academicJournals

Vol. 8(17), pp. 1763-1778, 23 April, 2014
DOI: 10.5897/AJMR2014.6603
Article Number: D01293344167
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Phenotypic and symbiotic characterization of rhizobia isolated from *Medicago ciliaris* L. growing in Zerizer from Algeria

Dahbia Cheriet*, Akila Ouartsi, Djamel Chekireb and Souad Babaarbi

[1]Department of Biochemistry, Faculty of Sciences, BadjiMokhtar University, Annaba Algeria.

Received 2 January, 2014; Accepted 4 April, 2014

Phenotypic characteristics of 37 rhizobia strains isolated from root nodules of *Medicago ciliaris* L. growing in soils collected from Zerizer (North Eastern Algeria) were studied. Tolerance to salinity, high temperatures, acid and alkaline pHs, drought and to antibiotics as well as symbiotic and cultural characteristics allowed the description of a wide physiological diversity among tested isolates. Thirteen (13) isolates from the total could grow at 45°C. Only six isolates grew at 4% NaCl. Furthermore, the isolates which showed tolerance to salinity stress also showed tolerance to water stress, indicating direct relationships between these two physiological pathways. High salt and water stress tolerant strains were isolated and tested for their ability to biological nitrogen fixation. However, seven isolates were categorized into *Agrobacterium.*

Key words: *Medica gociliaris*, stress tolerance, Algeria, symbiotic properties.

INTRODUCTION

Many species of the legume genus *Medicago* are native to the Mediterranean basin (Lesins and Lesins, 1979) and are important as agricultural crops (Irwin et al., 2001). Compared to *Medicago sativa*, the most important species for cultivation, and *Medicago truncatula*, the model chosen for studies in nitrogen fixation (Cook, 1999; de Billy et al., 2001; Ben Amor et al., 2003), investigations with *Medicago ciliaris* as the focus have been very limited (Laouar and Abdelguerfi, 2000) . The species *M. ciliaris* is an annual plant that is tolerat to salt stress (Ben Salah et al., 2009) and may show promise for cultivation in salt-affected soils. Due to the reason that this species grows in soils that are heavy with clay it has application as a cover crop, in pastures, or for producing forage (Laouar and Abdelguerfi, 2000).

Many species of this genus *Medicago* have significant and wide-ranging agricultural and environmental applications, such as the perennial species *M. sativa* L, alfalfa (Irwin et al., 2001). Alfalfa is one of the most important forage crops in the world because of its high nutritive quality, yield, drought-resistance and good adaptation to various climatic and soil conditions and, therefore, is reputed to be the “queen” of the forages, although it has been reported to be water use inefficient (Li et al.,

*Corresponding author. E- mail: cheriet_w@yahoo.fr.

2007). Furthermore, the annual species, collectively known as “medics”, are naturally distributed over a very wide range of environmental conditions in the Mediterranean basin, and the great importance in pastures in the Mediterranean and known to establish a nitrogen-fixing symbiosis with soil bacteria of the genus *Ensifer* (formerly *Sinorhizobium*) (Bena et al., 1998; Lesins and Lesins, 1979; Badri et al., 2008).

Nitrogen is a major limiting factor for plant productivity despite the inexhaustible reserve of atmosphere (78% N_2) (Foth, 1990). Biological fixation of molecular nitrogen (N_2) from the atmosphere is one of the main sources of nitrogen pool enhancement in agricultural soils (Bradic et al., 2003). The ability of legume species to establish nitrogen-fixing symbiosis with rhizobia makes them excellent candidates for use in sustainable agricultural systems (Howieson et al., 2000) .

Although most *Medicago* species form symbioses with the two species *Sinorhizobium meliloti* and *S. medicae* (Brunel et al., 1996; Rome et al., 1996), it is becoming evident that several different species of *Medicago* may have dissimilar affinities for infection by these 2 rhizobial species. For example, Garau et al. (2005) demonstrated that *S. medicae* frequently nodulated *Medicago* species that are adapted to acid soils, while *S. meliloti* formed symbioses with those growing in more alkaline to neutral soils. Bena et al. (2005) indicated that the geographic distribution of these rhizobial species appeared related to the incidence of the species of *Medicago* resulting from the characteristics of the soils.

In Algeria, diminution of pasture areas and deficit of forage production are major problems for development and extention of ovine and bovine breeding. Annual medics are grown as forage legume and regenerating pasture in the agro-pastoral Mediterranean systems or Australian ley-farming systems. However, the commercial Australian medic cultivars are not well adapted to most of agroecologicalzanes encountered in North Africa. Therefore, selection of better-adapted medics in association with appropriate symbiotic bacterial partners is agronomically important. Zerizer is an area of Algeria where most of *M. ciliaris* is represented, but less is known about their associated rhizobia. The advantages of this species are increased by the fact that, like most legumes, *Medicagociliarisis* able to form a symbiotic association with rhizobia and thus fix atmospheric nitrogen, which enriches the soil. The use of atmospheric nitrogen makes *M. ciliaris* a pioneer species capable of colonizing nitrogen poor soils.

This study is a preliminary step contributed to research efforts designed to uncover the biodiversity of rhizobia and, at the same time, select promising strains for the production of inoculants to improve *M. ciliaris* nitrogen fixation ability. We characterized 37 efficient rhizobia isolated from *M. ciliaris* L. collected from Zerizer area. The phenotypic characterization of these strains was conducted to evaluate their capacity to grow under abiotic stress such as severe temperatures, salinity, drought and high pH. Finally, the symbiotic properties of the representative strains were evaluated in terms of nodule numbers.

MATERIALS AND METHODS

Sampling zone

Thirty nodulated *M. ciliaris* plants were collected from from Zerizer (North Eastern Algeria). Healthy plants were uprooted carefully and those plants prossessing healthy nodules with pink colour were selected to isolate rhizobia.

Soil sample and isolation of rhizobia from *Medicago ciliaris*

Soil sample was collected from area Zerizer in 2010 and used for *M. ciliaris* cultivation as trap hosts (Soil samples were collected from the Zerizer area in 2010 and used for trapping rhizobia). Strains were isolated from naturally occurring root nodules collected on *M. ciliaris*. Nodules were washed several times with tap water and rinsed with sterile distilled water. They were surface sterilized by immersion for 30 s in ethanol (96% v/v), 3 min in 3% sodium hypochlorite and then washed ten times with sterile distilled water.

A single surface-sterilized nodule was placed into a Petri dish and crushed with a sterile glass rod in the presence of a sterile solution of sterile distilled water. A loopful of the resulting suspension was then streaked on Tryptone Agar medium surface containing 25 µg/ml Congo red (TA) in a Petri dish and incubated at 28°C.

Bromothymol blue (BTB) agar medium was used for differentiating of the isolates. The cultures were streaked on BTB agar plates. BTB agar was made by adding 5 ml of (0.5% BTB in ethanol) to 1 L of YEMA medium. The plates were incubated at 28°C for 4 days. The change in color of medium was observed. The isolates were classified as slow growers (medium turns blue) or fast growers (medium turns yellow) on their reaction on YEMA supplemented with BTB (Table 1) (Somasegaran and Hoben, 1994). Isolates were purified by repeated streaking of a single colony on TA medium and were checked for purity by light microscopic examination of living cells and Gram staining (Vincent, 1970). They were then stored at 4°C on TA slants and at -16°C in Tryptone yeast extract (TY) liquid culture aliquots in the presence of 20 and 50% glycerol (v/v).

Purification of isolates

a)-Growth on congo red medium

Rhizobia colonies appeared white, translucent, gummy, glistening elevated and comparatively small withenremargine were selected in contrast to of *Agrobacterium* on congo red medium which were red in color.

b)- Gram staining

Gram staining was done to ensure purity and freedom from Gram +ve bacteria. Gram-staining reaction was carried out by using a loopful of pure culture grown on Tryptone agar and stained as per the standard Gram’s procedure (Somasegaran and Hoben, 1994).

Table 1. Colony morphology of rhizobial isolates from *Medicago ciliaris*.

