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Diversity of *Acacia tortilis* rhizobia revealed by PCR/RFLP on crushed root nodules in Tunisia

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Abstract - Rhizobia are used exclusively in agricultural and agroforestry systems for enhancing the ability of legumes to fix atmospheric nitrogen. Knowledge on taxonomical characteristics and ecology of the indigenous *Rhizobium* population is necessary for the selection of inoculant strains. In this study, we have assessed the genetic diversity of rhizobia from the host plant, *Acacia tortilis* ssp. *raddiana* along different areas of Tunisia. Thirty-one nodules were collected both directly and by trap host plants. Genetic diversity of rhizobia strains was studied by restriction fragment length polymorphism (RFLP) analysis of PCR-amplified intergenic spacer (IGS) and 16S rDNA. The PCR analysis was performed on nodules extracted-DNAs. The approaches used in this study yielded consistent results, which revealed a high degree of heterogeneity among strains and detection of 20 distinct genetic groups. These rhizobia are related to *Sinorhizobium*, *Rhizobium*, *Mesorhizobium* and *Agrobacterium* genera. This is the first report on rhizobia nodulating leguminous trees in Tunisia.

Key words: *Acacia tortilis*, diversity, nodules extracted-DNAs, PCR-RFLP. Tunisia.

INTRODUCTION

The bacteria inducing nodules formation on leguminous plants (family *Fabaceae*) belong to at least 11 genera and 47 species. Nine of these genera belong to the alpha subdivision of the proteobacteria: *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Mesorhizobium*, *Allorhizobium, Methylobacterium*, *Blastobacter*, and *Devosia* (Willems, 2003). Two genera belonging to Beta proteobacteria were described; *Burkholderia* and *Ralstonia* (Willems, 2003). Recently, bacteria from Gamma proteobacteria have been isolated from root nodules of the genus *Hedysarum* (Benhizia *et al.*, 2004).

Acacia tortilis ssp. *raddiana* is the only wild and native acacia trees in Tunisia and have considerable potential for agroforestry, fuelwood production, forage and medicinal products. It contributes to the prevention of soil erosion and for improvement of impoverished soils. This leguminous tree is a good colonizer of poor soils in Tunisian arid zones. Indeed, the interactions that they have with root nodules bacteria can be responsible for substantial levels of nitrogen fixation (Nabli, 1989).

In contrast to 16S rRNA gene, which is remarkably well conserved throughout most bacterial species, DNA sequences in the 16S-23S spacer (IGS) are known to exhibit a great deal of sequence and length variation (Jensen *et al*., 1993). These variations are used to differentiate genera, species, and strains of prokaryotes (Harasawa *et al.*, 1993; Gürtler and Stanisich, 1996). PCR-restriction fragment length polymorphism (PCR-RFLP) of IGS has been applied for a rapid identification of rhizobia (Laguerre *et al.*, 1994).

Extraction of DNA directly from nodules and amplification by PCR have been previously used to study the phylogenetic relationships between *Frankia* reference strains and unisolated *Frankia* strains in nodules of *Casuarinaceae* family (Rouvier *et al.*, 1996; Gtari *et al.,* 2002) and rhizobia diversity (Ba *et al.*, 2004; Thiao *et al.*, 2004). This approach avoids the obstacle process of rhizobia isolation from field-collected nodules.

In this study we used the PCR technique, together with an RFLP analysis of ribosomal DNA (rDNA) intergenic region and 16S rDNA, to examine the genetic polymorphism of rhizobia. The present paper is, to our knowledge, the first report on the diversity of rhizobia nodulating *Acacia tortilis* ssp. *raddiana* in Tunisia.

MATERIALS AND METHODS

Soils were collected from 11 geographical sites throughout Tunisia covering a distance of 400 km. These sites, which cover different bioclimatic zones, are: INRGREF (sub-humid), Belkhir (arid), Feriane (arid), Gabes (arid), Menzel habib (arid) and Bouhedma with 6 locations (arid-saharian) (Fig. 1).

