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ORIGINAL ARTICLE

Genetic diversity of *Acacia tortilis* ssp. *raddiana* rhizobia in Tunisia assessed by 16S and 16S-23S rDNA genes analysis

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16S rRNA, 16S-23S rRNA spacer, *Acacia tortilis*, diversity, rhizobia, symbiotic efficiency.

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Abstract

Aims: In order to understand the genetic diversity of *Acacia tortilis* ssp. *raddiana*-rhizobia in Tunisia, isolates from nine geographical locations were obtained and analysed.

Methods and Results: Characterization using restriction fragment length polymorphism analysis (RFLP) of PCR-amplified 16S rRNA gene and the intergenic spacer (IGS) between the 16S and 23S rRNA genes was undertaken. Symbiotic efficiency of the strains was also estimated. Analysis of the 16S rRNA by PCR-RFLP showed that the isolates were phylogenetically related to *Ensifer* ssp., *Rhizobium tropicii*-IIA, and *Rhizobium tumefaciens* species. Analysis of 16S-23S spacer by PCR-RFLP showed a high diversity of these rhizobia and revealed eleven additional groups, which indicates that these strains are genetically very diverse. Full 16S rRNA gene-sequencing showed that the majority of strains form a new subdivion inside the genera *Ensifer*, with *Ensifer meliloti* being its nearest neighbour. Nodulation test performed on the plant host demonstrated differences in the infectivity among the strains.

Conclusion: Rhizobial populations that nodulate specifically and efficiently *Acacia tortilis* ssp. *raddiana* in representative soils of Tunisia is dominated by *E. meliloti*-like genomospecies.

Significance and Impact of the Study: This paper provides the first clear characterization and symbiotic efficiency data of rhizobia strains nodulating *A. tortilis* in Tunisia.

Introduction

Rhizobia are widespread soil bacteria able to induce the formation of root nodules and to fix nitrogen on cultivated and wild legumes. These rhizobia are of economic importance in low-input sustainable agriculture, agroforestry, and land reclamation. The taxonomy of bacterial endosymbionts of leguminous plants has experienced a profound series of extensions in the recent past (Young 2003). Currently there are five genera of rhizobia in the *α-Proteobacteria*, *Azorhizobium*, *Bradyrhizobium*, *Rhizobium*, *Mesorhizobium*, and *Ensifer* (Young 2003). New lines that contain nitrogen-fixing legumes symbionts include *Methylobacterium* (Jaftha *et al.* 2002; Jourand *et al.* 2004), *Devosia* (Rivas *et al.* 2003), *Blastobacter* (Van Berkum and Eardly 2002) and *Ochrobactrum* (Ngom *et al.* 2004; Trujillo *et al.* 2005) in the *α-Proteobacteria*; *Burkholderia* (Moulin *et al.* 2001), *Cupriavidus* (Vandamme and Coenye 2004) and *Ralstonia* (Chen *et al.* 2001) in the *β-Proteobacteria* and some unclassified strains in the *γ-Proteobacteria* (Benhizia *et al.* 2004) were recently described.

Both desertification and ecosystem degradation problems are common in Mediterranean regions, particularly

in the centre and the south of Tunisia, where low precipitation and human activities enhance the erosion and desertification processes. It is widely recognized that indigenous rhizobia play an important role in the dominance of *Fabaceae* in poor and arid soils (Zahran 2001) but, so far, only few wild legumes have been investigated for their nitrogen-fixing symbionts (Zakhia *et al.* 2004).

Acacia is widespread in arid regions of Africa and Middle East (Nabli 1989). They are well nodulated under drought stress conditions. Plants of the genus *Acacia* are pioneer plants, which play an important role for preservation and fertility of poor and eroded soils in Africa. These legumes produce extensive, deep root system, in addition to their potential to fix atmospheric N_2 (Nabli 1989). In Tunisia, *Acacia tortilis* ssp. *raddiana* is the only wild and native acacia, which grow spontaneously in arid and Saharan areas. This acacia is generally overused by local people, who have no other fuel wood. Regeneration of the tree is made difficult owing to overgrazing, which adds its limiting effects to those of aridity (Nabli 1989). The approach adopted to improve rehabilitation programs and soil fertility is to isolate rhizobia from these areas and obtain data on their diversity and nodulation efficiency.

