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Effect of *Azotobacter vinelandii* and Compatible Solutes on Germination Wheat Seeds and Root Concentrations of Sodium and Potassium under Salt Stress

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Abstract: The effect of Plant Growth-promoting Rhizobacteria (PGPR) and exogenous application of compatible solutes on seed germination and root concentrations of sodium and potassium of two wheat varieties (*Triticum durum* L.) were evaluated under saline stress. In this experiment, *Azotobacter vinelandii* strain DSM85, glycine betaine and proline were used. Inoculated seeds for each variety were placed on Whatman paper in 9 cm Petri dishes containing 15 mL of distilled water or NaCl solutions at various concentrations (control, 100, 200, 300 mM) supplemented with or without Glycine Betaine (GB) or proline at 5 mM. The results indicated that addition of proline (5 mM) stimulated the production of indol acetic acid and the growth of *A. vinelandii* at 200 and 300 mM NaCl, respectively. The germination rate index and the germination final percentage decreased significantly ($p<0.05$) with increasing salinity level. The germination was significantly diminished at 300 mM with significant variation among varieties and Waha variety had higher germination percentage than Bousselam variety. Inoculation of seeds by *A. vinelandii* and exogenous application of proline had significantly positive effect on the germination at this concentration of NaCl. The rate of accumulation of Na^+ in roots was important at 100 mM and increased at 200 mM. The concentration of K^+ decreased when salinity increased. The effect of inoculation or inoculation with proline decreased the accumulation of Na^+ and reduced the loss of K^+ under salt stress. From the present study we can conclude that the use of *A. vinelandii* strain DSM85 and external application of low concentrations of proline on seeds might be considered as a strategy for the protection of plants under saline stress.

Key words: *Azotobacter vinelandii*, glycine betaine, proline, germination, salinity, *Triticum durum* L.

INTRODUCTION

Salinity is a major environmental constraint to crop productivity throughout the arid and semi arid regions of the world. A third of arable land resources in the world are affected by salinity (Munns, 2000). The salt stress negatively affects plant growth and biological stability of ecosystems (Cordovilla *et al*., 1999). Seed germination is an important factor limiting plant growth. The saline conditions can decrease seed germination either by creating osmotic potential that prevents the absorption of water or by toxic effects resulting from high concentrations of Na^+ in the soil (Hussain *et al*., 2003). Thus, the limitation of water absorption can cause various structural, physiological and biochemical modifications of seeds (Ashraf and Foolad, 2007) that can reduce the rate of germination and retard plant development (Poljakoff-Mayber *et al*., 1994).

Several attempts have been made to reduce the drastic effect of salt stress on growth and productivity of plants. Most of them focus on the development of salt-resistant varieties. Indeed, these varieties have developed various biochemical and physiological mechanisms to combat this type of stress. Such a mechanism, ubiquitous in plants, is the accumulation of some organic metabolites of low molecular weight especially during germination and early growth (Bewley and Black, 1994; Bolarin *et al*., 1995; Turan *et al*., 2007).

The exogenous application of some of these compounds, such as proline and glycine betaine, to improve the capacity of stress tolerance of plants received the attention of many researchers for many years (Mansour, 1998; Rahman *et al*., 2002). Thus, the application of low concentrations of glycine betaine and proline maintains a high concentration of K^+ in leaves of tomato (Heuer, 2003) and in the roots of barley under salt stress (Cuin and Shabala, 2005). However, few studies on the role of exogenous application of the glycine betaine or proline on germination and growth of young seedlings are performed.

