polyphenols were analyzed as described Parida et al. (2002). For this, 0.5 g fresh leaves were frozen in liquid nitrogen and homogenized in 5.0 mL of 80% ethanol by using a chilled pestle and mortar, with subsequent centrifugation at 10,000 × *g* for 10 min. The supernatant was conserved and the residue re-extracted with 2.5 mL of 80% ethanol and centrifuged again. The supernatant was pooled and evaporated to dryness. The residue was dissolved in 5.0 mL of distilled and deionized water and analyzed. Supernatants were taken for analysis. Absorbance was measured at 650 nm. A standard curve was determined using pure gallic acid (Merck, USA) as standard.

2.10. Statistical analysis

Statistical analyses were carried out by using the software package SPSS version 17.0. Data were analyzed using Analysis of Variance (ANOVA) and the HSD Tukey pairwise comparisons. Associations among characters were examined by simple correlation analysis. All tests were subjected to a 95% confidence limit.

3. Results

3.1. Effect of NaCl on bacterial growth

High levels of NaCl repressed bacterial growth, where strains C5 and C9 tolerated a higher content of NaCl than C7 and C8 (Fig. 1). Optimal NaCl concentration for C5 and C9 was 11.7 g/L NaCl. Due to their high tolerances, C5 and C9 were selected for further studies. In addition, bacterial growth kinetics for C5 and C9 were evaluated at 0, 2.93, and 5.85 g NaCl/L. The results revealed that NaCl did not exert a negative effect on bacterial growth (Figs. 1 and 2).

3.2. Molecular identification of the C5 and C9 strains

Strains C5, C7, C8, and C9 were presumed to be *Azotobacter* sp. based on their biochemical and morphological characteristics (Table 2). We selected C5 and C9 for further tests due to their ability to tolerate high NaCl concentrations. Partial sequence of 16S rDNA of C5 and C9 showed 99% and 98% identity with the sequence of *Azotobacter chroococcum* strain IAM 12366, with fragment lengths of 1464 and 1377 base pairs, respectively. The partial 16S rDNA