Among the techniques developed to detect DNA polymorphisms in many different organisms including bacteria, PCR-RFLP of 16S rRNA genes is one of the quickest and easiest (Laguerre *et al.* 1994). This genomic technique has been applied for characterizing rhizobia at the level of species and genera (Laguerre *et al.* 1994; Lafay and Burdon 2001). The 16S rDNA sequence analysis, which is a conserved gene, supports the well-established subdivision of rhizobia into species and genera (Young and Haukka 1996). However, DNA sequences in the 16S-23S spacer are more discriminating and known to exhibit a great deal of sequence and length variation (Normand *et al.* 1996). These variations are used to differentiate genera, species, and strains of prokaryotes (Normand *et al.* 1996).

Very little is known about the bacterial symbionts of *Acacia* spp., especially *A. tortilis* in Tunisia (Ba *et al.* 2002). Our objective was to re-examine the genetic diversity among rhizobia isolated from root nodules of *A. tortilis* ssp. *raddiana* and to find their phylogenetic positions within the family *Rhizobiaceae* by using 16S and IGS rDNA analysis.

Materials and methods

Bacterial strains and growth conditions

Strains of rhizobia were isolated from root nodules of *A. tortilis* grown in different regions in Tunisia. The collected nodules were kept in closed containers over silica gel at room temperature until their isolation in the laboratory. Forty isolates were obtained from nine different locations in Tunisia (Fig. 1).

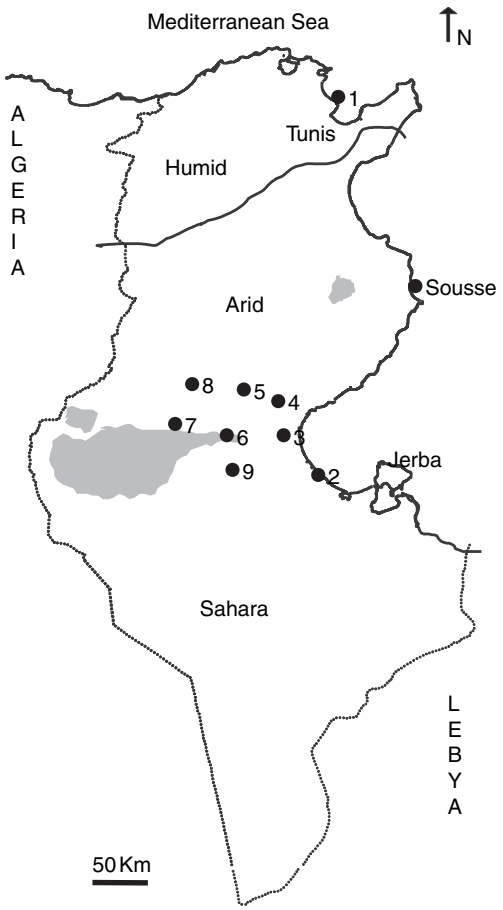

Figure 1 Map of Tunisia showing the sites prospected for nodulation of *Acacia tortilis* ssp. *raddiana*. 1, Ariana; 2, Gabes; 3, Belkhir; 4–9, Bouhedma sites.

Isolations were made according to the procedure described by Vincent (1970) using yeast-mannitol agar (YMA) supplemented with crystal violet (Collins and Lyne 1985), which inhibited the growth of gram positive contaminating-bacteria. Cultures used for further study were purified from single colonies on YMA agar plates pure cultures (or as the dominant colony type) after incubation at 28°C. All the rhizobia were maintained on YMA medium (Vincent 1970) and stored with 25% glycerol at −80°C.

