

Phenotypic and genotypic characterization of root nodules rhizobia of *Medicago littoralis* Rhode and *Melilotus indicus* (L.) All. growing in the Oasis of Touggourt, Oued Righ Valley, in the Algerian Sahara

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Abstract Forty rhizobial strains were isolated from root nodules of *Medicago littoralis* Rhode and *Melilotus indicus* (L.) harvested from the sandy soils of Touggourt's oases in the Oued Righ Valley, Algerian Sahara. The isolates were studied for their cultural, biochemical and symbiotic effectiveness. All of them were fast-growing bacteria; utilized a wide range of carbon sources, produced abundant extracellular polysaccharides, tolerated high concentrations of NaCl (up to 2.5 %), grew at temperatures between 28 and 45 °C and at pH values between 4.5 and 9. The isolates were sensitive to the antibiotics kanamycin, tetracycline and rifampicin but showed resistance to neomycin and erythromycin. All the isolates induced the formation of effective nodules on their host plants. On the basis of the physiological, biochemical and symbiotic effectiveness, we selected six strains MD05, MD09, MD12, ML08, ML17 and ML22 for genotypic characterization. Phylogenetic analysis of the selected strains based on 16S ribosomal RNA gene showed that these strains of bacteria were affiliated to the *Ensifer meliloti* group.

Keywords Rhizobia · *Medicago* · *Melilotus* · Oasis soil · Phenotype · Genotype

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1 Introduction

In Algeria, arid lands with a Saharan climate represent about 90 % of the total area of the country (Le Houérou 1975). The Algerian Sahara is characterized by the presence of particular regions called “oases” that represent biological diversity hotspots (Riou 1990; Chouaki et al. 2006). The most famous oases are those of Oued Righ Valley (Fig. 1) where more than 47 oases are located between 32°54' of latitude and 34°09' longitude. This valley has a hyper arid climate, with gritty soil characterized by a high salinity due to irrigation with artesian salty water (3 g/l). The chemical composition of these soils is variable but is generally very poor in nitrogen, phosphorous and potassium (Toutain 1974; Le Houérou 1975; Koull et al. 2013). Agriculture at these oases is characterized by small production units (Ferry and Toutain 1990) and the integration of fruit trees and tall crops to protect herbal plants against the strong sunlight. The oases of Oued Righ Valley have a beneficial microclimate due to the shade and the constant moisture provided by irrigation. These decrease the effect of the very hot Saharan climate (Riou 1990; Messar 1996).

The *Leguminosae* are one of three important families which are predominant in these agriculture units; the others being the *Poaceae* and the *Asteraceae*. *Medicago sativa* is the main annual fodder crop of the oases and occupies about 80 % of the land surface devoted to such crops and a third of the irrigated area (Janati 1990; Abdelguerfi et al. 1996; Chaabena and Abdelguerfi 2007; Chouaki et al. 2006; Chafi and Bensoltane 2009; Chaabena et al. 2011).

Plant productivity in these arid and semi-arid regions is low because of poor soil fertility (Toutain 1974; Le Houérou 1975; Djennane 1990; Zahran 1999; Priefer et al. 2001). Nitrogen is one of the main factors limiting plant growth, and soils are generally very poor on organic matter (Fitouri Dhane et al.

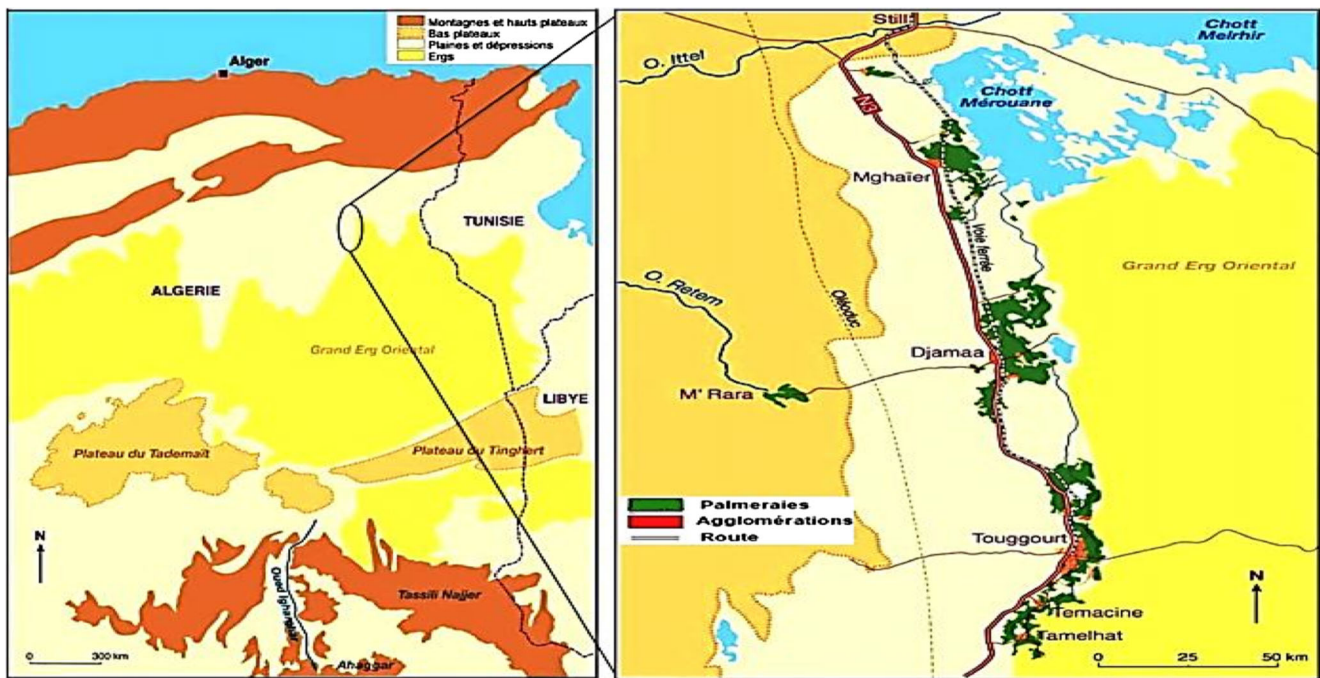


Fig. 1 The Geographic location of the oases of Oued Righ Valley

2012; Palma et al. 2013). Biological Nitrogen Fixation (BNF) is the sustainable source of nitrogen in the cropping systems (Serraj et al. 2004) and *Rhizobium*-legume symbioses provide soil enrichment (Zahran 1999, 2001). In Algeria, studies on rhizobia-legume symbiosis have only been completed in northern regions of the country while others have been carried out in the arid regions of Tunisia (Zakhia et al. 2004; Mnasri et al. 2007a, b; Djedidi et al. 2011; Rejjii et al. 2014), Morocco (Maâtallah et al. 2002; Thami-Alami et al. 2010) and Egypt (Zahran 1997; Abdel-Salam et al. 2010).

Rhizobia associated with wild legumes in the oases of Touggourt have not previously been investigated and the present work was carried out to study rhizobial strains associated with two indigenous legumes; *Medicago littoralis* and *Melilotus indicus* growing in salty soils of Touggourt's oases. The phenotypic characterization of these strains was conducted to evaluate their capacity to grow under environmental stresses which include high temperature, salinity, drought and pH. The phylogenetic positions of our strains were determined by an analysis of the 16S rRNA gene.

2 Materials and methods

2.1 Plant material

The wild legumes *Medicago littoralis* and *Melilotus indicus* were collected from the oases of Touggourt in Oued Righ Valley.

