African Journal of Microbiology Research Vol. 6(22), pp. 4687-4695, 14 June, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.1132
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Molecular study of bacteria associated with *Salicornia* symbiotic bacteria as a candidate for Hormozgan salty zone culturing by Persian Gulf water irrigation

Behboud Jafari[1]*, Morteza Hanifezadeh[2] and Maryam Saadat Jalali Parvin[1]

[1]Department of Microbiology, Ahar Branch, Islamic Azad University, Ahar, Iran.
[2]Department of Biology, Ahar Branch, Islamic Azad University, Ahar. Iran.

Accepted 17 November, 2011

Hormozgān is one of the 30 provinces of Iran. It is in the south of the country, facing Oman. Unfortunately, water extraction, in excess of the rate of replenishment, saline intrusion and inappropriate use of fertilizers have caused some increases in the salinity of many areas, which during the last two decades became a major problem in the production of traditional crops in many regions. *Salicornia* is a genus of succulent, halophyte (salt tolerant) plants that grow in salt marshes, on beaches, and among mangroves. Few studies on bacterial diversity within the rhyzosphere of salt marsh plants have been published. The most studied plant is *Spartina alterniflora.* This study was the first step in the promotion of this type of microorganisms as an efficient and reliable biological product for growth enhancement of halophyte such as *Salicornia*, as well as in the extension of the range of host plants for *Azospirillum halopraeferens,* besides the wild halophyte of *Salicornia* in where they live at the moment. Furthermore, studies on the association of *Rhodococcus fascians* and *Azospirillum* with *Salicornia* genus are recommended to determine their extent, so that these observations can be reproduced under field conditions.

Key words: Persian Gulf, molecular study, *Salicornia*, symbiotic bacteria, sea water irrigation and Iran.

INTRODUCTION

Hormozgān is one of the 30 provinces of Iran. It is in the south of the country, facing Oman. Its provincial capital is Bandar Abbas. The province has 14 islands located in the Persian Gulf, and 1,000 km (620 mi) of coastline. Bandar Abbas has a hot and humid, but dry climate. Maximum temperature in summers can reach up to 49°C (120°F) while in winters the minimum temperature drops to about 5°C (40°F). The annual rainfall is around 251 mm (10 in) and the relative humidity is 66% (Table 1).

Like other agricultural areas in the world, Bandar Abbas agricultural activities depend on groundwater from wells. Unfortunately, water extraction; in excess of the rate of replenishment, saline intrusion and inappropriate use of fertilizers have caused increases in the salinity of many areas, which during the last two decades became a major problem in the production of traditional crops in many regions (SAGAR, 1981; Rueda--Puente et al., 2003, 2004, 2010, 2009a, 2009b, 2009c, 2011). Producing alternatives including development of salt tolerant crops, and selection and evaluation of salt tolerant plants that already are adapted to salt flat areas, focusing on those that might make desirable crops (Ungar, 2000).

Halophyte plant spaces are found in various part of earth. They play the key roles in ecosystems preservation. As we know third millennium is the century of protection of blue planet. Halophytes, particularly *Salicornia* spp. (Chenopodiaceae), are promising plant resources in arid coastal zones because of their tolerance to highly saline conditions (Glenn et al., 1991). These potentially important plants might be incorporated

*Corresponding author. E-mail: b-jafari@iau-ahar.ac.ir. Tel: +989141287314 or +980426333545.

Table 1. Weather condition of Hormozgan.source: world meteorological organization.

Month	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sep.	Oct.	Nov.	Dec.
Minimum average temperature °C (°F)	12.1 (53.8)	14.0 (57.2)	17.5 (63.5)	20.9 (69.6)	24.7 (76.5)	28.0 (82.4)	30.3 (86.5)	30.1 (86.2)	27.7 (81.9)	23.5 (74.3)	18.0 (64.4)	13.5 (56.3)
Maximum average temperature °C (°F)	23.5 (74.3)	24.4 (75.9)	27.7 (81.9)	31.6 (88.9)	36.3 (97.3)	38.4 (101.1)	38.2 (100.8)	37.7 (99.9)	36.8 (98.2)	35.0 (95.0)	30.4 (86.7)	25.5 (77.9)
Rainfall mm (inches)	39.7 (1.56)	47.5 (1.87)	34.8 (1.37)	10.7 (0.42)	4.8 (0.19)	0.0 (0.00)	0.6 (0.02)	2.2 (0.09)	0.8 (0.03)	1.3 (0.05)	5.0 (0.20)	24.0 (0.94)
Days of rain	3.3	3.1	2.6	1.3	0.2	0	0.1	0.2	0.1	0.1	0.4	2.3