Isolate	Age of culture (hour)	Color and opacity	Size
MedS01	72	Slightly pink colony	Less than 2 mm Ø
MedS02	72	Whitish colony	1 mm Ø
MedS04	72	Milky translucent colony	1 mm Ø
MedS07	72	White circular colony	Less than 1 mm Ø
MedS08	72	Slightly pink mucilaginous colony	1 mm Ø
MedS09	72	Translucent colony	Less than 1 mm Ø
MedS10	72	White colony	Less than 1 mm Ø
MedS11	72	Pink slightly mucilaginous colony	Less than 2 mm Ø
MedS12	72	Pink mucilaginous colony	2 mm de Ø
MedS13	72	Transparent colony	1 mm Ø
MedS14	72	Whitish colony	1 mm Ø
MedS15	72	Milky gummy colony	Less than 2 mm Ø
MedS16	72	Pink slightly mucilaginous colony	1 mm Ø
MedS20	72	Milky mucilaginous colony	Less than 2 mm Ø
MedS23	72	Pink mucilaginous colony	1 mm Ø
MedS24	48	Whitish opaque colony	Less than 2 mm Ø
MedS25	48	Milky translucent colony	1 mm Ø
MedS26	48	Slightly pink opaque colony	1 mm Ø
MedS28	72	White plate colony	Less than 2 mm Ø
MedS29	48	White opaque colony	2 mm de Ø
MedS30	72	Translucent colony	Less than 1 mm Ø
MedS31	48	Pink gummy colony	Less than 2 mm Ø
MedS32	72	Transparent mucilaginous colony	2 mm de Ø
MedP01	72	Slightly pink opaque colony	Less than 3 mm Ø
MedP03	72	Transparent mucilaginous colony	Less than 2 mm Ø
MedP04	72	Pink slightly gummy colony	2 mm de Ø
MedP05	72	White opaque colony	1 mm Ø
MedP06	72	Translucent colony	1 mm Ø
MedP07	72	Slightly pink mucilaginous colony	1 mm Ø
MedP09	72	Whitish opaque colony	1 mm Ø
MedP10	72	Pink slightly gummy colony	Less than 3 mm Ø
MedP12	72	Pink slightly opaque colony	Less than 2 mm Ø
MedP13	72	slightly pink mucilaginous colony	2 mm de Ø
MedP14	72	Milky colony	1 mm Ø
MedP17	72	Translucent colony	1 mm Ø
MedP18	72	Milky opaque colony	Less than 2 mm Ø
MedP19	72	Translucent colony	Less than 1 mm Ø
S. meliloti	72	Slightly pink colony	Less than 1 mm Ø
S. meliloti	72	Whitish colony	Less than 2 mm Ø
S. fredii	72	Milky translucent colony	1 mm Ø
S. medicae	72	White circular colony	Less than 2 mm Ø
A. tumefaciens	72	Slightly pink mucilaginous colony	1 mm Ø
Rhizobium sp.	72	Translucent colony	Less than 1 mm Ø

c)*-Distinguishing test between *Rhizobium* and *Agrobacterium

The *A. tumefaciens* species complex (biovar 1) has the enzymatic ability to aerobically convert lactose to 3-ketolactose. This is tested by streaking on medium containing lactose. After 2 days of growth at 28°C, the plates are flooded with a layer of Benedict's reagents (17.3 g of sodium citrate and 10.0 g of Na_2CO_3, are dissolved by heating in 80 ml of distilled water; after filtration, the filtrate is added

to a 10 ml solution containing 17.3 g of $CuS0_4.5H_2O$, and the mixture is diluted to 100 ml). The presence of 3-ketolactose in the medium is indicated by the formation of a yellow ring around the growth of a positive strain (Table 1, Figure 3). Maximum intensity of the yellow ring (2-3 cm in diameter) of cuprous oxide around the bacterial spot. Around 3-ketolactose positive strains is reached in about 1-2 h after flooding with Benedict's reagent. Biovar 1 strains have the unique ability to oxidize lactose into 3-ketolactose (Bernaerts and Deley, 1963).

Morphological studies

The thick bacterial smear of all the isolates was Gram stained and morphological characterization was done on the basis of colony morphology including shape,color and surface margin (Table 2).

Biochemical studies

Biochemical characterization was done on the basis of oxidase, catalase.

Stress tolerance screening

The isolates characterized in this study were examined for growth under different stress conditions of high temperature, high salinity, alkaline pH and extreme drought. In the case of temperature tolerance, isolates were kept at 28 (as a control), 37, 40, 42 or 45°C on YMA plates for four to five days. To check the ability of isolates to grow under different concentrations of NaCl, the medium was supplemented with 0 (control), 1, 2, 3, or 4% NaCl. To test the tolerance to acid and alkaline pH, the pH of the medium was adjusted with 0.5 M HCl or 0.5 M NaOH to 4.5, 5.5, 6.8 (as a control), 8, and 9.

The salinity and pH test were performed on YMA plates kept at 28°C for 4-5 days. To test drought resistance, different concentrations of polyethylene glycol (PEG 4000) were applied to the sterile distilled water at 10, 15, 20, 25%. In this experiment, isolates were first grown in TA medium for 3 days at 28°C and the resulting bacterial suspensions containing approxi-mately 10^9 cells ml^{-1}, were transferred to YMA plates as previously indicated. The screening for stress tolerance was performed in Petri dishes divided into equal squares. Each square was spot inoculated with 10 µL of the cell suspensions at 10^9 cell ml^{-1} grown in Tryptone agar at an exponential phase. After incubation under different stressful conditions, the growth of isolates was estimated in comparison with that following control treatment, as follows: −, no growth; + weak growth (10-30% in relation to the control); ++, good growth (30-80% in relation to the control); and +++, very good growth (similar to the control).

Antibiotic susceptibility

Antibiotic resistance tests were performed by measuring the diameters of inhibition zones on YEM agar plates containing the following antibiotic discs: streptomycine (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), nalidicic acid (30 µg); kanamycin (30 µg); ampicillin (10 µg). The antibiotic resistance was detected by an inhibition zone measured over a seven day period for each disc. Determination of intrinsic antibiotic resistance was evaluated in plates of YEM with different concentrations of antibiotics (rifampicine, erythromycin and neomycin). Filter-sterilized aliquots of each antibiotic were added aseptically to sterile YEM medium at 50°C to give the final concentrations. Control plates contained no antibiotic (Van Berkum et al., 1998).

Plant test

To assess their abilities to generate root nodules on their original hosts, the isolates were grown on Tryptone agar for 3 days at 28°C and the resulting bacterial suspensions containing approximately 10^9 cells mL^{-1} were inoculated on aseptic *M. ciliaris* seeds. Seeds were surface sterilized in 3% sodium hypochlorite for 10 min, rinsed with sterile distilled water, and then scarified. These seeds were germinated for 72 h on water agar (0.7 w/v) and planted at the rate of two seedlings in plastic pots containing sterilized sand. As controls, two pots (T0) with non inoculated seedlings were tested. Plants were sprayed with sterile distilled water every two days, in addition to being provided once a week with a nitrogen-free nutrient solution.

Plants were inoculated with 1 ml of early stationary phaserhizobial culture (10^8-10^9 cells mL^{-1}) cultivated at 28°C in TA medium. Two replications were carried out for both inoculated and non-inoculated plants (negative controls). Plantlets were harvested six weeks growth. Nodulation was recorded by the existence of nodules and the efficiency was estimated by the presence of red coloring (leghemoglobin) inside the nodules (Vincent, 1970). Shoot weight and root nodule numbers in each plant were also determined. For each isolate, the inoculation effect was estimated by determining the relative index of dry weight increase according to the following formula: relative index of dry weight increase = (inoculated plant dry weight) / (control plant dry weight).

RESULTS

Isolation of rhizobia from *Medicago ciliaris*

A total of 37 isolates were isolated from root nodules of *M. ciliaris* . These rhizobia are Gramnegative, nonspore forming, rod-shaped, with circular and convex colonies having 1-3 mm in diameter when grown on TA plates at 28°C. Most strains are translucent and gummy, white coloured except the pink pigmented (Meds1, Meds8, Meds11, Meds12, Meds16, Meds23, Meds26, Meds31, Meds32, Medp1, Medp4, Medp7, Medp10, Medp12, and Medp13). Thirty two (32) strains can be regarded as fast-growers (Jordan, 1984) (visible colonies develop within (2-3 days). in contrast (Meds24, Meds25, Meds26, Meds29, Meds31) are very fast growers (visible colonies develop within (1-2 days).

Stress tolerance

The 37 isolates obtained from *M. ciliaris* and 6 reference strains of laboratory collection were first screened for resistance to high salinity, alkaline pH, high drought and high temperature conditions. According to this preliminary characterization, high diversity in stress resistance was observed. The data in Figure 1 show that *M. ciliaris* rhizobia exhibited a wide diversity in their salt tolerance. The salt inhibitory concentrations varied among strains

Table 1. Characterization of isolates.