Plant nodulation test and symbiotic efficiency

All isolates were tested for nodulation ability on *A. tortilis*-plants. The *Acacia* seeds were surface sterilized with concentrated sulphuric acid (H_2SO_4) for 90 min and rinsed with sterile water. The seeds were germinated for 72 h on semi-solid agar medium (8 g l^{-1}) in a growth

chamber at 28°C, and the seedlings were placed aseptically in Gibson tubes supplemented with a nitrogen free plant nutrient solution (Gibson 1980). Each tube was inoculated with a rhizobial suspension from an early stationary-phase culture. Uninoculated plants were used as controls. Three replicates were prepared to each treatment. After a month, the plants were harvested and the number of nodules was estimated. The nitrogen fixation ability of the strains was estimated from the pink colour of the nodules and the dark green colour of leaves compared to control plants that were not inoculated.

Morphological tests

Colony shape and colour were determined by using a magnifying glass. Cell dimensions and morphology were examined on living cells by phase-contrast microscopy, in order to confirm strain purity.

16S RNA gene amplification

The prokaryotic specific primers used for 16S rRNA gene amplification were fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGCTTAAGGTGATCCAG-CC-3′) (Weisburg *et al.* 1991). PCR amplification was carried out in a 25 μl reaction volume-containing template DNA (10–50 ng), reaction buffer, 1x freeze-dried marble (Ready-to-go PCR beads, Pharmacia Biotech) containing 1·5 U Taq polymerase, 10 mmol l^{-1} Tris–HCl, 50 mmol l^{-1} KCl, 1·5 mmol l^{-1} $MgCl_2$, 0·2 mmol l^{-1} dNTP and 1 μmol l^{-1} of each of the primers. PCR amplification was performed with a Perkin–Elmer model (GeneAmp PCR System 2400). The PCR temperature profile used was 95°C for 5 min followed by 35 cycles consisting of 95°C for 30 s, 50°C for 1 min, 72°C for 1 min, with a final extension step at 72°C for 1 min. Reaction efficiency was estimated by horizontal agarose-gel electrophoresis (1% w/v) and coloured in an aqueous solution of ethidium bromide (1 mg ml^{-1}).

16S-23S spacer amplification

Primers FGPS1490 (Navarro *et al.* 1992) and FGPL132′ (Ponsonnet and Nesme 1994) were used to amplify the IGS regions. The conditions for 16S-23S intergenic region amplification were the same as those used for 16S gene amplification, except that the annealing steps took place at 55°C.

Restriction fragment analysis

Eight microliter aliquots of PCR products were digested with restriction endonucleases (Pharmacia Biotech) as specified by the manufacturer in a total volume of 20 μl. Digestion was performed by four enzymes (*Hae*III, *Msp*I, *Cfo*I and *Rsa*I) for 16S rDNA and two enzymes (*Hae*III and *Msp*I) for 16S-23S spacer, used for their highly level of discrimination (Laguerre *et al.* 1994). The restriction fragments were separated by horizontal electrophoresis in TBE buffer with 2·5% (w/v) MetaPhor (FMC Bioproducts, Rockland, Maine, USA).

16S rRNA gene sequencing

The 16S rRNA gene of the isolate A1, chosen as a representative strain, was amplified. The amplified fragments were purified with the QIAEX II kit (Qiagen Inc., Chatsworth, CA, USA) and sequenced by the dideoxy chain termination method of Sanger *et al.* (1977). The six primers used for full sequencing of the 16S rRNA gene were fD1, FGPS485-292, FGPS1047-295, FGPS505′-313, FGPS910′-270 and rD1 (Weisburg *et al.* 1991).

DNA sequence analysis

The 16S rDNA sequences were aligned and analysed using Clustal X software. Phylogenetic analysis was inferred by using the neighbour-joining method (Saitou and Nei 1987) calculated by the Kimura method (Kimura 1980). The resulting tree was drawn with the Njplot software of Perrière and Gouy (1996).

Nucleotide sequence accession number

The newly determined 16S rDNA sequence was deposited in the GenBank Data Library under accession number DQ092342.

Results

Isolation and morphological characteristics

Forty strains (Table 1) were isolated from root nodules of *A. tortilis* spp. *raddiana*, the only native acacia tree in Tunisia. Approximately half of rhizobial isolates had the same colony morphology and growth rate on YMA medium. They were fast growing rhizobia and formed transparent to creamy colonies with 2–4 mm in diameter after 3 days incubation on Petri YMA plates.

