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Isolation and characterization of salt-tolerant rhizobia native to the desert soils of United Arab Emirates

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Abstract

The salinity tolerance of naturally occurring rhizobia, isolated from the root nodules of three leguminous plants, namely sesbania (*Sesbania sesban*), lablab (*Lablab purpureus*) and pigeonpea (*Cajanus cajan*), growing at a research farm in Dubai (United Arab Emirates) was studied. Eight isolates identified from colony morphology and gram staining reaction, when cultured on yeast extract-mannitol agar medium (YEMA) supplemented with different concentration of sodium chloride (NaCl), produced colonies even at salinities as high as 40 dS m^{-1}. The rhizobial isolates were also found to be effective in nodulating 21-day old seedlings grown in potting soil and irrigated with saline water of up to 12 dS m^{-1} after inoculation. The tolerance to high levels of salinity and the survival and persistence in severe and harsh desert conditions make these rhizobia highly valuable inoculums to improve productivity of the leguminous plants cultivated under extreme environments.

Key words: Desert soils, Lablab, Pigeonpea, Rhizobia, Salinity tolerance, Sesbania, United Arab Emirates

Introduction

Biological nitrogen fixation is an efficient source of nitrogen in the biosphere. Leguminous plants through their symbiotic relationship with certain gram-negative soil bacteria, collectively known as rhizobia, help to fix atmospheric nitrogen. Herridge et al. (2008) estimated that about 21 Tg of nitrogen is fixed annually through the crop legume-rhizobia symbiosis. The bacteria form nodules on the roots or rarely on the stem of legume hosts and by fixing atmospheric nitrogen into ammonia, they provide an easy and inexpensive way to enhance soil fertility and agricultural productivity. However, a number of factors affect the rhizobium-legume symbiotic relationship. These include the host symbiont compatibility and the physicochemical conditions of the soil, especially salinity and soil pH, nutrient deficiency, mineral and heavy metal toxicity, temperature extremes, insufficient or excessive soil moisture, etc. (Brockwell et al., 1995; Thies et al., 1995). Salinity in particular adversely affects the survival and proliferation of *Rhizobium* spp. in soil and rhizosphere, in addition to reducing plant growth, photosynthesis and demand for nitrogen from host plant. However, rhizobial populations are known to vary in their tolerance to major environmental factors (Wei et al., 2008). Singleton et al. (1982) showed that rhizobium strains can grow and survive at salt concentrations which are inhibitory to most agricultural legumes. Inoculation of such strains would enhance the nodulation and nitrogen fixing ability of the leguminous plants growing under saline conditions (Zahran, 1999; Ali et al., 2009). Furthermore, the ability of legume hosts to grow and survive in saline soils was also shown to improve when they were inoculated with salt-tolerant strains of rhizobia (Zou et al., 1995; Shamseldin and Werner, 2005).

The Arabian Peninsula is one of the driest and hottest regions in the world. The soils reflect the aridity of the climate - most being poorly developed, shallow and rich in lime, gypsum or salts. The region also lacks major river systems and many countries within it depend almost entirely on groundwater to irrigate farms. In several countries, an increase in farming area and large-scale extraction has depleted the groundwater reserves faster than the aquifer recharge from scanty rainfall.

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Making matters even more difficult, the growing urban areas are taking priority over the scarce freshwater, leaving agriculture to use low-value brackish or salty water with increased risk of soil salinization (Rao et al., 2009).

The naturally occurring soil rhizobia nodulating the leguminous plants in the desert regions are expected to have higher tolerance to prevailing adverse conditions such as salt stress, high temperatures and drought. For instance, Thrall et al. (2009) found that salt tolerance and growth were generally higher in rhizobial populations associated with *Acacia* spp., derived from saline soils than those from non-saline soils. Elanchezhian et al. (2009) reported tolerance to up to1000 mM of NaCl in *in v*itro studies of the rhizobium species associated with *Vigna marina*, a wild legume found growing in sandy seashores.

While nitrogen-fixing food and forage legumes tolerant of environmental stresses represents an important strategy to improve agricultural productivity, rhizobia with genetic potential for stress tolerance are equally vital for effective nodulation and enhanced productivity of the host-plants (Zahran, 1999). However, there are no previous reports on isolation and characterization of free living soils rhizobia from the desert soils of United Arab Emirates. In this paper, we present the results from a recent study to isolate and characterize the naturally occurring soil rhizobia nodulating different leguminous hosts.