2.2 Isolation of rhizobia

The nodules were surface-sterilized with sodium hypochlorite (2 %) for 4 min and rinsed five times with sterile water; then the nodules were crushed with glass rod in a sterile test tube. One loopful of the nodule content suspension was streaked on yeast extract mannitol agar medium (YEMA) containing Congo red dye and incubated at 28 °C until colonies achieved a maximum size (Somasegaran and Hoben 1985).

After incubation for 3 to 5 days at 28 °C; single colonies were selected and transferred several times on YEMA medium. Isolates were purified and maintained on YEMA medium and conserved at -20 °C in YEMB-glycerol (yeast extract mannitol broth supplemented with 20 % glycerol) until use (Somasegaran and Hoben 1985; Shetta et al. 2011).

2.3 Rhizobia presumptive tests

The absorption or not of the Congo Red dye (CR) and the production of acid or alkali by the isolates was estimated after 3 days of growth respectively on YEMA supplemented with CR (0.0025 %) and on YEMA supplemented with Bromothymol blue (BTB 0.0025 %) (Somasegaran and Hoben 1985; Shetta et al. 2011). *Ensifer meliloti* strain 1021, *Mesorhizobium ciceri* (M4) and *Agrobacterium tumefaciens* C58 were utilized as reference strains for the phenotypic tests. All the tests were done with suspension of isolates at the exponential growth phase.

2.4 Temperature and salt tolerance assays

The effect of the temperature and the tolerance to different concentrations of NaCl were determined by inoculating the isolates on YEMA medium supplemented with NaCl at different concentrations (*w/v*), 2.5; 3.5; 4.5 and 6.0 %. The plates were incubated at four different temperatures (28; 37; 40 and 45 °C) for 7 days (Maâtallah et al. 2002; Djedidi et al. 2011).

2.5 pH tolerance

The tolerance of isolates to large range of pHs (4.5; 5.5; 6.8; 8 and 9) was determined on YEMA medium. The plates were incubated for 3 days at 28 °C (Thami-Alami et al. 2010).

2.6 Tolerance to drought stress

The isolates were suspended in 1 ml of PEG 4000 solutions at final concentrations: 0; 10; 15; 20 and 25 % (*w/v*) and then 10 µl of each suspension were used for inoculating YEMA plates. The plates were incubated at 28 °C for 3 days; the tolerance was estimated by measuring the growth of isolates on plates at different concentrations of PEG 4000.

2.7 Intrinsic antibiotic resistance

In a first protocol, the test was performed by using antibiotics discs. A volume of 0.5 ml of an exponential culture of the isolates was spread on the surface of the YEMA plates; the plates were dried for 30 min and we put the antibiotics discs on the surface of the plates : Streptomycin (30 µg); ampicillin (30 µg); chloramphenicol (30 µg); tetracycline (30 µg); nalidixic acid (30 µg); kanamycin (30 µg). After 3 to 5 days of incubation at 28 °C, the inhibition zones were measured (Rome et al. 1996; Elbanna et al. 2009).

In a second protocol, filter-sterilized antibiotics were added aseptically to sterile YEM medium to give a final concentrations of rifampicin (30 µg/ml); erythromycin (30 µg/ml) and neomycin (30 µg/ml) (Somasegaran and Hoben 1985). Plates were incubated at 28 °C, after 3 days, results were recorded by the absence or the presence of bacterial growth (Shetta et al. 2011; Thami-Alami et al. 2010; Zhang et al. 1991).

2.8 Plant nodulation test

The ability of isolates to nodulate their host plants was tested. Seeds were hand-sorted for size uniformity and for absence of damages, surface-disinfected by soaking in sodium hypochlorite (2.5 %) for 10 min and rinsed several times with sterile distilled water, then left to germinate in the dark (Somasegaran and Hoben 1985; Amrani et al. 2010).

The nodulation tests were done using pots pre-filled with sterile sand. At least three seeds were used per pot for each

isolate tested (Abdel-Salam et al. 2010). The suspensions were prepared using isolates at the exponential growth phase. One ml of each suspension was used to inoculate young plants of *Medicago* and *Melilotus*. The plants were kept under natural conditions and watered with the solution of Rigaud and Puppò (Priefer et al. 2001; Djedidi et al. 2011). After 6 to 8 weeks of plant growth, the presence or absence of nodules on the roots was recorded. The efficiency of the nodules was estimated by the presence of red pigment (leghemoglobin) inside the nodules (Djedidi et al. 2011). The plants were dried at 50 °C after separating roots from aerial parts.

2.9 Test of 3-ketolactose

The determination of 3-ketolactase enzyme activity allows distinguishing between *Agrobacterium* and other rhizobial strains (Murugesan et al. 2010). Isolates were cultivated on nutrient glucose agar medium (NGA) for 48 to 72 h (Bouzar et al. 1995) and then suspensions were prepared with colonies grown on NGA. Drops of 10 µl were putted on lactose agar medium (LAM) with lactose (1 %), yeast extract (0.1 %), and agar (2 %). Three isolates per plate were tested. After incubation at 28 °C for 48 to 72 h, the plates were flooded with a thin layer of Benedict's reagent (Bouzar et al. 1995). The presence of 3-ketolactose in the medium was assessed by the formation of a yellow ring of cuprous oxide around the rhizobial colony after 15 to 20 min.

2.10 Biochemical and auxanographic tests

API 20NE and API 50CH are standardized systems, API 20 NE system combining 20 tests for identification of gram-negative rod and API 50CH gallery consists of 50 tests used for the study of carbohydrate metabolism. API 20 NE tests, the inoculation was done as recommended by the manufacturers (Shetta et al. 2011). The ability of isolates to metabolize carbohydrates was tested by using API 50 CH gallery (BioMérieux, France) as described by Kersters et al. (1984). After inoculation, galleries were incubated at 28 °C; the results were scored every day for 7 days.

2.11 Statistical analysis

The Principal Component Analysis (PCA) was performed to examine the relationships between the tolerance of isolates *in vitro* to both high temperature and salinity. Computations and graphical display were made using the XLSTATM software package (version 2004. 7. 5. 2, Addinsoft, Paris, France, <http://www.xlstat.com>). A computer cluster analysis of 206 phenotypic variables was carried out using STATISTICA 7.0 software package and a phenogram was constructed by the Unweighted Pair Group Method with Average (UPGMA) clustering method.

2.12 Genomic DNA extraction and phylogenetic analysis of rRNA genes

Genomic DNA was extracted using QIAamp DNA Mini Kit (QIAGEN, Düsseldorf, Germany) according to the manufacturer's instructions. The bacterial 16S rRNA gene fragment was amplified using the bacterial universal primers fD1 (5' AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') (Weisburg et al. 1991). The amplification reaction mixture consisted of 1x PCR reaction buffer, 100 nM of each primer, 200 mM of each deoxyribonucleotide triphosphate, 0.25 Units Hot Star Taq DNA polymerase (Qiagen, Hilden, Germany) and 20 ng of bacterial DNA.

The PCR program consisted of an initial step activation of the enzyme at 95 °C for 15 min, followed by a step of denaturation at 95 °C for 15 min; 30 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 55 °C and elongation for 1.5 min at 72 °C, followed by a final elongation step at 72 °C for 7 min. The amplicons were gel-purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and bidirectionally sequenced using primers rD1 and fD1. A single strand sequence was obtained for the strain ML08.

The 16S rRNA gene sequences were subjected to a NCBI nucleotide and 16S rRNA gene BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify sequences of the highest similarity. Phylogenetic analysis was inferred using the Maximum-likelihood method and bootstrap support for each node was evaluated with 1000 replicates. The multiple sequence alignment of nucleotide sequences for creating Maximum-likelihood was inferred using free software MEGA 5.2.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene are from KR476464 to KR476469.