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Recently, a biological approach using the inoculation of plants by rhizobacteria (Plant Growth Promoting Rhizobacteria: PGPR) has been attempted. Under salt stress, the most appropriate solution is the use a salt tolerant bacterial inoculum that can develop strategies to facilitate the growth of plants in saline soils (Bacilio *et al*., 2004). The PGPR are able to adapt to adverse conditions and to enhance plant growth at high osmolarity (Saleena *et al*., 2002; Biari *et al*., 2008). These salt-tolerant rhizobacteria can develop molecular mechanisms to survive and grow with the increase in salinity (Tripathi *et al*., 2002). According to many authors, most halotolerant bacteria can accumulate or synthesize organic compatible solutes, such as glutamine, proline and glycine betaine. Thus, the degeneration and the root exudation provide a supply of different amino acids and sugars readily available in the rhizosphere (Barber and Martin, 1976). A relevant example is the improvement of the growth of *Azotobacter* under the effect of root exudates of wheat and barley whose proline and essential sugars are among the most important components (Madkour *et al*., 1990). Under salt stress, the PGPR showed positive effects in plants, particularly on parameters such as the rate of germination, tolerance to drought, the weight of stems and roots (Kloepper *et al*., 2004; Kokelis-Burelle *et al*., 2006).

In view of these reports, it was postulated that the application of compatibles compounds and inoculation by rhizobacteria could alleviate the adverse effects of salt stress on the growth of wheat genotypes. Thus, the objectives of this study were to determine the effect of compatible solutes, glycine betaine and proline, on the growth and the capacity of production of indole acetic acid of a rhizobacteria : *Azotobacter vinelandii* DSM 85 in saline conditions. The consequences of inoculation by this strain and the beneficial effect of the exogenous application of glycine betaine and proline on the seed germination and on the ionic balance in two varieties of durum wheat under salt stress, were analyzed.

MATERIALS AND METHODS

Plant material: The seeds of two varieties of wheat salt-sensitive (*Triticum durum* L. cv. Waha and *Triticum durum* L. cv. Bousselam) were provided from the "Institut Technique des Grandes Cultures" (I.T.G.C) of Sétif-Algeria. Seeds were surface sterilized with a solution of sodium hypochlorite (2%) for 30 min and were rinsed several times with sterile distilled water.

Bacterial strain and inoculation: The strain of *Azotobacter vinelandii* (DSM 85) (A) provided by DSMZ was grown at 28°C/48 h on the nitrogen-free Winogradsky broth (Holt *et al*., 1994) containing mannitol (1%) as source of carbon. The cells were recovered by centrifugation (12000 xg 10^{-1} mn) and rinsed twice in PBS to obtain a density of 10^8 bacteria mL^{-1}. Sterile wheat seeds were immersed in the bacterial suspension for 30 min.

Growth of *A. vinelandii* DSM 85 in the presence of salt and/or compatible solutes: Culture of *A. vinelandii* DSM85 was performed in nitrogen free Winogradsky broth supplemented with NaCl concentrations from 0 to 600 mM. Glycine Betaine (GB) and proline (P) were added aseptically to the medium at a final concentration of 5 mM. Cultures were incubated at 28°C for 3, 6 and 9 days. Growth was measured at 630 nm.

Production of indole acetic acid (IAA) by *A. vinelandii* DSM 85: The effect of the addition of GB and P at 5 mM on the capacity of production of IAA by *A. vinelandii* DSM85 was determined on Winogradsky medium supplemented with tryptophan (5 g L^{-1}) at different concentrations of NaCl (0, 100, 200 and 300 mM). The cultures were incubated at 28°C/6 days. Presence of IAA was revealed by addition of the reagent of Salkowsky. The intensity of the color was measured at 530 nm (Sarwar *et al*., 1992).

Seed germination in the presence of salt and/or compatible solutes: The objective of this test was to evaluate the effects of inoculation with *Azotobacter vinelandii* DSM85 (A) and the addition of compatible solutes GB and P on seed germination of wheat under salt stress. 30 seeds of each variety of wheat were placed on Whatman (N°40) filter papers in 9 cm Petri dishes. 15 mL of distilled water or different solutions of NaCl were added. The Petri dishes were then divided into four groups and each group was subdivided into six subgroups. The four groups represented the concentrations of NaCl (control, 100, 200, 300 mM). The six subgroups indicated the type of treatment (control, +GB, +P, +A, +A+P, +A+GB). The solutions of sterile GB and P were added aseptically to a final concentration of 5 mM. The covered Petri dishes were incubated in the dark at 23±1°C. The experiment was performed in triplicate. The number of germinated seeds was counted after 3, 6 and 9 days of incubation. Seeds were considered germinated when the radicle reached at least 3 mm long (Al-Karaki, 2001). This test permitted to determine 2 parameters: final percentage and rate germination index. Final germination percentage was determined after 11 days of incubation when no further seeds germinated:

