Stress Tolerance and Symbiotic and Phylogenetic Features of Root Nodule Bacteria Associated with *Medicago* Species in Different Bioclimatic Regions of Tunisia

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(Received June 16, 2010—Accepted October 11, 2010—Published online December 4, 2010)

Thirty two rhizobial isolates were obtained from different bioclimatic regions of Tunisia using as trap plants, *Medicago sativa*, *Medicago ciliaris*, *Medicago polymorpha* and *Medicago minima*. To study their diversity and characterize them in relation to Mediterranean conditions, abiotic stress resistance, symbiotic properties and genetic diversity in terms of 16S rRNA and *nodA* sequences were assessed. Five isolates from *M. sativa*, three from *M. ciliaris* and three from *M. minima* could grow at 45°C. Only two isolates from *M. sativa* grew at 4% NaCl. The most stress tolerant isolates were obtained from arid soils. A phylogenetic analysis of 16S rRNA genes revealed 29 isolates to be closely related to *Ensifer* including one (Pl.3-9) that showed a 16S rRNA sequence similar to that of *Ensifer meliloti* and *nodA* sequence similar to that of *Ensifer medicae*. However, three isolates were categorized into *Agrobacterium* containing the *nodA* of *Ensifer*. Furthermore, these isolates developed nodules on original hosts. The results for the four isolates suggest horizontal gene transfer between the species.

Key words: *Ensifer*, stress tolerance, Tunisia, phylogeny, symbiotic properties

Legumes are frequently distributed in arid and semi-arid regions. They are pioneer plants, which can provide their own nitrogen in symbiosis with root nodule bacteria (rhizobia). In Tunisia, *Medicago* species as leguminous plants are naturally distributed and widely represented in the native flora (30). Many species of this genus have wide-ranging agricultural and environmental applications (15). Moreover, the annual species, collectively known as “medics”, are of great importance in pastures in the Mediterranean and known to establish a nitrogen-fixing symbiosis with soil bacteria of the genus *Ensifer* (formerly *Sinorhizobium*). In the last decade, wild legumes and their symbionts have attracted the attention of ecologists due to their tolerance of extreme environmental conditions including salinity, severe drought and elevated temperatures (23).

Several reports have described stress tolerant rhizobial strains. Payakapong *et al.* (25) isolated a highly salt tolerant *Ensifer* species (*Ensifer* sp.BL3), and Hung *et al.* (14) described strains of *Rhizobium*, *Bradyrhizobium* and *Agrobacterium* that could grow in a medium containing 4.5% NaCl and also grew well at 45°C. The diversity of rhizobia nodulating *Medicago* species has been widely reported (3, 4, 10, 38) including in Tunisia (1, 16, 44–46). Three different species, *Ensifer meliloti* (17), *Ensifer medicae* (27) and *Rhizobium mongolense* (37), were reported as microbial

symbionts of *Medicago* species. In Tunisia, the genetic diversity of natural populations of rhizobia nodulating *M. sativa*, *M. scutellata*, *M. truncatula* and *M. ciliaris*, was assessed by a polyphasic approach based on plasmid profiles, multilocus enzyme electrophoresis and a restriction fragment analysis of PCR-amplified DNA fragments (PCR-RFLP) of 16S rRNA, the inter genic spacer (IGS) and the nitrogen-fixing gene *nifDK* (16). Zribi *et al.* (45) characterized 299 isolates of rhizobia associated with *Medicago truncatula* in 10 Tunisian soil samples by PCR-RFLP of 16S rRNA genes. These reports indicated that *E. meliloti* is a predominant *Ensifer* species in Tunisia and has wide genetic diversity in Tunisian soils.

Recently, many *Agrobacterium* strains have been isolated from root nodules of legume plants and though most were non-symbiotic belonging to *Agrobacterium tumefaciens*, some may represent novel biovars (19). Moreover, Velazquez *et al.* (39) reported for the first time the occurrence of two strains (163C and ATCC 11325^T) belonging to *R. rhizogenes* able to nodulate *Phaseolus vulgaris*. These strains displayed symbiotic plasmids carrying *nod* and *nif* genes phylogenetically related to those of *E. meliloti*. In addition, Cumming *et al.* (7) characterized a bacterial strain, IRBG 74, isolated from root nodules of *Sesbania cannabina* with a 16S rRNA sequence closely related to that of *A. tumefaciens*. This strain contained also a symbiotic plasmid carrying *nodA* and *nifH* similar to those in other *Sesbania* symbionts and effectively nodulated *Sesbania cannabina* and even some

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other *Sesbania* species. On the other hand, horizontal gene transfer in bacteria is widely recognized as the mechanism responsible for the widespread distribution of antibiotic resistance genes, gene clusters encoding biodegradative pathways, and pathogenicity determinants. This mechanism is also responsible for speciation and sub-speciation in bacteria (6). In the case of rhizobia, Minamisawa *et al.* (22) suggested the potential horizontal transfer of *nod* genes among bradyrhizobia and other bacterial populations in soil environments. Similarly, Bailly *et al.* (2) suggested that during the evolution of *Medicago* symbionts, horizontal transfer of *nod* genes might have occurred either to the common ancestor of the two *Ensifer* species or to one *Ensifer* species followed by the other. Additionally, Suominen *et al.* (34), when describing the structure of the common nodulation genes of *Rhizobium galegae*, reported that lateral *nod* gene transfer might have occurred from fast growing rhizobia to agrobacteria.

Tunisia is facing increasingly serious environmental problems in arid and semi-arid regions. Salinity and drought in addition to soil degradation and desertification are severe limitations for agriculture. Furthermore, these abiotic constraints may have a detrimental effect on microbiological activity leading to a reduction in crop productivity. One way to overcome these serious environmental problems is to use native Tunisian species for revegetation in arid and semi-arid regions because the genus *Medicago* is distributed in all bioclimatic regions of the country including arid and semi-arid areas. To spread the genus *Medicago* into these parts of Tunisia, it is important to apply rhizobial inoculants that interact efficiently with *Medicago* species and are adapted to drought and salinity.