3 Results

3.1 Rhizobial isolation

In this study, 40 strains were isolated from nodules of the wild legumes *Medicago littoralis* Rhode and *Melilotus indicus* (L.) All. plants harvested from the field of Touggourt's Oases. Respectively, 15 isolates were from nodules of *M. littoralis* Rhode (MD1 to MD15) and 25 were from nodules of *M. indicus* (L.) All. (ML1 to ML25). All these isolates were

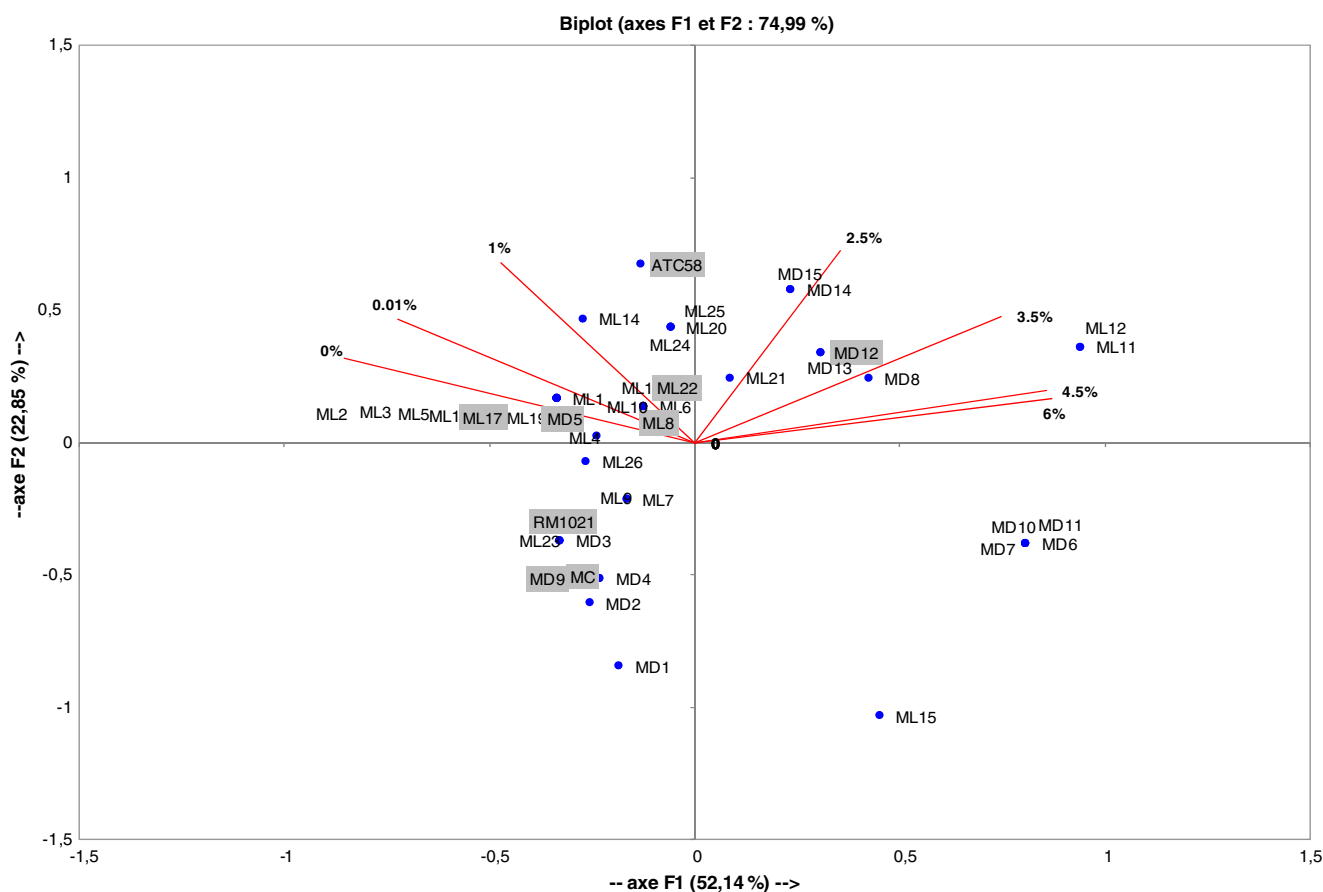


Fig. 2 PCA showing the relationship between tolerance of isolates to NaCl and their growth at 28 °C. RM1021: *E. meliloti* 1021, MC: *M. ciceri*, ATC58: *A. tumefaciens*. In grey isolates 16S rRNA sequenced and references strains

fast growing bacteria (72 h), with smooth, semi-translucent circular colonies and produced abundant quantity of polysaccharide. The diameter of the colonies varied between 3 and 4 mm after 3 days of incubation. The colonies of two isolates from *M. indicus* were yellow, smooth with 1 to 2 mm of diameter after 3 days of incubation and did not produce polysaccharide.

3.2 Phenotypic characterization

3.2.1 *Rhizobia* presumptive tests

None of the isolates absorbed Congo red dye and nearly all (97 %) caused a reduction in the pH of the medium during growth. The growth of isolates ML11, ML12, ML15 and MD07 slightly increased the pH of the medium.

3.2.2 Salt and high temperature tolerance

All of the isolates grew at temperatures between 28 and 40 °C and 98 % of them tolerated temperatures up to 45 °C. The reference strain isolates *Ensifer meliloti* strain 1021;

Mesorhizobium ciceri and *Agrobacterium tumefaciens* C58 were also able to grow at these temperatures.

The ability of isolates to tolerate NaCl was examined and the results show that 100 % of the isolates grow at low concentrations of NaCl (0.01 % w/v). Ninety eight percent of the isolates tolerated 1 % NaCl, 87 % 2.5 %, 75 % at 3.5 % and 52.5 % at 4.5 %. Only 45 % of the isolates tolerated high salinity (6 %). In comparison, *A. tumefaciens* C58, *E. meliloti* 1021 and *M. ciceri* did not tolerate more than 1 % of NaCl. The Principal Component Analysis graphs (PCA), showed that the direction of axis variables reflected tolerance to different concentrations of NaCl (Figs. 2, 3, 4 and 5).

Analysis of the combined effects of temperature and NaCl showed that temperatures between 30 and 37 °C improve the salt tolerance of isolates. Figures 2 and 3 show that the number of tolerant isolates varied, from 13 at 28 °C to 19 at 37 °C. Indeed, the optimal temperature of growth for our isolates was about 30 °C. It was interesting that incubation of isolates at high temperatures (40 and 45 °C) decreased the salt tolerance of isolates. At 40 °C, only 8 isolates showed a weak growth at 4 % of NaCl (Figs. 4 and 5), while at 45 °C isolates