- The final germination percentage was determined by the following equation:

$$\text{Final germination percentage} = \frac{\text{No. of germinated seeds}}{\text{Total No. of seeds}} \times 100$$

- The germination rate index was determined by using the modified formula described by Bouton *et al.* (1975):

$$\text{Germination rate index} = \frac{G3}{3} + \frac{G6}{6} + \frac{G9}{9}$$

G3, G6 and G9 were percentages of germination×100 at 3,6 and 9 days after initiation of germination.

Concentrations of Na^+ and K^+ in the roots: The dry matter (0.05 g) of roots was digested in 10 mL of H_2SO_4 at 98% and 3 mL of 30% H_2O_2 for 5 h using the method of Skoog *et al.* (2000). The content of Na^+ and K^+ was determined by flame spectrophotometer.

Statistical analysis: Each data point was the mean of three replicates for germination parameters and the root Na^+ and K^+ contents. All data were statistically treated by three-way of analysis of variance and at Least Significant Difference (LSD) test at probability level 0.05 was used to compare the means when the Tukey test indicated a significant effect of the treatments.

RESULTS

Salt tolerance of *A. vinelandii* DSM85 with compatible solutes: The effect of salt stress on growth of *Azotobacter vinelandii* DSM85 (A) was determined (Fig. 1). Optimal growth was observed at a concentration of 100 mM NaCl at 3 and 6 days of incubation. While for 9 days of incubation, the maximum absorbance was observed at 200 mM. During this incubation period, the growth of this bacterial strain was inhibited almost beyond 300 mM. The results of the effects of GB and P on the growth of *Azotobacter vinelandii* DSM85 were different. The effect of P was positive and significantly high during all periods of incubation at salinity levels where the growth was observed. However, GB had an influence, often minor, on the tolerance of *Azotobacter vinelandii DSM85* to salt stress.

Production of IAA under salt stress and with compatible solutes: The increase of salinity strongly inhibited the production of IAA by *Azotobacter vinelandii* DSM85 (Fig. 2). Production of this phytohormone was null at 300 mM even in the presence of GB or P. However, for other levels of salinity, P improved significantly the production of IAA where the concentrations were higher and as follow 19.81, 16.03 and 15.29 mg L^{-1} at 0, 100 and 200 mM, respectively. The effect of GB on the synthesis of the IAA showed the lowest results at different concentrations of NaCl.

Effect of salinity and treatment on seed germination: Analysis of variance of the results of the germination rate and final germination percentage of both wheat varieties showed that salinity had a significant negative impact on the growth parameters ($p \leq 0.01$) (Table 1). The treatment effect was also significant for both wheat varieties. They were significantly different for the two variables of germination. Significant interactions between treatment×salinity level, between level of salinity×variety

