academicJournals

Vol. 12(34), pp. 5278-5284, 21 August, 2013
DOI: 10.5897/AJB12.2750
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Ameliorative effects of salt resistance on physiological parameters in the halophyte *Salicornia bigelovii* torr. with plant growth-promoting rhizobacteria

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Accepted 27 May, 2013

Salicornia bigelovii **is a promising resource to cultivate under extreme climatic conditions of arid-desert regions. However, the production of** ***Salicornia*** **depends on the appropriate supplementation of nitrogen rich synthetic fertilizers. Application of specific halotolerant nitrogen-fixing bacteria associated with** ***S. bigelovii*** **could be an important practice for crop production in salt-affected regions. Seedlings of** ***S. bigelovii*** **were inoculated and developed with plant growth promoting rhizobacteria (*Klebsiella pnseumoniae*) at different salinities (0 and 0.25 M NaCl) grown under** ***in vitro*** **conditions. The inoculation increased growth and physiological activity at a high salinity. The major benefits of inoculation were observed on total seedlings biomass (320 and 175 g at 0 and 0.25 M NaCl, respectively) and adjacent branches of stem biomass (150 and 85 g at 0 and 0.25 M NaCl, respectively). The inoculation with** ***Klebsiella pneumoniae*** **also significantly improved seedlings salinity tolerance compared to the non-inoculated controls. In non-salinity conditions, the inoculated seedlings enhanced the CO_2 fixation and O_2 evolution. The non-inoculated controls were more sensitive to salinity than inoculated seedlings exposed to salinity, as indicated by several measured parameters. Moreover, inoculated seedlings had significantly increase on proline, phenolics content, but not significant in starch compared to non-inoculated controls. In conclusion,** ***K. pneumoniae*** **inoculation mitigates the salinity effects and promotes the** ***Salicornia*** **growth.**

Key words: *Salicornia bigelovii*, *Klebsiella pneumoniae*, halophyte, ecotype, stress salinity.

INTRODUCTION

Sustainable agricultural productivity in arid regions includes selection, evaluation and development of salt-tolerant plants, focusing on the desirable crops adapted to dry saline and desert areas (Jefferies et al., 1981; Glenn et al., 1999; Chatrath et al., 2000; Ungar, 2000). Increasing interest on the range of salt levels in soils to enhance the salt-tolerance crops (Stove, 1997); in Sonoran desert, *Salicornia bigelovii* has a wide distribution along the coast Peninsula (Bashan et al., 2000). *S. bigelovii* was identified among many halophytic species tested for possible domestication, because it is considered as a new promising economical resource of oilseed production

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(Glenn et al., 1999). *S. bigelovii* is a facultative annual halophyte that possesses a high potential for an agro-industrial commodity (Glenn et al., 1991). *S. bigelovii* is a leafless, jointed and succulent stems that form terminal fruit-bearing spikes, in which seeds are borne (Gallawa, 1996). However, the productivity of *S. bigelovii* is limited by available nitrogen (Loveland and Ungar, 1983).

Traditionally, farmers apply synthetic fertilizers to compensate for soil nitrogen deficiency. However, indiscriminate use of synthetic fertilizers might increase salinity and severely damage the soil microfloral structure and composition (Akhavan, 1991; Nahid and Gomah, 1991). Recent studies on bacterial diversity within the rhizosphere of salt marsh plants have been published, whereas, most studies being related with *Spartina alterniflora* (Lovell et al., 2000).

According to *Salicornia* spp., studies have been carried out by Rueda et al. (2003, 2004, 2007). In previous studies, to increase on the knowledge of known halotolerant nitrogen-fixing bacteria used as a biofertilizer (Rueda et al., 2003, 2004); currently, it is very important to know the physiological aspects behind the identified halotolerant nitrogen-fixing bacteria which help to reduce the major abiotic stresses like water logging, drought, temperature and salinity on desirable halophytes. In this study, was tested a known plant growth-promoting bacterium, *Klebsiella pneumoniae*, on *S. bigelovii* - Cerro Prieto ecotype. To address the next hypothesis: the colonization of *K. pneumoniae* may enhance the salinity tolerance expose to NaCl on *S. bigelovii* was examined on the levels of electrolytes, proline, phenolics, and starch content and related them to gas exchange and salinity tolerance on inoculated and non-inoculated seedlings under salinity stress.

