

Diversity of indigenous bradyrhizobia associated with three cowpea cultivars (*Vigna unguiculata* (L.) Walp.) grown under limited and favorable water conditions in Senegal (West Africa)

Tatiana Krasova-Wade^{1, 2, *}, Ibrahima Ndoye^{1, 2}, Serge Braconnier³, Benoit Sarr³, Philippe de Lajudie⁴ and Marc Neyra¹

¹Laboratoire de Microbiologie, IRD, BP 1386, Dakar, Senegal

²Département de Biologie Végétale, Université Cheikh Anta Diop, BP 5005, Dakar, Senegal

³Centre d'Etude Régional pour l'Amélioration de l'Adaptation à la Sécheresse (CERAAS), BP 3320, Thiès Escale, Thiès

⁴Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM), Campus de Baillarguet, TA10/J, 34398 Montpellier Cedex 5, France

Accepted 17 December 2002

The diversity of *Bradyrhizobium* strains nodulating three cowpea (*Vigna unguiculata* L. Walp.) cultivars in favorable and water-limited conditions occurring at flowering was analysed. PCR-RFLP analysis of 16S-23S rDNA intergenic spacer region (IGS) directly applied on 85 crushed nodules distinguished four genetic profiles, IGS types I, II, III and IV. The distribution of these IGS types according to water conditions and cowpea cultivars (B-21, TN 88-63 and Mouride) showed that nodulating strains appeared more diverse in water-limited condition. More than three quarters of prospected nodules presented the IGS type I. They were formed on all three cultivars and in both water conditions. Only a small part of nodules was distributed between the IGS type II, III and IV. Nodules showing the IGS types II and III were found mainly in limited conditions on TN 88-63 and Mouride cultivars, whereas nodules presenting the IGS type IV were collected only from cultivars B-21 and Mouride, in both water conditions. Strains corresponding to the different profiles were isolated. The phylogenetic analysis of 16S rRNA gene sequences showed that they belong to the genus, *Bradyrhizobium*. The sequence analysis of 16S-23S rDNA IGS revealed that the strains exhibiting IGS types II, III and IV were closely related to some *Faidherbia albida* isolates from Senegal. IGS type II can be assigned with at least 98% similarity to *Bradyrhizobium* genospecies IV. IGS types III and IV showed more than 96% similarity with genospecies VII and could belong to the same genospecies. IGS type I, the most frequent, exhibits low IGS similarity with reported sequences in the databases, and could represent a new genospecies.

Key words: *Bradyrhizobium*, *Vigna unguiculata*, water-limited condition, PCR-RFLP, 16S rDNA, 16S-23S rDNA IGS.

INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp.) is one of the earliest plants cultivated by man. In the Sahel region, it is an important grain legume crop for rural populations. It is used as vegetable, fodder, and textile resources. In Senegal, it is essentially cultivated for fresh or dried seeds and it plays a considerable role in the nutritional and economical balances of these populations.

Cowpea is particularly adapted to regions of limited rainfall. In Senegal, about 90% of the cultivated areas

are concentrated in the North and the Center-North parts, corresponding to the driest regions of the country. Cowpea is grown the rainy season. However, insufficient rainfall or irregular distribution can result in a decrease of yield. It was reported that cowpea is likely to drought during the period of nodulation and to a lack of moisture at mid-cycle before the flowering phase (Zablotowicz et al., 1981).

The low moisture content of the semi-arid soils limits the biological nitrogen fixation (Obaton, 1992; Sprent, 1976). Aridity can affect symbiotic partners, plant and rhizobium. The possibility of selecting *V. unguiculata* rhizobial strains according to their capacity to recover

*Corresponding author; phone: (221) 849 33 19, fax: (221) 832 16 75, e-mail: Tania.Wade@ird.sn

from water stress has been investigated. However, as for other plant species, it appears that the introduction of selected strains has often no effect on the growth of cowpea, because of the non-specificity of the bacteria, the occurrence and the resistance to stress of ineffective indigenous rhizobia strains in soils (Figueiredo et al., 1999). In fact, Thies et al. (1991) observed that in all sites of sampling on the island of Maui (Hawaii) indigenous bradyrhizobia isolated from cowpea nodules were more effective than the reference strains. Also on soybean, Hunt et al. (1988) found that several indigenous strains of *Bradyrhizobium japonicum* were superior inoculants than commercial strains under drought conditions. Rhizobial populations in tropical soils represent an important reservoir from which superior strains adapted to environmental stresses such as drought can be selected (Aurag and Brhada, 1995; Mpeperekki et al., 1997). The efficient exploitation of biological N₂ fixation to improve the agricultural productivity requires that the population of native rhizobia to be adequately characterized.

