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Possible Roles of Nitrogen Fixation and Mineral Uptake Induced by Rhizobacterial Inoculation on Salt Tolerance of Maize

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Abstract

Pot experiments were conducted to evaluate the possible roles of nitrogen fixation and/or enhanced mineral uptake by *Azospirillum lipoferum* and *Bacillus polymexa* inoculation in improving salt tolerance of maize plants. Plants were inoculated and grown under salt stress (osmotic potential: –0.3, –0.6, –0.9 and –1.2 Mpa). Both microorganisms were able to fix nitrogen up to –0.9 Mpa salinity level accompanied with increased total N-yield compared with the control plants. In order to investigate the role of bacterial inoculation on enhanced mineral uptake, the growth and some physiological parameters of inoculated plants were compared with plants fertilized by K and P foliar application. Plant inoculation with the N_2-fixers or plant spraying with KH_2PO_4 resulted in an increase in fresh and dry matter as well as water content of plants. Treated plants exhibited changed plant mineral content which was associated with increased Mg/K and decreased P/K, Ca/K and Na/K ratios. This was accompanied by accumulation of soluble sugars, amino acids in shoots and roots of plants resulting in a concomitant increase in the osmotic potential of the cell sap as a possible mechanism of adaptation to salinity.

Key words: Rhizobacteria, nitrogen fixation, salt tolerance.

Introduction

It has been argued that drought tolerance in plants can be enhanced by P and K nutrition (Safir and Nelson, 1995). The application of N fertilizers to plants growing in arid climate increases the salt tolerance of these plants (Cordovilla *et al.*, 1994). Physiological studies under well-watered conditions showed that suboptimal P and K levels reduce root hydraulic conductivity causing low water potential and stomotal conductance in cotton plants (Ackerson, 1985). Phosphorus fertilization increased plant hydraulic conductivity, plant water potential, and stomotal conductance in well-watered onion plants (Nelson and Safir, 1982).

Despite the importance of such minerals in improving plant growth specially under stress conditions, only a part (about 0.1%) of the total P and K in soil is available to plants (Scheffer and Schachtschabel, 1992). Moreover, the increasing costs and the danger of N fertilizers limit its application to rangelands. Therefore, the importance of inoculation of soil and plants with the beneficial microorganisms especially the nitrogen-fixing bacteria has been described by some researchers (Sarwar *et al.*, 1998).

To explore the importance of such beneficial microorganisms for plant growth, we have recently studied the possible role played by associative nitrogen fixing *Azospirillum* spp. and *Bacillus* spp. on growth, nitrogen fixation (using ^{15}N technique), nitrate reductase activity as well as mineral nutrition of maize and wheat plants grown under salt and drought stresses (Hamdia and El-Komy, 1998; El-Komy *et al.*, 2003). These investigations were focused mainly on the nitrogen-fixing and phytohormonal effects of such rhizobacteria, which were correlated with the exogenous application of GA3 (Hamdia and El-Komy, 1998).

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Inoculation of plants with growth promoting rhizobacteria (*e.g. Azospiillum* and *Bacillus* spp.) generally increases plant growth parameters, and eventually increases the yields of many crop plants (Bashan and Holguin, 1997; El-Komy and Abdel Wahab, 1998; Steenhoudt and Vanderleyden, 2000). These effects could be attributed to the enhancement of mineral uptake in different plant varieties rather than the nitrogen-fixing activity. Variations in the uptake of NO_3^-, NH_4^+ (Boddey and Dobereiener, 1988), P (Sarig *et al*., 1992; Murty and Ladha, 1988), K^+ (Lin *et al*., 1983) and Fe^{2+} (Barton *et al*., 1986) by plant inoculation have been demonstrated.

The objectives of the present work were to study the possible roles of nitrogen fixation and/or enhanced mineral uptake induced by *Azospirillium lipoferum* or *Bacillus polymexa* inoculation in improving salt tolerance of maize plants.