MATERIALS AND METHODS

Plant material and *in vitro* growth conditions

Seeds of *S. bigelovii*-Cerro Prieto (CP) ecotype were collected from salt marsh lands of Sonoran bay. Plants were sifted to separate mature seeds. The largest seeds remains cleaned and selected with uniform color, shape, size and structure with no visible damage. Seeds of CP ecotype were disinfected by immersion in sodium hypochlorite (3% active chlorine) for 30 s; they were then washed three times with sterilized distilled water. Germination was performed in sterilized Petri dishes, each with a cotton layer substrate (150 × 15 mm) covering the bottom of the dish. Dishes were moistened with uniform amounts of water sterile solution. Germination was performed inside a growth chamber at 27 ± 0.5°C and 35 ± 1% relative humidity (RH), with continuous white light (Environmental Chamber, Biotronette® Mark III, Melrose Park, IL, USA); 20 ml of the appropriate solution was added every 4 days to each dish.

Plant inoculation

The bacterial inoculum was produced by transferring loops of *K. pneumoniae* to 100 ml of OAB liquid medium (Okon and Labandera, 1994) in a 250-ml Erlenmeyer flask incubated at 28°C at 100 rpm for 48 h (Ait Barka et al., 2002). Bacteria were collected by centrifugation (3000 *g* for 15 min) and washed twice with phosphate-buffered saline (PBS) (10 mM, pH 6.5). The pellet was resuspended in PBS and used as inoculum. The bacterial concentration was estimated by spectrophotometry (600 nm) and adjusted to 10^6 CFU ml^{-1} with PBS (Ait Barka et al., 2002). The concentration was confirmed by plate counting. Seedlings, approximately 1 cm long taken from four weeks old stock seedling were immersed in the inoculum for 1 min, blotted with sterile filter paper, and placed in culture tubes as described previously (Ait Barka et al., 2002; Essaid et al., 2006). Non-inoculated control seedlings were dipped in PBS. The plants were incubated in the growth chamber as described earlier.

Salinity treatment

After 12 weeks, inoculated and non-inoculated *S. bigelovii* CP ecotype seedlings were divided into two sets: one set was transferred to a salinity growth chamber conditions (Environmental Chamber, Biotronette® Mark III, Melrose Park, IL, USA) maintained at 0.25 M of NaCl under a 15 h-photoperiod with light provided by white fluorescent lamps at an intensity of 200 µmol m^{-2} s^{-1}, and the other (control) set was kept under the conditions previously described. Each treatment consisted of 150 seedlings (repeated three times, each one of 50). Analyses were conducted after four weeks more of each treatment.

Electrolyte leakage

Adjacent branches of stem samples comprising the fifth and sixth branches from the basal end of the seedlings were taken from each seedling (n = 50), rinsed with distilled water and dried on filter paper. The adjacent branches were incubated in 30 ml mannitol (0.4 M) in 50-ml plastic bottles at 24°C for 20 h on a rotary shaker (80 rpm) described by Gognies et al. (2001). The conductance of the incubation medium was measured using a conductivity meter (Orion, model 150; Thermo Electron Corporation, Breda, The Netherlands). Samples were autoclaved at 120°C for 3 min and cooled to room temperature, and the final volume was adjusted to 30 ml. The conductivity of the samples was measured again to determine the total electrolyte content of the tissue. The degree of electrolyte leakage was calculated as described by Ait Barka and Audran (1996).