Cowpea rhizobia were first classified in an heterogeneous group of slow-growing rhizobia nodulating promiscuous tropical and subtropical legume species known as 'cowpea cross-inoculation group' (Allen and Allen, 1981). They were later transferred to the genus *Bradyrhizobium*. Currently this genus contains three named species, *B. japonicum* (groups I and Ia), *B. elkanii* and *B. liaoningense* (Hollis et al., 1981; Jordan, 1982; Kuykendall and Saxena, 1991; Xu et al., 1995). Most of the strains belonging to these species have been isolated from or are associated to *Glycine* nodules. Many other rhizobia isolated from other legume hosts have been assigned to *Bradyrhizobium* (Barrera et al., 1997; Doignon-Bourcier et al., 1999; Dupuy et al., 1994; Molouba et al., 1999; Vinuesa et al., 1998; Zhang et al., 1999). Recently Willems et al. (2001) showed, on the basis of DNA-DNA hybridizations and 16S-23S rDNA IGS sequence analysis, that *Bradyrhizobium* consists of a group of four highly related genospecies (*B. japonicum*, *B. liaoningense* and two other genospecies), and at least three other genospecies, one of which is *B. elkanii*.

We report here the genetic characterization and phylogeny of indigenous rhizobial populations isolated from cowpea cultivated in Senegal under favorable and water limited conditions occurring at flowering, a situation frequently occurring in Senegal. 85 nodules were analyzed by PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) of 16S-23S rDNA intergenic spacer region (IGS) directly on DNA extracted from crushed nodules. Strains corresponding to the different profiles were isolated, and their phylogenetic positions determined by 16S rDNA and 16S-23S IGS sequence analysis.

MATERIALS AND METHODS

Field experiment: The experimental site was located in Bambey (14° 42' N, 16° 28' W), Senegal, in experimental stations of CNRA

(Centre National de la Recherche Agronomique) during the dry season of 1999. The experimentation was carried out with three cultivars of cowpea: Bambey 21 (B-21), Mouride and TN88-63 (TN). The variety B21 has a mean vegetative cycle of 60 days, while it is 65-70 days for TN88-63 and Mouride (Sarr et al., 2001). Plants were subjected to two water treatments: favorable and deficit conditions during part of their vegetative growth. The trial was done in a randomized complete block designed with 24 plots of 6.5 m X 6.5 m (three blocs and four replications per treatment). Soil was sandy, pH 6-6.5 (vernacular name dior-modal), and had never been inoculated with rhizobial strains.

After ploughing, the sowing was carried out on March 19th in humid soil using three seeds per poquet with space of 0.25 m between poquets and 0.5 m between rows. Ten days after emergence 150 kg/ha of mineral fertilizer N-P-K (6-20-10) was applied. During cultivation, one herbicide treatment, dimethoat (300 g/l, 1.2 l/ha) was applied twice to flight infections of aphid and thrips. The plants were watered by sprinkling irrigation every three to five days. The water limitation was introduced 32 days after sowing at flowering initiation stage by stopping irrigation for 20 days.

Treatment of nodules: Nodules were collected 57 days after sowing. Two plots per treatment were evaluated. Nodules from three plants per plot were harvested and stored in collecting tubes containing CaCl₂ (Date, 1982). Soil samples were collected at 5-20 cm depth in the vicinity of plants and stored in plastic bags at 4 °C. Before analysis each nodule was treated individually. Nodules were rehydrated in sterile water and surface sterilised by immersion in 3.3% (w/v) Ca(OCl)₂ for 3 min, and rinsing in sterile water. This was followed by a second immersion in 96% ethanol for 2 to 3 min and rinsing in sterile water. From this stage the nodules were manipulated aseptically. Each nodule was crushed in 300 µl of sterile water with plastic pestle sterilised in 96% ethanol in a 1.5-ml Eppendorf tube. One aliquote of 50 µl of crushed nodule was mixed with 50 µl of 40% v/v Glycerol and stored for further bacterial isolation.