into traditional agriculture for helping the support of agricultural economy of those areas affected by salinity (Glenn et al., 1999). Elsewhere, actually there is an increasing interest about the remediation effects of saltbush on a range of salt levels in soils (Stove 1997). Halophyte plants can grow in dry, humid and flooded places. Salt average has strong effect on plants life cycle. There are many spaces of plant in Hormozgans' salty parts that has been shown in Table 2. In Hormozgan, Bandar Abbas is close to the Persian Gulf, *Salicornia* spp. has a wide distribution along with the coasts and over the Shoor River. Salicornia is a genus of succulent, halophyte (salt tolerant) plants that grow in salt marshes, on beaches, and among mangroves. *Salicornia* species are native to North America, Europe, South Africa, and South Asia. Common names for the genus are including glasswort, pickle weed, and marsh samphire; these common names are also used for some species not in *Salicornia* (Ávila and Delgadillo, 2007). *Salicornia* species can generally tolerate immersion in salt water. They use the c4 pathway to take in carbon dioxide from the surrounding atmosphere. *Salicornia* species are being used as food plants by the larvae of some Lepidoptera species including the *Coleophora* case-bearers *Coleophora atriplicis* and *Coleophora salicorniae* (the latter feeds exclusively on *Salicornia* spp). Because *Salicornia bigelovii* can be grown by using saltwater and its seeds that contains high level of unsaturated oil (30 percent, mostly linoleic acid) and protein (35 percent), (Anderson and Wellington, 2001; Arsac et al., 1990). It can be used for producing animal feedstuff and as a biofuel feedstock on coastal land where conventional crops cannot be grown. Adding nitrogen-based fertilizer to the seawater, leads to increase the rate of growth and the eventual height of the plant, (Bagwell et al., 2001) and it has been suggested that the effluent from marine aquaculture (e.g. shrimp farming) can be used for this purpose (Anderson and Wellington, 2001).

There are some experimental fields of Salicornia in Ras al-Zawr (Saudi Arabia) (Arsac et al., 1990), Eritrea (Northeast Africa) and Sonora (Northwest Mexico) (Baldani and Dobereiner, 1980) aimed for the production of biodiesel. This plant was iden-tified from among many halophyte species tested for possible domestication because of the promise as a new oilseed resource (Glenn et al., 1991; Glenn et al., 1999). It is a facultative halophyte that possesses a high potential as an agro industrial commodity (Glenn et al., 1991). Traditionally, farmers apply synthetic fertilizers to compensate for soil nitrogen deficiency. However, indiscriminately use of these fertilizers might increase salinity and severe damages to the soil micro-flora structure and composition (Kapulnik et al., 1981; Banwari and Rao, 1990; Nahid and Gomah 1991; Akhavan et al., 1991).

Just a few studies on bacterial diversity within the rhyzosphere of salt marsh plants have been published up to now. The most studied plant is *Spartina alterniflora* (Lovell et al., 2000). Recent molecular biology studies on *Spartina* rhyzosphere flora suggest a diazotrophic assembly and an apparently large number of unclassified micro-organisms (Lovell et al., 2000, Nielsen et al., 2001). In relation to *Salicornia* spp. Studies are limited to the mycoflora of *Spartina europaea* (Ito et al., 1999). It is important to increase the number of known salt-tolerant, nitrogen-fixing bacteria (Hamdi 1999; Whipps 2000) as potential bio fertilizer resources for salty production areas like Bandar Abbas, Iran. Several halophytes, including *S. bigelovii,* which already has considered a new plant resource for arid regional agriculture, can benefit with soil-enriched biological N_2-fixers (Bashan et al. 1992). The work

Table 2. Space of plant in Hormozgan salty parts.