Experimental

Material and Methods

Microorganisms and growth conditions. *Azospirillium lipoferum* strain Z4/1 isolated from maize histospere by El-Komy (1992), and *Bacillus polymexa* E.M. 1231 kindly provided by MIRCEN, Ain-Shams Univ., Cairo, Egypt, were used in this study.

1 ml sample of bacterial suspension prepared from a single colony was transferred into 50 ml of nutrient broth or DAS medium for *Bacillus* and *Azospirillium* respectively. Flasks were incubated in a shaking incubator at 200 rpm and 30°C for 20 h until the logarithmic phase has been reached. Bacterial cells were harvested by centrifugation (7,000 g, 10 min, 4°C) and then washed once in sterile distilled water. The bacterial suspension used for inoculation was adjusted to 10^7 CFU/ml.

Plant growth conditions. Seeds of maize (*Zea mays* L.) var. Giza 310 were disinfected by immersion in a mixture of ethanol 96% and H_2O_2 (1:1, V:V) for 3 minutes, followed by several washings with sterile distilled water and germinated on wet sterile filter papers in Petri dishes for 3 days. Each five germinated seeds were transplanted into a plastic pot containing 2 kg of washed pure quartz sand. Pots were divided into four groups. Seedlings of the first and second groups were inoculated with *Azospirillum lipoferum* or *Bacillus polymexa* (10^7 CFU/seedling). Plants of the third group were sprayed by KH_2PO_4 (100 µg g^{-1}) three times at intervals two weeks after sowing. The fourth group of pots were left without treating as control. Starting from the second week of sowing, all pots were irrigated every other day with NaCl solution at different salinization levels, corresponding to osmotic potential 0.0, –0.3,– 0.6, –0.9, and –1.2 MPa. In order to adjust the osmotic potential, the soil moisture content was kept near the field capacity using the corresponding salt solution. The salinized and non-salinized plants were irrigated every week once with nitrogen-free 1/10 Hogland minerals solution. Pots were arranged randomly with six replicates for each treatment. After 50 days of sowing, plants were harvested, dried in oven at 70°C to a constant mass, then weighed and grounded for further analysis.

Determination of total N-yield and fixed nitrogen. Shoot and root N-contents were determined after Kjeldahl digestion, and the total N-yield (mg N pot^{-1}) was calculated according to the following equation (Reinnie and Reinnie, 1983):

$$\text{Total N-yield} = \frac{\text{Dry matter (mg)} \times \text{\%N}}{100}$$

The percentage of nitrogen derived from air (%NdFa) and fixed nitrogen (mg N pot^{-1}) were calculated using the classical difference method (DM) according to the following equation (Reinnie and Reinnie, 1983; El-Komy and Abdel-Wahab, 1998):

$$\text{\% NdFa} = \frac{\text{N yield (Fx)} - \text{N yield (nFx)} \times 100}{\text{N yield (Fx)}}$$

$$N_2 \text{ Fixed (mg N)} = \text{N yield (Fx)} - \text{N yield (nFx)}$$

Where (Fx) and (nFx) are fixing (inoculated) and nonfixing (uninoculated) plants, respectively.

Analytical methods. Soluble sugars were determined by the anthrone-sulphuric acid method (Fales, 1951). Amino acids were assayed according to Moore and Stein (1948), soluble proteins according to Lowry *et al.* (1951) and proline according to Bates *et al.* (1973). Osmotic pressure was determined in water-soluble extract by wide range advanced Osmometer 3W2 (England). Sodium and potassium were determined by the flame photometeric method (Williams and Twine, 1960). Calcium and magnesium were determined by the versine titration method (Schwarzenbach and Biedermann, 1948). Phosphorus content was determined according to Woods and Mellon (1985).

Statistical analysis. Experimental data were subjected to one way analysis of variance and the means were separated by the least significant difference, L.S.D. (Steel and Torrie, 1960).