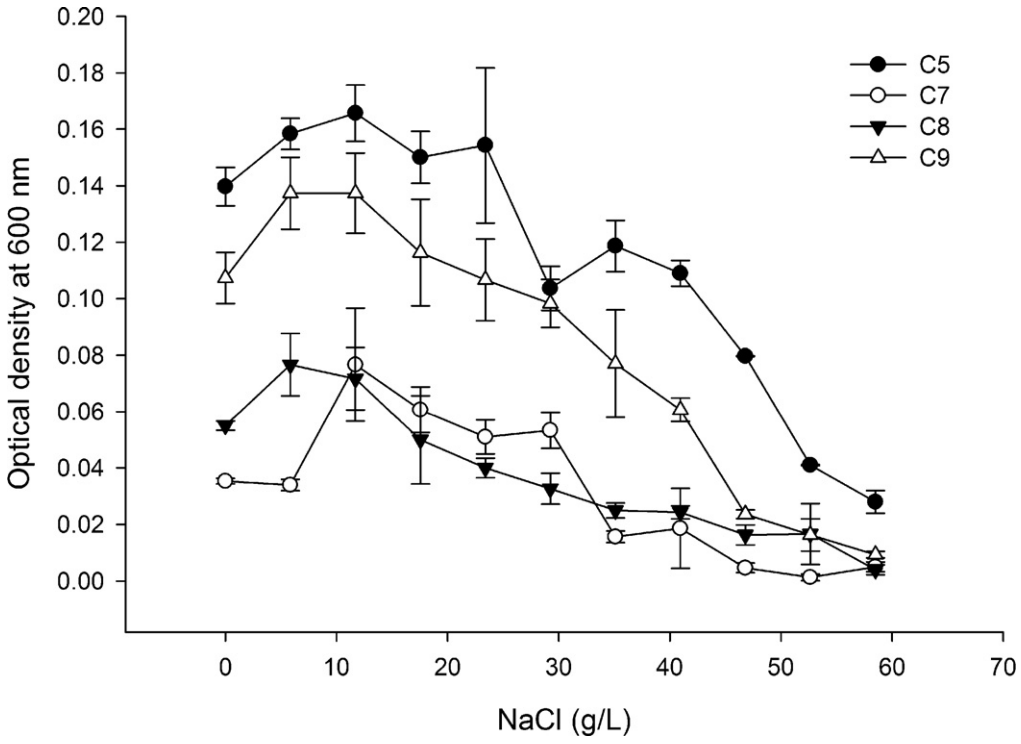

Fig. 1. Effect of NaCl on bacterial growth. Each value is the mean of three replicates. Error bars represent ±standard deviation.

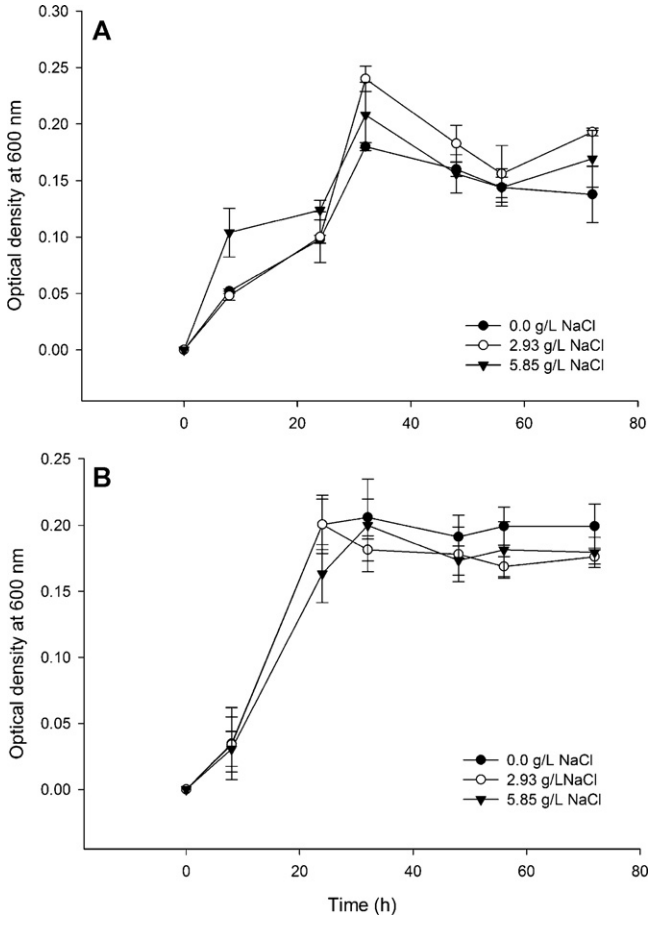

Fig. 2. Effect of NaCl on growth kinetics of *Azotobacter* sp. (A) C5 and (B) C9. Each value is the mean of three replicates. Error bars represent ±standard deviation.

Table 2
Morphological and biochemical description of strains.

Characteristics	C5	C7	C8	C9
Morphological				
Gram reaction	N	N	N	N
Cell shape	SR	SR	SR	SR
Brown pigmentation	+	+	+	+
Cyst formation	+	+	+	+
Biochemical reactions				
Aerobic nitrogen fixation	+	+	+	+
Nitrate reduction to nitrite	+	+	+	+
Catalase	+	+	+	+
Hydrolysis				
Urea	–	–	–	–
Esculine	+	+	+	+
Gelatine	–	+	+	+
Carbohydrate utilization				
Glucose	+	+	+	+
Arabinose	–	+	+	–
Mannose	+	+	+	+
Mannitol	+	+	+	–
N-acetyl-glucosamine	–	–	+	+
Maltose	+	–	+	+
Caprate	–	–	–	–
Malate	–	+	+	+
Citrate	–	–	+	+
Phenyl-acetate	–	–	+	–

+ Indicates a positive reaction while – indicates a negative reaction. N: negative Gram reaction; P: positive Gram reaction. SR: short rods.

sequences for these strains have been deposited with the EMBL-EBI/GenBank accession numbers JN683378 and JN683377 for the *A. chroococcum* C5 and C9 strains, respectively.