Isolate	Gram's nature	Color produced on BTB agar	Fast/Slow grower	3-ketolactose test	Catalase test	Oxidase test
MedS01	Gram -	Yellow	Fast	Negative reaction	+	+
MedS02	Gram -	slightlyyellow	Fast	Negative reaction	+	+
MedS04	Gram -	Yellow	Fast	Negative reaction	+	-
MedS07	Gram -	slightlyyellow	Fast	Negative reaction	+	-
MedS08	Gram -	Yellow	Fast	Negative reaction	+	-
MedS09	Gram -	Yellow	Fast	Negative reaction	+	+
MedS10	Gram -	Yellow	Fast	Negative reaction	+	+
MedS11	Gram -	Yellow	Fast	Negative reaction	+	+
MedS12	Gram -	slightlyyellow	Fast	Negative reaction	+	+
MedS13	Gram -	Yellow	Fast	Negative reaction	+	+
MedS14	Gram -	Yellow	Fast	positive reaction	+	+
MedS15	Gram -	slightlyyellow	Fast	Negative reaction	+	+
MedS16	Gram -	slightlyyellow	Fast	Negative reaction	+	+
MedS20	Gram -	slightlyyellow	Fast	Negative reaction	+	+
MedS23	Gram -	Yellow	Fast	Negative reaction	+	-
MedS24	Gram -	Yellow	Fast	positive reaction	+	-
MedS25	Gram -	Yellow	Fast	positive reaction	+	+
MedS26	Gram -	Yellow	Fast	positive reaction	+	+
MedS28	Gram -	Yellow	Fast	Negative reaction	+	+
MedS29	Gram -	Yellow	Fast	positive reaction	+	+
MedS30	Gram -	Yellow	Fast	Negative reaction	+	+
MedS31	Gram -	Yellow	Fast	positive reaction	+	+
MedS32	Gram -	Yellow	Fast	Negative reaction	+	+
MedP01	Gram -	Slightly yellow	Fast	Negative reaction	+	+
MedP03	Gram -	Slightly yellow	Fast	Negative reaction	+	+
MedP04	Gram -	Slightly yellow	Fast	Negative reaction	+	+
MedP05	Gram -	Yellow	Fast	Negative reaction	+	-
MedP06	Gram -	Yellow	Fast	Negative reaction	+	+
MedP07	Gram -	Yellow	Fast	positive reaction	+	-
MedP09	Gram -	Yellow	Fast	Negative reaction	+	+
MedP10	Gram -	Yellow	Fast	Negative reaction	+	+
MedP12	Gram -	Slightly yellow	Fast	Negative reaction	+	+
MedP13	Gram -	Slightly yellow	Fast	Negative reaction	+	+
MedP14	Gram -	Yellow	Fast	Negative reaction	+	-
MedP17	Gram -	Yellow	Fast	Negative reaction	+	+
MedP18	Gram -	Yellow	Fast	Negative reaction	+	+
MedP19	Gram -	Yellow	Fast	Negative reaction	+	+
S. meliloti	Gram -	Yellow	Fast	Negative reaction	+	+
S. meliloti	Gram -	Yellow	Fast	Negative reaction	+	+
S. fredii	Gram -	Yellow	Fast	Negative reaction	+	+
S. medicae	Gram -	Yellow	Fast	Negative reaction	+	+
A. tumefaciens	Gram -	Yellow	Fast	Positive reaction	+	+
Rhizobium sp.	Gram -	Yellow	Fast	Negative reaction	+	+

and salt nature. Indeed, tolerance to sodium chloride (NaCl) was found since than 100% of the tested rhizobia continued to grow with 1% NaCl. However, at higher concentrations, the percentage of tolerant strains decreased rapidly and only two isolates (4.65% of all isolates) were able to grow at 4% NaCl, while at 3% NaCl. All the isolates were sensitive to the high salinity level of 3 and 4% NaCl, however, they showed relatively

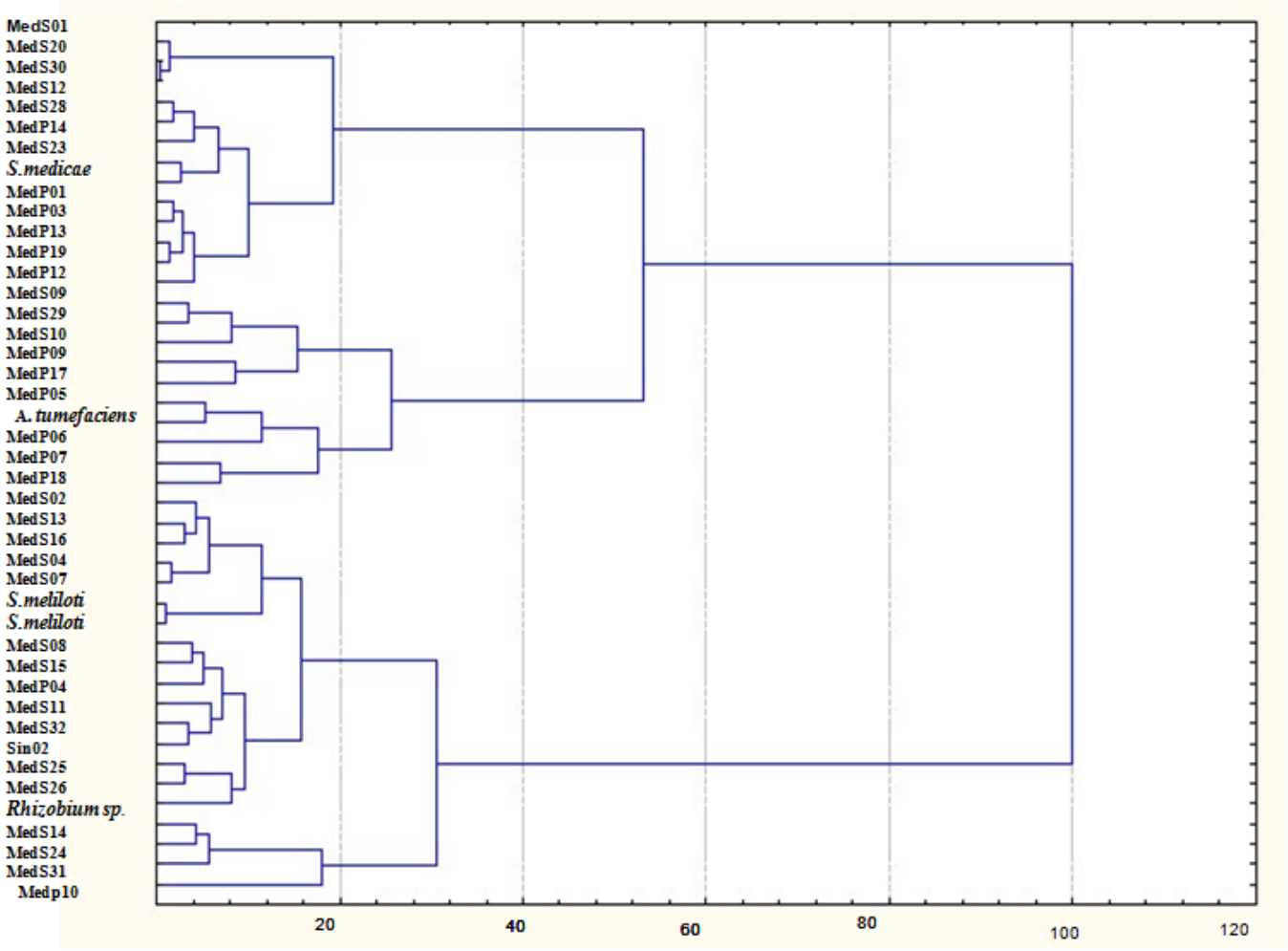

Figure 1. Dendrogram showing effect of different concentrations of Nacl and temperature on growth of *Medicago ciliaris* rhizobia.

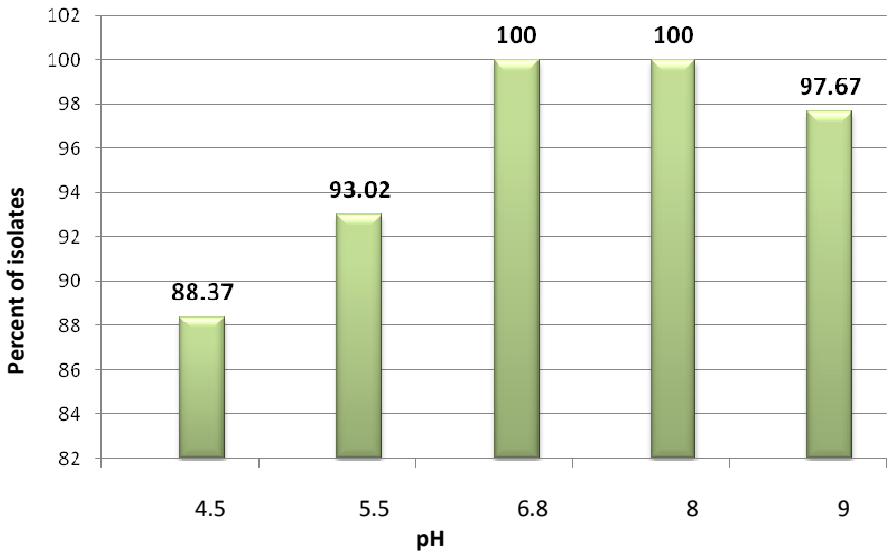

Figure 2. Effect of different concentrations of pH on growth of *Medicago ciliaris* rhizobia.