Results

Response of maize plants grown under salt stress to bacterization and foliar application by K and P. Response of maize plants grown under salt stress to bacterization and foliar application by K and P was evaluated by measuring plant dry weight, total N-yield and water content. Generally, control maize plants

Table I
The effect of foliar P and K fertilization and/or bacterial inoculation on fresh, dry matter (g plant –1) and water content as well as length Cm of shoots and roots of maize plants grown at different levels of NaCl

	NaCl [MPa]	Shoot		Root		Water content		Length	
		f.m.	d.m.	f.m.	d.m.	Shoot	Root	Shoot	Root
Control	–0.0	10.3	1.38	11.4	1.16	8.92	10.24	53.3	41.7
	–0.3	7.07	1.03	5.54	0.579	6.04	4.96	64.0	44.7
	–0.6	9.31	1.23	6.61	0.657	8.08	5.95	53.0	44.5
	–0.9	9.85	1.35	5.59	0.636	8.50	4.95	43.3	37.7
	–1.2	5.98	0.746	3.42	0.323	5.23	3.09	43.7	35.0
Azospirillum	0.0	17.5	2.19	15.0	1.28	15.3	13.7	68.7	50.0
	0.3	17.1	2.35	13.8	1.36	14.8	12.4	771.5	47.7
	0.6	8.91	1.85	5.99	0.602	7.06	5.39	60.5	40.0
	0.9	10.0	1.37	6.07	0.601	8.73	5.47	53.0	51.3
	1.2	4.59	.561	5.01	0.467	4.03	4.54	41.0	36.0
Bacillus	0.0	13.8	1.84	16.3	1.52	12.1	14.8	38.0	62.0
	0.3	14.9	4.82	11.9	1.38	10.1	10.5	75.0	39.0
	0.6	13.1	1.82	11.4	1.31	11.3	10.1	55.3	37.0
	0.9	10.1	1.29	10.9	1.23	8.81	9.67	45.0	32.7
	1.2	5.64	0.769	2.8	0.414	4.87	9.67	44.0	30.0
KH_2PO_4	0.0	12.2	1.65	9.13	0.925	10.6	8.21	57.3	48.3
	0.3	10.2	1.42	8.80	0.869	8.78	7.93	62.3	50.7
	0.6	10.5	1.31	6.17	0.598	9.19	5.57	38.0	62.0
	0.9	7.91	1.02	4.09	0.449	6.89	3.64	39.5	47.5
	1.2	3.53	0.493	2.07	0.301	3.04	1.77	48.0	32.5
LSD 5%		2.83	0.257	2.16	0.099	1.73	1.52	2.57	2.06

responded negatively to salt stress as shown in Table I. However, plant inoculation or foliar application with K and P significantly increased plant dry weight and water content up to –0.9 Mpa. Variations were observed in plant growth response to different treatments. *A. lipoferum* inoculation induced a more pronounced effect than that of *B. polymexa* inoculation which had a greater effect on growth than that of the foliar K and P application (Table I).

Nitrogen fixation associated with maize plants. Data in Figure 1 present the %NdFa in the shoots and roots tissues of plants inoculated by *Azospirillum* and *Bacillus* sp. Generally *Azospirillum lipoferum* inoculation resulted in higher plants %NdFa than *Bacillus polymexa* one. The percent of NdFa induced by *Azospirillum lipoferum* was 28 in shoot and 17.6 in root corresponding to 9.1 and 3.6 mg Npot^{-1}, respectively. However, the percent of NdFa induced by *Bacillus polymexa* was 18.8 in shoot and 20.4 in root

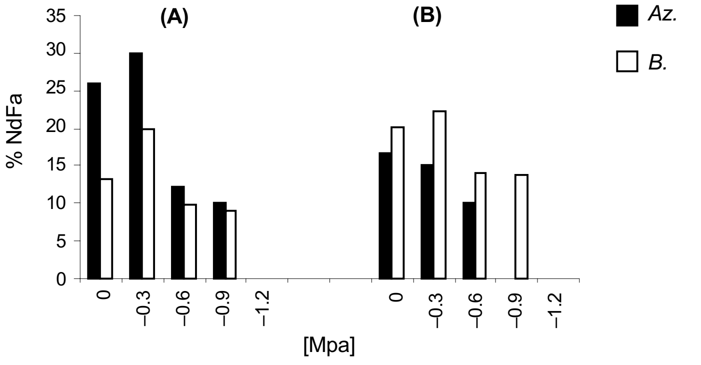

Fig. 1. % NdFa induced by *Azospirillum* sp. (Az) and *Bacillus polymexa* (B) in shoot (A) and root (B) of maize grown under different salt levels.