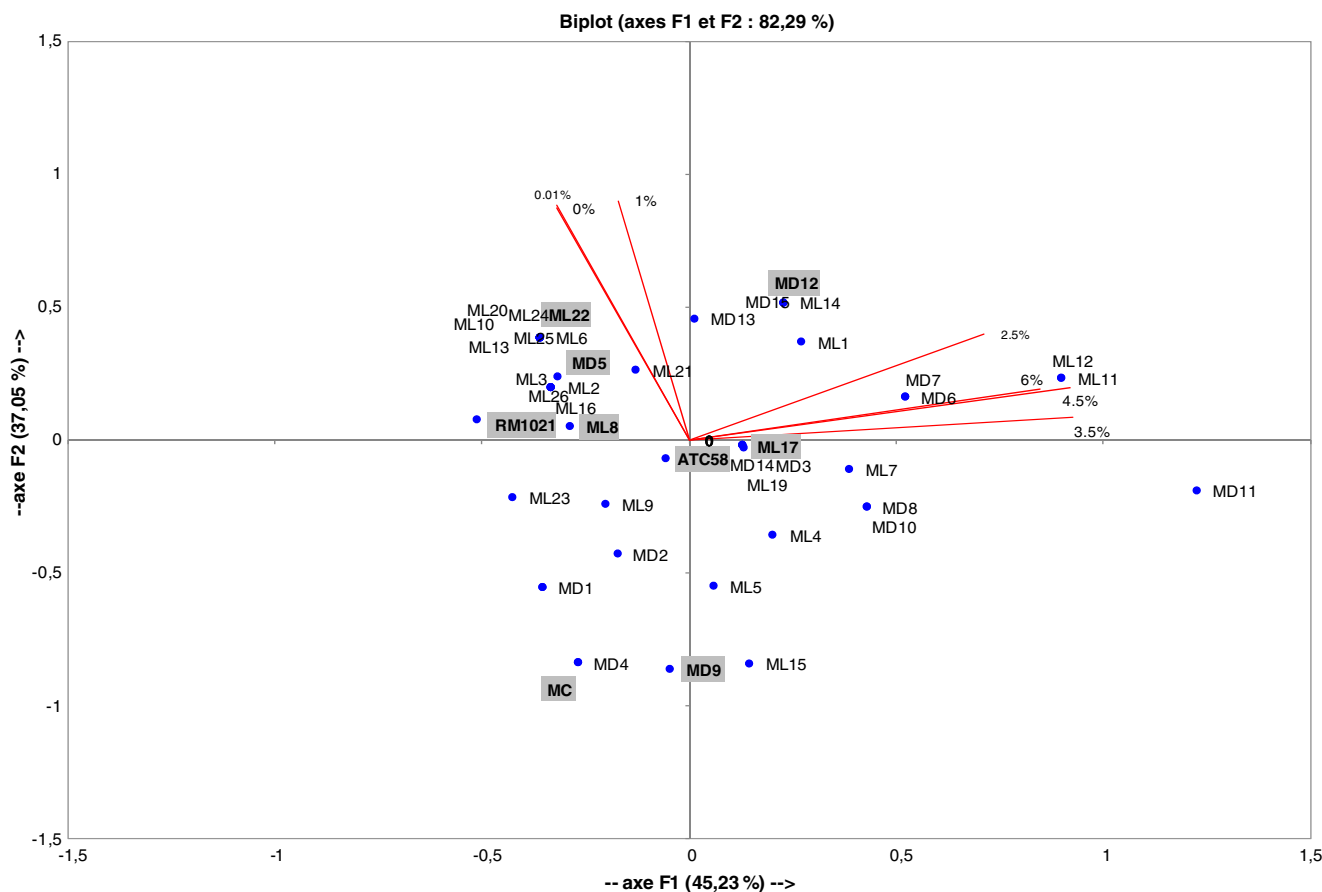


Fig. 3 PCA showing the relationship between tolerance of isolates to NaCl and their growth at 37 °C. RM1021: *E. meliloti* 1021, MC: *M. ciceri*, ATC58: *A. tumefaciens*. In grey isolates 16S rRNA sequenced and references strains

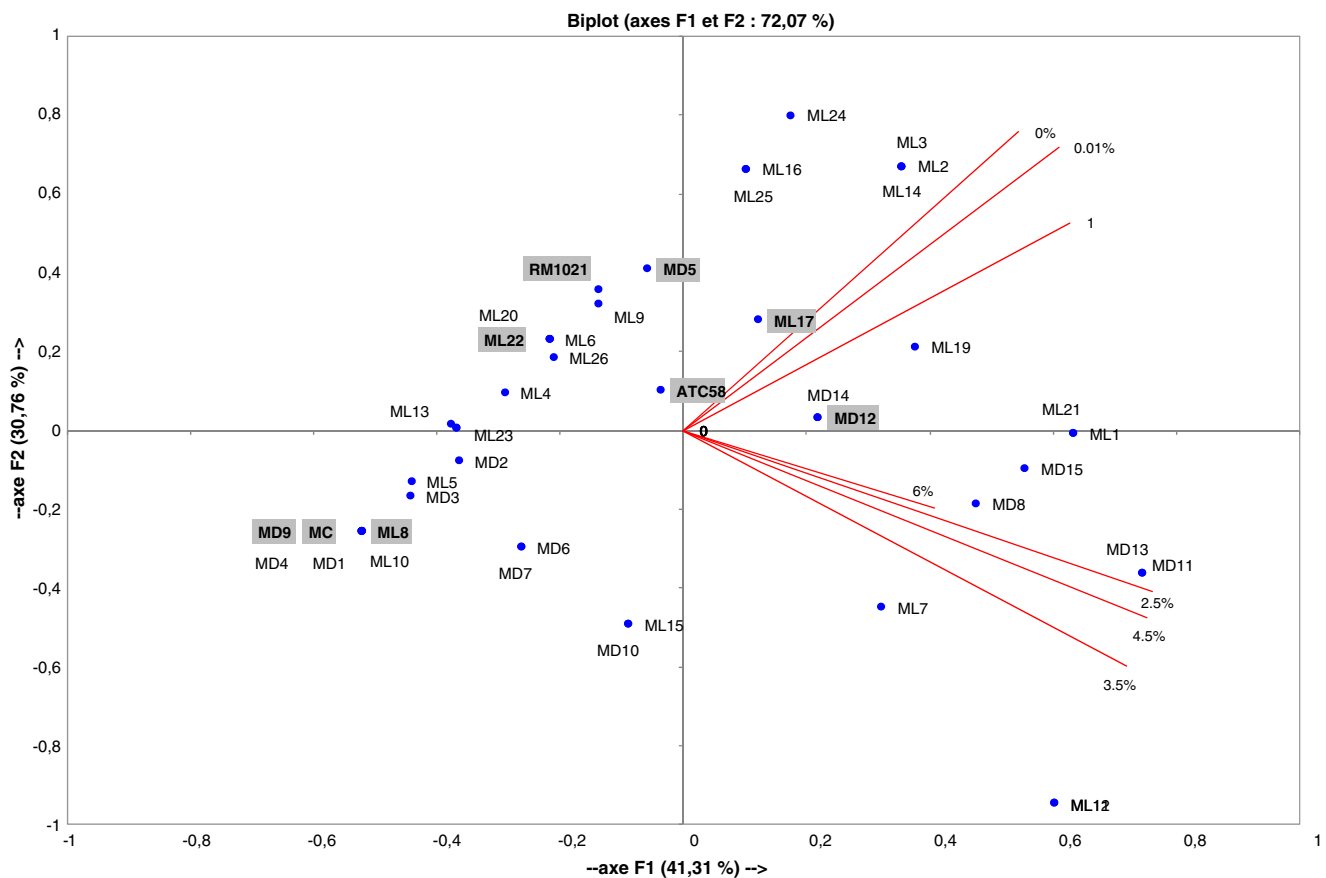


Fig. 4 PCA showing the relationship between tolerance of isolates to NaCl and their growth at 40 °C. RM1021: *E. meliloti* 1021, MC: *M. ciceri*, ATC58: *A. tumefaciens*. In grey isolates 16S rRNA sequenced and references strains

were only able to grow at low concentrations of salt. Only the strain ML12 had a remarkable tolerance to high salinity and high temperature.

3.2.3 pH and drought stress tests

Thirty-nine isolates and the references strains were able to grow on medium with pH values from 4.5 to 9.5. Only isolate ML15 did not grow at pH < 5.5. All the isolates tolerate 10 to 25 % of PEG4000 in the culture medium.