Materials and Methods

Three leguminous species namely sesbania (*Sesbania sesban* (L.) Merrill), lablab (*Lablab purpureus* (L.) Sweet), and pigeonpea (*Cajanus cajan* (L.) Millsp.) showing root nodulation with free living soil rhizobia were used in this study. Nine-month-old plants of these species growing at ICBA research station (25°05’49”N and 55°23’25”E) were gently uprooted, the roots were harvested, washed and transported to the laboratory in plastic bags. The nodules were separated from the roots, followed by washing in sterile distilled water several times, surface sterilization with 70% ethanol for 10 seconds and then 0.1% mercuric chloride solution for 1-2 minutes. Four to five nodules were crushed on a glass slide in Phosphate Buffer, streaked onto yeast extract mannitol agar medium (YEMA) containing Congo-red dye in Petri plates and incubated at 28°C for 96 hours. The bacterial colonies were repeatedly sub-cultured by streaking on YEMA plates until pure cultures were obtained. The bacterial isolates were examined for Gram-staining reaction and other morphological characteristics as described by Somasegaran and Hoben (1994). The ability of eight isolates, namely PP1W1a, PP2WR1a and PP3PF1a from pigeonpea, LL2R2, LL4RW1 and LL6RP from lablab and SS2W1a and SS3RW2b from sesbania to tolerate salinity was studied under *in vitro* conditions. For this, the isolates were grown in Petri plates containing YEMA medium supplemented with different concentrations of sodium chloride (NaCl) to obtain saline culture media with electrical conductivities (EC) of 2, 4, 8, 10, 12, 14, 16, 20, 30 and 40 dS m^{-1}. The salinity tolerance of the rhizobial isolates was assessed by examining the plates after 24 hours of incubation for growth and morphological characteristics of the colonies. Gram stained slides of the rhizobial cultures were examined under compound microscope (magnification x 1000) for changes in cell morphology of rhizobia in response to salinity stress.

The effect of salinity on nodulation was studied by inoculating 21-day old pigeonpea, lablab and sesbania seedlings with yeast mannitol broth cultures, prepared according to Cappuccino and Sherman (2007). In additions to the three field legumes, seedlings of *Acacia ampliceps* Maslin, a leguminous tree species from Australia, were also inoculated to find the nodulating ability of the isolates. The seedlings were raised under greenhouse conditions (25-30°C) in 1 liter polyethylene pouches (pigeonpea and lablab) or small paper cups (sesbania and *A. ampliceps*), containing commercial potting soil (Van Egmond) and by irrigating with fresh water with a salinity of 2 dS m^{-1}. Prior to planting, sesbania seeds were scarified by soaking in concentrated H_2SO_4 for 1 hour, followed by repeated washing with distilled water. Five isolates – two from pigeonpea (PP1W1a and PP3PF1a), two from lablab (LL2R2 and LL4RW1) and one from Sesbania (SS3RW2b) were used for the inoculation study. The broth cultures, adjusted to an optical density of 0.5 (4.13 x 10^5 CFU ml^{-1}), were diluted by a factor of 2 with water before inoculation. Lablab seedlings were inoculated with 50 ml, sesbania and pigeonpea with 20 ml and *A. ampliceps* with 10 ml of diluted broth, proportional to the size of seedlings after 3-weeks of growth. The inoculation of seedlings was repeated the following day. For each rhizobial isolate, a total of 16 seedlings, divided into four sets each of four seedlings was inoculated. While one set of seedlings served as negative control (not inoculated), the other three inoculated sets were irrigated with saline water at electrical conductivities (EC) of 2, 6 and 12 dS m^{-1}, each obtained from diluting saline ground water (~20 dS m^{-1}). The plants were harvested four weeks after inoculation and scored for the number of root nodules. In each treatment, the effectiveness of inoculation was determined from the mean number of nodules observed per plant and coded as: Effective (E) if the mean is higher than 5; Partially effective (P), if it is between 1 and 4; and Ineffective (I) if the mean number is <1. Since nodulation was ineffective in sesbania and absent in *A. ampliceps*, the nodulation data from only pigeonpea and lablab were analyzed statistically using GenStat (ver. 7.22 DE).