In this study, to characterize the root nodule bacteria associated with three wild *Medicago* species, *Medicago ciliaris*, *Medicago polymorpha* and *Medicago minima*, and a commonly cultivated species, *Medicago sativa*, in Tunisia, a polyphasic approach was used. 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. Their genetic properties were determined by an analysis of 16S rRNA and *nodA* sequences. Finally, the symbiotic properties of these representative strains were evaluated in terms of nodule numbers and acetylene-reducing activity and plant growth-promoting rates.

Materials and Methods

Isolation of *Rhizobium* from Tunisian soils using *Medicago* species as trap hosts

Five representative soil samples corresponding to different bioclimatic regions of Tunisia were used (Fig. 1). Four *Medicago* species; *M. sativa* cv. Gabes, *M. ciliaris* line TNC 1.8, *M. polymorpha* cv. Manguio and *M. minima* (natural population), were used as trap hosts. Seeds from these species were surface-sterilized with 96% H₂SO₄ to promote germination, incubated on 0.9% agar medium at 4°C for 24 h, and then kept at 25°C in the dark for germination. Germinated seeds were placed in a 300-mL glass bottle filled with sterilized vermiculite. Inoculation was performed using 1 g of soil sample dissolved in 5 mL of sterilized water per bottle. A sterilized N-free nutrients solution (5) was added to each pot corresponding to a 60% moisture level which was maintained

Fig. 1. Geographical locations of soils used for rhizobial isolation. 1, Mateur (texture: silty, annual rainfall: 600–800 mm, sub-humid); 2, El Kef (texture: silty, annual rainfall: 400–500 mm, superior semi-arid); 3, Soliman (texture: fine silt, annual rainfall: 500–700 mm, superior semi-arid); 4, Enfidha (texture: silt sandy, annual rainfall: 300–400 mm, inferior semi-arid); 5, Gabes (texture: sandy, annual rainfall: 180–220 mm, arid).

during the growing period. Plants were placed in a growth chamber under controlled conditions: A 16-h photoperiod at 25°C and 8 h of darkness at 18°C (16, 46). Root nodules were collected from roots of 60-day-old plants. Bacteria were isolated from these nodules according to Vincent (40). The root nodules were surface sterilized with 70% ethanol (v/v) for 30 s and 3% sodium hypochlorite for about 3 min and crushed in a 15% glycerol solution. A loopful of the root nodule solution was transferred to a yeast mannitol agar (YMA) medium. Single colonies were picked out and repeatedly streaked to check their purity and then pure colonies were maintained in YMA on slants at 4°C.

Stress tolerance screening

The isolates obtained from the *Medicago* species used in this study were examined for growth under conditions of high temperature, high salinity, alkaline pH and extreme drought. In the case of temperature tolerance, isolates were kept at 28°C (as a control), 37, 40, or 45°C on YMA plates for 4 to 5 days. The pH of the medium was adjusted with 0.5 M HCl or 0.5 M NaOH to 6.9 (as a control), 8, 9, or 10.5. This test was performed on YMA plates kept at 28°C for 4–5 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 then incubated at 28°C for 4 to 5 days in YMA plates. To test drought resistance, different concentrations of polyethylene glycol (PEG 6000) were applied to the medium at a final concentration of 0 (as a control treatment), 15, or 25%. In these experiments, isolates were first grown in YM broth for 4 days at 28°C and then transferred to YMA plates as previously indicated. The screening for stress tolerance was performed in Petri dishes divided into equal squares as shown in Fig. 2-a. Each square was spot-inoculated with 10 µL of the cell suspensions at 10⁹ cells mL⁻¹ grown in YM broth 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) as shown in Figs. 2-b and c. This experiment was carried out in triplicate for each isolate and repeated two or three times for confirmation.

Fig. 2. Screening for stress tolerance, a: plate divided into equal squares used for screening. b: growth scores were recorded as follows: 1; no growth: -, 2; weak growth: + (10–30% in relation to the control) 3; good growth: ++ (30–80% in relation to the control) and 4; very good growth: +++ (similar to the control). c: example of colony morphology after 4 days of incubation under stressful condition.

Isolation of genomic DNA

Genomic DNA of each isolate was prepared as described in Yokoyama *et al.* (42) with modifications. A loopful of bacterial cells was inoculated into 20 mL of YM broth. After 4 days at 28°C, the cells were harvested and washed twice with TNE (10 mM Tris, 0.1 M NaCl, and 1 mM EDTA, pH 8) solution. The pellets were suspended in 200 μ L of a lysozyme solution, pH 8 (25 mM Tris, 12.5 mM EDTA, 12.5% sucrose, and 2 mg of lysozyme per mL) and kept for 30 min at 37°C. Then, a 200- μ L aliquot of a lysing solution (1 mg mL⁻¹ proteinase K in 1% sodium dodecyl sulfate) was added and the mixture was incubated for 2 hours at 37°C. Thereafter, 70 μ L of 5 M NaCl was added and mixed thoroughly and 55 μ L of 10% cetyltrimethylammonium bromide (CTAB) solution with 0.7 M of NaCl were added to remove cell materials such as polysaccharides and kept for 10 min at 65°C. The genomic DNA was purified from lysates by phenol-chloroform extraction two times and then precipitated using 2-propanol. The precipitated DNA was washed two times with 70% ethanol. Finally, it was resuspended in sterilized water purified with a SimPak.1 (Millipore, Billerica, USA) and kept at 4°C overnight in order to dissolve. The DNA concentration was checked by spectrophotometer (Ultrospec 3300 pro, GE Healthcare Japan, Hino, Japan).