3.3. Plant growth-promoting features

Plant growth-promoting capabilities of the selected strains were studied (nitrogen fixation, phosphate solubilization, and indole synthesis) at 0, 2.93, and 5.85 g NaCl/L. *A. chroococcum* C5 and C9 were able to reduce acetylene in both the presence and absence

Table 3
PGP features of strains.

Strain	NaCl (g/L)	ARA (nmol ethylene/mL h)	Indole production (μg indole/mL)	Phosphate solubilization (μg PO_4^{3-}/mL)
C5	0	138.65 ± 9.77 a	0 a	161.11 ± 13.43 a
	2.93	130.18 ± 3.50 a	16.06 ± 1.07 b	140.12 ± 26.37 a
	5.85	132. 24 ± 1.96 a	16.01 ± 2.46 b	144.74 ± 5.26 a
C9	0	133.84 ± 3.18 a	0 a	151.32 ± 7.46 a
	2.93	139.33 ± 5.93 a	0 a	153.81 ± 17.89 a
	5.85	143.22 ± 6.49 a	0 a	179.83 ± 2.63 b

± shows standard deviation. Different letters represent significant statistical differences based on Tukey HSD test.

Table 4
Chlorophyll content.

Treatment	Chlorophyll (mg mL^{-1} g^{-1} FW)		
	0 g NaCl/kg soil	2.93 g NaCl/kg soil	5.85 g NaCl/kg soil
Non-inoculated	2.338 ± 0.023 a	4.063 ± 0.099 b	0.518 ± 0.019 a
C5	3.780 ± 0.085 b	3.359 ± 0.277 a	2.515 ± 0.111 b
C9	3.560 ± 0.313 b	5.292 ± 0.283 c	3.251 ± 0.281 c

± shows standard deviation. Different letters represent significant statistical differences based on Tukey HSD test.

of NaCl; however, we observed no statistical differences ($P > 0.05$). Nitrogen-fixation capacity of C5 and C9 was confirmed by nested PCR of *nifH* genes, which yielded a 370-pb DNA fragment by the successive use of primers *nifH*(forA) and *nifH*(rev), and after *nifH*(forB) and *nifH*(rev). Phosphate solubilization activity was exhibited by both strains C5 and C9; we only observed differences at 5.85 g NaCl/L with strain C9 ($P < 0.05$). *A. chroococcum* C9 did not synthesize auxins under the conditions tested, while C5 synthesized indole acetic acid but only under saline stress (Table 3).

3.4. Influence of PGPB and NaCl on maize growth and content of photosynthetic pigments

We tested the influence of PGPB inoculation on maize growth under conditions of slight and moderate salinity. The electrical conductivities were 3.054 and 6.004 dS/m for 2.93 and 5.85 g NaCl/kg soil, respectively. Parameters such as shoot and root length, and shoot and root dry weight were evaluated. Inoculation with strains C5 and C9 increased plant growth, but only under saline stress ($P < 0.05$) (Fig. 3). With 2.93 g NaCl/kg soil, the increases for shoot length were 45% and 58%, while at 5.85 g NaCl/kg soil the increases were 27% and 47% for C5 and C9, respectively (Fig. 3A). Similarly, promotion of shoot dry weight under salt stress was 100% and 95% at 2.93 g NaCl/kg soil, and 81% and 122% at 5.85 g NaCl/kg soil with strains C5 and C9, respectively (Fig. 3C). Increases in root length by bacteria were only exhibited when the concentration of salt was 5.85 g NaCl/kg soil (Fig. 3B). Root dry weight was increased by bacterial inoculation by 23% and 42% at 2.93 g NaCl/kg soil, and 44% and 40% at 5.85 g NaCl/kg soil, for the strains C5 and C9, respectively (Fig. 3D). Both bacteria were able to increase the chlorophyll content at 0 g NaCl/kg soil (Table 4). At 2.93 g NaCl/kg soil *Azotobacter* sp. C9 increased chlorophyll content by 30%, while C5 did not exhibit any effect ($P > 0.05$). And at 5.85 g NaCl/kg soil, both strains increased chlorophyll content by 4- and 6-fold with respect to the non-inoculated treatment (Table 4).