3.2.4 Antibiotics tests

Antibiotic resistance or sensitivity was performed using nine different antibiotics. All the isolates as well as *E. meliloti* 1021 and *A. tumefaciens* C58 showed a strong sensitivity to kanamycin, tetracycline and rifampicin. *M. ciceri* was only resistant to kanamycin and tetracycline. Ninety eight percent of the isolates were resistant to neomycin, 87 % to ampicillin, 85 % to the nalidixic acid, 80 % to erythromycin and 68 % to

chloramphenicol but resistance to other antibiotics varied from isolate to isolate (Fig. 6).

3.2.5 Nodulation test

After 8 weeks of plant growth, root nodules were collected and recorded and we observe that 87 % of MD isolates and 100 % of ML isolates induced nodulation of the hosts' plants. All formed pinky indeterminate nodules. The number of nodules obtained per host on *M. littoralis* plants inoculated with MD isolates varied from 0 to 21. In comparison, an average number of 26 nodules per plant were recorded for *E. meliloti* 1021 (no nodules were detected on negative controls of *Medicago* or *Melilotus* plants). In the case of *M. indicus* plants inoculated by ML isolates, the number of nodules per plant varied from 3 to 26. An average of 17 nodules were recorded on inoculated *M. ciceri* plants. Figure 7 shows the relationship between dry matter (dry weight of the aerial parts of the nodulated plants) and the number of nodules. We observed that the dry matter increased proportionally with the number of nodules and that dry matter and nodule numbers were higher for *Melilotus* plants than for *Medicago* plants.

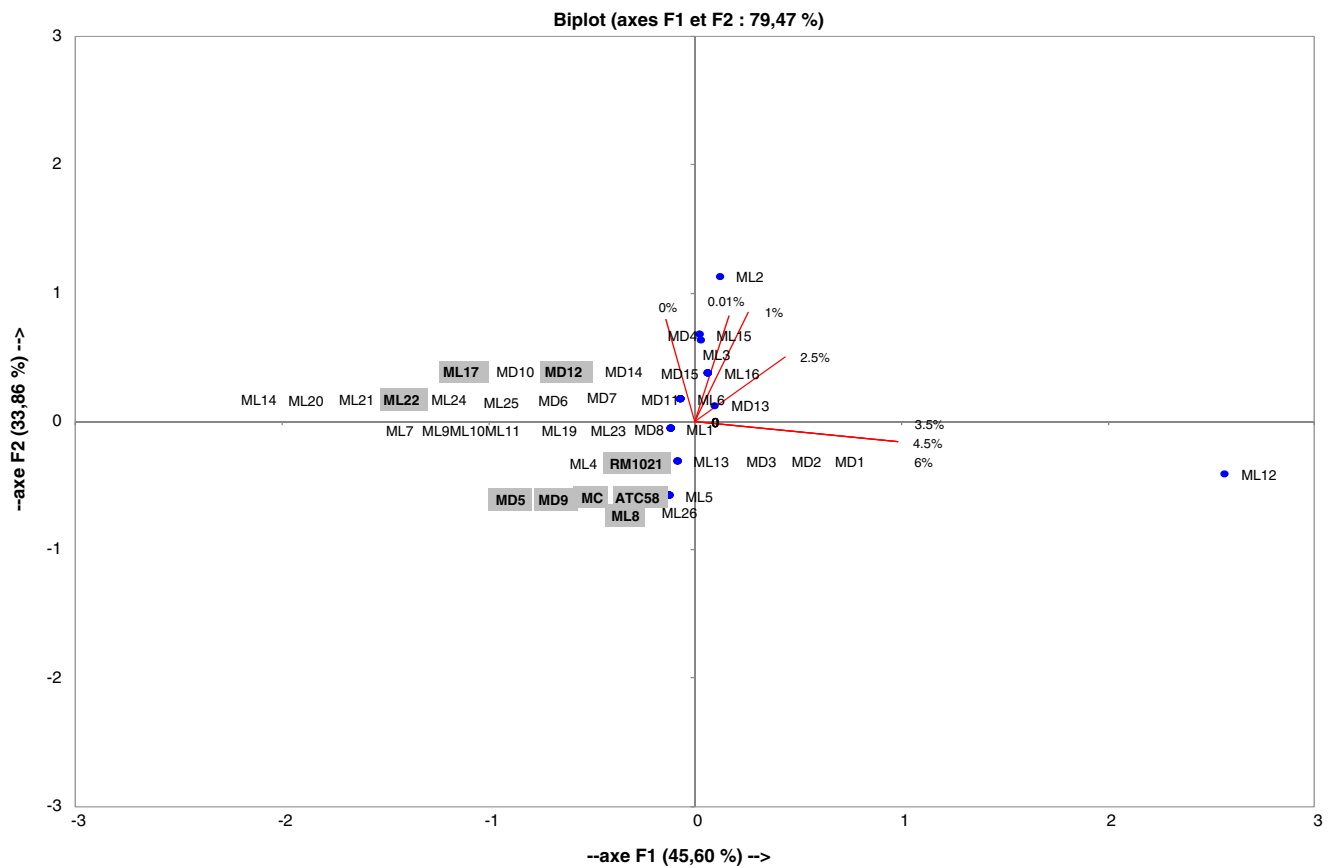


Fig. 5 PCA showing the relationship between tolerance of isolates to NaCl and their growth at 45 °C. RM1021: *E. meliloti* 1021, MC: *M. ciceri*, ATC58: *A. tumefaciens*. In grey isolates 16S rRNA sequenced and references strains

3.2.6 Test of 3-ketolactose

The 3-ketolactose enzyme activity distinguishes between *Agrobacterium* species and other rhizobial strains. We did not observe any 3-ketolactose enzyme activity in any of the isolates tested and concluded that the MD and ML isolates do not belong to the *Agrobacterium* group.

3.2.7 Carbohydrates assimilation

Fifteen isolates, nine ML and six MD, and *E. meliloti* 1021, *M. ciceri* and *A. tumefaciens* C58 were selected for phenotypic characterization by API20NE and API50CH tests. A total of 49 substrates were tested for the study of the carbohydrate metabolism. The analysis of phenotypic characters is presented as a dendrogram of dissimilarity in Fig. 8 which shows that the isolates comprise four groups. Group 1 includes ML1, MD3 and MD4; group 2 includes ML3, ML5, ML8, MD9 and MD12; group 3 includes ML17 and ML22 and the group 4 includes ML4, ML7 and MD13. The reference strains *E. meliloti* 1021, *M. ciceri*, *A. tumefaciens* C58 as well as the isolate ML12 constitute a single group and were used to confirm the phenotypic characterization.

3.3 Phylogenetic analysis of partial 16S rRNA genes

Based on the physiological characters and results of symbiotic effectiveness tests, six isolates (MD05, MD09 and MD12 from *Medicago littoralis*, ML08, ML17 and ML22 from *Melilotus indicus*) were selected for further genotypic characterization (Table 1). Genomic DNA extract were amplified by PCR using universal primers fD1 and rD1 and the amplicons obtained were sequenced. The sequences were BLAST analyzed and the results showed clearly that our isolates belong to the *Ensifer* group with 99 % identity (Table 2). The phylogenetic tree built from 16S rRNA genes sequences (Fig. 9) showed that all the six isolates were clustered with the *Ensifer meliloti* type strain and neighbor species.