PCR amplification and sequencing of regions of the *nodA* and 16S rRNA genes

The *nodA* and 16S rRNA genes were amplified by PCR using the pairs of primers shown in Fig. 3. The reaction was performed in a mixture of 50 μ L containing template DNA, 10 $\times$ enzyme buffer, deoxynucleoside triphosphates (dNTPs), 1 μ L of the primers (100 pmol) and 0.25 μ L of the enzyme (ExTaq polymerase 5 U mL⁻¹, Takara Bio, Otsu, Japan) according to the manufacturer's manual. Amplification was carried out with the following temperature profile: an initial cycle of denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 3 min, and a final extension at 72°C for 7 min. The PCR products were checked by horizontal electrophoresis in

a 1% (w/v) agarose gel with Tris-Acetate-EDTA (TAE) buffer. Subsequently, bands were excised and DNA was purified using a QIAEX II agarose gel extraction kit (Qiagen, Valencia, USA). Prior to sequencing, a nearly full *nodA* sequence and a partial 16S rRNA sequence were subjected to a sequencing reaction with an ABI Prism Big-Dye terminator v 3.1 cycle sequencing Kit (Applied Biosystems, Foster City, USA) using the same pairs of primers shown in Fig. 3. DNA sequences of the fragments were determined using ABI PRISM 310 and ABI PRISM 3130 genetic analyzers (Applied Biosystems).

Analysis of DNA sequences

The multiple sequence alignment of nucleotide sequences and boot-strapping for creating N-J phylogenetic trees were performed using Clustal X 1.81 (36) and MEGA 4.0 (35).

Nucleotide sequence accession numbers

The 16S rRNA and *nodA* sequences have been deposited in the DNA Data bank of Japan under the accession numbers AB535681–AB535712 for 16S rRNA and AB535160–AB535191 for *nodA*.

Plant test

To assess the symbiotic abilities of the isolates to generate root nodules on their original host plants, isolates were grown on YM broth for 3 days at 28°C and the resulting bacterial cells, approximately 10⁹ cells mL⁻¹ determined by a dilution plate count method described by Vincent (40), were inoculated to aseptic *Medicago* seeds. Plants were grown in a growth chamber with the same conditions indicated previously. Each isolate was tested on triplicate plants and uninoculated control plants were included. Eight weeks later, to evaluate the symbiotic performance of each isolate, ethylene production was evaluated by the acetylene reduction assay according to the method described by Hardy *et al.* (12). 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). Additionally, four isolates corresponding to the most stress-tolerant isolates originating from the four *Medicago* species were further tested for symbiotic efficiency in cross inoculation tests. Plant inoculation and growth conditions were as above and uninoculated control treatments were included. After 6 weeks of growth, nodulation was recorded by the existence of nodules and the efficiency was estimated by the presence of red coloring (leghemoglobin) inside the nodules (40).

Results

Isolation of rhizobia from *Medicago* species

A total of 256 isolates were isolated from root nodules of four *Medicago* species. The repartition of these isolates according

Fig. 3. Pairs of primers used for PCR amplification and sequencing of the *nodA* (A) and 16S rRNA genes (B). A: Sino-nodA (TGCRGTGGAAR NTRNCTGGGAA), start: nucleotide 14 of *nodA*, end: nucleotide 38 of *nodA* gene sequence of *E. meliloti*; Sino-nodB (GGNCCGTC RAAWGTARGTA), Start: nucleotide 88 of *nodB*, end: nucleotide 66 of *nodB* gene sequence of *E. meliloti*. B: 1F (AGTTTGATCTGGCTC), start: nucleotide 11 of 16S rRNA, end: nucleotide 26 of 16S rRNA gene sequence of *Escherichia coli*; 3R (AAGGAGGTGATCCAGCC), start: nucleotide 1489 of 16S rRNA, end: nucleotide 1472 of 16S rRNA gene sequence of *Escherichia coli*.