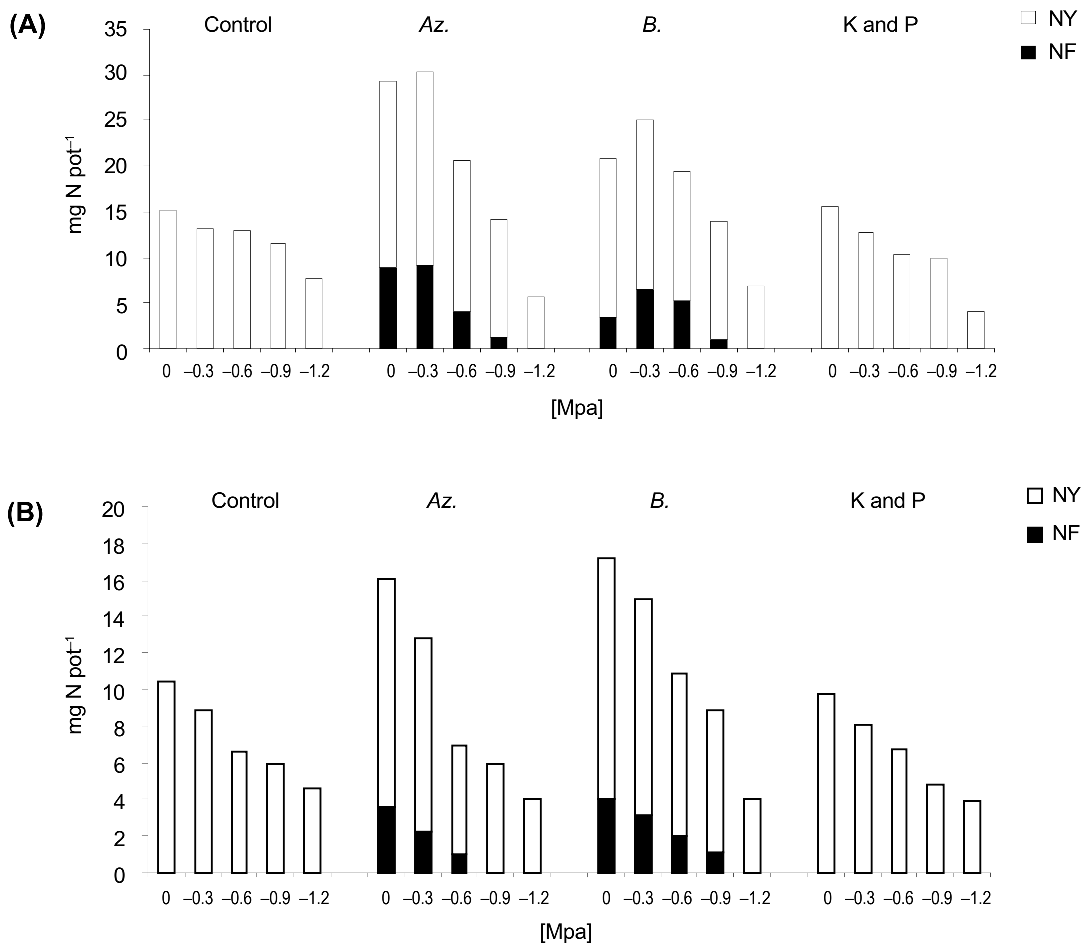

Fig. 2. Total N yield (NY) and fixed N (NF) induced by *Azospirillum* sp.(Az) and *Bacillus polymexa* (B) or P and K application in shoot (A) and root (B) of maize grown under different salt levels

corresponding to 6.5 and 4 mg N pot^{-1}, respectively (Fig. 2). Generally, NdFa was significantly decreased by increasing salt concentration as compared with nonsalinized plants. Higher %NdFa was obtained when plants were inoculated with *Azospirillum* or *Bacillus* at –0.3 Mpa NaCl. However, at high salinization level (–1.2 Mpa), nitrogen fixation was completely inhibited.