3.5. Effect of bacterial inoculation on Na^+, K^+, Ca^{2+} and Mg^{2+} uptake by maize

Increasing the NaCl concentration of soil resulted in increased Na^+ content in plants (Table 5). In roots, inoculation with bacteria caused a decrease of Na^+ content. Similarly, Na^+ content in shoots was decreased with inoculation of C5 and C9 at 2.93 g NaCl/kg soil, and of C9 at 5.85 g NaCl/kg soil. In contrast, the Na^+ content was unaltered by inoculation of bacteria in the non-saline soil.

K^+ uptake by roots and shoots was increased by bacterial inoculation, demonstrating a clear bacterial effect on potassium transport in plants. Accumulation of Na^+ and K^+ was negatively and positively correlated with plant biomass, respectively (Fig. 4). In most cases bacterial inoculation revealed no effects on Ca^{2+} uptake in roots, while the amount of Ca^{2+} in shoots was increased. Finally, the amount of Mg^{2+} in roots and shoots was diminished and increased by inoculation with C5 and C9 under saline stress, respectively (Table 5).

3.6. Polyphenol content in leaves

Changes in polyphenol content in maize plants were shown as response to both NaCl and bacterial inoculation (Table 6). The amount of total polyphenols was enhanced with increasing concentrations of salt. Similarly, when bacteria were present, an increase in content of polyphenols was also observed regardless of salt concentration (Table 6). *Azotobacter* sp. C5 caused the greatest effect on content of polyphenols in maize leaves (Table 6).

Table 5
PGPB and salt influence on Na^+, K^+, Ca^{2+} and Mg^{2+} uptake.

NaCl (g/kg soil)	Treatment	Root (mg/g DW)				Shoot (mg/g DW)			
		Na^+	K^+	Ca^{2+}	Mg^{2+}	Na^+	K^+	Ca^{2+}	Mg^{2+}
0	Non-inoc.	2.86 a	20.51 f	2.38 a	1.47 a	0.20 a	51.24 c	3.67 a	2.51 a
	C5	2.70 a	18.55 e	2.05 a	1.54 a	0.41 a	50.57 c	2.60 a	2.37 a
	C9	2.61 a	18.34 e	2.13 a	1.43 a	0.40 a	61.21 d	3.57 a	2.20 a
2.93	Non-inoc.	16.09 c	8.19 c	2.35 a	2.42 b	17.97 c	40.42 b	3.48 a	2.97 b
	C5	15.59 b	8.53 c	2.25 a	1.61 a	16.02 b	37.60 b	3.23 a	3.25 c
	C9	14.08 b	11.41 d	1.83 a	1.76 a	16.32 b	42.35 b	3.96 b	3.04 b
5.85	Non-inoc.	18.93 d	3.53 a	3.92 b	3.12 c	44.88 e	25.20 a	4.65 b	3.15 b
	C5	21.77 e	4.90 b	2.53 a	2.40 b	43.37 e	34.67 b	5.47 c	5.61 d
	C9	15.18 b	8.41 c	2.57 a	2.38 b	32.56 d	36.68 b	5.65 c	3.45 c

Different letters represent significant statistically differences based on Tukey HSD test.