Free proline analysis

Determination of free proline content was carried out by Ait Barka and Audran (1997). Seedlings were frozen in liquid N_2 and kept at -80°C until used. Adjacent branches of stems, and roots were ground separately and homogenized in 3% (w/v) sulfosalicylic acid. The homogenate was filtered through Whatman no. 1 filter paper. After the addition of ninhydrin reagent [1% ninhydrin in 60% acetic acid (w/v)], the mixture was heated at 100°C for 20 min. The reaction was then stopped by ice. The mixture was extracted with 1 ml toluene, and the sample was vigorously shaken for 15 s. After 4 h in darkness at room temperature, sample absorbance of the toluene layer was read at absorbance of 520 nm. Proline concentration was determined by using a calibration curve and the values expressed as µM proline g^{-1} fresh weight (FW).

Total phenolics determination

The content of total phenolics was determined by using a modified protocol of Folin-Ciocalteu colorimetric method (Singleton and

Rossi, 1965). Fresh leaf tissue (600 mg) was ground in 5 ml ethanol (80%) using a tissue homogenizer. Samples were placed in 50 ml tightly covered plastic tubes and incubated at 4°C for 2 h in dark and then filtered as aforementioned. The pellet was resuspended in 2.5 ml ethanol. Five replicates of 125 µl phenolic extract, 625 µl 1/10 diluted Folin-Ciocalteu reagent, and 250 µl 7.5% (w/v) Na_2CO_3 were vortexed for 10 s, and the mixture was incubated at 45°C in a water bath shaker for 15 min. Phenolics were measured at 750 nm using catechin as standard. Total phenolics were expressed as ng g^{-1} FW.

Gas exchange measurements

Carbon dioxide exchange rates were measured on 50 seedlings from each treatment using a Li-Cor 6200 portable photosynthetic system (Li-Cor, Lincoln, Neb.) and a 250-ml gas exchange chamber. During the measurement, the gas exchange chamber was illuminated with a white light source (1000 µmol photons m^{-2} s^{-1}) under a relative humidity of 25 to 35%, a CO_2 air concentration of 420 to 460 µl l^{-1}, and a flow rate of 1125 µmol min^{-1}. O_2 evolution was measured with an oxygen electrode (Hansatech, Cambridge, United Kingdom). Three adjacent branches of stem were detached from each plant and placed in the electrode chamber. Saturating CO_2 conditions were maintained using 2 M potassium carbonate-potassium bicarbonate buffer, pH 9.3. The electrode buffer contained saturated potassium chloride.

Starch extraction and analysis

Organs were sampled from each plantlet (n = 50) and homogenized individually at 4°C in a mortar containing 0.1 M phosphate buffer, pH 7.5. The homogenates were centrifuged at 12,000 *g* for 15 min, and the pellets were used for starch analysis. The collected pellets were resuspended in dimethyl sulfoxide-8 M hydrochloric acid (4:1, v/v). Starch was dissolved over 30 min at 60°C with agitation at 60 rpm. After centrifugation for 15 min at 12,000 g, 100 µl supernatant samples were mixed with 100 µl iodine-HCl solution (0.06% KI and 0.003% I_2 in 0.05 M HCl) and 1 ml distilled water. The absorbance was read at 600 nm after 15 min of incubation at room temperature.

Quantification of bacterial cells adhered

Vital staining microorganisms with floureseine diacetate on and in roots less than 1 mm in diameter was performed according to Puente et al. (2004), and observed with an episcopic fluorescent microscope (Olympus BX41 equipped with fluorescence accessories series 2B, with excitation at 450 to 480 nm, a filter U-MW 640, and a digital camera - Cool Snap-Pro Color, Japan) attached to an image analyzer (Image ProPlus 4.5, Media Cybernetics, Japan). Quantification of bacteria adhering to the root system of *S. bigelovii* was carried out at the final study (16 weeks after sowing), fifteen plants of each treatment were washed with sterile, distilled water and introduced during 1 min into Eppendorf tubes with sterile water. Tubes were agitated for 1 min to detach bacteria from the roots. Three samples of 100 µl were taken from the bacterial solution of each tube and sowed by dispersion on N-free media OAB in Petri dishes, which were incubated during 24 h at 30°C for CFU measurement.

Statistical analyses

Data were analyzed statistically using analysis of variance. Least significant differences (LSD) between mean values of treatments were separated by Duncan's multiple range tests at $P < 0.05$. All statistical tests were performed with SAS (SAS, 2001).