Table 1. Summary of polyphasic analysis

Isolates ^a	Host plants	Geographic Origins	Stress tolerance				Phylogenetic properties				Indexes of dry weight increase**
			Temperature range	NaCl (%)*	pH*	Drought PEG*	16S-rRNA group	Species	nodA group	Origin of nodulation genes	
St.1-2	<i>M. sativa</i>	Sub-humid	28–40°C	2% (++)	10.5 (+)	25% (+)	GIV	<i>E. meliloti</i>	GVII	<i>Ensifer</i>	6.2
St.1-3	<i>M. sativa</i>	Sub-humid	28–40°C	4% (+)	10.5 (++)	25% (+)	GIV	<i>E. meliloti</i>	GVII	<i>Ensifer</i>	9.8
St.1-15	<i>M. sativa</i>	Sub-humid	28–40°C	3% (++)	10.5 (+)	25% (+)	GIV	<i>E. meliloti</i>	GVI	<i>Ensifer</i>	7.3
St.2-5	<i>M. sativa</i>	Sup. Semiarid	28–40°C	2% (++)	10.5 (+)	25% (+)	GIV	<i>E. meliloti</i>	GVII	<i>Ensifer</i>	7.5
St.2-6	<i>M. sativa</i>	Sup. Semiarid	28–45°C	3% (+)	9 (+)	25% (+)	GIV	<i>E. meliloti</i>	GVI	<i>Ensifer</i>	8.3
St.3-1	<i>M. sativa</i>	Sup. Semiarid	28–45°C	3% (+)	9 (+)	25% (+)	GIV	<i>E. meliloti</i>	GVI	<i>Ensifer</i>	6.2
St.3-7	<i>M. sativa</i>	Sup. Semiarid	28–40°C	2% (++)	10.5 (+)	25% (+)	GIV	<i>E. meliloti</i>	GVI	<i>Ensifer</i>	5
St.3-13	<i>M. sativa</i>	Sup. Semiarid	28–40°C	4% (+)	10.5 (+)	25% (+)	GII	<i>A. tumefaciens</i> ***	GVI	<i>Ensifer</i>	5.7
St.4-2	<i>M. sativa</i>	Inf. Semiarid	28–45°C	2% (++)	10.5 (+)	25% (+)	GIV	<i>E. meliloti</i>	GVI	<i>Ensifer</i>	7.7
St.4-5	<i>M. sativa</i>	Inf. semiarid	28–40°C	2% (++)	10.5 (+)	25% (+)	GIV	<i>E. meliloti</i>	GVI	<i>Ensifer</i>	9.1
St.4-14	<i>M. sativa</i>	Inf. semiarid	28–45°C	2% (++)	9 (+)	25% (+)	GIV	<i>E. meliloti</i>	GVI	<i>Ensifer</i>	4.9
St.5-10	<i>M. sativa</i>	Arid	28–45°C	2% (++)	10.5 (+)	25% (+)	GIV	<i>E. meliloti</i>	GVIII	<i>E. meliloti</i>	3.6
Cl.1-14	<i>M. ciliaris</i>	Sub-humid	28–40°C	3% (+)	10.5 (+)	25% (+)	GVI	<i>E. medicae</i>	GX	<i>E. medicae</i>	2.2
Cl.2-12	<i>M. ciliaris</i>	Sup. Semiarid	28–40°C	1% (+++)	9 (++)	25% (+)	GIV	<i>E. meliloti</i>	GVII	<i>Ensifer</i>	2.9
Cl.3-1	<i>M. ciliaris</i>	Sup. Semiarid	28–40°C	2% (+)	10.5 (+)	25% (+)	GVI	<i>E. medicae</i>	GX	<i>E. medicae</i>	2.1
Cl.3-2	<i>M. ciliaris</i>	Sup. Semiarid	28–40°C	2% (+)	10.5 (+)	25% (+)	GVI	<i>E. medicae</i>	GX	<i>E. medicae</i>	1.7
Cl.4-7	<i>M. ciliaris</i>	Inf. Semiarid	28–45°C	2% (+)	9 (+)	25% (+)	GIV	<i>E. meliloti</i>	GVI	<i>Ensifer</i>	2
Cl.4-10	<i>M. ciliaris</i>	Inf. Semiarid	28–45°C	3% (+)	10.5 (+)	15% (+)	GIV	<i>E. meliloti</i>	GVI	<i>Ensifer</i>	1.4
Cl.4-12	<i>M. ciliaris</i>	Inf. Semiarid	28–45°C	2% (++)	10.5 (+)	15% (+)	GIV	<i>E. meliloti</i>	GIX	<i>Ensifer</i>	1.5
Pl.2-1	<i>M. polymorpha</i>	Sup. Semiarid	28–40°C	3% (++)	9 (+)	25% (+)	GII	<i>A. tumefaciens</i> ****	GX	<i>E. medicae</i>	1.2
Pl.3-6	<i>M. polymorpha</i>	Sup. Semiarid	28–40°C	3% (+)	10.5 (+)	25% (+)	GII	<i>A. tumefaciens</i> ****	GX	<i>E. medicae</i>	4
Pl.3-9	<i>M. polymorpha</i>	Sup. Semiarid	28–40°C	3% (+)	10.5 (+)	25% (+)	GIV	<i>E. meliloti</i>	GX	<i>E. medicae</i>	2.5
Pl.4-2	<i>M. polymorpha</i>	Inf. semiarid	28–37°C	1% (++)	9 (+)	25% (+)	GVI	<i>E. medicae</i>	GXI	<i>Ensifer</i>	7.5
Mn.2-8	<i>M. minima</i>	Sup. Semiarid	28–40°C	2% (+)	10.5 (++)	25% (+)	GIV	<i>E. meliloti</i>	GVII	<i>Ensifer</i>	3.3
Mn.3-1	<i>M. minima</i>	Sup. Semiarid	28–40°C	3% (++)	10.5 (+)	25% (+)	GIV	<i>E. meliloti</i>	GVI	<i>Ensifer</i>	3.1
Mn.3-12	<i>M. minima</i>	Sup. Semiarid	28–40°C	2% (++)	10.5 (+)	25% (+)	GIV	<i>E. meliloti</i>	GVI	<i>Ensifer</i>	3.2
Mn.4-1	<i>M. minima</i>	Inf. semiarid	28–45°C	3% (+)	10.5 (++)	25% (+)	GIV	<i>E. meliloti</i>	GVI	<i>Ensifer</i>	3.6
Mn.4-4	<i>M. minima</i>	Inf. semiarid	28–45°C	2% (++)	10.5 (++)	25% (+)	GIV	<i>E. meliloti</i>	GVI	<i>Ensifer</i>	6.7
Mn.4-9	<i>M. minima</i>	Inf. semiarid	28–45°C	2% (++)	10.5 (++)	25% (+)	GIV	<i>E. meliloti</i>	GVI	<i>Ensifer</i>	6.5
Mn.4-18	<i>M. minima</i>	Inf. semiarid	28–40°C	2% (++)	10.5 (+)	25% (+)	GIV	<i>E. meliloti</i>	GVI	<i>Ensifer</i>	3
Mn.5-1	<i>M. minima</i>	Arid	28–40°C	2% (++)	10.5 (+)	25% (+)	GIV	<i>E. meliloti</i>	GIV	unknown	4.3
Mn.5-4	<i>M. minima</i>	Arid	28–40°C	2% (++)	10.5 (+)	25% (+)	GIV	<i>E. meliloti</i>	GVII	<i>Ensifer</i>	3.1

* Growth scores were recorded 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); ** effects relative to the control were determined in terms of plant dry weight; *** this isolate showed horizontal gene transfer from *Sinorhizobium* to *A. tumefaciens*; **** these isolates showed horizontal gene transfers from *S. medicae* to *A. tumefaciens*. ^a the two letters in the isolate names indicate the host plant of origin (St: *M. sativa*, Cl: *M. ciliaris*, Pl: *M. polymorpha* and Mn: *M. minima*), the number following the letters indicates the soil of origin of the isolate (soil sample number in Fig. 1) and the last number indicates the isolate reference number.

to the host plants was as follows: ninety six isolates from *Medicago sativa*, sixty from *Medicago ciliaris*, sixty from *Medicago polymorpha* and forty from *Medicago minima* with combinations of five different soils covering several bioclimatic regions of Tunisia. All isolates had the same colony morphology and produced transparent and creamy colonies when grown on YM plates at 28°C. Almost all the isolates tested were fast growers, their colonies reaching 2 mm in diameter after 4 to 5 days at 28°C.