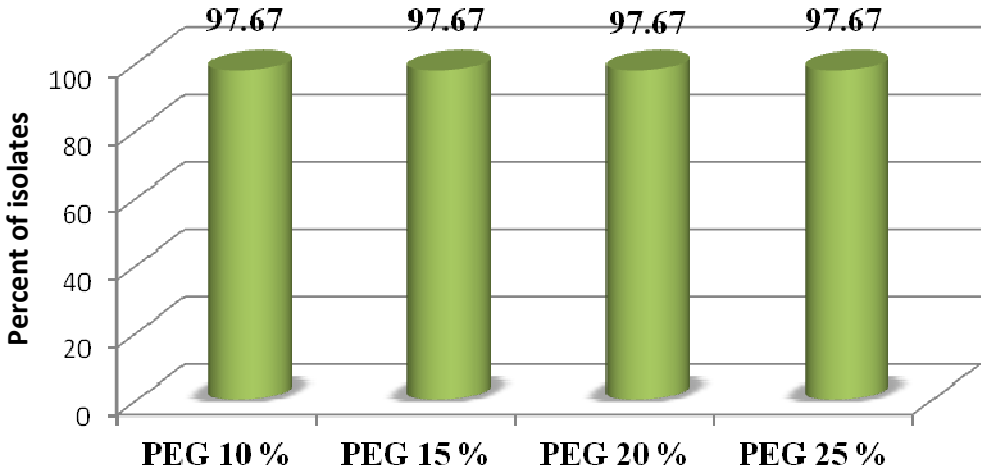

Figure 3. Effect of different concentrations of PEG 4000 on growth of *Medicago ciliaris* rhizobia.

good growth a 2% NaCl. Regarding high temperature resistance, response to extreme temperatures was positive for all the strains. Optimum temperature range for growth of culture is 28-30^{0}C. 100 % of the isolates were able to grow at 37°C. Above those values, the percentage of isolates that grew decreased to reach 69.76 % at 42°C and 27.90% at 45°C.

In the case of the pH test (Table 3, Figure 2), showed a wide diversity in their pH tolerance. From 93.02 to 100% of the isolates grew in lightly acid and neutral pH. At low pH, some isolates exhibited anacido-tolerant character. Above pH 8, 100% of the isolates grew in alkaline pHs. Osmotolerance of all isolates was tested in minimal medium YMA supplemented with increasing concentrations PEG 4000 (drought), as described in the Materials and methods. All strains were able to grow in YMA with PEG added. Nearly all isolates survived at 25% PEG 4000 with the exception of Medp01.

Distinguishing test between *Rhizobium* and *Agrobacterium*

Agrobacterium is common in soil and in the plant rhizosphere, but was never described inside root nodules. Distinguished *Agrobacterium* from *Rhizobia* by 3-ketolactose test, whereas the *Agrobacterium* produced yellow ring of precipitate of CuO_2 around the colonies of the bacterium when plates were flooded with Benedict's reagent. In the present study six isolates showed positive results for 3-ketolactose test (Table 1 and Figure 4).

Antibiotic susceptibility

Intrinsic resistance to antibiotics showed a general resistance to erythromycin, and 69.67% of the strains were also resistant to 10 µg ml^{-1} ampicillin. 18.60% of strains were scored resistant to 30 µg m^{-1} chloramphe-nicol and 55.81% were also resistant to 30 µg ml^{-1} nalidixic acid; 51.16 and 93.02% were also resistant to 10 and 30 µg ml^{-1} of streptomycin and neomycin, respectively. Nevertheless, all strains were highly sensitive to tetracycline and kanamycin; rifampicin the concen-tration of 30 µg ml^{-1} (Table 5 and Figure 5).

Plant tests

The 37 isolates and reference strains of laboratory collection were tested for their capacity to form root nodules on their original host plants under controlled laboratory conditions of temperature and relative humidity. All the isolates induced root nodules to form on their original hosts, and the uninoculated plants used as negative controls were not nodulated - root nodule numbers in the original host plants.

The mean number varied from 2.25 in *Agrobacterium tumefacians* (reference strain)to 19.50 in *S. meliloti* (reference strain). Seven isolates MedS14, MedS24, MedS25, Meds26, Meds29, and Meds31 and Medp07 have the enzymatic ability to aerobically convert lactose to 3-ketolactose, Strains Medp09 and *A. tumefaciens* were the less infective, with a respective average of 2 and 0 formed per plant. While strains MedS32 and *S. meliloti* were the most infective with 22 and 28 nodules formed per plant respectively. Relative indexes expressed as a shoot dry weight of the inoculated plants compared to the positive control plants, was largely variable (Table 4 and Figure 7). The most infective strains were also the most effective (Table 4 and Figure 6).

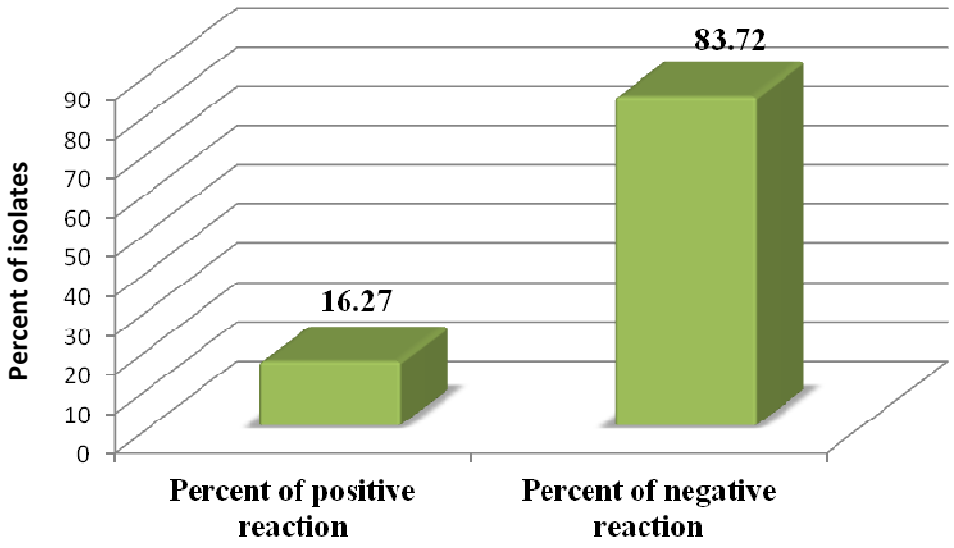

Figure 4. 3-ketolactose test reaction of *Medicago ciliaris* rhizobia.

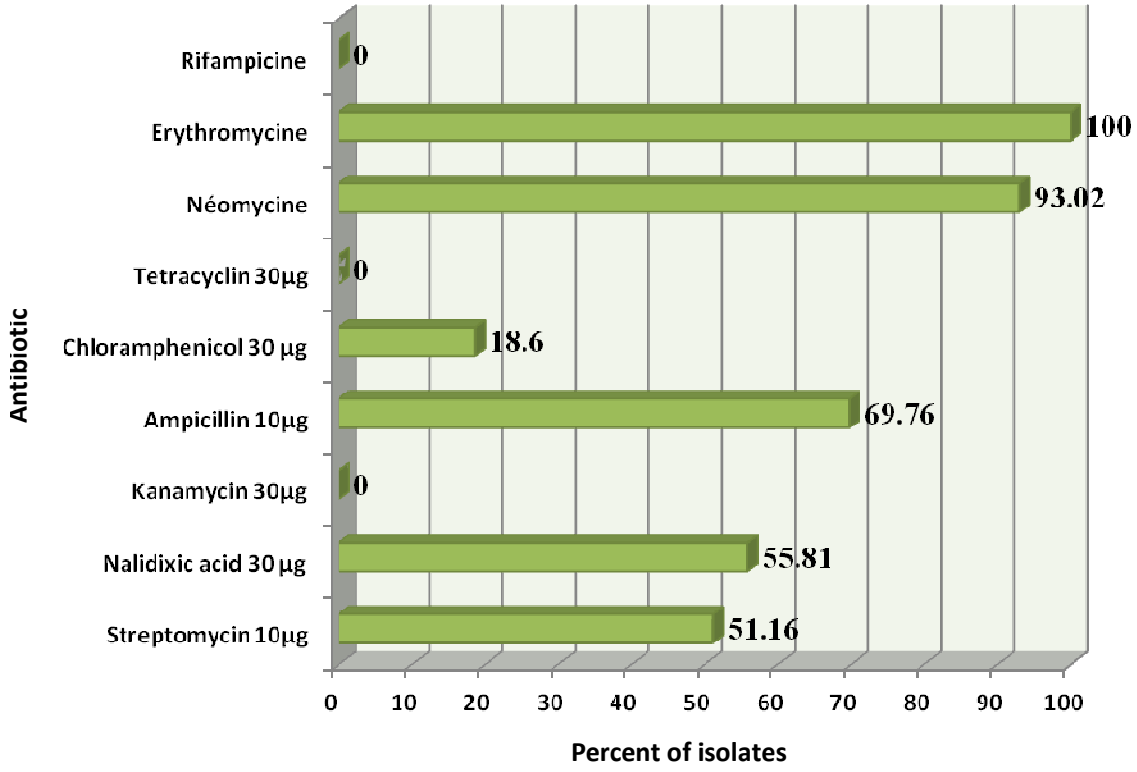

Figure 5. Effect of different antibiotics on growth of *Medicago ciliaris* rhizobia.