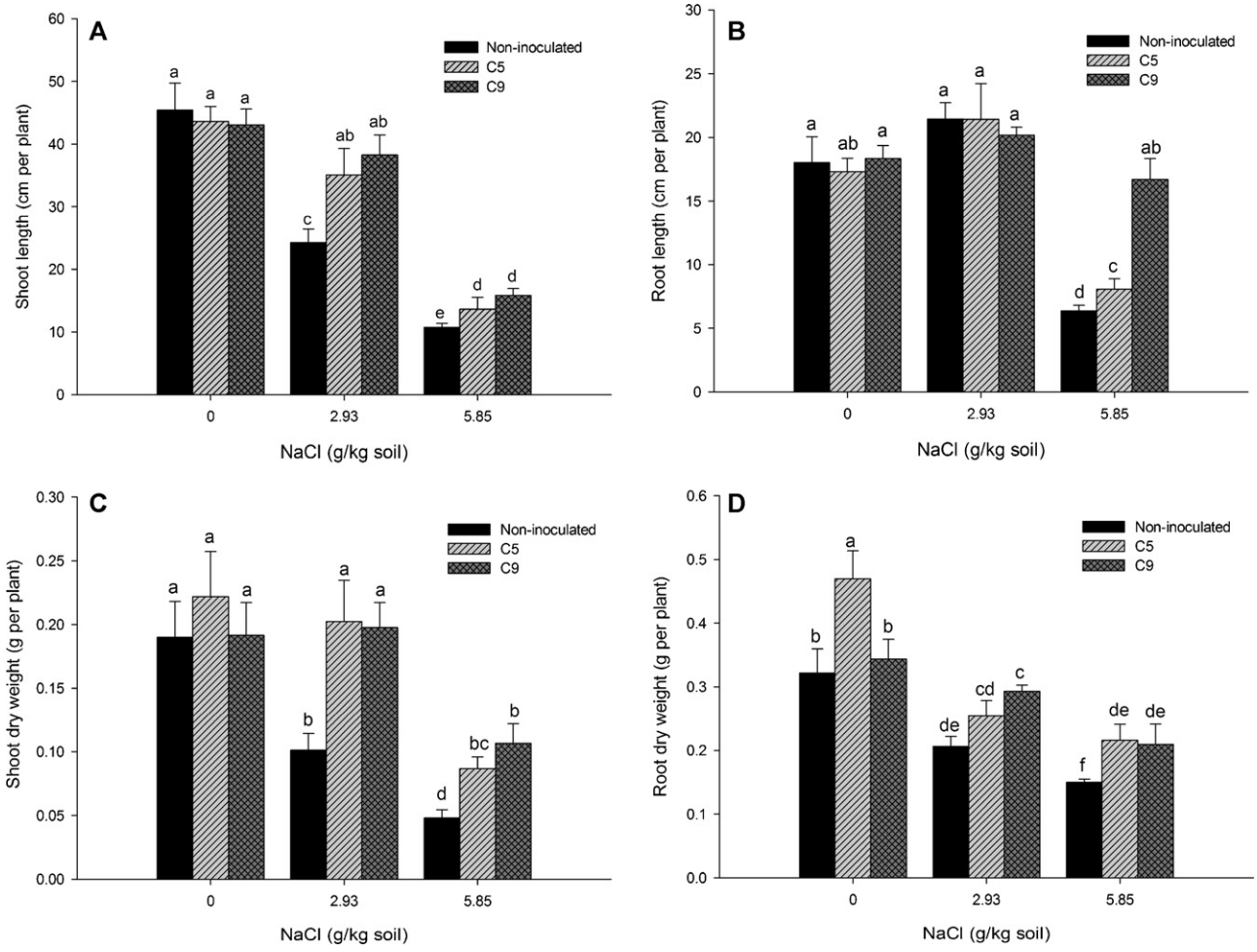

Fig. 3. Effect of NaCl and inoculation with C5 and C9 on plant biomass expressed as: (A) shoot length, (B) root length, (C) shoot dry weight, and (D) root dry weight. Each value is the mean of six replicates. Error bars represent ±standard deviation. Different letters represent significant statistical differences based on Tukey HSD test ($P < 0.05$).

Table 6
Measuring of total polyphenols in leaves.

Treatment	Total polyphenols (mg/g FW)		
	0 g NaCl/kg soil	2.93 g NaCl/kg soil	5.85 g NaCl/kg soil
Non-inoculated	2.858 ± 0.363 a	3.210 ± 0.340 a	3.834 ± 0.360 a
C5	3.468 ± 0.133 b	4.110 ± 0.036 b	4.937 ± 0.446 b
C9	3.612 ± 0.298 b	3.531 ± 0.256 a	4.902 ± 0.103 b

± shows standard deviation. Different letters represent significant statistical differences based on Tukey HSD test.