Changes in P, K, Mg, Ca and Na ion balance in maize plants induced by bacterization or foliar K and P application. Bacterial inoculation or foliar K and P application significantly changed the ion balance in both shoot and root tissues. However, no common pattern was observed in these changes among different treatments. Analysis of Mg/K balance revealed that *A. lipoferum* and *B. polymexa* inoculation induced significant increases in the ratio of these ions in shoot and root tissues at salinity levels up to –1.2 Mpa compared with those of the uninoculated plants. However, KH_2PO_4 foliar application lowered the Mg/K ratio (Fig. 3). On the other hand the P/K and Ca/K ratios in both shoot and root tissues were lower by plant bacterization or P and K application up to –1.2 Mpa in comparison to those of uninoculated and untreated plants (Fig. 3). The reduction of P/K ratio in the shoot system was more evident when plants were sprayed with P and K compared with bacterial inoculation. The Na/K ratio in both shoot and root tissues of control plants was increased with the increase in salt stress. Inoculation by *Azospirillum* sp. or *Bacillus* sp. and P and K application lowered this ratio in both shoot and root tissues (Fig. 3).

The soluble saccharides accumulation increased in shoots and decreased in roots as the salt stress increased in control plants (Table II). However, *Azospirillum* sp. inoculation significantly increased the soluble sugar content in both shoots and roots up to –0.6 and –1.2 Mpa salinity levels, respectively. On the other hand *B. polymexa* and P and K application had no marked effect on the soluble sugars content in plants. In control plants soluble protein concentration decreased in roots but increased in shoots as the salt stress increased. Though, inoculation with *Azospirillum* sp. or *Bacillus* sp. and KH_2PO_4 application markedly enhanced protein accumulation in the roots but not in shoots (Table II). Protein accumulation was more prominent in shoots by KH_2PO_4 application.