RESULTS

Seedlings inoculation and growth promotion and quantification of bacterial cells adhered

In general, as expected, all seedlings, *Salicornia* roots were colonized by *K. pneumoniae*. The presence of *K. pneumoniae* increased significantly ($P < 0.05$) the root seedling growth, total biomass, stems and adjacent branches weight (Figure 1). A greater number of bacterial cells adhered to the roots with *K. pneumoniae* was observed without salinity; the quantification of bacteria adhering at the final study indicate presence of 3535 $CFU \cdot ml^{-1}$ (colony-forming bacterial units) without salinity and 3387 $CFU \cdot ml^{-1}$ at 0.25 M NaCl declining trend when increasing salinity. Also, it was detected that the bacterium initially colonized the root surface, followed by tissue penetration and colonization of the root interior, translocation via stem xylem vessels, and then endophytic colonization of adjacent branches tissues. These results are in agreement with Dong et al. (2001, 2003); they carried out a study under the same *in vitro* culture and bacterial inoculation conditions and demonstrated that *K. pneumoniae* establishes endophytic populations. At 0.25 M NaCl, inoculated seedlings had two fold higher total biomasses compared to those non-inoculated controls (Figure 1). Although, there was a significant ($P < 0.05$) enhancement of the biomass of all plants organs, the stimulation of root growth was the greatest approximately 25%.

After 10 days of salinity conditions treatment, the total biomass decreased for both treatments, and a significant difference between inoculated and control treatments was recorded for only root biomass.

Electrolyte leakage

Electrolyte liberation results showed that inoculation significantly ($P< 0.05$) improved the salinity tolerance of *Salicornia* seedlings (Table 1). Prior to seedlings exposure to the salinity treatment, ion leakage from branches of stem of both inoculated and non-inoculated control seedlings were similar (15 and 16%), respectively of the total adjacent branches electrolytes. However, after Salinity, the electrolyte leakage was approximately twice as great in the controls as in inoculated seedlings (45 and 23%), respectively.

Free proline, total phenolics and starch content

The inoculated treatments with and without salinity were significantly ($P < 0.05$); they increased free proline