3.7. Proline content in leaves

Our findings indicated that under saline stress plants synthesized proline to a greater extent (Table 7). However, the content of proline with both slight and moderate salinity was decreased by bacterial inoculation. Proline content in bacterial-inoculated plants was enhanced in the non-saline soil by 76 and 84% for C5 and C9, respectively.

Table 7
Proline in leaves.

Treatment	Proline (μmol/g FW)		
	0 g NaCl/kg soil	2.93 g NaCl/kg soil	5.85 g NaCl/kg soil
Non-inoculated	3.156 ± 0.058 a	5.781 ± 0.580 b	6.283 ± 0.552 c
C5	5.569 ± 0.219 b	3.995 ± 0.087 a	4.797 ± 0.261 a
C9	5.800 ± 0.472 b	5.355 ± 0.291 b	5.443 ± 0.376 b

± shows standard deviation. Different letters represent significant statistical differences based on Tukey HSD test.

4. Discussion

Salinity affects plant growth by imposing both ionic and osmotic stresses (Shabala and Cuin, 2008). We observed that regardless of biological treatment salinity negatively affected plant growth (length and weight). Because of the osmotic gradient generated, elevated Na^+ levels in the soil solution drive water out of the cell reducing almost instantaneously cell turgor, leaf area, and consequently the photosynthetic activity and carbon fixation (Yeo et al., 1991). In the current study, *A. chroococcum*-inoculated plants had significantly higher biomass than their respective controls in the saline soil; however, no effects were observed under non-restrictive growth conditions. Moreover, the effect of salinity on synthesis of photosynthetic pigments depended on the specific concentration of NaCl. Nevertheless, the inoculation with C5 and C9 enhanced the content of chlorophyll revealing a positive effect on growth and plant development. Early studies have shown that synthesis and activity of photosynthetic pigments could be repressed by excessive concentrations of Na^+ (Parida et al., 2004); However, studies reported by Hamdia et al. (2004) showed that inoculation with *Azospirillum lipoferum*, also a PGPB, ZA/I improved plant dry weight and leaf area in maize under high salinity. Similarly, Mayak et al. (2004) observed that tomato plants inoculated with