DISCUSSION

Although phenotypic and genotypic approaches provided very different information on the *M. ciliaris* rhizobia strains, they were similarly sensitive in demonstrating the large diversity found amongst these bacteria. The phenotypic characterization of the sampled 37 isolates and six reference strains of laboratory collection for above

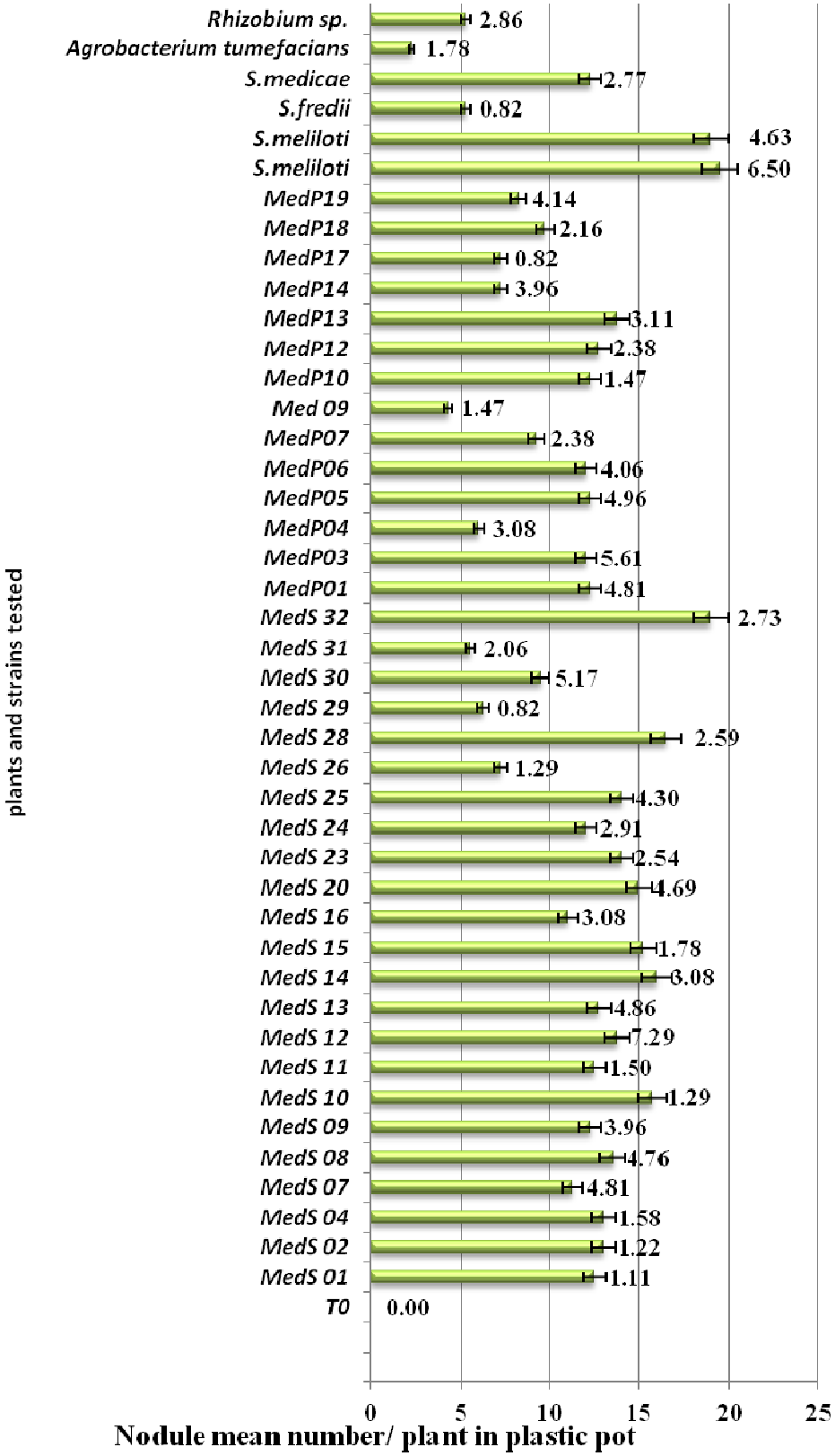

Figure 6. Infectivity of *Medicago ciliaris* rhizobia of nodulation test in plastic pot.

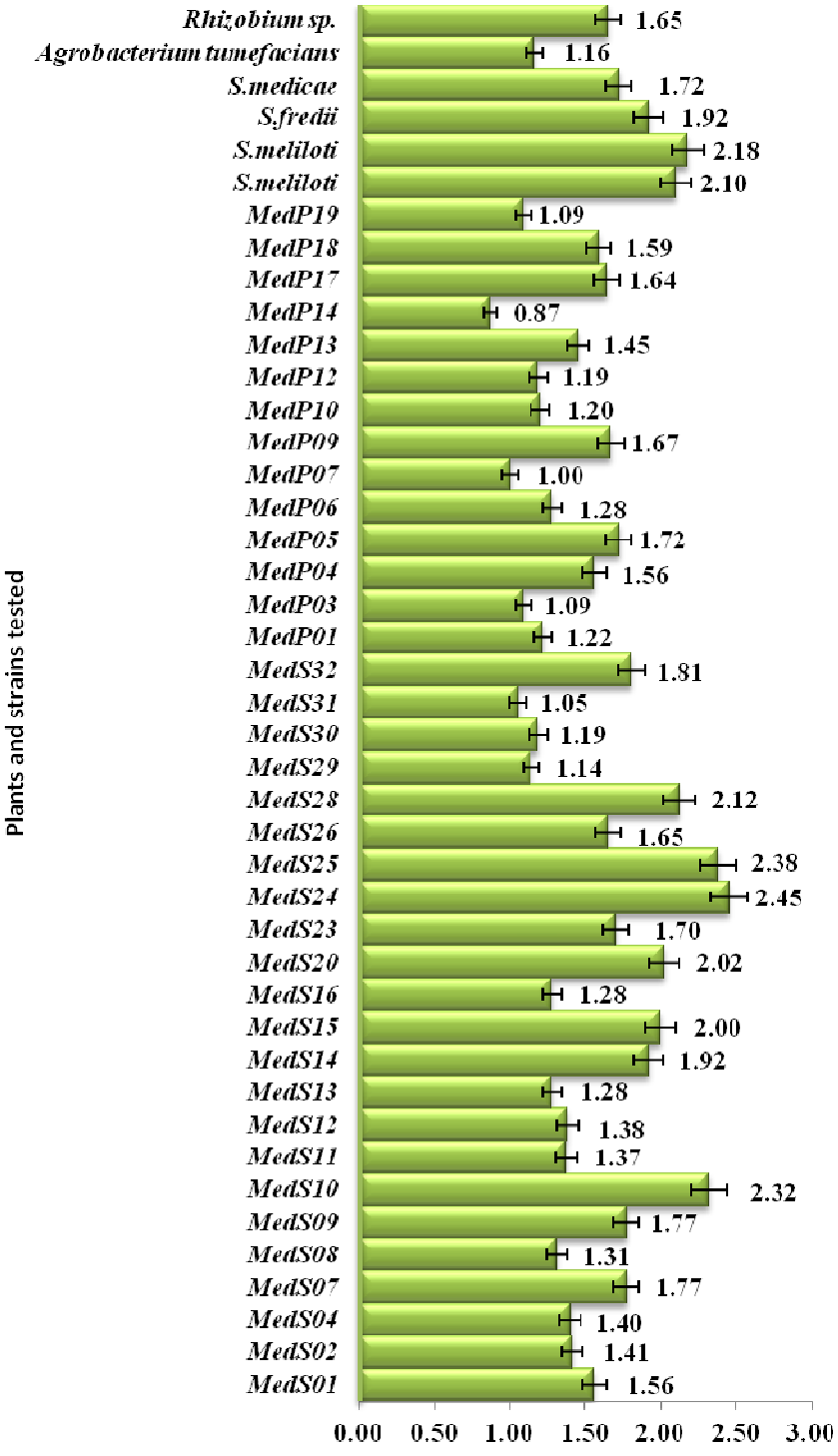

Figure 7. Relative index of dry weight increase of nodulation test in plastic pot.