Plant infection test and symbiotic efficiency

We tested the infectivity and symbiotic efficiency of 40 strains of rhizobia on *A. tortilis* spp. *raddiana*. Only half of the strains could induce root nodules on their plant host, appearing after 2 weeks of inoculation with dia-

Table 1 *Acacia tortilis* strains used in this study

Geographic origin and climate	Isolate	16S groups (PCR-RFLP)	IGS groups (PCR-RFLP)	16S rDNA analysis	Nodulation test and NFA
Ariana, humid	A12	1	5	*Ensifer meliloti*-like	E
	A13	1	5	*E. meliloti*-like	E
	A6	2	2	*Rhizobium tumefaciens*	NN
	A23	1	11	*E. meliloti*-like	E
	A56	3	6	NI	I
	A54	1	5	*E. meliloti*-like	E
	A51	1	5	*E. meliloti*-like	E
	A34	4	18	*Rhizobium tropicii*-IIA	PN
	A5	2	2	*R. tumefaciens*	NN
	A25	4	13	*R. tropicii*-IIA	NN
Bouhedma-S1, arid	A26	8	14	NI	PN
	A45	5	19	NI	NN
	A22	4	10	*R. tropicii*-IIA	NN
	A10	1	3	*E. meliloti*-like	E
Bouhedma-S2, arid	A1	1	1	*E. meliloti*-like	E
	A2	1	1	*E. meliloti*-like	E
	A3	1	1	*E. meliloti*-like	E
	A52	1	1	*E. meliloti*-like	E
Bouhedma-S3, arid	A46	5	19	NI	PN
	A33	4	18	*R. tropicii*-IIA	PN
	A19	2	8	*R. tumefaciens*	NN
	A20	2	8	*R. tumefaciens*	NN
Bouhedma-S4, arid	A32	7	17	NI	PN
	A28	1	16	*E. meliloti*-like	NN
	A29	6	16	NI	PN
Bouhedma-S5, arid	A21	1	9	*E. meliloti*-like	NN
	A18	2	8	*R. tumefaciens*	NN
	A35	4	18	*R. tropicii*-IIA	NN
	A8	1	3	*E. meliloti*-like	E
	A9	1	5	*E. meliloti*-like	E
	A14	1	5	*E. meliloti*-like	E
	A7	1	3	*E. meliloti*-like	E
	A53	1	5	*E. meliloti*-like	E
Bouhedma-S6, arid	A11	1	4	*E. meliloti*-like	NN
	A55	9	20	NI	I
Gabes, arid	A17	2	7	*R. tumefaciens*	NN
	A4	1	1	*E. meliloti*-like	E
	A57	7	15	NI	E
Belkhir, arid	A24	4	12	*R. tropicii*-IIA	I
	A47	6	19	NI	NN

A, *Acacia*; *E*, *Ensifer*; NI, not identified; NFA, nitrogen fixation ability; E, effective nodulation; I, ineffective nodulation; NN, no nodule formation; PN, occasional formation of pseudonodules.

meters of 1–4 mm (Table 1). About 42·5% of the inoculated strains give pink-nodules and the leaves of the corresponding plants were dark-green, while the control noninoculated plants were yellow–green.

PCR-RFLP analysis of amplified 16S rDNA genes

The 16S rDNA were amplified and we have obtained a single band of about 1500 bp for all the strains.

Aliquots of PCR products were digested with four restrictions enzymes and separated by electrophoresis. The length of PCR product estimated by summing the sizes of the restricted fragments was shorter than or equal to 1500 bp. The patterns of these isolates were compared, by computer-simulated RFLP analysis, with those of reference strains published sequences (Neyra *et al.* 1998). A total of nine different combinations, each corresponding to one genotype, were identified among the 40 strains analysed by RFLP in this study (Table 1). Three groups correspond to reference species and six groups did not match with any of the reference species into the family of *Rhizobiaceae*. Results obtained showed that the majority

of the strains showed restriction profiles closer to those of *Ensifer meliloti* species (47·5%). About 15% of the strains showed restriction patterns identical to those of *Rhizobium tropici*-IIA. 15% of the strains were related to *Rhizobium tumefaciens* species. Finally, they were 22·5% of the isolates which were not identified and remained unclassified.