Scientific name	Family	Vegetative form
Aerva persica	**Amaranthaceae**	**ch**
Calotropis procera	Asclepiadaceae	Ph
Heliotropium bacciferum	Boraginaceae	He
Capparis decidua	Capparidaceae	Ph
Sphaerocoma ancheri	Caryophllaceae	Ch
Anabasis setifera	Chenopodiaceae	Ch
Atriplex Leucoclada	Chenopodiaceae	Ch
Bienertia cycloptera	Chenopodiaceae	th
Halopeplis perfoliata	Chenopodiaceae	Ch
Halocharis sulfurea	Chenopodiaceae	Th
Halocnemum strobilaceum	Chenopodiaceae	Ch
Halothamnus iranicus	Chenopodiaceae	Ch
Hammada salicornica	Chenopodiaceae	Ch
Salsola baryosma	Chenopodiaceae	Ch
Salsola vermiculata	Chenopodiaceae	Ch
Seidlitzia rosmaninus	Chenopodiaceae	Ch
Suaeda vermiculata	Chenopodiaceae	ch
Grantia aucheri	Compositae	He
Cressa cretica	Convolvulaceae	Th

presented here focuses on the composition of natural bacteria associated with the root system of the halophyte *Salicornia* spp. that is being grown in Bandar Abbas Iran desert, to isolate and collect endemic bacteria able to fix atmospheric nitrogen, and to measure the effect of isolated bacteria on germination of *Salicornia*. In this case, we want to corroborate (our hypothesis) the association between benefits micro organisms with halophytes as a *Salicornia* using conventional technique to isolate them in coastal and inland saline environments, with the objection to enlarge the range of this kind of micro-organisms and know the possibilities to employ as a tool into the agriculture organic and reforest Bandar Abbas Iran desert.

MATERIALS AND METHODS

Microbial isolation and growth conditions

Samples of root system and soil of *Salicornia* plants in pre-flowering stage were taken; a portion of the roots system was extracted (50 g) and samples of the soils were gathered (1 kg). The samples of soils and root were developed around Bandar Abbas desert, at approximately 27°12′ 06.61′′ N- 56°24′51.40′′ E meridians. The annual maximum average temperature of this region is 49°C in continuous periods, the annual precipitation is 251 mm, and the temperature can descend to about -5.3°C. Four samples were gathered from the areas: 1) 27°11′28.84′′ N 56°24′09.10′′E, elevation 3 msnm; 2) 27°10′ 51.08′′ N 56°24′15.79′′E, elevation 2 msnm; 3) 27°12′20.19′′ N 56°24′39.55′′E, elevation 5 msnm; 4) 27°31′ 01.93′′ N 56°25′ 49.73′′E, elevation 7 msnm (Figure 1); the samples of soil and root system were at 30 cm of deep of the superficies of soil and 50 cm of distance from the principal root. The samples were placed in black plastic bags and labelled with date and location of the collection. During collection, plants were kept on an ice-cold recipient for 12 h and immediately processed in laboratory.

Isolation from roots

Roots were cut into one or two cm pieces in the laboratory, and two separate groups were placed in different N-free media: OAB (Reinhold et al., 1987) and Rennie´s medium (Rennie, 1981), for 48 h at 30°C with 150 rpm agitation. The resulting solutions were diluted 10^{-1} six times, in 0.85% saline solution, to achieve total dilution of 10^{-6}. Three replicates of dilution from both media weredispersed in four NaCl concentrations each (0, 0.25, 0.50, and 0.75 M) by spreading in 12 plates containing the same liquid and solid N-free media. The plates were incubated at 30°C (12 plates) and 55°C (12 plates) for 4 days.

Isolation of samples from soil

Four grams of soil, obtained from each root sample, in sterile distilled water (1:100 soil: water, by volume) were diluted. After five 10^{-1} dilutions to reach total dilution of 10^{-5}, a volume of 0.1 ml to each N-free medium, in three replicates for each, then they were applied in plates with four NaCl concentrations (0, 0.25, 0.50, and 0.75 M). The 12 plates were incubated at 30°C and others 12 plates at 55°C.

Vigorous colony growth was observed in all plates containing 0, 0.25, 0.5 and 0.75 M NaCl at 30 and 55°C. Colonies from these 21 samples were isolated again, plated and purified on N-free media (both AOB and Rennie's) containing 0.5 M NaCl.