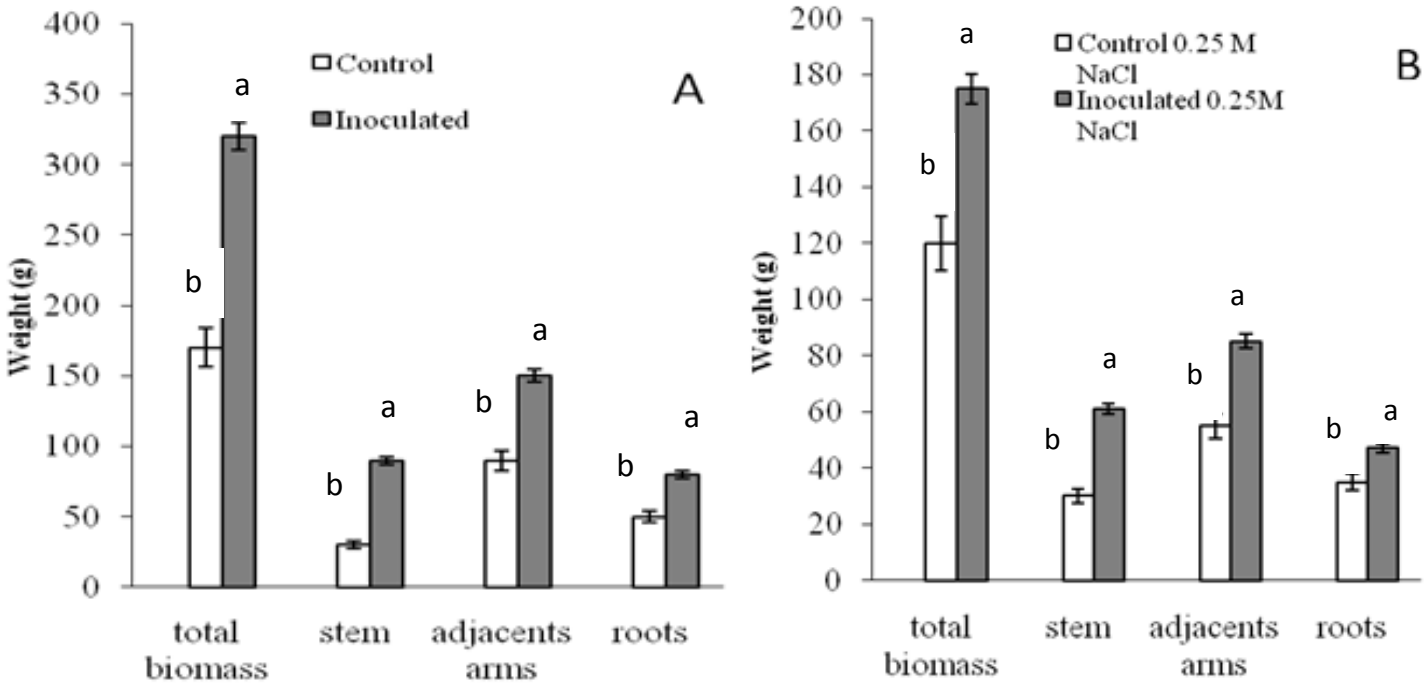

Figure 1. Effect of salinity on the weight of *Salicornia bigelovii* Cerro Prieto ecotype seedlings. (a) Seedlings without salinity conditions. (b) Under salinity conditions at 0.25 M NaCl. Error bars indicate standard deviations. For each histogram, the values by the same lower-case letters are not significantly different at $P < 0.05$.

Table 1. Effect of salinity on electrolyte leakage from aerial biomass-adjacent branches, adjacent branches-free proline, total phenolics and starch content of *Salicornia bigelovii* - Cerro Prieto ecotype seedlings.

Treatment	Electrolyte leakage* (%)	Free proline (µmoles g^{-1} FW) branches	Roots of stem	Total phenols (nanogram g^{-1} FW)	Starch content (mg g^{-1} FW) branches	Roots of stem
Control+	15.00 ±3^{c}	1.30 ±0.2^{cd}	0.09 ±0.04^{b}	21.21 ±3^{bc}	0.014±0.02^{bc}	0.016 ±0.08^{a}
Control++	45.00 ±10^{a}	3.50 ±0.7^{b}	0.23 ±0.15^{b}	23.65 ±2^{b}	0.013±0.03^{bc}	0.009 ±0.04^{ab}
Inoculated +	16.00 ±4^{c}	2.10 ±0.5^{c}	0.50 ±0.9^{a}	25.98 ±2^{ab}	0.019±0.02^{a}	0.010 ±0.04^{ab}
Inoculated++	23.00 ±7^{b}	5.45 ±1^{a}	0.45 ±0.7^{a}	29.56 ±3^{a}	0.016 ±0.02^{ab}	0.009 ±0.04^{ab}

+Treatments without salinity conditions; ++ Treatments at 0.25 M NaCl. In each row, the values represented by the same lowercase letters were not significantly different at a $P < 0.05$; *Expressed as percentage of total electrolyte content.

content in adjacent branches and stems in all tissues of the control seedlings (Table 1). No proline content was found in the roots of the control seedlings subjected to salinity 0.09 and 0.50 µmol g^{-1} FW in inoculated treatments. Similarly, the inoculation under salinity treatment showed significantly ($P < 0.05$) and enhanced the total phenolic contents in adjacent branches (Table 1). Independently of salinity condition, the inoculation increases the total phenolic contents by approximately 19% and without salinity the treatments enhanced 20%. Compared to the non-inoculated controls, the enhanced starch content on adjacent branches of stem and roots of the inoculated seedlings results showed were not significant ($P > 0.05$) (Table 1).