Results and Discussion

Nodule characteristics

The number of plants sampled per species, the location of nodules on the roots and the number of nodules per unit weight of the root systems together with the morphological data such as colour, shape, and size of the nodules are presented in Table 1. In both sesbania and pigeonpea the number of nodules was found to be proportional to the root mass. However, no such relationship was found in lablab. The nodules were light yellow to cream colored in all species. The shape was predominantly globose and semi-globose with smooth surface in sesbania, globose and semi-globose with striated surface in lablab, and semi-globose to irregular and coralloid in pigeonpea. While the nodules were found to be associated with primary and secondary roots in sesbania, they were found mostly on secondary roots in lablab and pigeonpea (Table 1). Mahmood and Iqbal (1994) observed that nodules in sesbania and lablab were globose with or without reticulate surface, elongated in pigeonpea and distributed both on primary and secondary roots in all. Nodules formed on primary roots are considered to be more efficient than those on the secondary roots and in cowpea, and a gradual shift was observed in the spatial distribution of nodules from primary roots to secondary roots under increased salt-levels (Predeepa and Ravindran, 2010).

Rhizobia isolation

The primary cultures isolated from sesbania and lablab showed a mixture of red, white and pink colored colonies. However, pigeonpea also showed slightly yellowish colonies in addition to the above colors. Pure cultures obtained from the primary culture showed varying characteristics in terms of color (white, pink and red), elevation (flat, raised), margins (entire, wavy, grainy and translucent) and colony size (1-10 mm) (Table 2). Twenty isolates were studied for colony morphology and Gram-staining reaction, among which eight isolates – three from pigeonpea (PP1W1a, PP2WR1a and PP3PF1a), three from lablab (LL2R2, LL4RW1 and LL6RP) and two from sesbania (SS2W1a and SS3RW2b) were identified as potential rhizobial strains.

In vitro test for salinity tolerance

Irrespective of the salt concentration of the YEMA medium, all the eight isolates grew within 24 hours. In lablab and sesbania, no significant differences were observed in colony size and morphology at different salt concentrations (Figure 1A-D). Microscopic observation of the Gram-stained slides of the cultures showed that some isolates underwent morphological changes in response to salt stress. Thus, the rhizobial cells in both the isolates of sesbania became shorter and more compact (Figure 2A, B). In the lablab isolate LL2R2, besides shortening of length, some cells became spherical at the high salinity (40 dS m^{-1}) (Figure 2C). In contrast to lablab and sesbania, the colonies from pigeonpea isolates were observed to be small with irregular margins but without any obvious changes to cell morphology at the higher salt concentration (40 dS m^{-1}). The rhizobial cells are known to respond to stress conditions by changing their morphology and size (Zahran, 1999). For instance, Kulkarni and Nautiyal (2000) found that in mesquite, the shape of temperature-stressed cells changed to spherical, compared with rod-shaped cells in non-stressed control.

Seedling inoculation test

In all the species, while the un-inoculated plants remained free of nodules, salinity had variable effect on the ability of the rhizobial isolates to infect and nodulate the host plants. For instance, pigeonpea showed consistent nodulation at all salinities (2, 6 and 12 dS m^{-1}), compared to the other legumes (Table 3). Lablab on the other hand, had a much higher level of nodulation than pigeonpea when irrigated with fresh water (2 dS m^{-1}), but showed a decrease with increase in salinity of the irrigation water. Sesbania showed slight nodulation up to 6 dS m^{-1} salinity but no nodules were observed at 12 dS m^{-1} salinity. Since the number of nodules formed is dependent on the compatibility of both the host plant and the infecting strains, it can be concluded that pigeonpea is a better host species than the others studied. The results also showed that the rhizobial isolates have considerable tolerance to salinity, confirming the observations from *in vitro* studies.

Table 1. Characteristics of nodules isolated from leguminous host-plants growing at a research farm in Dubai.

Host	Fresh weight of roots (g) Mean ± SD	No. of nodules Mean ± SD	Fresh weight of nodules (g) Mean ± SD	Weight of nodules/ weight of roots (g) Mean ± SD	Color	Shape and surface	Size (mm)	Location on roots
Sesbania	49.6±21.5	129.4±71.5	0.78±0.64	0.014±0.01	Cream	Globose, semi-globose, smooth surface	1-4	Primary and secondary roots
Lablab	34.1±24.3	21.0±10.7	0.50±0.40	0.037±0.06	Cream	Globose, semi-globose, striated	1-4	Secondary roots and tertiary roots
Pigeonpea	108. 8±26.8	64.7+74.5	2.01±2.57	0.016±0.02	Cream	Semi-globose, irregular and coralloid	1-2	Primary, secondary roots and tertiary roots

Table 2. Colony morphology of rhizobial isolates from three leguminous species.