Acetylene reduction assay

Pure isolate cultures were grown in appropriate bottles with OAB and Rennie´s N-free media at 0.5 M NaCl, which were sealed and

Figure 1. Map physiographic of Bandar Abbas area indicating the sampled area (*Salicornia* spp) (sample 1, 2, 3 and 4).

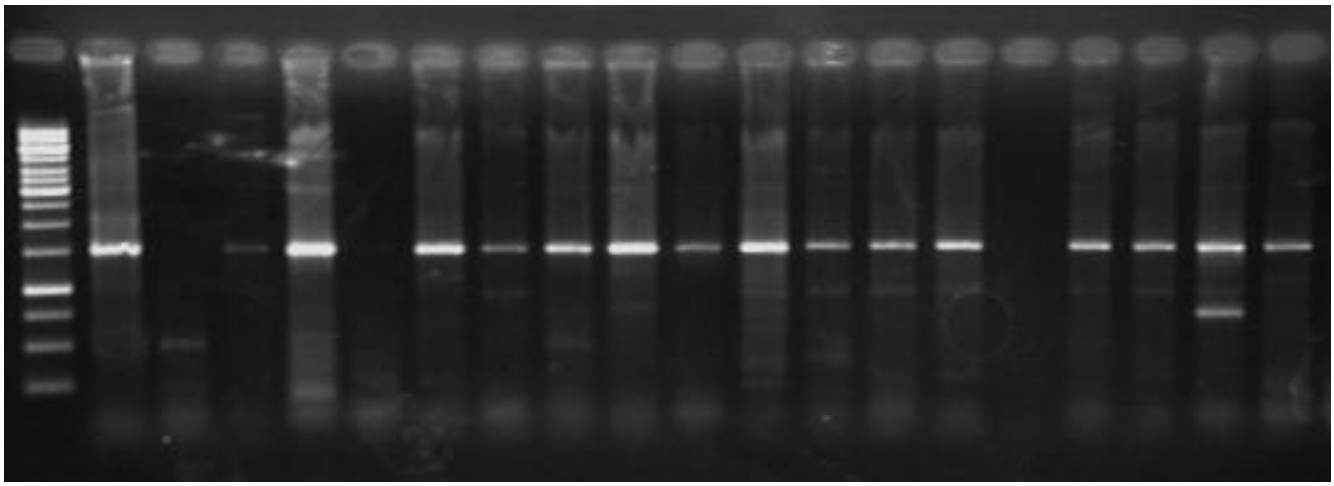

Figure 2. Electrophoresis of amplification sequences 16S rDNA 16 S *rDNA*.

incubated at 30°C for 5 days. After incubation, 1 ml of air was extracted with a syringe and 1 ml of acetylene was injected. Culture stocks were incubated for 48 h at 30°C after this procedure. Five replicates for validating results and as control of an ATCC strain (*Azospirillum halopraeferens* AU10) were used, which it is a nitrogen-fixing bacterium with capacity to develop in roots (Castellanos, 1998; Tyler, 1967; Reinhold et al., 1987; Gamo and Sang, 1990; Troch and Vanderleyden, 1996).

Subsequently, nitrogen activity was assayed by gas chromatography. Ethylene analysis was performed with a gas chromatograph (Vary 6000, Vary Instrument Group, USA) equipped with hydrogen flame ion detector (FID). A different set of 5 bottles was used for each reading. C_2H_2 reduction was evaluated after 120 h. The amount of produced C_2H_4 was expressed in nanomoles C_2H_4 per culture (Holguin et al., 1992).

Bacteria identification

Molecular bacteria characteristics were analysed by gas chromatography of cell fatty acid methyl esters (FAME) (Sasser, 1990) and 16S rRNA sequencing *(*ACCULAB, Newark, DE). For analyzing with 16S rRNA sequencing deoxyribonucleic acid (DNA) was extracted from isolated bacteria according to protocols listed in the previous section, about 5 ml of the DNA extracted in 0.8% agaroz gel was generated. After electrophoresis for 1 h with 100 V voltages, quality of bonds under UV radiation was investigated and samples of DNA which have good quality of their bands were selected.