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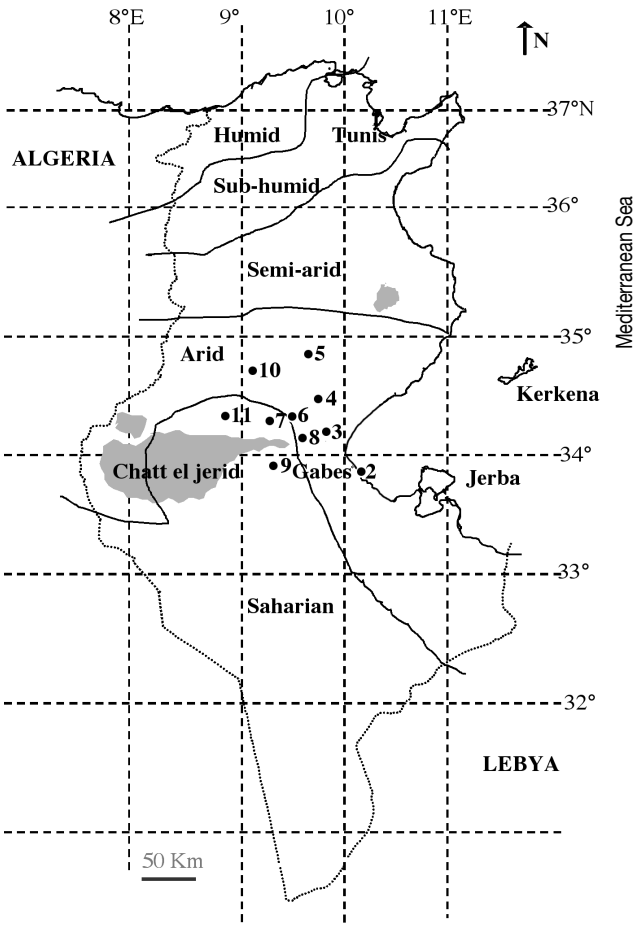

FIG. 1 – Map of Tunisia showing the sites prospected for nodulation of *Acacia tortilis* ssp. *raddiana*. 1, INRGREF; 2, Gabes; 3, Belkhir oued el hadj; 4, Feriane-rimani; 5, Saidi menzel habib; 6-11, Bouhedma-sites.

Seeds of *Acacia tortilis* were surface sterilized by immersing in pure sulphuric acid for 90 min and rinsed abundantly with sterile distilled water then allowed to germinate. Seedlings were transferred to 25 cm sterile plastic pots containing soil samples. The plants were grown in the greenhouse for 2 months at 25 °C.

Field root systems were excavated to a depth of 1 m. Nodules were excised from roots *in situ*, brushed free of soil debris then immediately desiccated over silica gel held in plastic (5 ml) screw-capped vials topped with cotton wool. Healthy, non-ruptured nodules were collected and stored at 4 °C for future use in the isolation of root-nodules bacteria. The nitrogen fixation ability of the strains was estimated from the pink colour of the nodules.

Direct DNA-extraction from nodules was defined by Rouvier *et al.* (1996) and optimised by Demba (1999). This extraction is done on the following steps: The nodules were surface sterilized in saturate calcium hypochlorite and ethanol 96° then rinsed with sterile water. The sterilized nodules were crushed individually in 150 µl of ultra-pure water and added to 150 µl of extraction buffer 2X (TrisHCl 100 mM, NaCl 1.4 M, EDTA 20 mM, cetryltrimethyl ammonium bromide (CTAB) 2%, PolyVinyl PolyPyrrolidone (PVPP) 1%). The mixture was incubated at 65 °C for 60 min and centrifuged for 10 min at 13,000 rpm to remove cellular debris. Supernatant was then extracted with an equal volume (300 µl) of phenol-chloroform-isoamyl alcohol (25:24:1 v/v/v) and centrifuged for 15 min at 13,000 rpm. DNA from the aqueous phase was purified from phenol with 300 µl of chloroform-isoamyl alcohol (24:1 v/v) and centrifuged for 15 min at 13,000 rpm. Supernatant was centrifuged one more time for 5 min. DNA from the aqueous phase was precipitated overnight at –20 °C with the addition of 0.1 volume of sodium acetate and 2.5 volumes of absolute ethanol. The samples were centrifuged for 30 min at 13,000 rpm at 4 °C. The resulting DNA pellet was washed with 70% v/v ethanol by centrifugation for 15 min at 13,000 rpm at 4 °C, vacuum dried, and solubilized in 20 µl of ultrapure water. The purity and the quantity of DNA extracted were estimated by a spectrophotometer UV/VIS (Pharmacia Biotech) in the range of 200 nm to 340 nm.