characters revealed a large degree of variation. Consequently, a total of the strains was isolated from root nodules. Based on differences in high salinity, alkaline pH, high temperature and drought stress resistance. Concerning the symbiotic partner of *M. ciliaris* has a preference for *E. medicae*. Out of thirty seven isolates and six reference strains, seven shown a positive reaction3-ketolactose test. In the case of high salt resis-

tance, Meds09, Meds10, Meds29, Medp06 and Medp10 survived at 4% NaCl. Shamseldin et al. (2009) reported that *E. meliloti* strains from faba bean root nodules survived at 3% NaCl. Payakapong et al. (2006) also reported that an Ensifer strain of BL3 obtained from root nodules of *Phaseolus lathyroides* could survive at 3.5% NaCl. Shamseldin et al. (2006) reported the proteomic characterization of *Rhizobium etli* at 4% NaCl. In the case of high temperature resistance, out of 37 isolates, and six of reference strains thirteen isolates and two of reference strains survived at 45°C as shown in Table 3. Concerning high temperature resistance, some tolerant rhizobial isolates have been described. For instance, Fall et al. (2008) isolated rhizobia from *Acacia Senegal* that showed good growth at 45°C. Furthermore, Ge-Hong et al. (2008) reported a temperature tolerant strain of *Mesorhizobium* at 35°C. However, there is no report about *E. meliloti* surviving at 45°C.

Moreover, two isolates, Meds13 and Meds14 survived at both 45°C and 2% NaCl. Nonetheless, we could not find an isolate able to survive abilities at 45°C and 3% NaCl. Out of these 37 isolates, only 2 (5.40% of the total) could survive at both 45°C and 2% NaCl as shown in Figure 1 and Table 3**.** This shows that the frequency of isolates having both high temperature resistance and high salt resistance is low. For salinity tolerance, we observed a wide variability for tolerance (0-4%). The isolates showed variation for NaCl tolerance, indicating that the rhizobia nodulating *Medicago* spp. are more tolerant compared to other rhizobia species (Struffi et al., 1998; Zahran, 1999). However, as suggested by El Sheikh and Wood (1989) and Odee et al. (1997), we found that fast growing strains were generally more tolerant to high salt concentrations than slow-growing strains. Salinity imposes both ionic and osmotic stresses. Indeed, the imposition of any stress to rhizobia results in adaptive responses, which lead to changes in the regular metabolic processes that are then reflected in protein profiles. The tolerant rhizobia to osmotic stress accumulate the osmolytes, and changes their morphology and dehydration of cells (Buss and Bottomley, 1989). There was a good correlation between the tolerances to both stressors; strains that were halotolerant were in general also tolerant to PEG, suggesting that common osmo-adaptation mechanisms were operating. Hypersaline stress, most bacteria synthesize and accumulate small organic molecules called compatible solutes, as they compensate for hyperosmotic stress without interfering with cellular metabolism. As drought also imposes osmotic stress, it is plausible that at least part of the cell response to drought involves the synthesis and accumulation of compatible solutes. Our results showed that all the isolates grew at 25% of PEG 4000. This result showed that Zerizer soils contained rhizobial strains well adapted to dry conditions. For the most rhizobia, optimum temperature range for growth of culture is 28-31°C, and many cannot grow even at 37°C (Graham, 1992). At 28, 37 and 40°C, the isolates grew well like most *Sinorhizobium* species (Lindstrom and Lehtomaki, 1988; De Lajudie et al., 1994), could grow above 40°C. There was a varied response of the isolates tested to pH. All the isolates tested grew in alkaline pH (pH 8 and 9). At very low pH (pH 4.5), isolates grew normally with the exception of Meds09, Medp01, Medp04 and Medp05 *S. fredii.* According to Jordan (1984), slow-growing strains appear to be more tolerant to low pH than fast-growing strains. Some fast-growing strains such as *Rhizobium tropici* and *Mesorhizobium loti* can grow at a pH as low as 4 (Cooper, 1982; Cunningham and Munns, 1984; Graham, 1992; Gao et al., 1994). It has been reported that *S. melilot*i grows at a pH range of 5.0-9.5 (Jordan, 1984) and is tolerant to 2.0% (w/v) NaCl. Another *Medicago*-nodulating species, *S. medicae*, can grow at pH 5.0-10.0 and is resistant to 2.0% NaCl (Rome et al., 1996). Growth at pH 5.0 has been recorded for only some strains (Jordan, 1984) from acidic soils. Regarding the intrinsic resistance to antibiotics, it has been reported that fast-growing strains are more sensitive to antibiotics (Jordan, 1984) than slow-growing rhizobia. The evaluation of intrinsic resistance to antibiotics showed that most tested isolates had high resistance to erythromycin and nalidixic acid, chloramphenicol, and streptomycin. However, the degree of resistance to antibiotics was higher than in other species of rhizobia (Wei et al., 2003), indicating that *S. meliloti* and *S. medicae* had higher levels of tolerance to these antibiotics.

All tested strains were able to infect their host plant and to fix atmospheric nitrogen leading to plant shoot production above the noninoculated controls. Strains Meds10, Meds14, Meds15, Meds28, Meds32 developed 15.75, 16, 15.25, 16.50 and 19 mean number of root nodules respectively, while *S. meliloti* developed 19.50 mean number of root nodules with the same host plant. This result shows that the root nodule forming ability of the symbiotic *Agrobacterium* is significantly lower than that of the reference strain *S. meliloti.* The root nodule number inoculated with trap host isolates is clearly lower than that of host plants inoculated with the remaining isolates. Sullivan and Ronson (1998) reported that a symbiotic element of *M. loti* was transferred into three non symbiotic species. Bailly et al. (2007) reported that several interspecific horizontal gene transfers occurred during the diversification of *Medicago* symbionts. Similarly, Wong and Golding (2003) reported that a large portion of pSym B genes in *E. meliloti* are most closely related to genes in *A. tumefaciens* linear chromosomes. These reports support the existence of symbiotic *Agrobacterium* isolates produced by horizontal transfer of symbiotic genes.

We conclude that rhizobia strains isolated from *M. ciliaris* nodules in Algerian soil are both phenotypically diverse. To verify this suggestion, we need to complete

Table 3. Stress tolerance screening.

Isolate	Temperature range (°C)	NaCl (%)*	pH*	Drought PEG*
MedS01	28-42	1%(++)	9 (++)	25% (++)
MedS02	28-45	1%(++)	9 (++)	25% (++)
MedS04	28-45	0.01%(++)	9 (++)	25% (++)
MedS07	28-45	0.01%(++)	9 (++)	25% (++)
MedS08	28-42	1%(++)	9 (++)	25% (++)
MedS09	28-42	4%(++)	9 (++)	25% (++)
MedS10	28-42	4%(++)	9 (++)	25% (++)
MedS11	28-45	1%(++)	9 (++)	25% (++)
MedS12	28-40	0.01%(++)	9 (++)	25% (++)
MedS13	28-45	2%(++)	9 (++)	25% (++)
MedS14	28-45	2%(++)	9 (++)	25% (++)
MedS15	28-42	2%(++)	9 (++)	25% (++)
MedS16	28-45	0.01%(+)	9 (++)	25% (++)
MedS20	28-40	1%(++)	9 (++)	25% (++)
MedS23	28-45	1%(+)	9 (++)	25% (++)
MedS24	28-45	0%(+)	9 (++)	25% (++)
MedS25	28-45	0.01%(+)	9 (++)	25% (++)
MedS26	28-40	1%(++)	9 (++)	25% (++)
MedS28	28-42	4%(+)	9 (++)	25% (++)
MedS29	28-40	1%(++)	9 (++)	25% (++)
MedS30	28-45	1%(+)	9 (++)	25% (++)
MedS31	28-42	2%(++)	9 (++)	25% (++)
MedS32	28-42	2%(++)	9 (++)	25% (++)
MedP01	28-40	2%(++)	9 (++)	25% (+)
MedP03	28-40	2%(++)	9 (++)	25% (++)
MedP04	28-42	2%(++)	9 (++)	25% (++)
MedP05	28-42	1%(++)	9 (++)	25% (++)
MedP06	28-45	4%(+)	9 (++)	25% (++)
MedP07	28-42	2%(++)	9 (++)	25% (++)
MedP09	28-42	4%(++)	9 (++)	25% (++)
MedP10	28-45	4%(+)	9 (++)	25% (++)
MedP12	28-42	2%(++)	9 (++)	25% (++)
MedP13	28-42	2%(++)	9 (++)	25% (++)
MedP14	28-37	3%(++)	9 (++)	25% (++)
MedP17	28-40	3%(++)	9 (++)	25% (++)
MedP18	28-42	2%(++)	9 (++)	25% (++)
MedP19	28-42	1%(++)	9 (++)	25% (++)
S.meliloti	28-37	3%(++)	9 (2+)	25% (2+)
S.meliloti	28-45	1%(+)	9 (2+)	25% (2+)
S.fredii	28-45	1%(++)	9 (2+)	25% (2+)
S.medicae	28-40	1%(++)	9 (2+)	25% (2+)
A.tumefaciens	28-42	2%(++)	9 (2+)	25% (2+)
Rhizobium sp.	28-42	3%(++)	9 (2+)	25% (2+)

*Growth scores were recorded as follows: −, no growth; +, weak growth (10-30% in relation to the control); ++, good growth (30-80% in relationto the control); and +++, very good growth (similar to the control).

this study using molecular techniques such as REP/PCR or RFLP/PCR, sequencing of 16S rDNA genes and

Table 4. Symbiotic properties of isolates on *M. ciliaris* nodulation test in plastic pots.