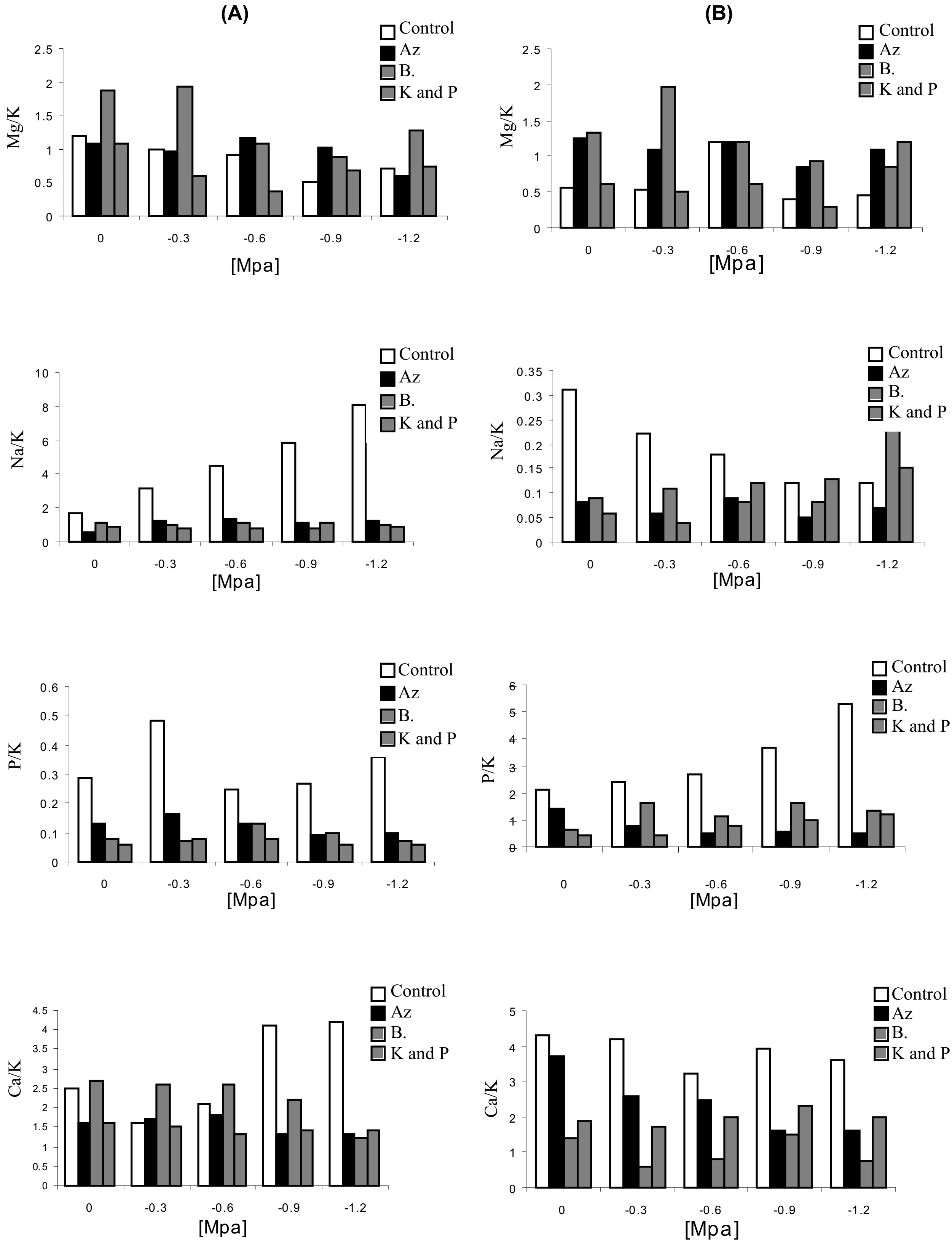

Fig. 3: Changes in Mg/K, P/K, Na/K and Ca/K ratios induced in shoot (A) and root (B) in corn by *Azospirillum* sp. (Az) and *Bacillus polymexa* (B) inoculation or K and P application grown under salt stress

Total amino acids was almost unchanged by increasing salt stress in control uninoculated plants. However, it was decreased by increasing salt stress in the root system (Table II). Plant bacterization or foliar application with K and P significantly enhanced total amino acids accumulation in both root and shoot

Table II
The effect of foliar P and K fertilization and/or bacterial inoculation on soluble sugar [mg g^{-1} (d.m.)], soluble protein [mg g^{-1} (d.m.)], amino acids [mg g^{-1} (d.m.)] and proline content [mg g^{-1} (d.m.)] of shoots and roots of maize plants grown at different levels of NaCl

	NaCl [Mpa]	Soluble sugar		Soluble protein		Amino acids		Proline	
		Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Control	0.0	13.31	18.9	45.4	38.5	6.92	4.78	1.60	2.87
	0.3	15.6	17.9	25.9	15.9	4.51	6.46	2.44	2.64
	0.6	17.8	12.7	26.7	11.4	6.19	5.86	4.57	3.45
	0.9	29.7	7.43	52.3	13.7	8.17	3.99	2.66	5.39
	1.2	24.8	1.6	85.5	4.87	6.05	2.03	3.27	5.24
Azoapirillum	0.0	15.6	18.27	21.71	20.95	13.6	15.61	4.40	1.89
	0.3	19.5	19.9	26.6	21.6	13.3	15.49	3.62	2.37
	0.6	22.9	19.9	30.2	20.2	12.9	14.40	4.88	4.83
	0.9	20.8	18.9	25.9	20.9	12.0	9.79	5.41	4.66
	1.2	23.8	16.2	18.4	24.2	14.2	3.97	2.49	1.96
Bacillus	0.0	9.27	7.93	30.3	25.6	6.15	6.74	4.35	1.55
	0.3	19.9	8.00	31.81	25.6	4.23	4.99	2.71	2.25
	0.6	12.9	12.1	24.9	20.2	8.37	7.83	4.49	1.53
	0.9	14.9	13.9	22.8	26.6	10.0	7.25	3.99	1.74
	1.2	15.5	11.8	27.3	27.7	10.6	8.32	2.44	5.41
KH_2PO_4	0.0	12.6	16.9	16.2	53.6	7.97	7.20	3.62	1.67
	0.3	20.3	16.5	15.1	55.5	6.58	8.56	1.99	3.36
	0.6	19.9	25.2	11.6	52.6	7.59	6.75	3.28	3.75
	0.9	19.1	19.4	19.1	48.9	9.98	8.11	4.73	3.91
	1.2	15.8	22.0	15.9	51.2	15.52	6.60	3.60	5.73
LSD 5%		1.73	1.52	2.24	2.59	2.81	2.53	0.238	0.753