The intergenic region between the 16S and 23S rDNA was amplified by PCR with primers derived from the 3' end of the 16S rDNA (FGPS 1490-72. 5'-TGCGGCTGGATCCCCTCCTT-3') (Navarro *et al.*, 1992) and from the 5' end of the 23S rDNA (FGPL 132'-38; 5'-CCGGGTTTCCCCATTCGG-3') (Ponsonnet and Nesme, 1994). These primers are derived from conserved regions of the 16S and 23S rRNA genes, respectively, and can be used to amplify the IGS of all procaryotes DNAs tested so far. The oligonucleotides were purchased from Pharmacia. PCR amplification was carried out in a 25 µl reaction volume containing template DNA (10 to 50 ng), reaction buffer, 1X freeze-dried marble (Ready-to-go PCR beads, Pharmacia Biotech) containing 1.5 U Taq polymerase, 10 mM TrisHCl, 50 Mm KCl, 1.5 mM $MgCl_2$, 200 µM dNTP and 1 µM of each of the primers. PCR amplification was performed with a Perkin-Elmer model (GeneAmp PCR System 2400) using the following programme: initial denaturation for 5 min at 95 °C, 35 cycles of denaturation (30 s at 95 °C), annealing (30 s at 55 °C) and extension (1 min at 72 °C) and a final extension (7 min at 72 °C). PCR-amplified DNAs were visualized by electrophoresis of 3 µl of the amplified mixture on 1% (w/v) horizontal agarose gel (type II; Sigma) in TBE buffer (89 Mm Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0) at 80 $V.cm^{-1}$ for 1 h. The gels were stained in an aqueous solution of 1 mg ethidium bromide l^{-1} and photographed under UV illumination with an apparatus Gel Doc BIO-RAD.

Primers fD1 and rD1 (Weisburg *et al.*, 1991) were used to amplify the 16S rDNA gene. The conditions for 16S gene amplification were the same as those used for 16S-23S intergenic region amplification, except that the annealing steps took place at 50 °C.

Aliquots (7 to 10 µl) of PCR products were digested with restriction endonucleases (Pharmacia Biotech) as specified by the manufacturer in a total volume of 20 µl. Three enzymes (*Hae*III, *Msp*I and *Cfo*I) were used for their highly level of discrimination. The restriction fragments were separated by horizontal electrophoresis in TBE buffer (89 Mm Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0) with 2.5% (w/v) MetaPhor (FMC Bioproducts, Rockland, Maine, USA). The gels were run at 100 $V \cdot cm^{-1}$ for 3 h, stained in an aqueous solution of ethidium bromide (1 mg/ml) and photographed under UV illumination with an apparatus Gel Doc BIO-RAD.

RESULTS

A collection of 31 nodules was established (Table 1). Nodules were collected both directly and by trap host plants.