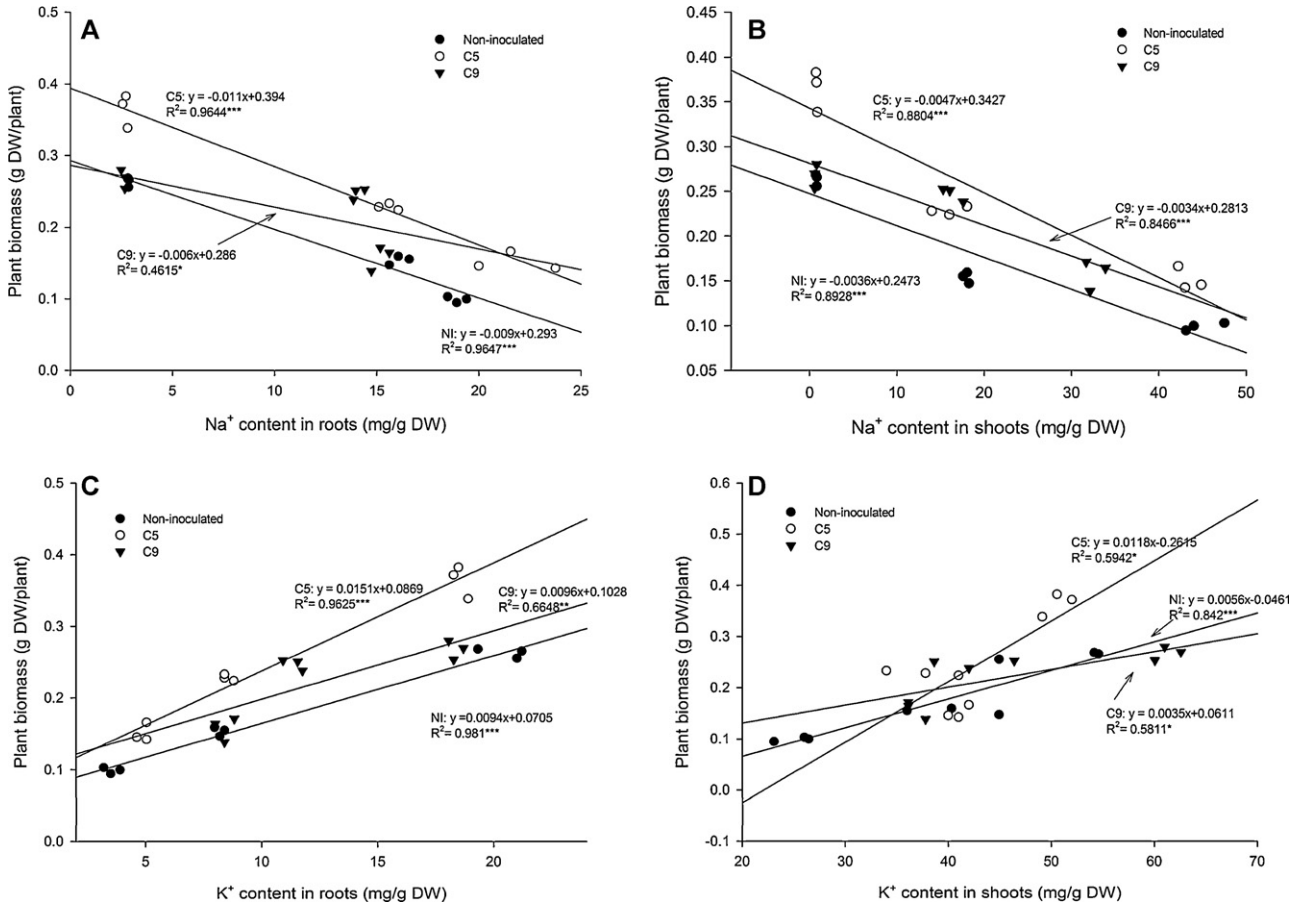

Fig. 4. Correlations of plant biomass (root weight + shoot weight) with Na^+ in (A) roots and (B) shoots, and K^+ content in (C) roots and (D) shoots. *, **, *** significant at $P < 0.05$, 0.01 and 0.001, respectively. At equations, C5, C9, and NI indicate inoculation with A. chroococcum C5 and C9, and non-inoculated treatment, respectively.

Achromobacter piechaudii ARV8 had increased plant biomass under 120 and 207 mM NaCl. These results indicated that inoculation with the selected bacterium could decrease the injurious effects caused by salinity.

High salinity can also affect the growth and role of bacteria in the environment (Steinborn and Roughley, 1974). However, we observed that growth and PGP features of strains C5 and C9 were not negatively influenced by salinity. Bacteria can synthesize compatible solutes (sugars, amino acids, or derivatives) that act as osmolytes and help organisms to survive when there is extreme osmotic stress (Bacilio et al., 2004; da Costa et al., 1998; Parida and Das, 2005). Bacterial traits, such as nitrogen fixation, phosphate solubilization, and IAA synthesis, have exhibited an influence on plant growth by increasing nutrient availability and by influencing plant development (Glick, 2010). Therefore, growth promotion by strains C5 and C9 may be mediated by these traits. *A. chroococcum* C5 and C9 were able to solubilize tricalcium phosphate and to fix nitrogen, but only C5 was able to synthesize IAA, indicating plant growth-promoting characteristics.