systems up to –1.2 Mpa salinity level compared with the control plants. Proline content significantly increased in shoots and roots with elevating NaCl levels. Proline content was rather unchanged in shoots while tended to decrease in roots by plant bacterization or KH_2PO_4 application.

Fig. 4 shows the results of the osmotic pressure of the root and shoot cell-sap. These results indicate that *Azospirillum* inoculation or foliar application with K and P significantly enhanced the shoot osmotic pressure up to –1.2 Mpa salinity level. On the other hand, *B. polymexa* inoculation stimulated root osmotic pressure at high salinity levels (–1.2 Mpa) while, K and P application resulted in sharp stimulatory effect at all salinity levels (Fig. 4).

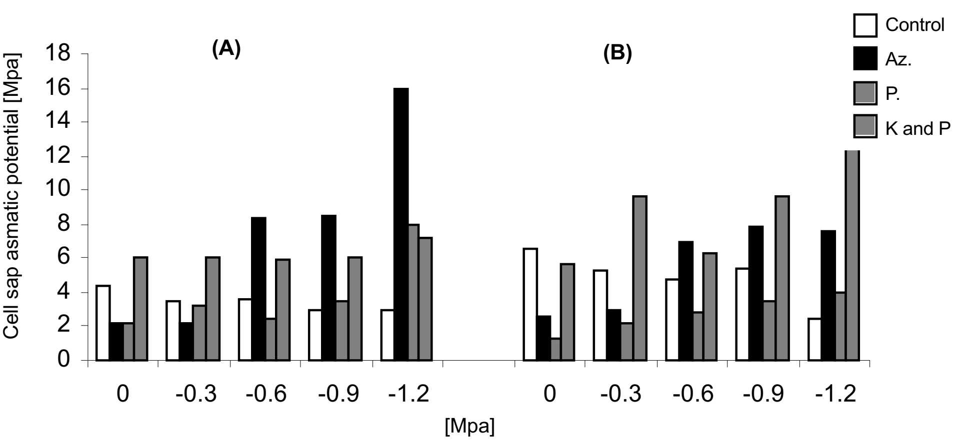

Fig. 4. Cell sap osmotic potential in shoot (A) and root (B) of maize grown under different salt levels

Discussion

Published experiments on the growth response of plants to the growth promoting rhizobacteria (PGPR) raise several questions regarding the mode of action of these bacteria when inoculated into the plant rhizosphere (Bashan and Holguin, 1997). Decreased total N-yield in control plants by increasing salt stress (Fig. 2) was attributed to the reduction in plant dry mass rather than the N%, since N-yield is a product of dry mas and N%. This finding was also reported by Hamdia and El-Komy (1998) using different methods of ^{15}N- techniques. In this study, estimation of N_2 fixation was based on the difference method (DM). Although, this method requires Kjeldal N analysis and therefore is time consuming, it is more informative in terms of N_2-fixed and dry matter content (Handarson and Danso, 1993; El-Komy and Abdel-Wahab, 1998).