4 Discussion

Legume species, within the genera *Medicago* and *Melilotus*, are important as foliage and as a source of green manure, they are naturally distributed or cultivated in many regions of the world within arid regions (Yan et al. 2000; Chouaki et al. 2006; Bruning and Rozema 2012; Palma et al. 2013). Wild legumes

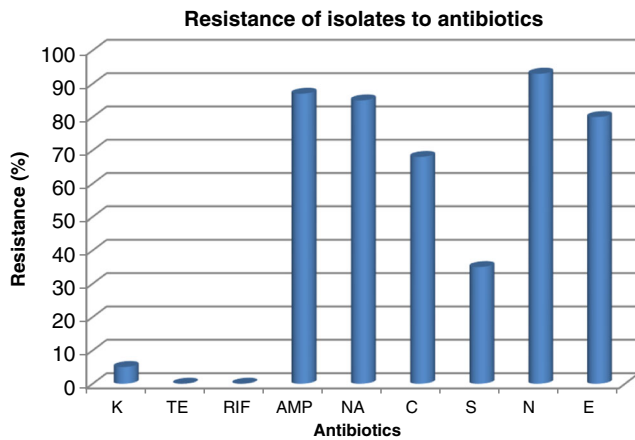


Fig. 6 The antibiotic resistance of isolates, *K* kanamycin, *TE* tetracycline, *RIF* rifampicin, *AMP* ampicillin, *NA* nalidixic acid, *C* chloramphenicol, *S* streptomycin, *N* neomycin, *E* erythromycin

often have a higher nitrogen fixing activity and are more tolerant to stress conditions than crop legumes. They are also better able to establish symbiotic relationships (Zahran 2001; Serraj et al. 2004; Bruning and Rozema 2012; Palma et al. 2013).

Our objective was to characterize the nitrogen fixing bacteria associated with two wild legumes *Medicago littoralis* Rhode and *Melilotus indicus* (L.) All., collected in the arid regions in Algeria, for which there are few data in the literature. We studied 40 isolates from saline soils of Touggourt oases in the Algerian Sahara. All our rhizobia strains isolated from *Medicago* nodules, and most from *Melilotus* nodules had typical colony and growth characteristics, when grown on standard YEMA medium (Somasegaran and Hoben 1985). Rhizobia generally do not absorb Congo red dye when plates are incubated in the dark. The fast-growing rhizobia that nodulated *Medicago* and *Melilotus* also showed the usual acidification of the culture medium. (Somasegaran and Hoben 1985; Shetta et al. 2011).

In arid regions, high soil temperature affects both the free-living and symbiotic rhizobia (Zahran 1999). For most of rhizobia, the optimum temperature range for growth is 28 to 31 °C. Many are unable to grow at 38 °C (Zahran 2001; Priefer et al. 2001; Cacciari et al. 2003; Thami-Alami et al. 2010; Shetta et al. 2011). In the present study 98 % of our isolates were able to grow at 45 °C. These strains were isolated

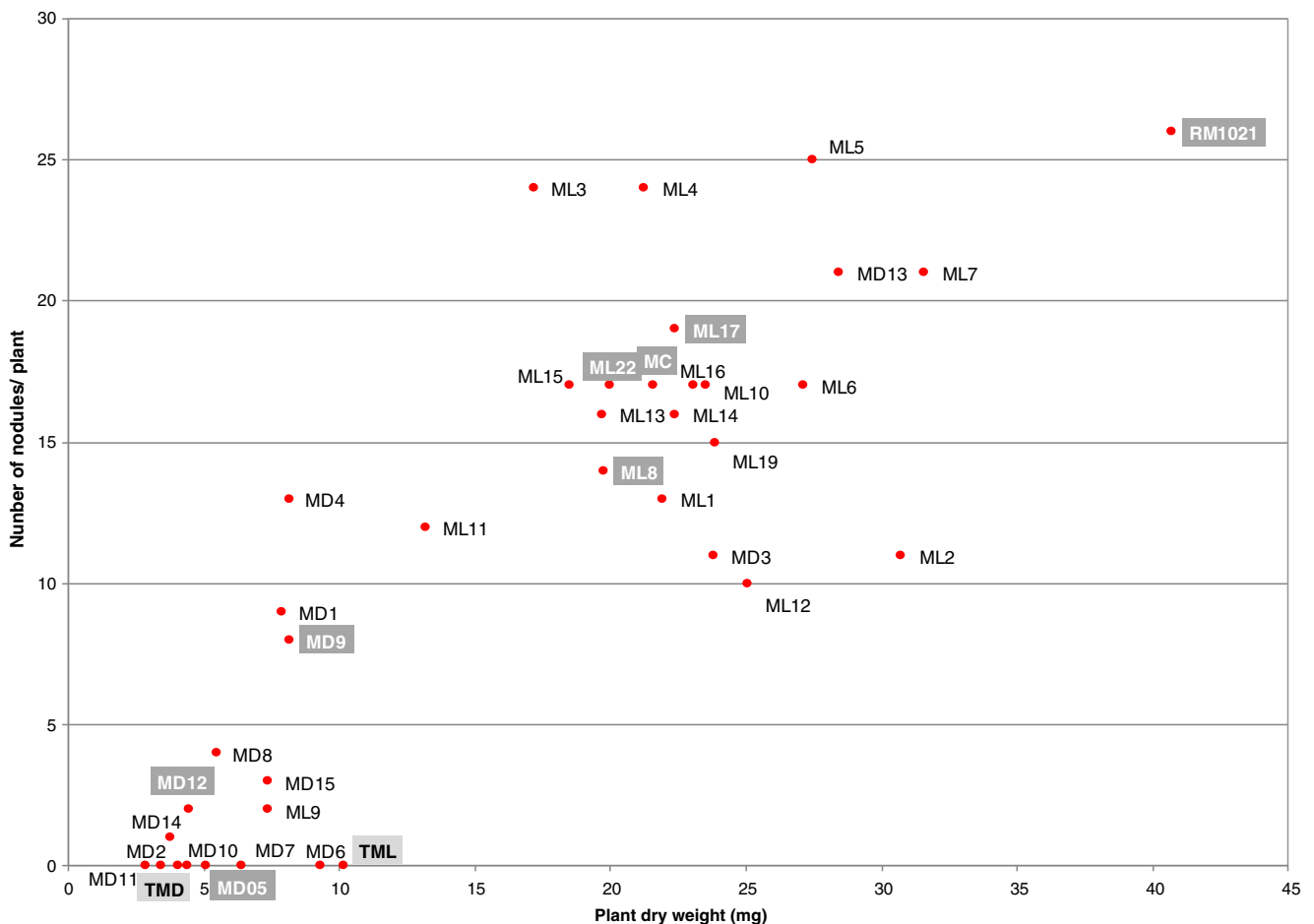


Fig. 7 The Number of nodules induced by MD and ML isolates versus plant dry matter. In grey: reference strains; identified isolates; TMD and TML control plants

from a hot arid region which suggests that temperature tolerance is correlated with the isolation site. Indeed, Rejii et al. (2014) identified *Ensifer* species from wild lotus nodules in arid Tunisian soil that were able to grow between 35° and 42 °C.