The length of the amplified region was different among

TABLE 1 – Sampling sites, strains used in this study and rhizobial groups

Strain	Site/origin	16S rDNA group	IGS size (pb)	IGS group (PCR/RFLP)	Assignation (PCR/RFLP)
AT4F	INRGREF	1	1350	1	*Sinorhizobium* sp.
AT5F	INRGREF	1	1350	1	*Sinorhizobium* sp.
AT6F	INRGREF	4	1100	4	*Agrobacterium tumefaciens*
AT7F	INRGREF	1	1300	5	*Sinorhizobium* sp.
AT10	INRGREF	5	1300	2	NI
AT5	INRGREF	1	1280	6	*Sinorhizobium* sp.
AT37	INRGREF	2	1200	8	*Rhizobium tropici* IIa
AT4	INRGREF	1	1300	9	*Sinorhizobium* sp.
AT9	INRGREF	3	1050	11	*Mesorhizobium* sp.
AT1	INRGREF	5	1150	18	NI
AT18	S1-Bouhedma	1	1300	2	*Sinorhizobium* sp.
AT32F	S2-Bouhedma	1	1300	12	*Sinorhizobium* sp.
AT28	S3-Bouhedma	3	1050	3	*Mesorhizobium* sp.
AT33	S3-Bouhedma	4	1100	4	*Agrobacterium tumefaciens*
AT34	S3-Bouhedma	6	1200	17	NI
AT36	S3-Bouhedma	2	1150	13	*Rhizobium tropici* IIa
AT16	S4-Bouhedma	5	1250	16	NI
AT23	S5-Bouhedma	5	1250	16	NI
AT35F	S5-Bouhedma	1	1300	10	*Sinorhizobium* sp.
AT27	S5-Bouhedma	1	1300	2	*Sinorhizobium* sp.
AT33b	S5-Bouhedma	4	1100	4	*Agrobacterium tumifaciens*
AT37b	S7-Bouhedma	2	1200	8	*Rhizobium tropici* IIa
AT29	Belkhir	2	1200	7	*Rhizobium tropici* IIa
AT32	Belkhir	1	1300	2	*Sinorhizobium* sp.
AT19	Belkhir	6	1200	17	NI
AT13	Ferjane	1	1300	2	*Sinorhizobium* sp.
AT12	Ferjane	5	1150	19	NI
ATG6	Gabes	1	1300	10	*Sinorhizobium* sp.
AT15F	Menzel Habib	3	1050	14	*Mesorhizobium* sp.
AT25	Menzel Habib	5	1050	20	NI
AT15	Menzel Habib	2	1200	15	*Rhizobium tropici IIa*
Reference strains					
CIAT 299	CIAT	A	1200	G1	*Rhizobium tropici IIa*
CIAT 899^T	CIAT	B	1200	G2	*Rhizobium tropici IIb*
USDA 1002^T	USDA	C	1300	G3	*Sinorhizobium meliloti*
USDA 4894	USDA	D	1400	G4	*Sinorhizobium terangae*
LMG 6123	LMG	E	1000	G5	*Mesorhizobium loti*
LMG 14107	LMG	F	1000	G6	*Mesorhizobium huakuii*
USDA 3383	USDA	G	1100	G7	*Mesorhizobium ciceri*
LMG 8321	LMG	H	1000	G8	*Bradyrhizobium japonicum*
USDA 3622	USDA	I	1000	G9	*Bradyrhizobium liaoningense*
IAM 13570^T	IAM	J	1100	G10	*Agrobacterium rhizogenes*
IAM 13129^T	IAM	K	1400	G11	*Agrobacterium tumefaciens*

^{T}, type strain; AT, *Acacia* de Tunisie; NI, Not Identified. A-K and G1-G11 are PCR-RFLP-profiles of reference strains for 16S rDNA and IGS, respectively.
INRGREF, Institut National de Recherches en Génie Rural, Eaux et Forêts, Tunis, Tunisie; CIAT, Centro International de Agricultura Tropical, Cali, Columbia; USDA, *Rhizobium* Culture Collection, Beltsville Agriculural Research Center, USDA, Beltsville, MD, USA; LMG, Collection of Bacteria of the Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium; IAM; Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan.