16S-23S spacer analysis

To investigate further the genetic differences among the 40 rhizobia isolated from *A. tortilis* ssp. *raddiana* in humid and arid areas of Tunisia, we analyse the 16S-23S spacer by PCR/RFLP. The electrophoresis of the undigested PCR products showed that the majority of the isolates possess one band. The length of the IGS amplified region was between 1000 and 1350 bp (Fig. 2). Therefore, we were able to identify 18 groups in this natural population of rhizobia.

The amplification products were digested separately with endonucleases *Msp*I and *Hae*III for all the isolates (Fig. 3). The representative isolates within each cluster showed the same patterns, but different patterns were observed among different clusters. After comparing the restriction patterns obtained by the two endonucleases, we have obtained 20 groups (Table 1).

Sequence analysis

We sequenced full length of 16S rRNA gene for A1 strain, which belonged to the biggest group (group 1 of the 16S rDNA) of the collection. The sequence was aligned and compared with the 16S rDNA sequences of other members of the family *Rhizobiaceae* available in the GenBank database. Figure 4 is a dendrogram, which shows the phylogenetic relationships of these unclassified rhizobia and

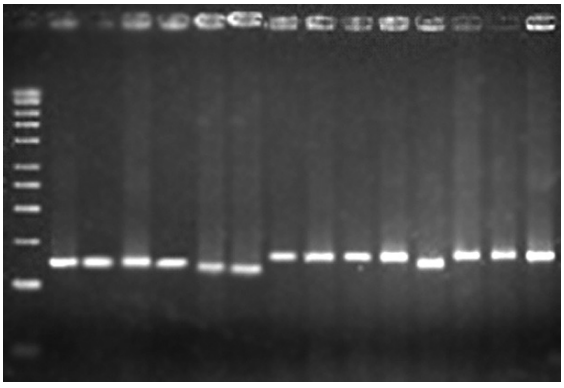

Figure 2 Electrophoresis of PCR products obtained with the universal primers FGPS1490-72 and FGPL 132′-38, which target the ribosomal IGS of Rhizobium strains that nodulate *Acacia* spp.

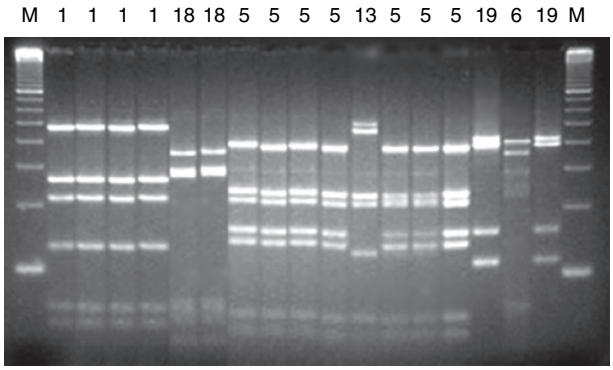

Figure 3 Examples of RFLP of PCR-amplified 16S-23S rRNA genes digested with *Hae*III and separated by electrophoresis in 2·5% (w/v) metaphor gel. Numbers 1, 5, 6, 13, 18 and 19 are IGS groups. M, molecular mass marker (100 bp ladder) from Pharmacia Biotech; the smallest band of the marker is 100 bp.

the previously named species of *Rhizobiaceae*. The tree showed that the majority of identified strains form a new subdivion inside the genera *Ensifer*, with *E. meliloti* being its nearest neighbour. This strain showed a 16S rDNA sequence similarity of 99·23% with *E. meliloti*, 99·22% with *Ensifer medicae* and 99·09% with *Ensifer arboris* type strains. DNA–DNA hybridization would be needed to verify whether these strains represent separate species or not.