Photosynthetic capacity

Independently, the presence or absence of salinity condition, the inoculation significantly enhanced the photosynthetic activity of *Salicornia*, compared with the non-inoculated control (Table 2). When the seedlings were subjected to salinity conditions, the photosynthetic activities of both inoculated and non-inoculated seedlings were reduced; although, the salinity treatment affected the non-inoculated seedlings significantly ($P < 0.05$) (Table 2). According to O_2 evolution, it was also negatively affected by 0.25 M NaCl treatment. Under salinity and without salinity, O_2 evolution and CO_2 fixation were significantly ($P < 0.05$) greater in seedlings inoculated.

Table 2. Effect of salinity and inoculation of *Klebsiella pneumoniae* on photosynthetic activity (CO_2 fixation and O_2 production) and photosynthesis of *Salicornia bigelovii* Cerro Prieto ecotype seedlings.

Treatment	CO_2 fixation ($\mu mol\ m^{-2}\ s^{-1}$)	O_2 production ($nmol\ min^{-1}\ cm^{-2}$)	Photosynthesis level ($\mu moles\ CO_2\ cm^{-2}\ s^{-1}$)
Control^{+}	4.45 ± 0.87^{a}	0.33 ± 0.10^{bc}	0.51 ± 0.18^{b}
Control^{++}	2.81 ± 0.45^{b}	0.21 ± 0.07^{cd}	0.23 ± 0.11^{c}
Inoculated^{+}	4.78 ± 0.67^{a}	0.67 ± 0.12^{a}	0.90 ± 0.25^{a}
Inoculated^{++}	3.92 ± 0.23^{ab}	0.42 ± 0.14^{ab}	0.49 ± 0.13^{b}

+ Treatments without salinity conditions; ++ Treatments at 0.25 M NaCl. In each row, the values represented by the same lowercase letters were not significantly different at $P < 0.05$.

DISCUSSION

The results related to plant-bacteria interaction with and without salinity conditions agree with the findings of Baldani and Dobereiner (1980) and Arsac et al. (1990), who indicated that positive effects of bacteria on the experimental *S. bigelovii* Cerro Prieto ecotype possibly suggested the production of plant-growth-promoting substances, which are often reported as responsible for enhancement of plant growth depend on strain-host plant specificity. With this specificity, the bacterium also enhances the fungal resistance of tissue culture seedlings (Ait Barka et al., 2002; Mariita et al., 2011) and temperature (Ashraf and Harrisb, 2004). Moreover, this plant-microbial interaction may express a high level of 1-aminocyclopropane-1-carboxylate deaminase (Pablo et al., 2008), an enzyme that hydrolyzes the ethylene precursor 1-aminocyclopropane-1-carboxylate to ammonia and α-ketobutyrate. By lowering the production of this hormone in plant, the bacterium can decrease inhibitory effects of ethylene on root elongation and its stimulation of senescence under stress (Pablo et al., 2008; Dong et al., 2001). Despite the ability of plants to adapt partially to low-salinity stress in arid-saline areas, plant growth and overall productivity generally decline under high salinity conditions (Rueda et al., 2007). The extent of a plant's ability to withstand such stress is determined by metabolic alterations (Pablo et al., 2008; Aghaleh et al., 2010). However, the result disagrees with Okon and Labandera (1994), who stated that the effect is not strain-dependent among different plant species.

The inoculation significantly ($P < 0.05$) elevated the level of proline, phenolics and the rate of photosynthesis but not to starch deposition. This last, due to the halophytes produced more fatty bodies to diminish the injuries by strong tides; these fatty bodies may have contributed energy to the stimulation of growth, development and adaptation to stress salinity. The results show the contrary with glycophytes reports the accumulation of starch in grapevine buds subjected to low temperatures (Ait Barka and Audran, 1997), indicating that starch may also play a role in protecting plant tissues against chilling. Also, the proline is a dominant organic molecule that accumulates in many organisms upon exposure to environmental stress (Aghaleh et al., 2010) and plays multiple roles in plant adaptation to stress (Nanjo et al., 1999; Aghaleh et al., 2010). Also, was found a significant correlation between salinity tolerance and an increase of proline concentration in adjacent branches tissue in the seedlings apexes of Salicornia after been exposed to salinity conditions. However, *K. pneumoniae* was significantly ($P< 0.05$) increased proline accumulation in Salicornia seedlings under salinity conditions. The enhancement of proline accumulation in leaf tissues was also reported with a virulent strain of *Pseudomonas syringae* pv. in tomato, but not with the isogenic virulent bacteria (Fabro et al., 2004).