DNA extraction: 150 µl of 2X CTAB/PVPP buffer (0,2 M Tris-HCl, pH 8; 0,04 M EDTA pH 8; 2,8 M NaCl; 4% w/v CTAB (hexadecyltrimethylammonium bromide); 2% w/v PVPP (polyvinylpyrrolidone)) was added to 150 µl of crushed nodule. The homogenate was incubated at 65° C for 60 min and centrifuged for 10 min at 15000 x g 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 15000 x g. 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 15000 x g. 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 13000 rpm at +4° C. The resulting DNA pellet was washed with 70% v/v ethanol by centrifugation for 15 min at 13000 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 spectrophotometry (Pharmacia Biotech) in the range 200 nm to 340 nm. Bacterial genomic DNA was extracted from 1.5 ml of stationary phase bacterial cultures grown in YM (Yeast extract Mannitol medium). Cells were pelleted by centrifugation and resuspended by homogenisation in one volume of 1X CTAB/PVPP buffer. Total genomic DNA was recovered and purified as described above for crushed nodule DNA.

PCR amplification of 16S-23S rDNA spacer region: Two primers FGPS1490-72 (5'-TGCGGCTGGATCCCTCCTT-3') (Normand et al., 1996), and FGPL132-38 (5'-CCGGGTTTCCCCATTCGG-3') (Ponsonnet and Nesme, 1994), were used for PCR amplification. PCR was carried out in 25 µl reaction volume containing 50 ng of

pure total DNA extract, one dried bead (Ready-to-Go PCR beads, Pharmacia Biotech) containing 1.5 U of *Taq* polymerase, 10 mM Tris-HCl, (pH 9 at room temperature), 50 mM KCl, 1.5 mM MgCl₂, 200 μM of each dNTP and 1.0 μM of each primer. PCR amplification was performed in GeneAmp PCR System 2400 (Perkin Elmer) thermal cycler adjusted to the following temperature profile: initial denaturation at 95°C for 5 min; 35 amplification cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 1 min, and extension 72°C for 1 min; and final extension at 72°C for 3 min. After electrophoresis of 3 μl on a 1% (w/v) agarose gel in TBE buffer (1.1 w/v Tris-HCl; 0.1% w/v Na₂EDTA·2H₂O; 0.55% w/v boric acid), the gel was stained for 30 min in an aqueous solution of ethidium bromide (1 μg/ml) and photographed under UV illumination with Gel Doc (BIO-RAD) software.

Restriction fragment analysis of 16S-23S intergenic spacer region: Aliquots (6 to 10 μl) of PCR products were digested with restriction endonucleases *Hae*III and *Msp*I as specified by the manufacturer (Amersham Pharmacia Biotech) with an excess of enzyme (10 U per 20 μl reaction volume) for 2 h. Restricted DNA was analysed by horizontal electrophoresis in 2.5% (w/v) agarose Metaphor^R (FMC BioProducts, Rockland, Marine USA). Electrophoresis was carried out at 80 V for 3 h in 11 by 14-cm gels. Gel was stained and photographed as described above.

Sequencing of 16S rDNA and 16S-23S rDNA IGS: The 16S rDNA of each isolate was amplified using the primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGCTTAAGG-AGGTCATCCAGCC-3') (Weisburg et al., 1991). Products of amplification were purified with a QIAquick PCR purification kit (Qiagen) before sequencing. The purified PCR products were sequenced by Genome Express (Grenoble, France), using primers fD1, rD1, FGPS485-292 (5'-CAGCAGCCGCGGTAA-3'), FGPS1047-295 (5'-ATGTTGGGTTAAGTC-3'), FGPS505'-313 (5'-GTATTACCGCGGCTGCTG-3') (Normand et al., 1996), MBAS-1 (5'-CGTCATCCCCACCTTCCTC-3') (this study). 16S-23S IGS was sequenced using primers FGPS 1490-72, FGPL 132-38, BR4 (5'-CGAACCGACGACCTCATGC-3') and its antiprimer *BR4 (5'-GCATGAGGTCGTCGGTTCG-3') (Willems et al., 2001).