Classification of isolates based on differences in stress resistances under different salt concentrations, pH, temperatures and PEG 6000 concentrations

The 256 isolates were first screened for resistance to high salinity, alkaline pH, high drought and high temperature conditions as described in Fig. 2. According to this preliminary characterization, high diversity in stress resistance was observed among the 256 isolates. In the high salinity test, only two isolates from *M. sativa* (about 2% of the isolates from this plant) were able to grow at 4% NaCl, while at 3% NaCl, the percentage of tolerant isolates was 25% for *M. sativa*, 26.6% for *M. ciliaris*, 26.6% for *M. polymorpha*

and 42.5% for *M. minima*. Regarding high temperature resistance, 5.2% of the isolates from *M. sativa*, 5% from *M. ciliaris* and 7.5% from *M. minima* were able to grow at 45°C, however none of the isolates obtained from *M. polymorpha* survived at this temperature. Similarly, most of the isolates tolerated a pH of 10.5: i.e. 70% of *M. sativa* isolates, 75% of *M. ciliaris* isolates, 66.6% of *M. polymorpha* isolates and 72.5% of *M. minima* isolates. Similarly, almost the same percentages of tolerant isolates were recorded in the drought stress test with 25% PEG 6000.

Twelve isolates from *M. sativa*, seven from *M. ciliaris*, four from *M. polymorpha* and nine from *M. minima* were selected based on stress resistance as shown in Table 1. To confirm their resistance, these 32 isolates were subjected to the same stress test described previously.

Concerning high temperature resistance, all the isolates tested grew at 40°C with one exception, Pl.4-2. Five isolates from *M. sativa*, three from *M. ciliaris* and three from *M. minima* survived at 45°C. However, none of the four isolates from *M. polymorpha* survived at 45°C.

Regarding high salt resistance, only two isolates obtained from

Table 2. Reference strains used in this study

Species/strains	Source	Reference
<i>Agrobacterium tumefaciens</i>		
C 58	<i>Prunus</i> sp., United States	26
CCBAU 85015	<i>Caragana sinica</i> , China	13
<i>Ensifer adhaerens</i>		
LMG 20216 ^T	Soil, United States	20
ORS 529	<i>Lotus arabicus</i> , Senegal	21
<i>Ensifer arboris</i>		
LMG 14919 ^T (=HAMBI 1552 ^T)	<i>Prosopis chilensis</i> , Sudan	20
<i>Ensifer fredii</i>		
USDA 205 ^T	<i>Glycine max</i> , China	20
<i>Ensifer garamanticus</i>		
ORS 1400 ^T	<i>Argyrobolium uniflorum</i> , Tunisia	21
<i>Ensifer medicae</i>		
A321 ^T	<i>Medicago truncatula</i> , France	20
WSM419	<i>Medicago murex</i> , Italy	10
<i>Ensifer meliloti</i>		
1021	<i>Medicago sativa</i> , Australia	9
USDA 1002 ^T	<i>Medicago sativa</i> , United States	24
LMG 6133 ^T	<i>Medicago sativa</i> , United States	20
<i>Ensifer numidicus</i>		
ORS 1444 ^T	<i>Argyrobolium uniflorum</i> , Tunisia	21
<i>Ensifer saheli</i>		
LMG 7837 ^T (=ORS 609 ^T)	<i>Sesbania cannabina</i> , Senegal	20
<i>Ensifer species</i>		
ORS 1407 ^T	<i>Argyrobolium uniflorum</i> , Tunisia	21
<i>Rhizobium etli</i>		
CNF 42 ^T	<i>Phaseolus vulgaris</i> , Mexico	24
<i>Rhizobium mongolense</i>		
USDA 1844 ^T	<i>Medicago ruthenica</i> , China	37
<i>Rhizobium species</i>		
Glm 16	<i>Glycine max</i> , Korea	18

M. sativa were able to grow at 4% NaCl as shown in Table 1. At 3% NaCl, five isolates from *M. sativa*, two from *M. ciliaris*, three from *M. polymorpha* and two from *M. minima* were able to grow.

In the case of high pH resistance, almost all the isolates tested were able to grow at pH 9 and 25 isolates survived at pH 10.5 as shown in Table 1. Similarly, in the case of drought resistance, nearly all isolates survived at 25% PEG 6000 with the exception of Cl.4-10 and Cl.4-12 from *M. ciliaris*.

Phylogenetic analysis of the 32 isolates in terms of differences in 16S rRNA gene sequences

The nearly entire sequence of the 16S rRNA gene of the 32 isolates was phylogenetically analyzed using the Neighbour-joining method (28). The 32 isolates and 15 reference strains (Table 2) were classified into 9 groups shown in Fig. 4. *R. etli* CFN 42^T and *R. mongolense* USDA 1844^T were classified into GI without any isolate from this study. Similarly, *E. fredii* USDA205^T and *E. saheli* LMG 7837^T were classified into GVIII and no isolates existed in this group. *E. sp.* ORS 1400^T, *E. sp.* ORS 1407^T, *E. arboris* LMG 14919^T and *E. adhaerens* LMG 20216^T consisted of singletons named GIII, GV, GVII and GIX, respectively. Pl.2-1, Pl.3-6 and St.3-13 from *M. polymorpha* and *M. sativa* were classified into GII and this group included *A. tumefaciens* CCBAU85015 and *A. tumefaciens* C58. The 16S rRNA gene St.3-13 was identical in sequence to *A. tumefaciens* C58 and the 16S rRNA genes of Pl.2-1 and Pl.3-6 were 99% similar to *A. tumefaciens* CCBAU85015. Twenty five isolates were classified into GIV with the reference

strains *E. meliloti* 1021 and *E. meliloti* LMG 6133^T. The 16S rRNA genes of these isolates and reference strains were 99.7 to 100% similar. Therefore, these isolates had close relationships to *E. meliloti*. Three isolates obtained from *M. ciliaris* and one from *M. polymorpha* were grouped into GVI. This group contained 2 reference strains of *E. medicae*. Therefore, these 4 isolates had close relationships to *E. medicae*. Notably, the 16S rRNA sequences of Cl.1-14, Cl.3-1 and Pl.4-2 were identical to those of *E. medicae* WSM419 and *E. medicae* A321^T.