The accumulation observed in phenolic compounds were induced by the inoculation of *K. pneumoniae* confirms other results which includes other abiotics stresses (Compant et al., 2005; Essaid et al., 2006). This phenolmenon is linked to the host defense response, which also includes the strengthening of cell walls in the exodermis and several cortical cell layers. The activation of secondary responses associated with the onset of induced resistance, including the oxidation and polymerization of pre-existing phenols and the synthesis of new phenolic compounds via the activation of the phenylpropanoid pathway has been demonstrated with another endophytic bacterium, *Serratia plymuthica*, in cucumbers (Benhamou et al., 2000) and with two plant growth-promoting rhizobacteria (PGPR) strains (*Pseudomonas fluorescens* strain *Pf4* and *P. aeruginosa* strain *Pag*) in chickpeas (Singh et al., 2003). Others studies with *B. phytofirmans* strain PsJN protects the grapevines against *Botrytis cinerea* (Ait Barka et al., 2000, 2002). The mechanism of this protection was not localized but systemic. This type of response usually confers an enhancement of plant resistance to both biotic and abiotic stress (Shevyakova et al., 1998; Nowak and Shulaev, 2003). This phenomenon is known as rhizobacteria-mediated induced systemic resistance and was described as the mode of action of disease suppression by nonpathogenic rhizosphere bacteria (van Loon et al., 1998; Mariita et al., 2011).

Host defense response pathways are pre-induced by the colonizing beneficial bacteria, allowing a much faster

response to pathogen infection, that is, formation of structural barriers such as thickened cell wall papillae due to the deposition of callose and the accumulation of phenolic compounds at the site of pathogen attack (Benhamou et al., 2000). The results indicated that the plant's reaction to salinity stress could be similar to one of the previous report on pathogenic attacks. Thus, the present results linked the biotic stress to the abiotic stress in the way by which plants react to the stress. In this study, it was demonstrated that the plant growth-promoting bacteria colonizing Salicornia seedlings can significantly influence the resistance to salinity. Seedlings inoculation with *K. pneumoniae* had a pronounced effect on Salicornia growth, development, and responses to 0.25 M of NaCl, that is, diminished rates of biomass reduction and electrolyte leakage during salinity conditions. The beneficial effect of rhizobacterium like *K. pneumoniae* may be through their induction of the synthesis of proteins, which reduces the development of symptoms, and also through the prevention of some sets of reactions, which produce the symptoms of salinity injury.

Based on the fact that the salinity nucleation of plants increases with increasing population sizes of halobacteria, pre-emptive competitive exclusion of halobacteria with naturally occurring without salinity nucleation-active bacteria could be an effective and practical means of salinity control. The management of salinity injury by reducing halobacterial populations might become an important new method of salinity control. In this sense, it is important to understand molecular mechanisms behind the salinity-induced effects on photosynthesis by examining the myriad changes in gene expression will be the next step towards a better understanding role of PGPR in high salinity acclimation.

Conclusions

Positive interactions between the bacterial endophytes and their host plants can result in a range of beneficial effects, which includes increase in plant growth and development, as well as resistance to disease, and improvements in the host plant's ability to withstand environmental stress (for example, salinity). These findings indicated that this mechanism could also enhance the *S. bigelovii* resistant to salinity stress.

ACKNOWLEDGMENTS

We thank Dr. Carlos Mota Urbina, because he was the first person to introduce us to halophyte studies. This research is part of the project CD-DCACA-0904-14. Thanks to Apoyos complementarios para la consolidación institucional de grupos de investigación (estancias de consolidación) 2007: code: 74592 of Dr. Bernardo Murillo Amador- CIBNOR -level 2 of the National System of Researchers in Mexico.

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