Discussion

In this research, we characterized 40 nodules isolates from *A. tortilis* ssp. *raddiana* in representative soils of Tunisia. We have used PCR/RFLP analysis of 16S rDNA to characterize these natural rhizobia. We found a high diversity among rhizobial strains. Our results corroborated several studies, which revealed a high heterogeneity in the populations of rhizobia nodulating *Acacia* spp. (Ndiaye 1996; De Lajudie *et al.* 1998; Khbaya *et al.* 1998; McInroy *et al.* 1999; Mohamed *et al.* 2000; Lafay and Burdon 2001; Odee *et al.* 2002; Toledo *et al.* 2003).

We have used PCR-RFLP of the 16S rDNA to characterize all the isolates of our collection. The results obtained have discriminated nine groups covering three genera; genus *Ensifer* was the most represented in our collection. To our knowledge, there are many data showing that a high proportion of tree-nodulating rhizobia in Africa, particularely *Acacia* spp., are more closely related to *Ensifer* species (Ndiaye 1996; Khbaya *et al.* 1998; Ba *et al.* 2002).

Full 16S rRNA gene-sequencing of the strain A1 which belong to the biggest group (group 1) have showed that the majority of the identified strains that nodulate *A. tortilis* ssp. *raddiana* in Tunisia belonged to the genus *Ensifer*