Plant inoculation resulted in significant increases in shoot and root total N-yield (Fig. 2) up to –0.9 and –1.2 salinity levels, respectively. Increased total N-yield at low and moderate salinity levels (–0.3 to –0.9 Mpa) could be attributed to nitrogen fixation by rhizobacteria. However, increased total N-yield at high salinity levels (–1.2 Mpa) could be attributed to factors other than N_2-fixation, since nitrogen fixation was completely inhibited at such NaCl levels (Fig. 1). Inhibition of production and activity of nitrogenase at high salinity stress was reported by Tripathi *et al.* (2002). Moreover, it has been reported that salinity blocks the expression of *nifH* gene (Jofre *et al.*, 1998). The promotion of plant growth at high salinity levels by rhizobacterial inoculation could be due to its hormonal effect, enhanced mineral uptake (Stancheva *et al.*, 1995) and nitrate reductase activity (Boddey and Dobereiner, 1988; El-Komy *et al.*, 2003).

Enhanced mineral uptake in inoculated maize plants was studied as a possible mechanism of plant growth promotion by rhizobacteria spp. The major element involved was suggested to be nitrogen in the form of NO_3^- in wheat, sorghum, and corn plants (Boddey and Dobereiner, 1988), or NH_4^+ in rice plants (Murty and Ladha, 1988). However, other elements such as P and K were also suggested to play a key role in this plant-bacteria interaction (Sarig *et al.*, 1992).

Results of the present investigation indicated that bacterial inoculation or K and P application enhanced plant water content which was concomitant with increased plant dry weight up to –0.9 Mpa salt level. These findings agree with those by Creus *et al.* (1997), who reported that wheat inoculation with *Az. brasilense* sp-245 reversed part of the negative effects of NaCl stress by increasing plant water uptake. This was reflected by faster shoot growth in seedlings exposed to severe osmotic stress.

Results also showed that bacterial inoculation enhanced shoot and root Mg/K ratio significantly compared to control plants. The increased Mg/K ratio was accompanied by an increase in total soluble sugars accumulation in plant tissues specially those inoculated with *Azospirillum*. These results agree with those of Stancheva *et al.*, (1995) who postulated that *Azospirillum* inoculation enhanced the plant photosynthetic efficiency through mineral uptake specially Mg^{+2}. In addition, inoculation with rhizobacteria enhances the accumulation of photosynthetic pigments (Hamdia and El-Komy, 1998).

P/K and Ca/K ratio decreased in plants inoculated with *Azospirillum* sp. and *Bacillus* sp. or sprayed by KH_2PO_4 when compared to control plants (Fig. 3). Surprisingly foliar K and P application gave the lower shoot P/K ratio. This could be related to high K uptake rather than P accumulation as described by Marschner (1995). Na/K ratio increased progressively in both shoot and root tissues of control plants as salinity increased (Fig. 3). However, *Azospirillum* and *Bacillus* inoculation as well as K and P application retarded the accumulation of Na^+ in maize tissues. It is well known that the toxicity of NaCl at high concentrations contributed to the specific ion effects of both Cl^- and Na^+ which have antagonistic effects to other nutrients (Wallace and Berry, 1981).

Results also showed that plant bacterization had stimulatory effect on amino acids accumulation compared to control uninoculated plants as well as KH_2PO_4 application up to –1.2 Mpa salinity level. Such results were recorded previously at our laboratory (Hamdia and El-Komy, 1998). Thus plant bacterization could play an important role in protein biosynthesis either directly through nitrogen fixation (up to –0.9 Mpa) or indirectly by enhancing uptake of nitrogen and other minerals required for protein biosynthesis.

The obtained results and those of other investigations (Garcia *et al.*, 1997; Pelah *et al.*, 1995) indicated that inoculation of maize plants with nitrogen fixing rhizobacteria as well as K and P fertilization increases the efficiency of water utilization under stress condition. Hence some organic and/or inorganic osmotically active components of the cell sap were accumulated resulting in a concomitant increase in the osmotic pressure (Fig. 4) as a possible mechanism of adaptation to salinity.

It could be concluded that, both the nitrogen-fixing capacity (especially at low and moderate salinity levels) and the enhancement of mineral uptake by rhizobacterial inoculation, contribute to the tolerance of maize plants to salt stress.

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