Na^+ exclusion and K^+ influx are the most important plant strategies for alleviating salt-induced stress (Fortmeier and Schubert, 1995; Shabala and Cuin, 2008). We observed that high levels of Na^+ in roots and shoots were negatively correlated with maize biomass, regardless of bacterial treatment employed. Conversely, K^+ content in plants was correlated positively with plant biomass. The present results showed that salinity increased Na^+ and decreased K^+ concentration, thus decreasing the K^+/Na^+ ratio with increasing salinity stress. However, bacterial inoculation resulted in significantly decreased Na^+ and increased K^+ concentration, improving the K^+/Na^+ ratio. In this case, *A. chroococcum* C9 exhibited the greatest effect. In addition, accumulation of Ca^{2+} and Mg^{2+} in roots decreased as the concentration of Na^+ in soil increased. Likely, Na^+ exerted ionic competence in soil, diminishing the ability for ion uptake by the plant. In shoots, however, an increase in the content of Ca^{2+} and Mg^{2+} mediated by bacterial inoculation was observed, which may be explained by an increase in mineral availability mediated by the bacterial metabolism (e.g. releasing of organic acids). Similar results were reported by Ashraf et al. (2004), who found that inoculation with exopolysaccharide-producing bacteria could restrict Na^+ influx into roots. Further, Zhang et al. (2008) reported that inoculation with *Bacillus subtilis* GB03 could mediate the level of salt tolerance in *Arabidopsis thaliana* through regulation of the potassium transporter HKT1. These results supported the idea that bacteria can mediate the expression of an ion high-affinity K^+ transporter in *Arabidopsis*.

Salinity decreases carbon uptake by limiting photosynthesis, causing an over-reduction of photosynthetic electron chain, and redirecting the photon energy into processes that favor the production of Reactive Oxygen Species (ROS) (Hichem et al., 2009; Johnson et al., 2003). Synthesis of polyphenols by plants constitutes one of the adaptive mechanisms for reducing oxidative damage (Hichem et al., 2009; Nautiyal et al., 2008). In this study, salinity significantly increased polyphenols content in maize leaves and bacterial inoculation also improved the amount of polyphenols compared with the respective non-inoculated control. Polyphenols can eliminate radical species, thus preventing the propagation of oxidative chain reactions (Rice-Evans et al., 1997). Nautiyal et al. (2008) similarly showed that inoculation with PGPB *Bacillus lentimorbus* NRRL

B-30488 could mediate induction of dietary antioxidant in vegetables and fruit expressed as total polyphenol content. However, few data are available about the mechanisms involved in bacterial-mediated plant antioxidative protection.

Studies carried out by Hamdia et al. (2004) and Nadeem et al. (2007) showed that plant proline contents are increased by saline stress, but decreased by inoculation with PGPB. In this study, we also found that proline content in leaves increased with increasing NaCl concentration. However, inoculation with strains C5 and C9 significantly decreased the proline concentration in leaves. To date, there is evidence indicating a positive correlation among proline accumulation and adaptation to salt stress, but the results are still controversial (Ashraf and Foolad, 2007; Chandler and Thorpe, 1987). Our observation revealed that under salt-mediated stress, inoculation with C5 and C9 decreased proline concentration concomitantly with increased plant biomass. Notably, in the absence of salinity, bacteria significantly increased the proline content in leaves indicating that bacteria could generate some kind of stress on plant growth. This information may support the results obtained in pot experiments where no effects on plant biomass were observed under non-saline conditions. Sziderics et al. (2007) reported that the strains EZB4 of *Arthrobacter* sp. and EZB8 of *Bacillus* sp. increased the content of proline even in the absence of abiotic stress. They argue that bacteria likely exerted some biotic stress that triggered proline biosynthesis in plants.

5. Conclusions

We demonstrated that inoculation with *A. chroococcum* strains C5 and C9 protected plants against the inhibitory effects of NaCl. We argue on the basis of our findings that bacterial amelioration of saline stress could be the integration of several aspects including increasing plant antioxidative capacity, improving plant mineral nutrition, and promoting plant growth by bacterial synthesis of plant hormones or regulation of ions transporters that resulted in an improved K^+/Na^+ ratio in plants. However, extensive research is needed to elucidate how bacteria mediate this effect on maize growth. In summary, results indicated that bacteria could ameliorate saline stress of maize plants caused by high levels of NaCl in soil. Hence, use of selected PGPB may be important inputs to decrease the deleterious effects of saline soils.

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