Phylogenetic analysis of the 32 isolates in terms of differences in nodA gene sequences

The 32 isolates and 12 reference strains (Table 2) were classified into 13 groups as shown in Fig. 5. GI only consisted of the reference strains *E. fredii* USDA205^T and *R. etli* CNF 42^T, while GV consisted of *E. nimidicus* ORS1444^T and *E. garamanticus* ORS1400^T. *E. saheli* ORS 609^T, *E. arboris* HAMBI 1552^T, *E. meliloti* 1021 and *R. mongolense* USDA1844^T consisted of singletons, named GII, GIII, GXII and GXIII, respectively. Sixteen isolates from *M. sativa*, *M. ciliaris* and *M. minima* were classified into GVI but there were no reference strains in this group. GVII comprised 6 isolates from *M. sativa*, *M. minima* and *M. ciliaris* without any reference strains. One isolate of St.5-10 was grouped into GVIII with the reference strain *E. meliloti* USDA1002^T, and DNA sequence of this isolate was 99% similar to that of the reference strain. Two isolates, Cl.4-12 and Pl.4-2, consisted of singletons. The remaining 6 isolates were categorized into GX with 2 reference strains assigned to *E. medicae*. DNA sequences of the 6 isolates were identical to that of *E. medicae* WSM419 and *E. medicae* A321^T which is a type strain of *E. medicae*. The 16S-rRNA gene sequences of isolates categorized into GVI, GVII, GVIII and GIX had a close relationship to those of *E. meliloti* as shown in Figs. 4 and 5 with the exception of one isolate, St.3-13. This means that *E. meliloti* had extensive genetic diversity in terms of DNA sequences of *nodA*. Concerning GX, 3 isolates were categorized into *E. medicae* based on DNA sequences of the 16S rRNA gene. Interestingly, the remaining 3 isolates of this group were assigned to *A. tumefaciens* and *E. meliloti*.

Plant tests

The 32 isolates 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 as shown in Fig. 6. Root nodule numbers in the original host plants, are shown in Fig. 6-A. The mean number varied from 6 in Mn.5-1 to 44 in Cl.2-12. Isolates from *M. ciliaris* formed more root nodules than the other isolates. In general, the isolates from *M. minima* formed fewer nodules. Three isolates, St.3-13, Pl.2-1 and Pl.2-6, categorized as *A. tumefaciens* based on 16S rRNA sequences clearly showed nodulation in the original host plants.

The acetylene reducing activities of the 32 isolates when applied to their original host plants respectively are shown in Fig. 6-B. One isolate from *M. sativa* (St.3-1) displayed the highest level of activity. This isolate was assigned to *E. meliloti* according to 16 rRNA gene sequencing. St.3-13, Pl.2-1 and Pl.3-6 categorized as *A. tumefaciens* displayed acetylene reduction activities which were not significantly different in comparison with the remaining isolates classified into *E. meliloti* or *E. medicae*, except for St.3-1 as shown in Fig. 6-B.

Relative indexes of dry weight increase in original host plants inoculated with the 32 isolates are shown in Fig. 7. Generally, inoculations of isolates obtained from *M. sativa* to the original host plants had clearly induced increases of shoot dry weights. The relative indexes with isolates obtained from *M. sativa* were higher than those of remaining isolates.

The symbiotic effectiveness of the four *Medicago* species with a sample of four isolates representing the most stress tolerant isolates

Fig. 4. Phylogenetic tree derived from neighbour-joining analysis of 1400-bp 16S rRNA sequences from *Medicago* nodulating isolates and 15 representative type strains from GenBank: *Agrobacterium tumefaciens* C58, *Agrobacterium tumefaciens* CCBAU85015, *E. adhaerens* LMG 20216^T, *E. arboris* LMG 14919^T, *E. fredii* USDA205^T, *E. medicae* A321^T, *E. medicae* WSM419, *E. meliloti* 1021, *E. meliloti* LMG 6133^T, *E. saheli* LMG7837^T, *E. sp.* ORS 1400^T, *E. sp.* ORS 1407^T, *R. etli* CNF 42^T, *R. mongolense* USDA 1844^T and *R. sp.* GLM16. The numbers at the branch nodes indicate bootstrap values based on 1000 replicates. The scale bar indicates substitutions per site.

(salinity and high temperature especially) was estimated. The four isolates selected for this cross inoculation test were St.1-3, Cl.4-10, Pl.3-9 and Mn.4-1 from *M. sativa*, *M. ciliaris*, *M. polymorpha* and *M. minima*, respectively (Table 3). Each isolate was able to nodulate and fix nitrogen efficiently when applied to its host plant and none of the four isolates was effective with the four *Medicago* species. Three isolates from *M. sativa*, *M. ciliaris* and *M. minima* (St.1-3, Cl.4-10 and Mn.4-1) with a *nodA* originating from *Ensifer* species (according to sequencing of the *nodA* region) were able to form effective nodules (estimated by the red color of nodules) on *M. sativa*, *M. ciliaris* and *M. minima*. Though, in the case of *M. polymorpha*, only ineffective nodules characterized by a white color were observed when three previous isolates were applied to this plant. Additionally, in the case of Pl.3-9 from *M. polymorpha* with a *nodA* gene sequence categorized as *E. medicae*, effective nodules

were observed only when this isolate was applied to *M. ciliaris*. However this isolate from *M. polymorpha* was ineffective when applied to *M. sativa* and *M. minima*.