The sequences were analyzed with Sequence Navigator software and the double strands were aligned in the consensus sequences with program AutoAssembler (Applied Biosystems, Foster City, CA 94404 USA). GenBank was scanned for related sequences by using the algorithm, BLAST (Altschul et al., 1997) and the closely related sequences found were included in phylogenetic analysis using the CLUSTAL X software (Thompson et al., 1997). Matrix pairwise comparisons were corrected for multiple base substitutions by the two-parameter method of Kimura (Kimura, 1980) by using the DNADIST program of the PHYLIP package (supply reference). Phylogenetic trees were constructed by the neighbour-joining method (Saitou and Nei, 1987). A bootstrap confidence analysis was performed on 1000 replicates to determine the reliability of the distance-tree topology obtained (Felsenstein, 1985). Graphic representation of the resulting trees was done using NJPLOT software (Perriere et al., 1996).

The nucleotide sequences of the 16S rRNA genes of ORS 3257, ORS 3259, ORS 3260 and ORS 3262 have been deposited in the GenBank database under accession numbers AY039014, AY039015, AY039016 and AY039017, respectively. The nucleotide sequences of the 16S-23S IGS of the same strains have been deposited in under accession numbers AY039018, AY03019, AY039020 and AY03021, respectively.

Isolation of rhizobial strains: Rhizobial strains were isolated from crushed nodules stored at -20° C in glycerol 20% by streaking onto YMA plates (Vincent, 1970). Purity of colonies showing the morphological characteristics of rhizobial strains was checked for by

repeated streaking on YMA plates and by microscopic examination of living cells. Bacterial DNA were analysed by PCR-RFLP of 16S-23S rDNA IGS with the restriction enzymes *Hae*III and *Msp*I. In some cases, nodules were obtained *in vitro* by cultivating cowpea seedlings (Mouride cultivar), grown aseptically in Jensen slant agar tubes (Vincent, 1970) and inoculated with one ml of stirred soil suspension in sterile water. Three week-old nodules were collected, washed, surface sterilised with 96° v/v ethanol for 2 min. They were aseptically cut and samples were directly picked out from the centre of the nodule and streaked onto YMA plates. Individual isolates were established from single colonies and used to inoculate cowpea seedling in tube to test their infectivity.

Nodulation test: *V. unguiculata* seeds (cultivar Mouride) were surface-sterilised with 96% ethanol for 5 min and rinsed with sterile water. Then they were sterilised with 3.3% w/v Ca(OCl)₂ for 10 min. *Faidherbia albida* seeds were scarified and surface-sterilized with concentrated sulfuric acid for 30 min. After treatment, the seeds were washed several times with sterile water to eliminate any trace of Ca(OCl)₂ and acid. The seeds were germinated by incubation in sterile Petri dishes containing 1 % water agar for 24-48 h in the dark. They were then transferred into tubes containing Jensen seedling slant agar for root nodulation trials (Vincent, 1970). Four plants were routinely tested with each isolate. Plants were grown under intermittent light (12h/12h, day/night). After two days, the plants were inoculated with 1 ml of exponential phase bacterial culture (grown at 28° C for 5-6 days in YM) of each cowpea rhizobial isolate (ORS 3257, ORS 3259, ORS 3260, and ORS 3262) and *F. albida* rhizobial strains (ORS 111, ORS 114, ORS 116, ORS 117, ORS 146, ORS 186, and ORS 188). Uninoculated controls were used to check for cross-contamination.

RESULTS

PCR-RFLP of 16S-23S rDNA IGS

Nodules were observed on each cultivar and for all treatments. No difference in appearance in the number of nodules per plant was found between treatments (results not shown). PCR amplification of 85 nodules yielded single IGS PCR products ranging from 910 bp to 1034 bp (Table 1). Digestions with restriction enzymes *Hae*III and *Msp*I gave similar pattern. The two RFLP profiles obtained for each nodule allowed for the distribution of the 85 nodules into four distinct restriction patterns named IGS types I, II, III and IV (Table 1; Figures 1A and 1B).