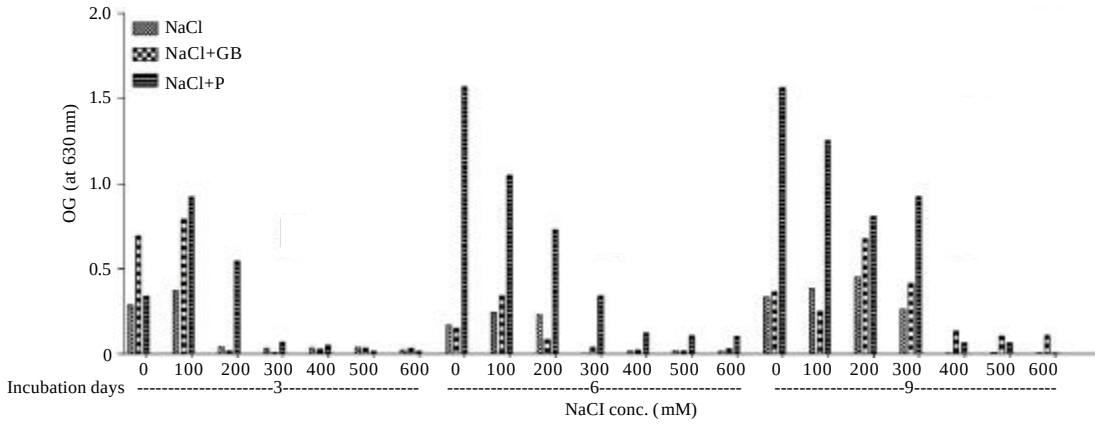

Fig. 1: Effects of salt stress and compatible solutes (GB or P) on growth of *Azotobacter vinelandii* DSM85, OG: Optimal growth

Table 1: Analysis of variance summaries (mean squares) of data for germination rate index, final germination percentage, concentrations of Na^+ and K^+ in roots

Source of variation	df	Germination rate index	Final germination (%)	Na^+ in roots (mg g^{-1} DW)	K^+ in roots (mg g^{-1} DW)
Salinity (S)	3	80.0262**	325.0229**	1487.2427**	198.8724**
Treatment (T)	5	25.8154**	8.9112**	31.2709**	66.3863**
S×T	15	1.2664^{ns}	4.3725**	36.9184**	5.7105**
Variety (V)	1	61.9809**	36.4525**	40.8804**	101.8317**
S×V	3	6.1897**	13.3150**	19.0453**	15.2819**
T×V	5	5.5264**	25.5972**	23.7978**	28.5050**
S×V×T	15	1.7441^{ns}	9.1883**	34.2187**	4.5115**
Error	48	0.00247	0.00454	9.51581	0.25584

*,**Significative at a level of 5% ($p \leq 0.05$) and 1% of probability ($p \leq 0.01$), respectively, ns: Non-significative ($p \geq 0.05$), df: Degree of freedom

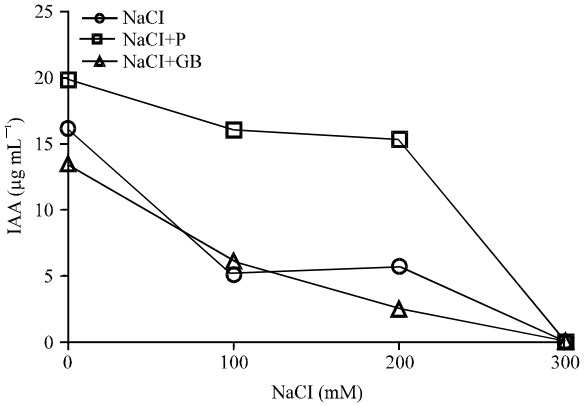

Fig. 2: Production of IAA (µg mL^{-1}) by *Azotobacter vinelandii* DSM85 with compatibles solutes (GB or P) before and after exposure to 100, 200 and 300 mM NaCl stress

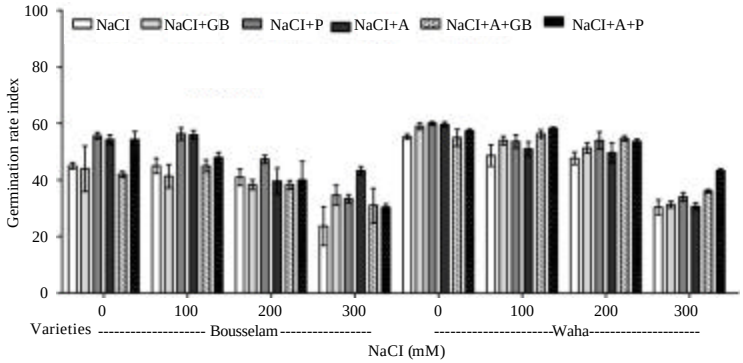

Fig. 3: Germination rate index in two wheat durum varieties at different treatments on before and after exposure to 100, 200 and 300 mM NaCl stress, results are shown as Mean±standard error ($p \leq 0.05$) from three replicates

and between treatment x variety on final germination percentage were also observed.