the *Acacia tortilis* nodules extracted-DNA. The electrophoretic analysis of the undigested PCR products showed, in most cases, a single band of length variable from 1050 bp to 1350 bp (Fig. 2). Some crushed-nodules (five), of which the purity of DNA was carefully checked, showed one or two additional bands (data not shown). Therefore, we were able to identify 8 groups corresponding to 8 different bands (Table 1). Group 1 contains two strains, which have an IGS of 1350 bp. Group 2 contains the majority (10 nodules) of the strains, which are characterized by an IGS of 1300 bp. Group 3, which is represented by one strain, have an IGS slightly lesser than 1280 bp. Group 4 contains 3 strains of 1250 bp IGS-long. Group 5 is characterized by an IGS of 1200 bp. Groups 6 and 7 contain each 3 strains having an IGS of 1150 and 1100 respectively. Finally, group 8 contains 4 strains, which have an IGS of 1050 bp.

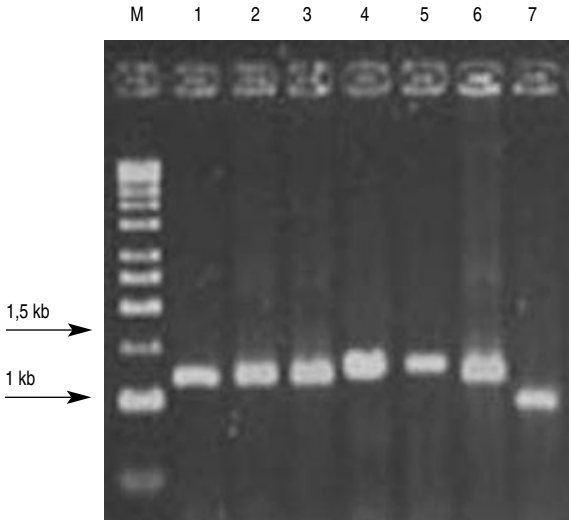

FIG. 2 – Electrophoresis of PCR products obtained with the universal primers FGPS1490-72 and FGPL 132'-38, which target the ribosomal IGS of rizobial strains that nodulate *Acacia* spp. Lanes 1-7, different strain-patterns (1-3, 1200 pb; 4-5, 1300 pb; 6, 1280 pb; 7, 1050 pb). M, 1 kb ladder (Promega).

The PCR product derived from each nodule was digested separately by three enzymes, and the resulting fragments were separated by electrophoresis. The length of PCR product estimated by summing the sizes of the restricted fragments was equal or longer to the size estimated before enzymatic digestion (Fig. 3A, 3B). In fact, in some cases, we found a superimposition of 2 or 3 different patterns that make difficult their identification. The patterns were compared with those of strains belonging to recognized species (data not shown). A total of 20 different combinations, each corresponding to one genotype, were identified among the 31 nodules analysed by RFLP in this study.

Results obtained by PCR/RFLP of the IGS were confirmed by 16S rRNA analysis (Fig. 4) and showed that our collection was represented by 3 rhizobial genera (Table 1). There were 38.7% of the strains belonging to the genus *Sinorhizobium*. IGS sequences and 16S rDNA phylogenetic analysis showed that these strains were related to but distinct from *Sinorhizobium meliloti*. The genus *Rhizobium* was represented by 16.12% of the strains and was closely related to *Rhizobium tropici* IIa. 9.6% of the strains belonged to the genus *Mesorhizobium* and were related but different from *Mesorhizobium huakuii*. For clarity, in the following text we will consider unnamed strains as *Sinorhizobium* sp. and *Mesorhizobium* sp. However, group 4 showed 3 restriction profiles closer to the genus *Agrobacterium* and represented 9.6% of the strains, which were related to *Agrobacterium tumefaciens* species. Finally, PCR/RFLP of the IGS and 16S rDNA genes could not discriminate 25.8% of the strains that were not identified. Gene sequencing and DNA-DNA hybridisation would be needed to clarify their phylogenetic positions and to establish whether the unclassified strains represent new species or not.