Isolate	Mean No. of nodules/ plant * nodulation test in plastic pot	Plant dry weight (mg)	Relative indexes
Control	00	37.34 ± 17.94 [a]	/
MedS01	12.5 ± 1.11[a]	58.61 ± 16.81	1.56
MedS02	13 ± 1.22	52.79 ± 9.27	1.41
MedS04	13 ± 1.58	52.44 ± 16.73	1.40
MedS07	11.25 ± 4.81	66.13 ± 18.49	1.77
MedS08	13.5 ± 4.76	49.25 ± 9.37	1.31
MedS09	12.25 ± 3.96	66.33 ± 7.12	1.77
MedS10	15.75 ± 1.29	86.67 ± 33.39	2.32
MedS11	12.5 ± 1.50	51.35 ± 2.43	1.37
MedS12	13.75 ± 7.29	51.71 ± 16.76	1.38
MedS13	12.75 ± 4.86	47.80 ± 8.51	1.28
MedS14	16 ± 3.08	72.02 ± 24.77	1.92
MedS15	15.25 ± 1.78	74.75 ± 12.4	2.00
MedS16	11 ± 3.08	47.91 ± 8.76	1.28
MedS20	14 ± 2.54	63.51 ± 10.78	1.70
MedS23	12 ± 2.91	91.77 ± 30.88	2.45
MedS24	14 ± 4.30	88.90 ± 39.91	2.38
MedS25	7.25 ± 1.29	61.85 ± 10.61	1.65
MedS26	16.5 ± 2.59	79.45 ± 9.24	2.12
MedS28	6.25 ± 0.82	42.69 ± 5.43	1.14
MedS29	9.5 ± 5.17	44.73 ± 7.87	1.19
MedS30	5.50 ± 2.06	39.57 ± 2.75	1.05
MedS31	19 ±2.73	67.65 ± 18.15	1.81
MedS32	19 ±2.73	67.65 ± 18.15	1.81
MedP01	12.25 ± 4.81	45.73 ± 16.19	1.22
MedP03	12 ± 5.61	40.96 ± 12.11	1.09
MedP04	6 ± 3.08	58.61 ± 3.81	1.56
MedP05	12.25 ± 4.96	64.44 ± 11.82	1.72
MedP06	12 ± 4.06	48.13 ± 26.49	1.28
MedP07	9.25 ± 2.38	37.57 ± 7.32	1.00
MedP09	4.25 ± 1.47	62.73 ± 5.19	1.67
MedP10	12.25 ± 1.47	44.98 ± 4.03	1.20
MedP12	12.75 ± 2.38	44.62 ± 6.49	1.19
MedP13	13.75 ± 3.11	54.38 ± 5.26	1.45
MedP14	7.25 ± 3.96	32.77 ±11.23	0.87
MedP17	7.25 ± 0.82	61.52 ± 15.86	1.64
MedP18	9.75 ± 2.16	59.55 ± 13.61	1.59
MedP19	8.25 ± 4.14	40.93 ±11.49	1.09
S. meliloti	19.5 ± 6.5	78.51 ±17.61	2.10
S. meliloti	19 ± 4.63	81.75 ± 13	2.18
S. fredii	5.25 ± 0.82	71.95 ± 27.91	1.92
S. medicae	12.25 ± 2.77	64.40 ± 20.37	1.72
A. tumefaciens	2.25 ± 1.78	43.61 ± 8.35	1.16
Rhizobium sp.	5.25 ± 2.86	61.70 ± 11.49	1.65

[a]Average ± standard deviation.

Table 5. Effect of different antibiotics on growth of *Medicago ciliaris*L. rhizobia.

Strain	Str 10 µg	Nal. ac 30 µg	Km 30 µg	Amp 10 µg	Chl 30 µg	Tet 30 µg	Rif	Neo	Ery
MedS01	R	R	S	R	R	S	S	R	R
MedS02	R	R	S	R	R	S	S	R	R
MedS04	R	R	S	S	R	S	S	R	R
MedS07	R	R	S	R	R	S	S	R	R
MedS08	R	R	S	R	R	S	S	R	R
MedS09	S	S	S	R	S	S	S	R	R
MedS10	S	R	S	R	R	S	S	R	R
MedS11	R	R	S	R	S	S	S	R	R
MedS12	R	R	S	R	S	S	S	S	R
MedS13	S	R	S	R	S	S	S	R	R
MedS14	R	S	S	R	S	S	S	R	R
MedS15	S	R	S	R	R	S	S	R	R
MedS16	S	R	S	S	S	S	S	R	R
MedS20	S	R	S	S	S	S	S	R	R
MedS23	R	S	S	R	S	S	S	R	R
MedS24	R	S	S	R	S	S	S	R	R
MedS25	R	S	S	R	S	S	S	S	R
MedS26	R	S	S	R	S	S	S	R	R
MedS28	S	S	S	S	S	S	S	R	R
MedS29	R	S	S	R	S	S	S	R	R
MedS30	S	R	S	R	S	S	S	R	R
MedS31	R	S	S	R	S	S	S	R	R
MedS32	R	S	S	S	S	S	S	R	R
MedP01	S	R	S	R	S	S	S	R	R
MedP03	R	S	S	S	S	S	S	R	R
MedP04	R	R	S	R	S	S	S	R	R
MedP05	S	S	S	S	S	S	S	R	R
MedP06	S	S	S	R	R	S	S	R	R
MedP07	S	S	S	S	S	S	S	S	R
MedP09	S	S	S	S	S	S	S	R	R
MedP10	R	R	S	R	S	S	S	R	R
MedP12	S	R	S	R	S	S	S	R	R
MedP13	R	R	S	R	S	S	S	R	R
MedP14	S	R	S	R	S	S	S	R	R
MedP17	S	R	S	S	S	S	S	R	R
MedP18	S	R	S	S	S	S	S	R	R
MedP19	S	S	S	R	S	S	S	R	R
S. meliloti	S	R	S	R	S	S	S	R	R
S. meliloti	R	S	S	S	S	S	S	R	R
S. fredii	S	R	S	S	S	S	S	R	R
S. medicae	S	R	S	R	S	S	S	R	R
A. tumefaciens	R	S	S	R	S	S	S	R	R
Rhizobium sp.	R	S	S	R	S	S	S	R	R

R, Resistant to antibiotic; S, sensivite to antibiotic; Str: Streptomycin; Nal. ac: nalidicic acid; Km: kanamycin; Amp: Ampicillin; Chl: chloramphenicol; Tet: tetracycline; Rif: Rifampicin; Neo: Neomycin; Ery: Erythromycin.

DNA/DNA hybridization.

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES

Badri M, Zitoun A, Soula S, Ilahi H, Huguet T, Aouani ME (2008). Low levels of quantitative and molecular genetic differentiation among natural populations of *Medicago ciliaris* Kroch. (Fabaceae) of different Tunisian eco-geographical origin. Conserv. Genet. 9(6):1509-1520.

Bailly X, Olivieri B, Brunel JC, Cleyet-Marel, Bena G (2007). Horizontal gene transfer and homologous recombination drive the evolution of the nitrogen-fixing symbionts of Medicago species. J. Bacteriol. 189:5223-5236.

Ben Amor B, Shaw SL, Oldroyd GED, Maillet F, Penmetsa RV, Cook D, Long SR, Denarie J, Gough C (2003). The NFP locus of Medicagotruncatula controls an early step of Nod factor signal transduction upstream of a rapid calcium flux and root hair deformation. Plant J. 34:495-506.