Discussion

Symbiotic relationship among 4 *Medicago* species and *Ensifer*

In Tunisia, *M. sativa* is widely cultivated, *M. ciliaris* is widely distributed from humid to semi-arid climatic zones and *M. minima* and *M. polymorpha* are distributed from semi-arid to saharian climatic zones. To obtain *Ensifer* isolates, these four *Medicago* species were used in combination with five soil samples collected from different climatic zones in Tunisia. Consequently, a total of 256 isolates was isolated from root nodules. Based on differences

Fig. 5. Neighbour-joining tree of *nodA* gene sequences of root nodule bacteria associated with *Medicago* species. Twelve representative type strains from GenBank were used as references: *E. adhaerans* ORS529, *E. arboris* HAMBI 1552^T, *E. fredii* USDA205^T, *E. garamanticus* ORS1400^T, *E. medicae* A321^T, *E. medicae* WSM419, *E. meliloti* 1021, *E. meliloti* USDA 1002^T, *E. nimidicus* ORS1444^T, *E. saheli* ORS 609^T, *R. etli* CNF 42^T, and *R. mongolense* USDA1844^T.

The numbers at the branch nodes indicate bootstrap values based on 1,000 replicates. The scale bar indicates substitutions per site.

in high salinity, alkaline pH, high temperature and drought stress resistance, we selected 32 isolates as shown in Table 1.

Based on frequencies of *Ensifer* species from root nodules of each host plant, *M. sativa* and *M. minima* show a preference for *E. meliloti*. Concerning the symbiotic partner of *M. minima*, Sebbane *et al.* (29) first isolated *E. meliloti* from root nodules of *M. minima* in Algeria. We also confirmed that *E. meliloti* seems to be the predominant symbiont of *M. minima* in Tunisia. In contrast, *M. ciliaris* has a preference for *E. medicae*. In the case of *M. polymorpha*, out of four isolates, two were categorized as *A. tumefaciens* and one each as *E. meliloti* and *E. medicae*, respectively. Van Berkum *et al.* (38) reported that the predominant symbiont of *M. polymorpha* is *E. medicae*.

Relationship between high salt and high temperature resistance of *Ensifer* isolates and their geographical origin

To isolate *Ensifer* isolates, we used five different soil samples

from four bioclimatic regions (Fig. 1). Accordingly, we found that *M. sativa* is one of the best trap hosts to isolate *Ensifer* displaying high abiotic stress resistances as indicated in Table 2. In the case of high salt resistance, St.1-3 and St.3-13 from *M. sativa* survived at 4% NaCl. Shamseldin *et al.* (32) reported that *E. meliloti* strains from faba bean root nodules survived at 3% NaCl. Payakapong *et al.* (25) also reported that an *Ensifer* strain of BL3 obtained from root nodules of *Phaseolus lathyroides* could survive at 3.5% NaCl. Shamseldin *et al.* (31) reported the proteomic characterization of *Rhizobium etli* at 4% NaCl. In the case of high temperature resistance, out of 32 isolates, eleven isolates survived at 45°C as shown in Table 2. Concerning high temperature resistance, some tolerant rhizobial isolates have been described. For instance, Fall *et al.* (8) isolated rhizobia from *Acacia senegal* that showed good growth at 45°C. Furthermore, Ge-Hong *et al.* (11) reported a temperature tolerant strain of *Mesorhizobium* at 35°C. However, there is no report about *E. meliloti* surviving at 45°C. Interestingly, out of the

Fig. 6. Root nodule numbers and Acetylene reduction assay of *Medicago* plants inoculated with the 32 isolates. *1: uninoculated control plants, *2: isolates with high temperature tolerance, *3: isolates with high salinity tolerance, *4: isolates categorized as *A. tumefaciens*, *5: isolates assigned to *E. medicae*, *6: isolate categorized as *E. meliloti*; however, the sequence of *nodA* was identical to that of *E. medicae*. Different small letters on histograms represent statistically significant differences at $P<0.05$ based on the Tukey-Kramer test.

Fig. 7. Relative indexes of dry weight increase when the 32 isolates were applied to their original host plants, respectively: relative index of dry weight increase=(inoculated plants dry weights (mg))/(control plants dry weights (mg)). *1: uninoculated control plants, *2: isolates with high temperature tolerance, *3: isolates with high salinity tolerance, *4: isolates categorized as *A. tumefaciens*, *5: isolates assigned to *E. medicae*, *6: isolate categorized as *E. meliloti*; however, the sequence of *nodA* was identical to that of *E. medicae*.

eleven isolates, five were obtained from root nodules of *M. sativa* shown in Table 1; this also confirmed that *M. sativa* is a useful trap host to isolate *Ensifer* having high abiotic stress resistance. For *Ensifer* isolation, we used five different soils from four climatic zones in Tunisia, sub-humid, superior semi-arid, inferior semi-arid and arid zones. The geographic origins of these eleven isolates were all categorized into superior semi-arid, inferior semi-arid and arid zones. This suggests that the distribution of *Ensifer* with resistance

Table 3. Cross inoculation tests of most stress tolerant isolates from *Medicago* species

Isolate	Original host	Origin of nodulation genes (<i>nodA</i>)	Nodulation and nitrogen fixation on host plant ^a			
			<i>M. sativa</i>	<i>M. ciliaris</i>	<i>M. polymorpha</i>	<i>M. minima</i>
Control			nod–/fix–	nod–/fix–	nod–/fix–	nod–/fix–
St.1-3	<i>M. sativa</i>	<i>Ensifer species</i>	nod+/fix+	nod+/fix+	nod+/fix–	nod+/fix+
Cl.4-10	<i>M. ciliaris</i>	<i>Ensifer species</i>	nod+/fix+	nod+/fix+	nod+/fix–	nod+/fix+
Pl.3-9	<i>M. polymorpha</i>	<i>Ensifer medicae</i>	nod+/fix–	nod+/fix+	nod+/fix+	nod+/fix–
Mn.4-1	<i>M. minima</i>	<i>Ensifer species</i>	nod+/fix+	nod+/fix+	nod+/fix–	nod+/fix+

^a: nod–, no nodules; fix–, inefficient nodules (white color); fix+, efficient nodules (red color).

to high temperature is closely related to climatic conditions.