Distribution of PCR-RFLP 16S-23S IGS types according to water conditions and cowpea cultivars

The 85 restriction profiles were grouped according to cowpea cultivars and water conditions (favorable and limited) (Table 2). Sixty-nine nodules presenting the IGS type I was formed on all three cultivars and both water conditions. The IGS type II was found only once, within a nodule of a stressed TN 88-63 cultivar. Seven of the eight nodules presenting the IGS type III were formed on TN 88-63 and Mouride cultivars plants under limited conditions. The IGS type IV was observed from one nodule collected on well-irrigated plant of B-21 cultivar

Table 1. IGS types and restriction patterns determined by PCR-RFLP analysis of 16S-23S rDNA IGS regions obtained directly on crushed nodules DNA.

rDNA IGS types ^a	Size IGS PCR product (bp) ^b	Fragments sizes (bp) ^c of amplified rDNA IGS digested with:	
		<i>HaeIII</i>	<i>MspI</i>
I	910	44, 82, 85, 114, 585	129, 223, 558
II	944	44, 82, 220, 233, 365	129, 163, 223, 429
III	1034	47, 83, 236, 264, 404	6, 11, 48, 99, 131, 216, 225, 298
IV	1032	47, 83, 102, 236, 263, 301	6, 86, 99, 131, 216, 224, 270

^aAn rDNA IGS type corresponds to a grouping of samples which presented the same restriction patterns obtained with two enzymes.

^bThe size of the PCR product.

^cThe sizes of bands after digestion.

Table 2. Distribution of PCR-RFLP 16S-23S rDNA IGS genotypes obtained on crushed nodules according to water conditions and cowpea cultivars.

Cowpea cultivars	Water conditions ^a	Number of analyzed nodules	Number of nodules with IGS types ^b			
			type I	type II	type III	type IV
B-21	non limited	11	10	0	0	1
	limited	9	9	0	0	0
TN 88-63	non limited	21	20	0	1	0
	limited	17	15	1	1	0
Mouride	non limited	12	9	0	0	3
	limited	15	6	0	6	3
Total		85	69	1	8	7

^aIn the field the cultivars were submitted to two water conditions: non limited or limited, induced during vegetal stade by stopping irrigation for 20 days.

^bThe IGS types refers to the 16S-23S rDNA IGS PCR-RFLP patterns obtained on total DNA of crushed nodules with the restriction enzymes *HaeIII* and *MspI*.

and from six nodules of Mouride cultivar plants either in limited or non-limited water conditions. Nodules with IGS types II, III and IV were always found on plants bearing other nodules exhibiting IGS type I profile.

Isolation of rhizobial strains and analysis of rDNA

Only pure cultures exhibiting profile similar to IGS type II was obtained from rehydrated crushed nodules streaked

on YMA. (Figures 1A and 1B). Strains corresponding to the three other IGS profiles (I, III and IV) were isolated from nodules obtained on axenic seedlings grown in tubes. The pure isolates were deposited in the strain collection bank in Laboratory of Microbiology of IRD (Senegal) under numbers ORS 3257 and ORS 3258 (IGS type I), ORS 3259 (IGS type II), ORS 3260 and ORS 3261 (IGS type III), ORS 3262 and ORS 3263 (IGS types IV). PCR-RFLP of 16S-23S rDNA IGS profiles of two isolates of each IGS types I, III and IV were identical, and

Table 3. Effect of cross-inoculation of *Bradyrhizobium* strains isolated from nodules of *V. unguiculata* and *F. albida* on nodulation.

Isolates ^a	IGS types ^b	<i>Bradyrhizobium</i> genospecies ^c	Host plant or source of isolation	Origin	Inoculation effect on Infectiveness of ^d	
					<i>F. albida</i>	<i>V. unguiculata</i>
ORS 3257	I		<i>V. unguiculata</i>	Bambey	+	+
ORS 3259	II		<i>V. unguiculata</i>	Bambey	+	+
ORS 3260	III		<i>V. unguiculata</i>	Bambey	+	+
ORS 3262	IV		<i>V. unguiculata</i>	Bambey	+	+
ORS 111 (LMG 10667)		VII	<i>F. albida</i>	Louga	+	+
ORS 114 (LMG 10670)		VII	<i>F. albida</i>	Louga	+	+
ORS 116 (LMG 10672)		VII	<i>F. albida</i>	Louga	+	+
ORS 117 (LMG 15181)		VII	<i>F. albida</i>	Louga	+	+
ORS 146 (LMG 10702)		IV	<i>F. albida</i>	Dioukoul	+	+
ORS 186 (LMG 10725)		IV	<i>F. albida</i>	Dagana	+	+
ORS 188 (LMG 10727)		IV	<i>F. albida</i>	Dagana	+	+