From the mean results of the germination rate and final germination percentage (Fig. 3, 4), it is clear that these two parameters had decreased by increase of salinity. Germination was significantly reduced at the highest NaCl concentration (300 mM). Inoculation or inoculation with P significantly decreased the effect of salt stress on seed final germination percentages at this concentration of NaCl. The results of the germination rate of seeds inoculated or uninoculated in the presence of GB were similar but lower. However, the effect of treatment on the germination rate at different NaCl concentrations was not clearly observed. The differences between the two varieties were low but Waha was more tolerant than Bousselam at all levels of salinity.

Effects of salinity and treatment on Na^+ and K^+ in roots: Salinity also showed a significant effect on the concentration of Na^+ in roots of both varieties of wheat

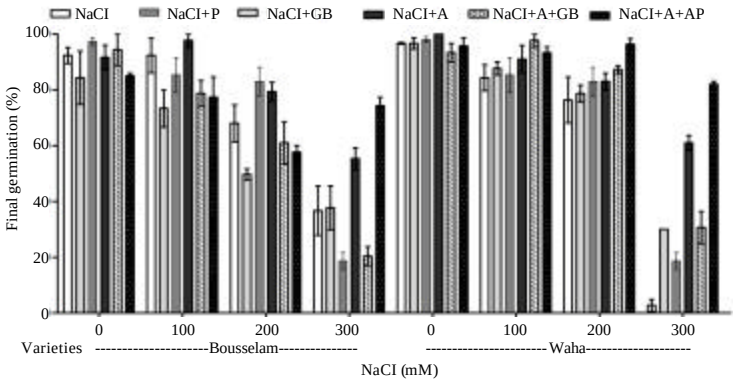

Fig. 4: Final germination percentage (%) in two wheat durum varieties at different treatments before and after exposure to 100, 200 and 300 mM NaCl stress, results are shown as Mean±standard error ($p \leq 0.05$) from three replicates

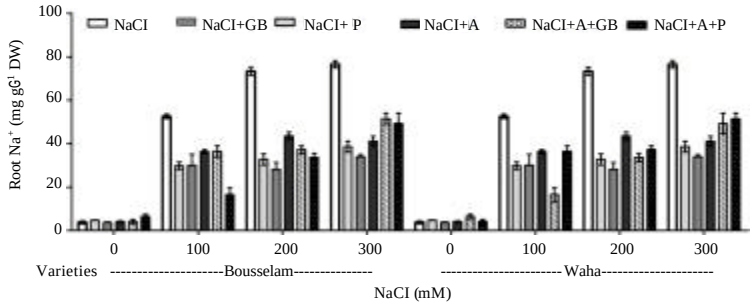

Fig. 5: Root Na^+ content in two wheat durum varieties at different treatments before and after exposure to 100, 200 and 300 mM NaCl stress, results are shown as Mean±standard error ($p < 0.05$) from three replicates

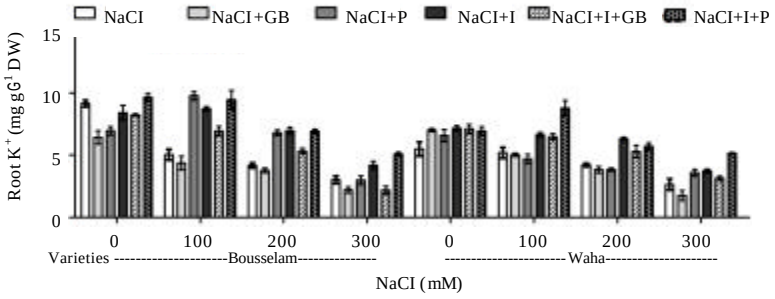

Fig. 6: Root K^+ content in two wheat durum varieties at different treatments before and after exposure to 100, 200 and 300 mM NaCl stress, results are shown as Mean±standard error ($p \leq 0.05$) from three replicates