Nitrogen fixation ability was estimated and showed different degree of symbiotic efficiency between the strains. All the strains belonging to *Sinorhizobium* sp. species gave pink-nodules, while those obtained by the remaining strains were yellow-green. Rhizobia-isolation would be needed to

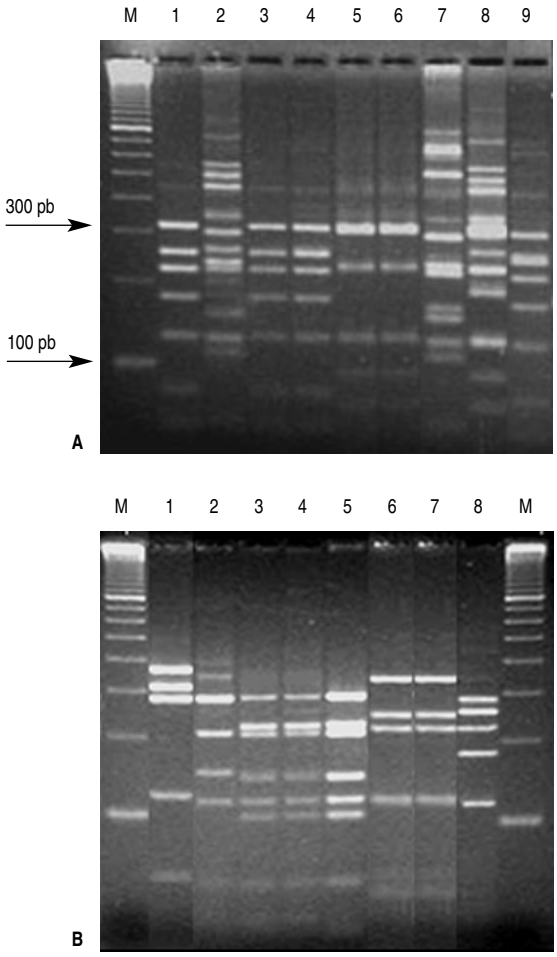

FIG. 3 – A and B: examples of PCR-RFLP of 16S-23S rDNA genes, on crushed nodules, digested with MspI and separated by electrophoresis in 2.5% (w/v) metaphor gel. M, 100 pb ladder (Pharmacia Biotech); the smallest band of the marker is 100 pb. A: lanes 2, 7, 8, superimposition of multiples patterns.

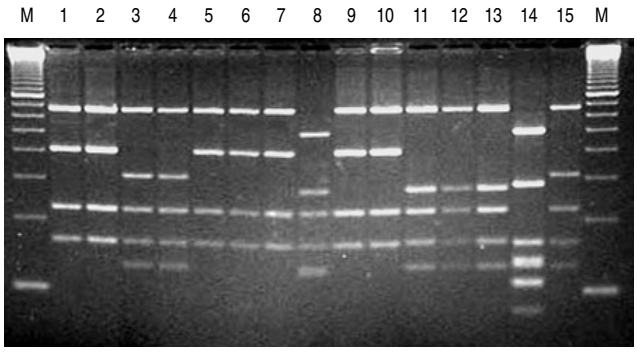

FIG. 4 – PCR/RFLP profiles of 16S rRNA genes digested with MspI and separated by electrophoresis in 2.5% (w/v) metaphor gel. M, molecular mass marker (100 pb ladder, Pharmacia Biotech); the smallest band of the marker is 100 pb.

select the most efficient strains in order to use for inoculation of degraded area.

DISCUSSION

DNA extracted directly from nodules was used to assess genetic diversity of rhizobia strains from *Acacia tortilis* in Tunisia. We have used PCR with RFLP analysis to evaluate 16S-23S spacer variation among these rhizobia. Our results showed that the IGS between the 16S and 23S rRNA genes was a good marker for detecting genetic diversity. The approaches used were easy, rapid and powerful.