Ben Salah I, Albacete A, Martinez Andujar C, Haouala R , Labidi N, Zribi F, Martinez V, Perez-Alfocea F, Abdelly C (2009). Response of nitrogen fixation in relation to nodule carbohydrate metabolism in *Medicago ciliaris* lines subjected to salt stress. J. Plant Physiol. 166:477-488.

Bena G, Lyet A, Huguet T, Olivieri I (2005). Medicago-Sinorhizobium symbiotic specificity evolution and the geographic expansion of Medicago. J. Evol. Biol. 18:1547-1559.

Bena G, ProsperiJM , Lejeune B,Olivieri I (1998). Evolution of annual species of the genus *Medicago*: a molecular phylogenetic approach. J. Mol. Evol. 9:552-559.

Bernaerts MJ, De Ley J (1963). A biochemical test for crown gall bacteria. Nature 197:406-407.

Bradic M, Sikora S, Redzepovic S, Stafa Z (2003). Genetic identification and symbiotic efficiency of an indigenous *Sinorhizobium meliloti* field population. Food Technol. Biotech. 41(1):69-75.

Brunel B, Rome S, Ziani R, Cleyet-Marel JC (1996) Comparison of nucleotide diversity and symbiotic properties of Rhizobium meliloti populations from annual Medicago species. FEMS Microbiol. Ecol. 19:71-82.

Buss MD, Bottomley PJ (1989). Growth and nodulation responses of *Rhizobium melilot*ito water stress induced by permeating and nonpermeating solutes. Appl. Environ. Microbiol. 55:2431- 2436.

Cook D (1999).Medicagotruncatula: a model in the making. Curr. Opin. Plant Biol. 2: 301-304.

Cooper JE (1982). Acid production, acid tolerance and growth rate of lotus rhizobia in laboratory media. Soil Biol. Biochem. 14:127-131.

Cunningham SD, Munns DN (1984). The correlation between extra cellular polysaccharide production and acid tolerance in Rhizobium. Soil Biol. Biochem. 14:127-131.

De Billy F, Grosjean C, May S, Bennett M, Cullimore JV (2001). Expression studies on Auxl-like genes in Medicagotruncatula suggest that auxin is required at two steps in early nodule development. Mol. Plant Microbe Interact. 14:267-277.

De Lajudie P, Willems A, Pot B, Dewettinck D, Maestrojuan G, Neyra M, Collins M.D, Dreyfus B, Kersters K, Gillis M (1994). Polyphasic taxonomy of rhizobia: emendation of the genus Sinorhizobium and description of Sinorhizobiummeliloti comb. nov., Sinorhizobiumsaheli sp. nov., and Sinorhizobiumteranga sp. nov. Int. J. Sys. Bacteriol. 44:715-733

El Sheikh EAE, Wood M. (1989). Salt effects on survival and multiplication of chickpea and soybean rhizobia. Soil Biol. Biochem. 22:343-347.

Fall D, Diouf D, Ourarhi M, Faye A, Abdelmounen H, Neyra M, Sylla SN, El Idrissi MM (2008). Phenotypic and genotypic characteristics of *Acacia senegal* (L.) Wild.root-nodulating bacteria isolated from soils in the dryland part of Senegal. Lett. Appl. Microbiol. 47:85-97.

Foth HD (1990). Fundamentals of soil science (eighth ed.), Wiley, New York, 384. Gagnon, M., W. Hunting and W. B. Esselen. 1959. A new method for catalase determination. Anal. Chem. 31:144.

Gao JL, Sun JG, Li Y, Wang ET, Chen WX (1994). Numerical taxonomy and DNA relatedness of tropical rhizobia isolated from Hainan Province. China. Int. J. Syst. Bacteriol. 44:151-158.

Garau G, Reeve WG, Brau L, Deiana P, Yates RJ, James D, Tiwari R, O'Hara GW, Howieson JG (2005). The symbiotic requirements of different Medicago spp. suggest the evolution of *Sinorhizobium meliloti* and *S. medicae* with hosts differentially adapted to soil pH. Plant Soil 276:263-277.

Ge-Hong, WXue-Ying Y, Zhi-Xin Z, Ya-Zhen Y, Lindsrom K (2008). Strain *Mesorhizobium* sp. CCNWGX035: A stress stolerant isolate from Glycyrrhiza glabra displaying a wide host range of nodulation. Pedosphere 18:102-112.

Graham PH(1992). Stress tolerance in *Rhizobium* and *Bradyrhizobium*, and nodulation under adverse soil conditions. Can. J. Microbiol. 38: 475-484.

Howieson JG, O'Hara GW, Carr SJ (2000).Changing roles of legumes in Mediterranean agriculture; developments from an Australian perspective. Field Crops Res. 65:107-122.

Irwin JAG, Lloyd DL, Lowe KF (2001). Lucerne biology and improvement - an analysis of past activities and future goals in Australia. Aust. J. Agric. Res. 52:699-712.

Jordan DC (1984). Rhizobiaceae. In: Bergey's Manual of Systematic Bacteriology ed. Krieg NR , Holt JG, pp. 234-242. Baltimore:The Williams & Wilkins.

Laouar M, Abdelguerfi A (2000). Study of complex species Medicago Medicago ciliaris - intertexta: characterizations of different types of seed heads. Cahiers Options Mediterraneennes 45: 39-41.

Lesins KA, Lesins I (1979). Genus *Medicago*(Leguminosae): A Taxogenetic Study. The Hague, The Netherlands.

Li X, Su D, Yuan Q (2007). Ridge-furrow planting alfalfa (Medicago sativa L.) for improved rainwater harvest in rainfed semiarid areas in Northwest China. Soil Till. Res. 93:117-125.

Lindstrom K, Lehtomaki S (1988). Metabolic properties, maximum growth temperature and phage sensitivity of Rhizobium sp. (Galega) compared with other fast-growing rhizobia. FEMS Microbiol. Lett. 50:277-287.

Odee DW, Sutherland JM ,Makatiani ET, McInroy SG, Sprent JI (1997). Phenotypic characteristics and composition of rhizobia associated with woody legumes growing in diverse Kenyan conditions. Plant Soil 188:65-75.

Payakapong W, Tittabutr P, Teaumroong N, Boonkerd N, Singleton PW, Borthakur D (2006). Identification of two clusters of genes involved in salt tolerance in Sinorhizobium sp. strain BL3. Symbiosis 41:47-51.

Rome S, Fernandez MA, Brunel B, Normand P, Cleyet-Marel JC (1996). Sinorhizobiummedicae sp. nov., isolated from annual Medicago spp. Int. J. Syst. Bacteriol. 4:972-980.

Shamseldin A, El-Saadani M, Sadowsky MJ, An CS (2009). Rapid identification and discrimination among Egyptian genotypes of *Rhizobium leguminosarum* bv. viciae and *Sinorhizobium meliloti* nodulating faba bean (*Vicia faba* L.) by analysis of nodC, ARDRA, and rDNA sequence analysis. Soil Biol. Biochem. 41:45-53.

Shamseldin A, Nyalwidhe J, Werner D (2006). A proteomic approach towards the analysis of salt tolerance in *Rhizobium etli* and *Sinorhizobium meliloti* strains. Curr. Microbiol. 52:333-339.

Struffi P, Corich V, Giacomini A, Benguedouar A, Squartini A, Casella S, Nuti MP (1998). Metabolic proprieties, stress tolerance and macromolecular profiles of rhizobia nodulating *Hedysarum coronarium*. J. Appl. Microbiol. 84:81-89.

Sullivan GT, Ronson CW(1998). Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a phe-tRNA gene. Proc. Natl. Acad. Sci. USA. 95:5145-5149.

Van Berkum P, Beyene D, Bao G, Campbell TA, Eardly BD (1998). Rhizobium mongolense sp. nov. is one of the three rhizobial genotypes identified which nodulate and form nitrogen fixing symbiosis with *Medicago ruthenica* [(L.) Ledebour]. Int. J. Syst.

Bacteriol. 48:13-22.
Vincent JM (1970). A Manual for the Practical Study of Root Nodule Bacteria. IBP Handbook, No. 15. Oxford: Blackwell Scientific Publications Ltd.
Wei GH, Tan ZY, Zhu ME, Wang ET, Han SZ, Chen WX (2003). Characterization of rhizobia isolated from legume species within the genera *Astragalus* and *Lespedeza* grown in the Loess Plateau of China and description of Rhizobium loessense sp. nov. Int. J. Syst. Evol. Microbiol. 53(Pt 5):1575-83.
Wong K, Golding GB (2003). A phylogenetic analysis of the pSymB replicon from the Sinorhizobiummeliloti genome reveals a complex evolutionary history. Can. J. Microbiol. 49:269-280.
Zahran HH (1999). Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in arid climate. Micobiol. Mol. Biol. Rev. 63:968-989.