Moreover, four isolates, St.2-6, St.3-1, Cl.4-10 and Mn.4-1, survived at both 45°C and 3% NaCl. Nonetheless, we could not find an isolate able to survive abilities at 45°C and 4% NaCl. Out of these 256 isolates, only 4 (1.6% of the total) could survive at both 45°C and 3% NaCl as shown in Table 1. This shows that the frequency of isolates having both high temperature resistance and high salt resistance is low. Therefore, this finding suggests that in order to look for suitable inoculants with multi-tolerance of temperature and salinity for leguminous crops located in semi-arid and arid areas, a large number of isolates is needed.

Relationship between horizontal transfer of symbiotic genes and symbiotic Agrobacterium isolates producing effective root nodules in Medicago species

Based on sequencing of the 16S rRNA genes of 32 isolates, 25 isolates were categorized as *E. meliloti* and 4 isolates were assigned to *E. medicae*. However, the remaining three isolates were categorized as *A. tumefaciens* (Fig. 4 and Table 1). Furthermore, to confirm the 16S rRNA and *nodA* sequences of these isolates, we performed additional analysis. We confirmed that the *nodA* sequences of Pl.2-1 and Pl.3-6 were identical to that of *E. medicae*, while the *nodA* sequence of St.3-13 was categorized into *Ensifer* species as shown in Fig. 5. These results suggest that horizontal gene transfer occurred between *Ensifer* and *A. tumefaciens*. In the case of Pl.2-1 and Pl.3-6, it seems that symbiotic genes of *E. medicae* were transferred into *A. tumefaciens*. Whereas, in the case of St.3-13, it seems that symbiotic genes of *Ensifer* were transferred. Pl.2-1 and Pl.3-6 developed 11 and 12 root nodules with *M. polymorpha* respectively, while Pl.4-2 categorized into *E. medicae* developed 33 root nodules with the same host plant as shown in Fig. 6-A. This result shows that the root nodule forming ability of the symbiotic *Agrobacterium* is significantly lower than that of the *E. medicae* isolate. Similarly, regarding ethylene production by acetylene reduction assays, those of the two *Agrobacterium* isolates were clearly lower than that of *E. medicae* isolates shown in Fig. 6-B. Regarding St.3-13, the *nodA* sequence of this isolate was closely related to that of *E. meliloti*. The root nodule number of *M. sativa* inoculated with this isolate is clearly lower than that of host plants inoculated with the remaining isolates. Sullivan and Ronson (33) reported that a symbiotic element of *Mesorhizobium loti* was transferred into three non symbiotic species. Bailly *et al.* (2) reported that several interspecific horizontal gene transfers occurred during the diversification of *Medicago* symbionts. Similarly, Wong and Golding (41) reported that a large portion of *pSym B* genes in *E. meliloti* are most closely related to genes in *Agrobacterium tumefaciens* linear chromosomes. These reports support the existence of symbiotic *Agrobacterium* isolates produced by horizontal transfer of symbiotic genes. In this context, Velazquez *et al.* (39) reported for the first time the occurrence of two strains, named 163C and ATCC 11325^T, belonging to *R. rhizogenes* able to nodulate *Phaseolus vulgaris*. The two strains displayed symbiotic plasmids carrying *nod* and *nif* phylogenetically related to those of *E. meliloti*. Interestingly, Cumming *et al.* (7) characterized one bacterial strain IRBG 74 isolated from root nodules of *Sesbania cannabina* with a 16S rRNA sequence closely related to that of

A. tumefaciens. This strain contained also a symbiotic plasmid carrying *nodA* and *nifH* similar to those in other *Sesbania* symbionts and this strain effectively nodulated *Sesbania cannabina* and even some others *Sesbania* species.

Moreover in this study, one isolate from *M. polymorpha* (Pl.3-9) showed a 16S rRNA sequence closely related to that of *E. meliloti* however its *nodA* sequence was similar to that of *E. medicae*. For this isolate, the sequencing of 16S rRNA and *nodA* genes was repeated several times and the results were the same. This finding suggests gene transfer but further characterization of this isolate is needed to confirm its taxonomic status. Out of 4 isolates obtained from *M. polymorpha*, symbiotic genes of 3 isolates, Pl.2-1, Pl.3-6 and Pl.3-9, seemed to undergo horizontal transfer from other species; however we have no clear answer to understand such a phenomenon.

Nitrogen-fixing abilities of isolates having high abiotic stress tolerance

According to Fig. 6-b, St. 1-3 and St.3-1 having high temperature resistance at 45°C and/or high salt resistance at 3% NaCl showed relatively high nitrogen-fixing ability. These isolates were obtained from root nodules of *M. sativa*; therefore this demonstrated that *M. sativa* may be a convenient trap host to obtain candidate inoculants having both high abiotic stress tolerance and high nitrogen-fixing abilities. On the other hand, the most stress tolerant isolates from the four *Medicago* species showed promise as inoculants with the exception of Pl. 3-9 from *M. polymorpha* which showed a restricted spectrum of nitrogen fixation with *Medicago* species (Table 3). Furthermore, these stress tolerant isolates have been assigned to *E. meliloti* according to the 16S rRNA gene sequencing. This finding suggests the application of these isolates as effective inoculants to *Medicago* species.

However, to obtain effective inoculants for *Medicago* species in arid and semi-arid climatic regions of Tunisia, we need further studies such as developing efficient screening methods for isolates using *Medicago* plants as trap hosts.

Acknowledgements

The authors are grateful to Tarek Slatni, Saber Kouas, Kais Zribi and Mounawer Badri (Biotechnology Center, Borj Cedria Techno-park, Tunisia) for kindly providing seeds and soil samples. Salem Djedidi is supported by a grant from the Tunisian Ministry of Higher Education, Scientific Research and Technology.

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