Amplified 16s rRNA sequences

16s rRNA sequences isolated using designed primers during the polymerase chain reaction was reproduced. Pieces of the size of approximately 1500 pairs of nucleotides, in the reaction polymerase chain were acquired as shown in Figure 2.

Amplified products with approximate size of 1500 bp nucleotides and the single tape were obtained. To show the size of amplified fragments of 1 kb DNA Ladder was used.

The bacterial identifications assigned in this report are based on percent genetic distances (% GD), which are defined as the number of nucleotide differences between two sequences, expressed as a percent. All DNA sequences were generated using PE Biosystems' MicroSeq 16S rRNA Gene Kit or MicroSeq 500 rDNA Bacterial Sequencing Kit. Samples were then identified using the MicroSeq Microbial Identification software and databases. Phylogenetic tree was generated using the Neighbour Joining Algorithm (Saitou and Nei, 1987).

All isolates, including diazotrophs, were stored at 4°C. Both the ATCC stock and the nitrogen-fixing isolate were stored in 15% glycerol at -70°C (Carrillo et al., 1998).

RESULTS

Isolation of nitrogen-fixing bacteria in the *Salicornia* spp. rhyzosphere

In order to consider abundance and morphologic characteristics, from bacteria in the root system and soil of *Salicornia* 21 colonies were selected. These 21 morpho-types were present at the four sites sampled. When they were grown in different concentrations of sodium chloride, the 21 colonies of nitrogen-fixing bacteria selected grew most quickly in 0.25 M NaCl at 30 and 55°C. At this concentration, sufficient growth of bacteria occurred in 8 to 24 h, while at 0, 0.5, and 0.75 M NaCl, growth was slower considering the two temperatures. These results indicate the ability of the bacteria to grow in a dry arid saline environment, which has 0.3 M NaCl (Velarde et al., 2003).

N_2-fixing by bacteria isolated and Identification

During acetylene reduction assays, only two of the 21 isolated species showed significant acetylene reduction activity; these nitrogen-fixing bacterium with high acetylene reduction ability were identified and the results showed identity with *Rhodococcus fascians* (Figure 3), and *Planococcus antarcticus*, which are occur in the soil and water, associated with plants and has significant nitrogen-fixing ability (Vazquez et al., 2000; Puente, 2004; Villegas et al., 2010). The values of acetylene reduction assays of *R. fascians* were: 6.09 ± 0.28 nmole culture^{-1} h^{-1}, in contrast to the control *A. halopraeferens*, with 7.11 ± 1.9 nmole culture^{-1} h^{-1}. To acetylene reduction of *P. antarcticus* was 5.95 ± 0.34 nmole culture^{-1} h^{-1}.

Determination of phosphate–solubilising capacity

After 19 h of incubation, both bacterial species were capable of dissolving insoluble phosphate. However, in phosphate solubilisation among the tested species, *P. antarcticus* showed significant differences compared with other genus studied (*Rhodococcus fascians*), considering the salinity at 0, 0.25 and 0.75 M of NaCl at 30 and 55°C (Table 3). The contrariwise occurred in 0.5 M at 30°C where *R. fascians* showed higher values, while at 50°C was *P. artracticus*.

According to the phosphate-solubilization of four phosphate solubilizing bacterial species in cultural medium containing insoluble calcium phosphate, the greatest solubilization was 17 h after of incubation. Among the treated bacterial species, *R. fascians* was the best genus and the worst was *Planococcus antarcticus* (101 ±13 mg·mL at 16 h, 143 ±11 mg·mL solubilised phosphate at 16 h, respectively)

DISCUSSION

N_2-fixing by the isolated bacterium

Because *Salicornia* has being improved for reforesting disturbed zones and has been used by food and agricultural industries (FAO, 1998), it is important to consider strategies for its reproduction and cultivation. So, it was assumed that *Salicornia* spp. might get benefits from beneficial microorganisms present in the rhyzosphere, because its rhyzosphere presents many