We found great diversity in the length of IGS bands. Therefore, we were able to distinguish 8 groups on the basis of IGS length. In this study, our results corroborate these of Khbaya *et al.* (1998), which show that classification of rhizobia strains to any of these IGS groups appeared to be independent to the site of origin. We must increase the number of strains from each location to confirm this observation.

Digestion of the amplified 16SrDNA and 16S-23S spacer with three restriction enzymes (*Msp*I, *Hae*III and *Cfo*I) allow us to distinguish 6 and 20 different combinations inside nodules, respectively. In few cases, the digestion of the amplified intergenic spacer gave us a superimposition of two or three patterns originating from two or three amplified band. In fact, the rRNA operon is present in multiple copies in bacteria. At least three copies occur in *Rhizobium* species (Geniaux *et al.,* 1993; Huber and Selenska-Pobell, 1994). The multiple PCR products observed for some strains suggest variations in the length of IGS regions between the different copies, which could be partly explained by insertion of various tRNA genes in IGS regions (Jensen *et al.,* 1993). In few cases, the nodule can be invaded by a mixed population of very related bacteria whose 16S rDNA are identical (Benhizia *et al.,* 2004).

Our results showed that Tunisian acacia-rhizobia belong to *Sinorhizobium* sp., *Rhizobium tropici* IIa and *Mesorhizobium* sp. species. However, Ba *et al.* (2002) found that *Acacia tortilis* were nodulated by only *Sinorhizobium* sp. and *Mesorhizobium* sp. strains. In Kenya, *Acacia* sp. was nodulated by rhizobia belonging to four genera, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium;* but *Acacia tortilis* was nodulated by *Mesorhizobium* species only (Odee *et al.*, 2002). Whereas, in Morocco soils, *Acacia* sp. were nodulated by only *Sinorhizobium* species (Khbaya *et al.*, 1998). This high diversity of rhizobia nodulating *Acacia tortilis* from diverse countries in Africa may be in relation with climatic, and edaphically conditions.

Our results showed that 9.6% of the strains had high rDNA identity with *Agrobacterium tumefaciens* species. In fact, *Agrobacterium* spp. is genetically related to *Rhizobium* genomic species and several *Agrobacterium* strains were isolated from the root nodules of common bean in Tunisia (Mhamdi *et al.*, 2002). Other agrobacteria have been isolated from tropical legumes in Africa (De Lajudie *et al.*, 1999), from nodules of Acacia spp. in Morocco (Khbaya *et al.*, 1998) and from soybean (Chen *et al.*, 2000).

Further more, 25.8% of the strains from our collection were not identified. In fact, several strains nodulating wild legumes were not identified and were with characters, which were totally different from the existing one, a finding which points out that new species and genera of root-nodule bacteria may emerge (Zahran, 2001; Chen *et al.*, 2003). Moreover, new bacteria species belonging to alpha- beta- and gamma proteobacteria have been recently found in root nodules of leguminous plants (Willems, 2003; Benhizia *et al.*, 2004). Other molecular techniques (genes sequencing and DNA-DNA hybridisation) are needed for further characterization of the non-identified root nodule bacteria.

In symbiotic rhizobacteria species, the evolution of the population structure may be influenced by environmental conditions like biological barriers to gene exchange or geographical isolation and also by the type of soil or the genotype of the host plant (Demezas *et al.*, 1995). One additional element that can also play a critical role in the evolution of rhizobial populations is the occurrence of large plasmids that can have an evolutionary history different from that of the strains living in (Schofield *et al.*, 1987).

This study has been particularly constructive in revealing a hitherto hidden diversity of rhizobia capable of nodulating *Acacia tortilis* ssp. *raddiana* in Tunisia. This study provides the first demonstration of genetic diversity among rhizobial strains nodulating *Acacia tortilis* in Tunisia; the large number of different genotypes obtained suggests that Tunisian regions may hide a wide rhizobial diversity, still largely unexplored, and with an important potential for improving the growth of *Acacia tortilis* in arid soils.

Acknowledgments

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