($p \leq 0.01$) (Table 1). Our results also indicated that the content of Na^+ was strongly affected by different levels of salinity. This ion increased with the level of salt in the medium (Fig. 5). The rate of accumulation of Na^+ was important at 100 mM and increased at 200 mM. The effect of inoculation or inoculation with P or GB decreased significantly the accumulation of Na^+. A higher concentration of Na^+ was observed in the variety Waha at 300 mM of NaCl. The concentration of K^+ was also significantly affected by salt stress (Table 1). Generally, concentration of K^+ decreased when salinity increased (Fig. 6). This decrease was lower for Waha at different

concentrations of NaCl. The effect of various treatments reduced the loss of K^+ under salt stress. In addition, inoculation in the presence of P allowed a better accumulation of this ion mainly at 300 mM.

DISCUSSION

The germination rate index and final germination percentage of two wheat varieties decreased with increase of NaCl concentration. Significant variation in salt tolerance was observed in two varieties where Waha had a higher salt tolerance revealed by the two parameters of germination. These results were similar to those reported by Othman *et al.* (2006) and Li (2008) who found that the high level of salinity of the medium could significantly inhibit seed germination. The decrease in germination rate particularly under drought and salt stress conditions may be due to the fact that seeds develop an osmotically enforced "dormancy " under water stress conditions. This may be an adaptive strategy of seeds to prevent germination under stressful environment thus ensuring proper establishment of the seedlings (Gill *et al*., 2003). The results showed that seeds inoculated and inoculated with proline improved significantly germination under salt stress, however, the effect of seed treatment by glycine betaine was not as important. Significant increases in the growth and crop yield in response to inoculation with PGPR have been reported by Asghar *et al.* (2002), Bashan *et al.* (2004) and Biswas *et al.* (2000). Nelson (2004) noted that the PGPR were able to exert a beneficial effect on plant growth such as increased germination rate. This positive effect of PGPR on seed germination and emergence could be attributed to bacterial ability to produce or modify plant hormones including gibberellins which play a key role in germination (Sarkar *et al*., 2002; Gaber, 2003; Barassi *et al*., 2006). For example, cultures of *Azotobacter* used as inoculants were reported as producers of gibberellic acid, indole 3 acetic acid and cytokinins which promote germination and plant growth (Brown and Burlingham, 1968; Barea and Brown, 1974; Keyeo *et al*., 2011).

On the other hand, many studies demonstrated the improvement of seed germination of different plant species under both normal conditions and under stress with the exogenous application of plant growth hormones and other organic substances (Ashraf and Foolad, 2005; Egamberdieva, 2009). In wheat, the negative effect of salinity was reduced by soaking seeds with indole acetic acid (Akbari *et al*., 2007). Abbas and Okan (1993) suggested that the indole acetic acid and other plant hormones were responsible for an increase in the growth of canola, tomato (*Lycopersicon esculentum* Mill.) and wheat (*Triticum turgidum* L.) in unsterile soil inoculated by *Azotobacter paspali*. In our study, *A. vinelandii* DSM85 was able to produce IAA in salinity levels from 0 to 200 mM. Concentrations of IAA were large enough when the proline was present in the environment that allowed *Azotobacter vinelandii* DSM85 had a PGPR activity in saline medium.

Shaukat *et al.* (2006) reported that inoculation with *Azotobacter* increased the final germination percentage of wheat seeds about 58.6%. Kloepper *et al.* (2004) showed that wheat yields increased up to 30% with inoculation with *Azotobacter*. According to Jagnow (1987), inoculation of wheat and maize with *Azotobacter* increased the aboveground portion of the plant (26-50%) and yield (19-30%).