Salinity is a serious threat to agriculture in arid and semi-arid regions (Le Houérou 1975; Djennane 1990; Zahran 2001; Palma et al. 2013). Nearly 40 % of the world’s land surface can be categorized as having potential salinity problems, most of these areas are confined to the tropics and Mediterranean region (Toutain 1974; Le Houérou 1975; Djennane 1990; Zahran 1997; Koull et al. 2013). Successful rhizobia-legumes symbiosis under salt stress requires the selection of salt tolerant rhizobia. It has been found that fast-growing strains are more tolerant than slow-growing rhizobia (Zahran 1999; Cacciari et al. 2003; Thami-Alami et al. 2010). Our results showed a strong relationship between the tolerance (*in vitro*) of isolates to salt and high temperature conditions which was clearly confirmed by the PCA study. Most of our strains appear to be tolerant to more than 2 % NaCl, the highest level of tolerance reported for most strains by Priefer et al. (2001); Maâtallah et al. (2002); Cacciari et al. (2003); Mnasri et al. (2007a) and Rejii et al. (2014). This may be different from the level of tolerance exhibited in nature when plants are irrigated by water

with increased salt levels, (Djennane 1990; Tabouche and Achour 2004; Koull et al. 2013). Rhizobia associated with *Medicago* and *Melilotus* are usually able to grow in presence of more than 2 % NaCl (Rome et al. 1996; Yan et al. 2000; Thami-Alami et al. 2010; Djedidi et al. 2011). However, the *in vitro* tolerance of rhizobial strains to NaCl in pure culture was not correlated with the salinity of the soils from which they were isolated (Boukhatem et al. 2012). There was also no correlation between laboratory assays and isolation conditions. This could be due to the fact that rhizobia are located in microniches at the rhizospheric level and are inside nodules, and thus are protected.

Analysis of the combination between temperature and salt stress results showed that only one isolate, ML12, survived at both of 45 °C and more than 3 % of NaCl. This may reflect the fact that oases have a distinctly different temperature and humidity from the arid hot surrounding areas. Djedidi et al. (2011) found that the frequency of resistance to both stresses is low among isolates. With respect to water stress, we found that all of the ML and MD isolates were drought-tolerant. Amrani et al. (2010) reported the good correlation between salt and drought stress, and that halotolerant strains were also tolerant to PEG, suggesting common osmo-adaptation mechanisms.

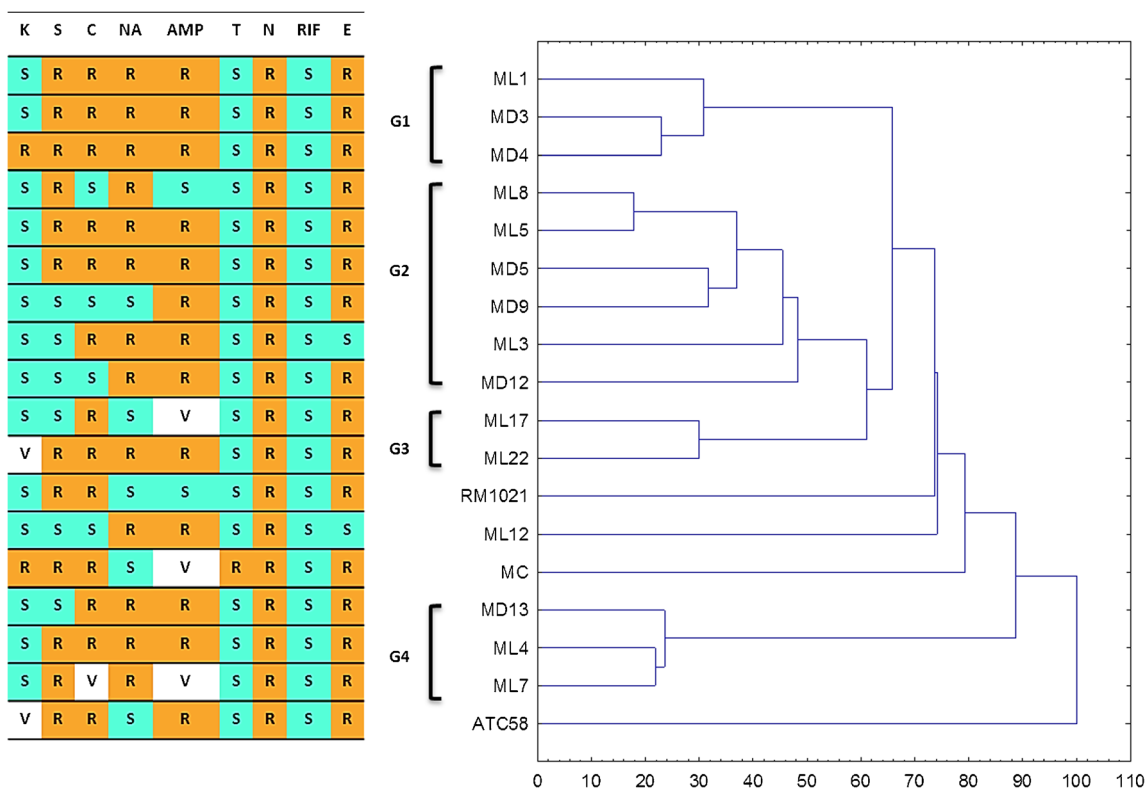


Fig. 8 UPGMA dendrogram of phenotypic characters of MD and ML root-nodulating isolates and their resistance/sensitivity profile to antibiotics: K Kanamycin, TE tetracycline, RIF rifampicin, AMP

ampicillin, NA nalidixic acid, C chloramphenicol, S streptomycin, N neomycin, E erythromycin, S sensitive, R resistant, V variable

Table 1 The origin of the strains with respect to plants and accession numbers

Strains	Original host plant	Sequence length (bp)	GenBank Accession number
ML08	<i>Melilotus indicus</i>	1042	KR476464
ML17	<i>Melilotus indicus</i>	1386	KR476465
ML22	<i>Melilotus indicus</i>	1398	KR476466
MD05	<i>Medicago littoralis</i>	1391	KR476467
MD09	<i>Medicago littoralis</i>	1391	KR476468
MD12	<i>Medicago littoralis</i>	1404	KR476469

In the present study, ML and MD isolates were tolerant of pH and grew on media ranging from 4.5 to 9. This agrees with Thami-Alami et al. (2010) who reported that some fast-growing *E. meliloti* strains, isolated from semi-arid soils of Morocco were able to grow normally at pH 3.5. Low pH appears to be a major factor limiting survival and activity of microorganisms in soils (Maâtallah et al. 2002; Shetta et al. 2011). Fast-growing rhizobia strains are generally considered to be less tolerant to acidic pH than the slow growing strains (Yan et al. 2000; Priefer et al. 2001) although there are some exceptions (Zahran 1999). In addition, Somasegaran and Hoben (1985) reported that fast-growing rhizobia could metabolize a wider range of carbon substrates than slow-growing strains. Carbohydrate utilization assays indicated that our isolates are able to use 44 of 49 carbohydrate sources tested, and may be able to produce important enzymes. The utilization of L-glutamine was a characteristic that can distinguish between strains of *Rhizobium* and *Ensifer* (Hartmann et al. 2006; Shetta et al. 2011).

Fast-growing rhizobia are more sensitive to antibiotics (Jordan 1984). In the present study, ML and MD isolates which are fast-growers and showed sensitivity tetracycline and rifampicin. However, they showed a high resistance to chloramphenicol, nalidixic acid, ampicillin, neomycin and erythromycin. Bromfield et al. (2010) report that *E. meliloti* strains are susceptible to about 5 µg of kanamycin and tetracycline, but could resist more than 50 µg of erythromycin and 20 µg of nalidixic acid. Elbouthiri et al. (2010) noted that *E. meliloti* and *E. medicae* from Morocco soils were resistance to chloramphenicol (up than 25 µg/l).

ML and MD isolates were able to infect their host plant and to fix atmospheric nitrogen, but we observed that the number of nodules formed by our strains are relatively low than *Ensifer meliloti* 1021, *Mesorhizobium ciceri*. Zahran (2001) suggested that rhizobia strains surviving under heat stress may lose their infectivity, due to plasmid curing or to alterations in cellular polysaccharides necessary for infection. High soil temperature (35–40 °C) usually results in the formation of ineffective nodules. Jebara et al. (2001) reported that plant growth and symbiotic efficiency in sand culture, were 5 to 8 time lower than that in hydro-aeroponic culture. The establishment and activity of legumes-rhizobia symbiosis *in natura* is particularly sensitive to environmental constraints (Zahran 1999, 2001; Drevon et al. 2004; Serraj et al. 2004; Bruning and Rozema 2012; Palma et al. 2013).