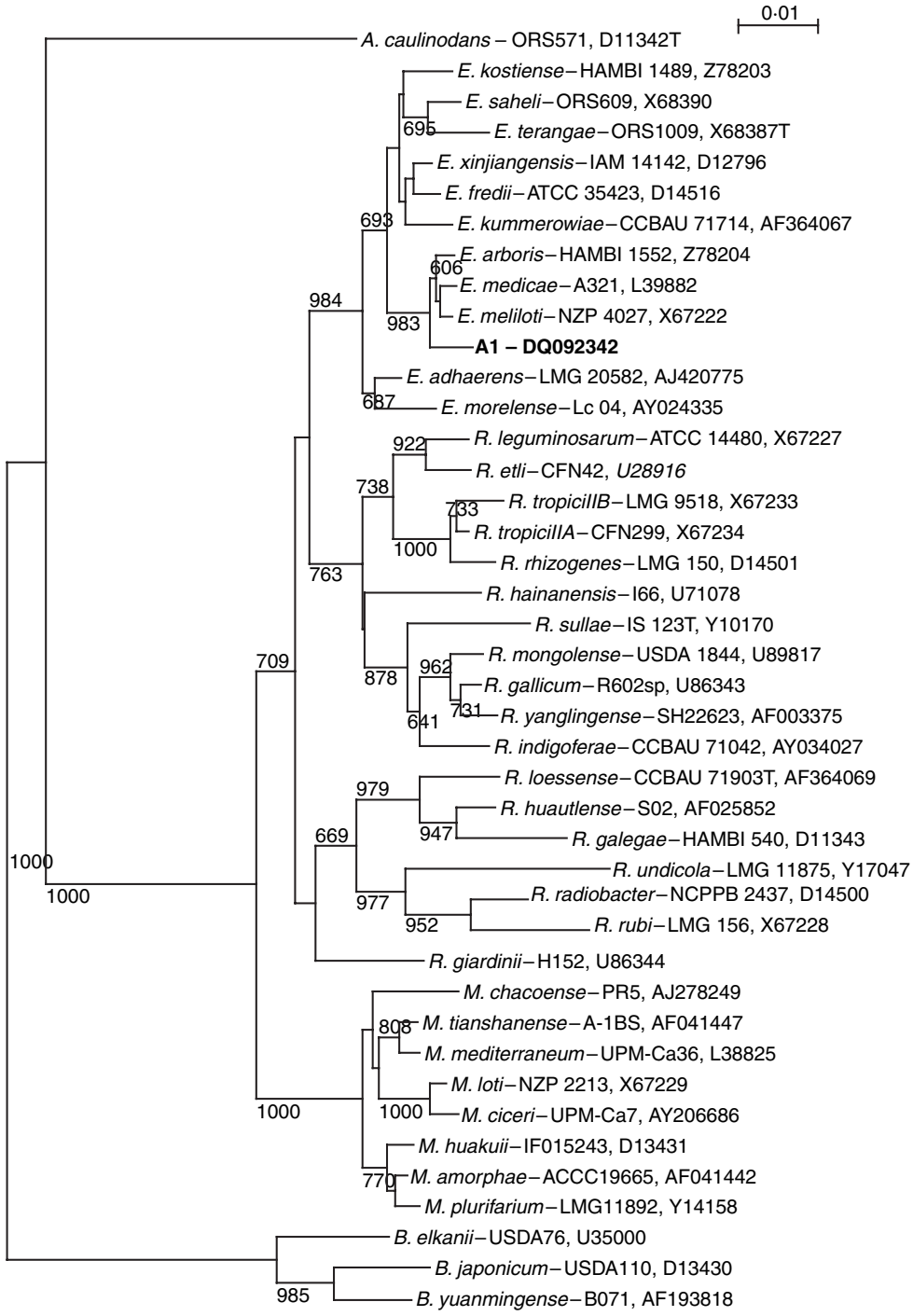

Figure 4 Phylogenetic tree based on 16S rDNA complete sequences of *Acacia raddiana* strains and references type strains of *Rhizobiaceae*. Numbers into brackets following strains names are Genbank accession numbers or strain designation. Bootstrap values were calculated from 1000 trees and the levels of support for the presence of nodes above a value of 600 are indicated.

within they can form new species. This strain showed a 16S rDNA sequence similarity of 99·23% with *E. meliloti*, 99·22% with *E. medicae* and 99·09% with *E. arboris* reference strains but occupied separate phylogenetic position within the Ensifer genus. Similar results were found by Zakhia *et al.* (2004) on rhizobia nodulating wild legumes

in Tunisia. DNA–DNA hybridizations and GC% are needed to clarify their taxonomic status inside *Rhizobiaceae* family. Our results thus confirm and extend the large diversity of fast-growing *A. tortilis*-rhizobia within the *Ensifer-Rhizobium* branch.

Our results showed that the majority of rhizobial strains were related to *E. meliloti*-like species (47·5%) and only 15% of the strains were related to *R. tropici*-IIA. However, rhizobia nodulating *A. tortilis* in Morocco were related to *E. meliloti* and *Ensifer fredii* species (Khbaya *et al.* 1998). Ndiaye (1996) have characterized, by SDS-PAGE, a collection of *A. tortilis*-rhizobia from diverse countries in Africa and he had found that they were related to *Ensifer teranga*, *E. fredii*, *R. tropici*, *Mesorhizobium huakuii* and *Mesorhizobium plurifarium* species. Strains from Tunisia, Senegal and Mauritania were grouped into *Ensifer terangae* lineage and into two novels groups (Ndiaye 1996). Ba *et al.* (2002) studied eight Tunisian strains that were more related to *E. medicae*. However, De Lajudie *et al.* (2003) found that these rhizobia were related to *E. terangae*. Recently, Zakhia *et al.* (2004) investigated one *A. tortilis* strain and was found to be related to *E. meliloti*. This high diversity of *A. tortilis*-rhizobia in Tunisia seemed to be related to the soil-origin of plant host.

Our results together with past surveys of nodule bacteria in Tunisia (Aouani *et al.* 2001; Jebara *et al.* 2001; Mhamdi *et al.* 2002; Zakhia *et al.* 2004; Zribi *et al.* 2004) suggest that Tunisian soils are dominated by fast growing rhizobia, especially by *E. meliloti* and *E. medicae* within they can form new genomospecies.