The application of exogenous compatible solutes on inoculated and uninoculated seeds allowed a significant reduction of the effect of salinity on two wheat varieties with, however, better final germination percentages for Waha variety. These results were similar to those reported by many authors in which they showed that the exogenous application of P had led to a significant increase the performance and morphological parameters of wheat growth i.e., the height and the number of ears. Thus, the accumulation of proline should avoid the adverse effects of low osmotic potential of cells, without interfering with protein synthesis (Raggi, 1994; Wyn Jones, 1981).

Seed treatment with GB could mitigate the negative effects of salt stress on several varieties of wheat but the response of exogenous application of GB of seeds was specific to the variety. Given the existing literature, it was clear that the effect of GB could be positive or negative on plant growth, for example, the exogenous application of GB enhanced the growth and yield of wheat (Borojevic *et al*., 1980) and *Gossypium hirsutum* (Naidu *et al*., 1998; Gorham *et al*., 2000). However, other studies found that the effect of exogenous application of GB on wheat and *Gossypium hirsutum* was not significant (Agboma *et al*., 1997; Meek *et al*., 2003).

The effect of salt stress increased the absorption of Na^+, whereas, the absorption of K^+ decreased in the roots for the two wheat varieties tested. Bhivare and Nimbalkar (1984) found that reducing the amount of K^+ and increased the content of Na^+ could be attributed to the effect of competition between Na^+ and K^+ on the sites of absorption in the plant. The toxicity of salt in the seeds would generally result in a marked decrease in K^+ concentration that caused a decrease in growth by reducing the ability of plants to maintain turgor and osmotic adjustment and to have many negative effects on metabolic functions such as protein synthesis

(Mudgal *et al*., 2010). Inoculation with *Azotobacter* increased the accumulation of K^+ but concentration of Na^+ was reduced. This finding was similar with the results of Elshanshoury (1995) and could be attributed to a decrease in the concentration of Na^+ in the medium by bacterial inoculation. The role of *A. vinelandii* in the production of substances that promote growth and nitrogen fixation could also be considered (Kader *et al*., 2002). It was shown that PGPR strains producing bacterial exopolysaccharides that could bind with some cations, including Na^+ (Geddie and Sutherland, 1993; Han and Lee, 2005).This notion was further supported by the findings of Ashraf *et al*. (2004) where the increase in population density of PGPR in the root zone could reduce the concentration of Na^+ available for the absorption of the plant.

An exogenous application of low concentrations of GB and P under salt stress, maintained the concentration of K^+ to a higher level in tomato leaves (Heuer, 2003) and induced a decrease in the loss of K^+ in roots of barley (Cuin and Shabala, 2005, 2007).

According to Lutts (2000), the application of GB at 1 mM through a nutrient solution was effective in improving the growth and reduction the accumulation of Na^+ in rice under salt stress. While in barley, exogenous application of P resulted in a decrease of the accumulation of Na^+ and Cl-in the cultures of embryos and the increase of the growth (Lone *et al*., 1987). Our results indicated that a concentration of 5 mM resulted in a decrease in the accumulation of Na^+ and reduced the loss of K^+. However, in rice, at P concentration of 30 mM was most effective in improving germination and seedling growth under salt stress and at increased the ratio K^+/Na^+ (Roy, 1993). Thus, these compatible solutes were generally effective in reducing the loss of K^+ in response to salinity (Cuin and Shabala, 2005, 2007).

From the results obtained in this study, we can conclude that salinity causes a reduction in the growth of *A. vinelandii* DSM 85 and its production capacity of the IAA. The inoculation of A. *vinelandii* and exogenous application of P improved germination and reduced the loss of K^+ in roots of the seeds of two varieties of wheat used under salt stress. Therefore, the protective role of GB on germination was less pronounced and it was negative on the production of IAA under salt stress.

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