Host	Isolate	Age of Culture (hr)	Color	Elevation	Size (mm)	Opacity	Margin
Sesbania	SS2W1a	120	White	Raised	2	Opaque	Wavy
	SS3RW2b	24	White	Raised	4	Translucent	Irregular
Lablab	LL2R2	120	Red	Raised	1	Opaque	Very smooth
	LL4RW1	24	White	Raised	5	Opaque	Very smooth, slight grainy appearance
	LL6RP	48	Pink	Raised	2.5	Opaque	Smooth wavy, grainy appearance
Pigeonpea	PP1W1a	72	White	Raised	10	Opaque	Grainy appearance
	PP2WR1a	72	White	Raised	9	Opaque	Wavy, grainy appearance, filamentous growth outside margin
	PP3PF1a	72	Pink	Raised	6	Opaque	Wavy

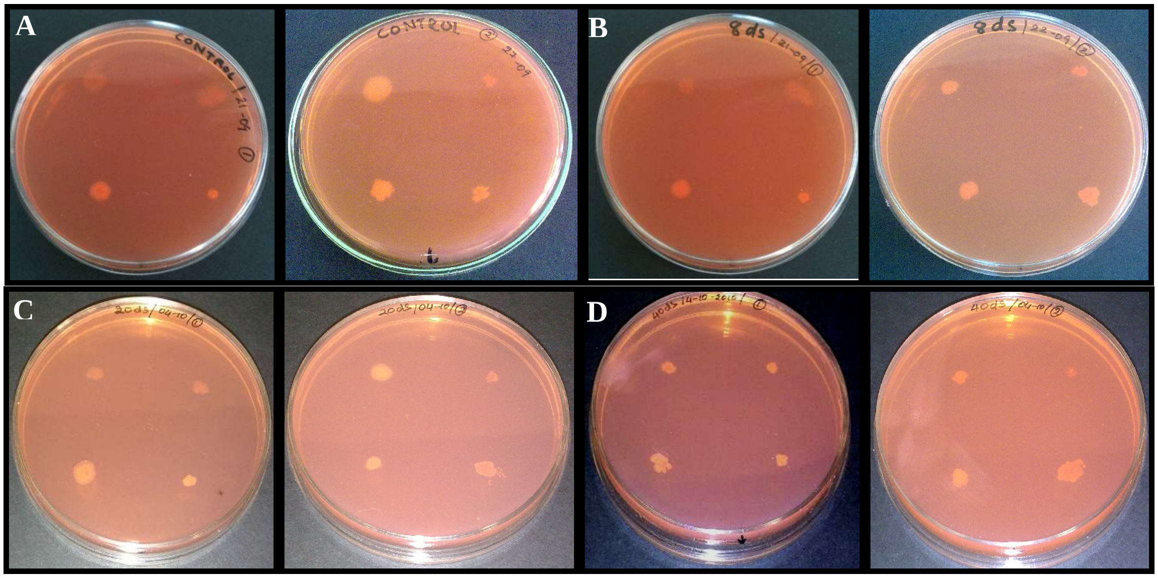

Figure 1. Rhizobial isolates growing on YEMA media supplemented with NaCl. Sets: A) Control, (B) 8 dS m^{-1}, (C) 20 dS m^{-1} and (D) 40 dS m^{-1}. Isolates: Sesbania (SS3RW2b, SS2W1a– upper two, left and right respectively in first plate of each set), lablab (LL2R2, LL4RW1 – lower two, left and right respectively in first plate and LL6RP – upper right in second plate of each set) and pigeon pea (PP1W1a, PP3PF1a, PP2WR1a – upper left, lower left and right respectively in second plate of each set).

In both pigeonpea and lablab, nodulation occurred with all the five isolates of rhizobia indicating their non-specific action. Also, the maximum number of nodules was observed when inoculated with rhizobia from other legumes than its own. In contrast to pigeonpea and lablab, sesbania showed ineffective nodulation, following inoculation. Interestingly, nodulation occurred with the two isolates from pigeonpea but not with its own, the reasons for which are not clear. *A. ampliceps* also did not show any nodulation, possibly because of cross-incompatibility of the rhizobia. In pigeonpea, the ability of different rhizobial isolates to nodulate the plants was found to be more or less similar. In lablab, although the differences in nodulating ability of the isolates were statistically insignificant, PP3PF1a and SS3RW2b exhibited more effectiveness than the others (P>0.05).