Furthermore, 22·5% of the isolates had no identity with any of rhizobial strains, but some of them (7·5%) could induce nodule formation. In fact, unknown associations were previously found and several strains may represent new genomospecies nodulating wild legumes in Tunisia (Zakhia *et al.* 2004). This non-nodule formation can be explained by several hypothesis (i) host specificity of strains to plant genotype (Paffetti *et al.* 1998). Strains would be better tested on their original host plants. (ii) Nodulation test experiment, used here, was not optimal for these strains. Using sterilized gravel or soil will be tested to verify this hypothesis. (iii) These strains are only accidentally associated with *A. tortilis* but are in fact better adapted for nodulation of another legume host species. In fact, in the natural environment, *A. tortilis* grow intermingled with other leguminous plant (perennial or annual) that might utilize nonidentified-rhizobia (Nasr, personal communication). Surveys of how strains are distributed on hosts in natural environments, together with cross inoculation experiments using additional legume species, will be necessary to clarify patterns of symbiotic specificity in these organisms. (iv) These bacteria originally symbiotic have lost their unstable symbiotic gene during laboratory experiments (isolation, purification and conservation) (Laguerre *et al.* 1993). Sutherland *et al.* (2000) have found that all strains had lost infectivity during storage. The symbiotic plasmid has been previously shown to be unstable under laboratory conditions (Romero *et al.* 1991). (v) These strains have acquired symbiotic genes by lateral gene transfer, which were lost during laboratory experiments. Several papers reported that soils contain a large diversity of nonsymbiotic bacteria which can acquire symbiotic properties by lateral gene transfer between bacteria in the soil (Segovia *et al.* 1991; Sullivan *et al.* 1995, 1996).

Moreover, several new bacterial strains, belonging to α- β- and γ-proteobacteria, were isolated from the root nodules of tropical legumes (Benhizia *et al.* 2004; Young 2003).

Further studies will help to classify these symbiotic bacteria and to understand their phylogenetic relationships to other rhizobial species.

Our results showed that 15% of the isolates were related to *R. tumefaciens* species, but could not induce tumour formation. In fact, several *Rhizobium* strains lacking their pathogenic gene were isolated from root nodules of tropical legumes in Africa (De Lajudie *et al.* 1999; Khbaya *et al.* 1998) and cultivated legumes in Tunisia (Mhamdi *et al.* 2002, 2005). It seems likely that under soil conditions, *R. tumefaciens* behaves as a pathogen or a symbiont according to its plasmid content. The symbiotic state could be unstable under laboratory conditions (Mhamdi *et al.* 2002).

We have used PCR with RFLP analysis to analyse 16S-23S spacer variation among our rhizobial isolates. We found a high diversity in the length of the amplification bands among the rhizobium strains. Therefore, we were able to distinguish 18 groups on the basis of IGS length. Furthermore, restriction digestion of the amplified IGS allowed us to distinguish two other new patterns. A similar result was also found by Paffetti *et al.* (1996) when investigating several strains belonging to *E. meliloti* by using PCR with RFLP of the IGS. However, digestion of the amplified 16S-23S spacer with nine restriction enzymes did not allow Khbaya *et al.* (1998) to distinguish additional groups. PCR-RFLP of the IGS have discriminated 11 supplementary groups than obtained by PCR-RFLP of the 16S rDNA gene. Thereby, our results confirmed the higher discriminating level for the IGS than for the 16S rDNA (Navarro *et al.* 1992; Normand *et al.* 1996; Yu and Mohn 2001).

Distribution of rhizobial strains appeared to be independent to the site of origin and to site-climate. These results corroborated with those of Khbaya *et al.* (1998) and Ndiaye (1996).

Results obtained from nodulation test showed that 50% of the isolates failed to renodulate their host plant. Non-nodulating or erratic-in-nodulation *Acacia* species have been reported (Odee *et al.* 1995; Frioni *et al.* 1998; Mohamed *et al.* 2000). Most (80%) of our rhizobial strains, which could renodulate their host plant were related to *E. meliloti* species. Similar results were found by Ndiaye (1996) and Ba *et al.* (2002) on rhizobia nodulating *A. tortilis* in Africa.

A collection of *A. tortilis* spp. *raddiana*-nodulating bacteria has been characterized, and had revealed a high diversity. Some strains may represent new genomospecies to be further characterized to clarify their phylogenetic positions. These rhizobia can be used to develop efficient inoculants in order to restore acacia forest and then soil fertility in arid and Saharan regions. This study provides the first clear characterization and symbiotic efficiency of rhizobia strains nodulating *A. tortilis* in Tunisia.

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