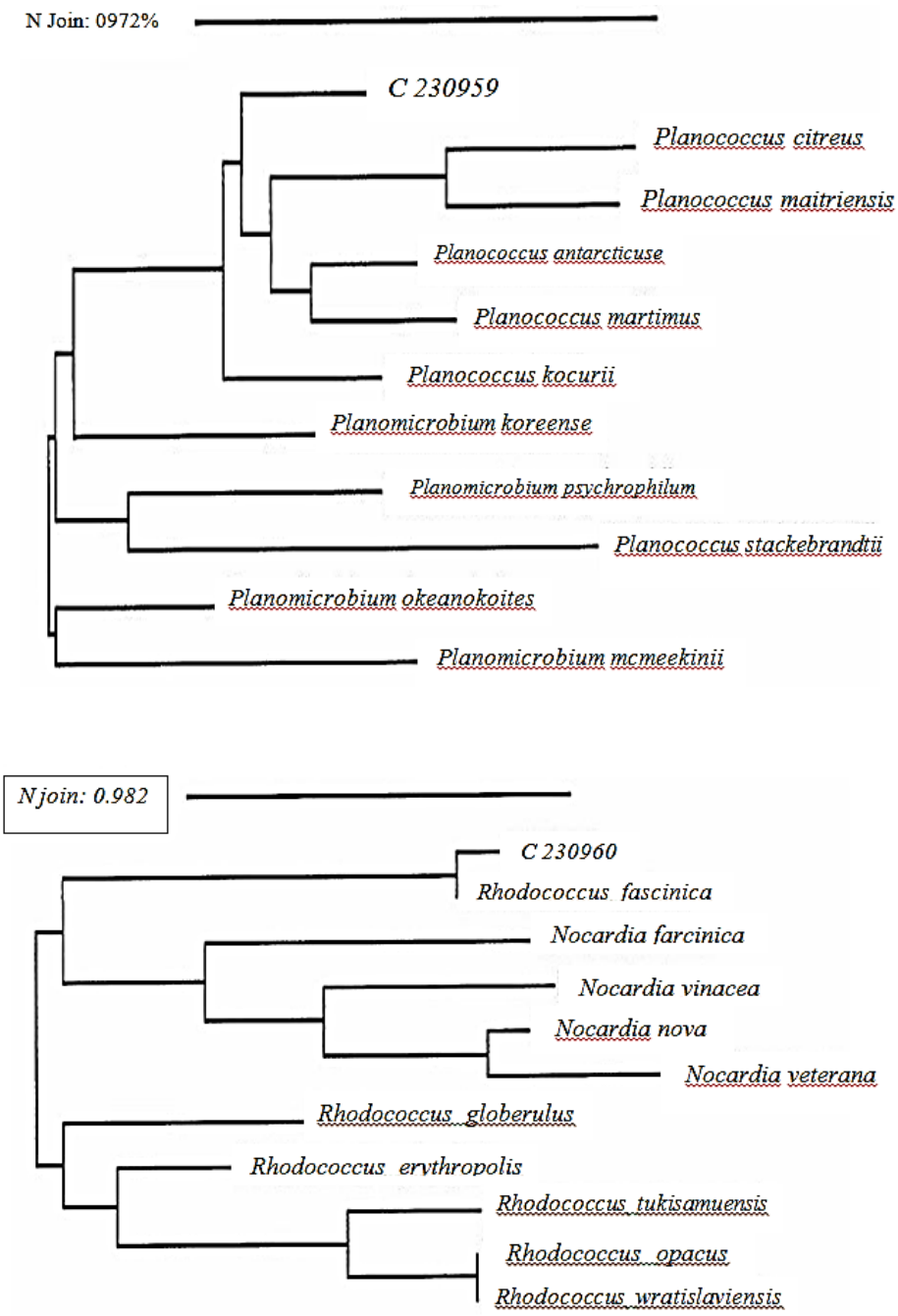

Figure 3. Phylogenetic tree of 16 rRNA sequence from bacterium isolation (*R. fascians* and *P. antarcticus*) from *Salicornia* spp., rhyzosphere in Bandar Abbas, Iran.

Table 3. In phosphate solubilisation among the tested species, *P. antarcticus* showed significant differences compared with other genus studied (*Rhodococcus fascians*), considering the salinity at 0, 0.25 and 0.75 M of NaCl at 30 and 55°C.