Legumes have immense value due to their capacity to enhance soil fertility by fixing atmospheric nitrogen through the symbiotic relationship with rhizobia. However, salinity, water deficit and temperatures stress are serious threats to rhizobium-legume symbiosis. Thus, while strategies to improve legume production in saline environments include selection of host genotypes that are tolerant to high salt conditions, inoculation with salt-tolerant strains of rhizobia could constitute another approach to improve legume productivity under symbiosis (Keneni et al., 2010). The rhizobia isolated in this study were able to grow at high salt concentration (40 dS m^{-1}) in *in vitro* cultures and formed nodules on seedlings irrigated with saline water at an EC of 12 dS m^{-1}.

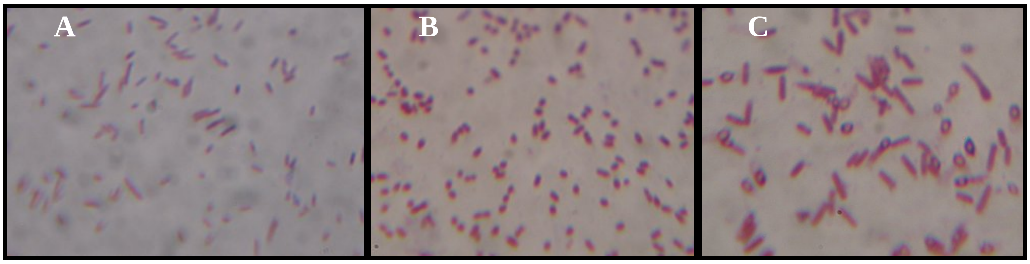

Figure 2. Changes in morphology of rhizobial cells in response to salinity. (A) Control (SS3RW2b), (B) 40 dS m^{-1} (SS3RW2b), (C) 40 dS m^{-1} (LL2R2) (x 1000).

Table 3. Effectiveness of rhizobial isolates in nodulating 21-day old seedlings of different leguminous species.

Plant	Salinity (dSm^{-1})	PP1W1a	PP3PF1a	LL2R2	LL4RW1	SS3RW2b
Sesbania	2	I	I	0	0	0
	6	I	0	I	0	I
	12	0	0	0	0	0
Lablab	2	E	P	E	E	E
	6	P	E	P	P	P
	12	I	P	I	P	P
Pigeonpea	2	P	P	P	P	P
	6	P	P	P	P	P
	12	P	P	P	P	P
A. ampliceps	2	0	0	0	0	0
	6	0	0	0	0	0
	12	0	0	0	0	0

E = effective, P = partially effective, I = ineffective and 0 = no nodulation.

Since the seed of the leguminous host-plants under study were not inoculated with rhizobia prior to sowing and there was no recent history of cultivation of other legumes in the same field, the rhizobia could be naturally occurring native strains probably associated with wild legumes such as *Indigofera colutea* and *Rhynchosia schimperi*, that grow as weeds in fallow fields. Although temperature tolerance of the rhizobial isolates was not investigated in this study, the fact that the rhizobia were isolated from the soils which experience high temperatures exceeding 50°C showed signs of their ability to survive and persist under extreme conditions. Another significant outcome of this study is that the isolated rhizobial strains did not seem to have any host specificity, which will be very beneficial when developing inoculants at a commercial level. Because of their natural adaptation to the harsh environmental conditions – especially salinity and heat stress, the rhizobial isolates will be highly beneficial as inoculums to improve productivity of compatible leguminous host plants grown in marginal environments.

Conclusions

Drought and salt-stress are the major constraints to plant productivity in desert environments and isolation of effective rhizobia to inoculate the leguminous crop plants could be an important strategy to improve the efficiency of rhizobium-legume symbiosis and thereby productivity. The results from this study showed that the rhizobia isolated from the desert soils are able to survive, grow and effectively nodulate their leguminous hosts even at high salt concentrations. Additional research to precisely identify the rhizobial strains through molecular characterization (16S-rDNA gene sequencing) and evaluate their growth performance, symbiotic efficiency and nodulating ability against other important environmental stresses such as temperature, pH and heavy metals is currently in progress.

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