Remarkably, *E. meliloti* isolates showed a broader host-range among wild legumes species and we found that *Melilotus indicus* represent one of the best host traps to isolate rhizobial strains which display high abiotic stress resistance in oases soils. The osmotolerance, fast growth and nitrogen fixation efficiency suggests that ML and MD isolates may be good candidates as biofertilizers and provide promising strains for developing inoculants.

Analysis of 16S rDNA sequences is widely used to define the taxonomic position and trace the evolutionary history of nodule bacteria (Shamseldin et al. 2013; Gnat et al. 2014). In order to clarify the taxonomic status and the phylogenetic relationship of *M. littoralis* and *M. indicus* nodules isolates and other rhizobia, PCR amplification and sequencing of nearly full-length 16S rRNA gene (about 1400 bp) of six

Table 2 The Sequence analysis of partial 16S rRNA gene of six isolates from nodules of *Medicago* and *Melilotus* plants

Isolates	BLAST best match (n.r. database)	Identity (%)	GenBank Accession number	BLAST best match (16SrRNA database)	Identity (%)	GenBank Accession number
ML08	<i>Ensifer</i> sp. LILM4H41	99	DQ394805.2	<i>Ensifer meliloti</i> strain 1021	99	NR_074279.1
ML17	<i>Ensifer</i> sp. LILM4H41	99	DQ394805.2			
ML22	<i>Ensifer</i> sp. LILM4H41	99	DQ394805.2			
MD05	<i>Ensifer</i> sp. XJ22DR2	99	AB773226.1			
MD09	<i>Ensifer meliloti</i> strain W44	99	JF730142.1			
MD12	<i>Ensifer</i> sp. LILM4H41	99	DQ394805.2			

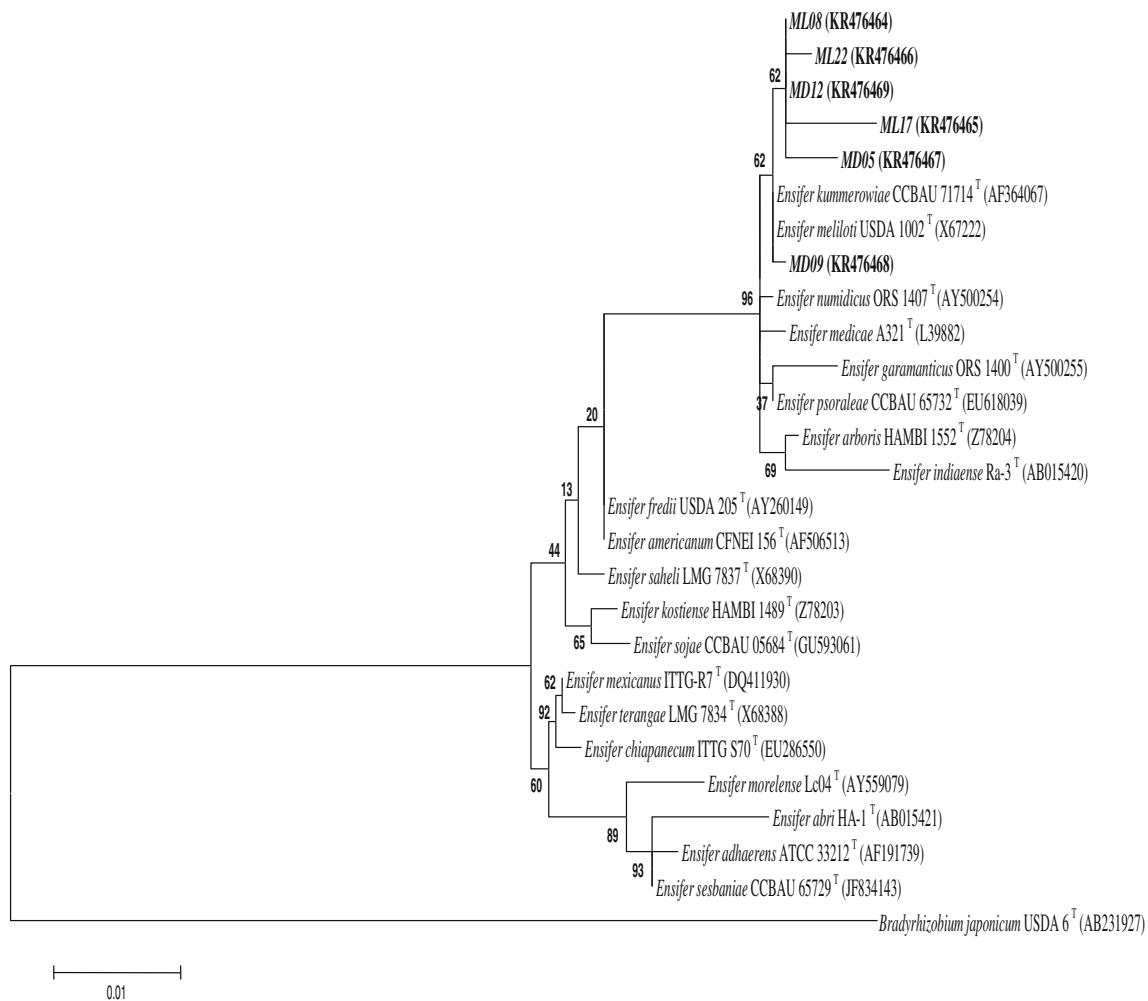


Fig. 9 The maximum likelihood phylogenetic tree showing the relationships of the 16S rRNA gene sequences of ML07, ML08, ML22, MD05, MD09 and other species of rhizobia

symbionts were performed. The comparative 16S rRNA gene sequence analysis of *M. littoralis* and *M. indicus* nodule isolates indicate that they are grouped on the phylogram into a separate cluster with known *E. meliloti* strains with more than 95 % of similarity. The type strains *E. meliloti* USDA 1002 and *E. kummerowiae* were localized on the phylogram between our strains suggesting that they belong to one of this species. The preliminary results the blast shows clearly that our isolates belong to the *Ensifer meliloti* but they could also be a new species. More research is needed to confirm this result. Our strains clustered near to the *Ensifer* strains that have been isolated from neighboring regions with similar environments, e.g. *E. numidicus* ORS 1407 and *E. garamanticus* ORS 1400, which were isolated from Tunisian soil (Merabet et al. 2010). Rome et al. (1996); Yan et al. (2000); Zahran (2001); Zribi et al. (2004); Badri et al. (2007). It appears that the majority of wild legumes are nodulated by strains belonging to *Ensifer* and *Rhizobium* genus and few by *Mesorhizobium* and *Bradyrhizobium* (Thami-Alami et al. 2010; Rejii et al.

2014). The wild legumes particularly *Medicago* and *Melilotus*, show a great variation in nodulation and nitrogen fixation ability in relation to location (Zahran 2001).

In conclusion, we showed that *Melilotus indicus* and *Medicago littoralis* nodule bacteria, from the oases of Touggourt, were grouped within the *Ensifer meliloti* cluster. Our study provides the basis for further research on the phylogeny of rhizobial strains nodulating wild legumes in Sahara oases of Algeria and we suggest that these strains could be used as inoculants to improve plant growth and nitrogen fixation in arid lands. These strains may represent new genospecies that need further characterization to assess their taxonomical status.

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