Bacterium	Salinity (NaCl)							
	0 M		0.25M		0.5M		0.75M	
	Temperature and size of halos (mm)							
	30°	50°	30°	50°	30°	50°	30°	50°
Rhodococcus fascians	1.32b	3.32a	1.66b	1.66b	1.32b	1.06c	0.32c	1.00d
Planococcus artarcticus	2.32b	1.66b	2.66b	1.32c	0.66c	1.32c	0.32c	1.32c

beneficial microorganisms. In this sense, it can be noticed that other microorganisms are associated with *Salicornia* roots. It was observed that 19 colonies that grew in N_2-free media in our findings were unable to reduce acetylene in large quantities except only two. This could be due to the dependence of it on other bacterium, a phenomenon that is quite common among microorganisms (Drozdowiez and Ferreira, 1987; Will and Sylvia, 1990; Isopi et al., 1995; Holguin et al., 1992; Holguin and Bashan, 1996; Rönkkö et al., 1993; Rojas et al., 2001).

R. fascians has an ability to fix nitrogen and to produce phytohormones (Haahtela et al., 1990; Kozyrovskaya et al., 1990; Conrad et al., 1992; Rodelas et al., 1996; Zexun and Wei, 2000); hence it was proposed to evaluate the possible effects of unknown beneficial bacterium on the halophyte *Salicornia* spp. This will increase knowledge on microorganisms beneficial to *Salicornia*, by using the well-known bacterium *A. halopraeferens* (Puente and Bashan, 1993; Puente et al., 1999) as a control. Also it was observed that both *A. halopraeferens* and *R. fascians* are good nitrogen-fixers and plant growth promoters for *Salicornia bigelovii* SOS-7.

Evaluation of inoculants on germination and seedling growth

Several experiments on the germination of *Salicornia* are carried out (Allen and Allen, 1981; Felker and Clark, 1981; Felker et al., 1981b; Felger, 1992). Other similar studies in *Prosopis* spp., were those carried out by Villagra and Bruno (2005) and Villegas et al. (2008). Our results considering plant growth promoting bacteria under salinity conditions in Bandar Abbas, Iran are not reported. *Salicornia* could get benefits from *R. fascians* and *A. halopraeferens* abilities, as are shown by our results.

Similar results were obtained for other plants and beneficial microorganisms (Puente et al., 1999; Rozema et al., 1975; Goodfriend et al., 2000). Although assays were carried out with other plants and other beneficial microorganisms, some inhibitive effects on germination were observed (Díaz et al., 2001). However, other studies reported positive effects from this kind of microorganisms (Arsac et al., 1990; Puente and Bashan, 1993), researches that are agreed with our results. The positive effects of bacterium on *Salicornia* plant possibly suggested the production of plant-growth-promoting substances, which are often reported as being responsible for enhancement of plant growth (Booth et al., 1988; Holguin et al., 1992; Arsac et al., 1990; Haahtela et al., 1990; Turyanitsa et al., 1995; El-Khawas and Adachi, 1999; Vázquez et al., 1999; Ito et al., 1999; Hildebrandt et al., 2000; Goodfriend et al., 2000; Bagwell et al., 2001).

This study was a first step in the promotion of this type of microorganisms as an efficient and reliable biological production for growth enhancement of halophyte such as Salicornia, as well as in the extension of the range of host plants for *A. halopraeferens,* beside the wild halophyte of Salicornia on where they live at the moment. Furthermore, studies on the association of *R. fascians* and *Azospirillum* with *Salicornia* genus are recommended to determine the extent to which these observations can be reproduced under some field conditions.

As the higher salinity of water in this kind of zones is a problem, due to that *Salicornia* is a plant for salty soils; it can be contributed to arid-zone management with irrigation with salty water (Felker et al., 1981b; Velarde et al., 2003). *Salicornia* SOS-7 can be used for agriculture and silvi-culture principally where there is a possibility for investment in sustainable forest management, which can contribute to arid-zone management where irrigation with salty water, including sea water, is now a necessity.

However, more studies are necessary under environmental conditions of Bandar Abbas, Iran. At the same time, *Salicornia* genus cultivation would help for balancing the carbon cycle, and to reduce the global warming, as this plant thrives in dry zones (Little, 1950; Hastings et al., 1972; Rosenthal, 1977). In this work, a reliable biological method based on beneficial bacteria, was assayed to contribute to maintain or improve the fertility of soils sustaining *Salicornia* fields.

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