^aOriginal isolate number and her synonyme. ORS, ORSTOM Collection, Institut de Recherche pour le Développement, BP 1386, Dakar, Sénégal; LMG, Laboratorium voor Microbiologie, Ghent, Belgium.

^bA rDNA IGS type corresponds to a grouping of samples which presented the same restriction patterns obtained with two enzymes, *Hae*III and *Msp*I.

^c*Bradyrhizobium* genus genospecies defined by Willems et al. (2001).

^dResults are given as the mean of three replicates. (+), formed nodules; (-), absence of nodules.

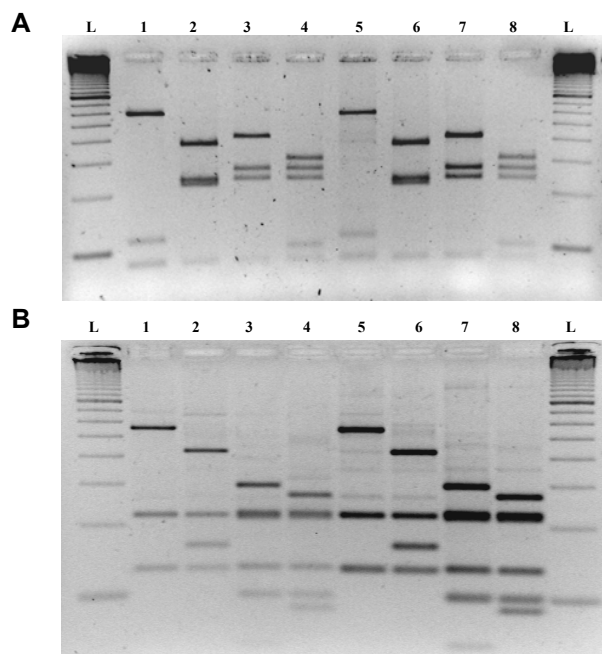


Figure 1. Comparison of restriction patterns of four PCR-amplified 16S-23S rDNA IGS region types of crushed nodules DNA and DNA of cowpea strains isolated from these nodules, obtained with *Hae*III (A) and *Msp*I (B). Lane L, 100 bp DNA size marker (Pharmacia Biotech), lane 1, 2, 3 and 4, IGS types I, II, III and IV, respectively, obtained on crushed nodules DNA; 5, 6, 7, and 8, IGS types I, II, III and IV, respectively, obtained on bacterial isolates DNA.

only one isolate of each IGS type was used for further analysis. All isolates are typically slow growing on YMA, with colony development appearance in 5 to 6 days.

Sequence analysis of 16S rDNA genes

The 1444 bp fragments of 16S rDNA of isolates ORS 3257, ORS 3259, ORS 3260 and ORS 3262 were sequenced. BLAST analysis confirmed that the cowpea isolates belong to the *Bradyrhizobium*. A phylogenetical tree was constructed based on full 16S rDNA gene sequences of the cowpea isolates and *Bradyrhizobium* reference strains. Two clusters with high confidence values from bootstrap analysis (Figure 2) were obtained. One of these include *B. japonicum* strains USDA 6^T, USDA 110, USDA 123, USDA 129 and *B. liaoningense* strain USDA 3622^T, as well as isolates ORS 3257 (IGS type I) and ORS 3259 (IGS type II). The second cluster includes *B. elkanii* strains USDA 76^T and USDA 94, ORS 3260 (IGS type III) and ORS 3262 (IGS type IV). This phylogenetic analysis suggests that strains ORS 3257 and ORS 3259 are closely related to *B. japonicum*, while ORS 3260 and ORS 3262 shares more similarities with *B. elkanii*.

Sequence analysis of 16S-23S rDNA IGS

Alignments of full 16S-23S rDNA IGS sequences of the cowpea isolates with related IGS sequences from

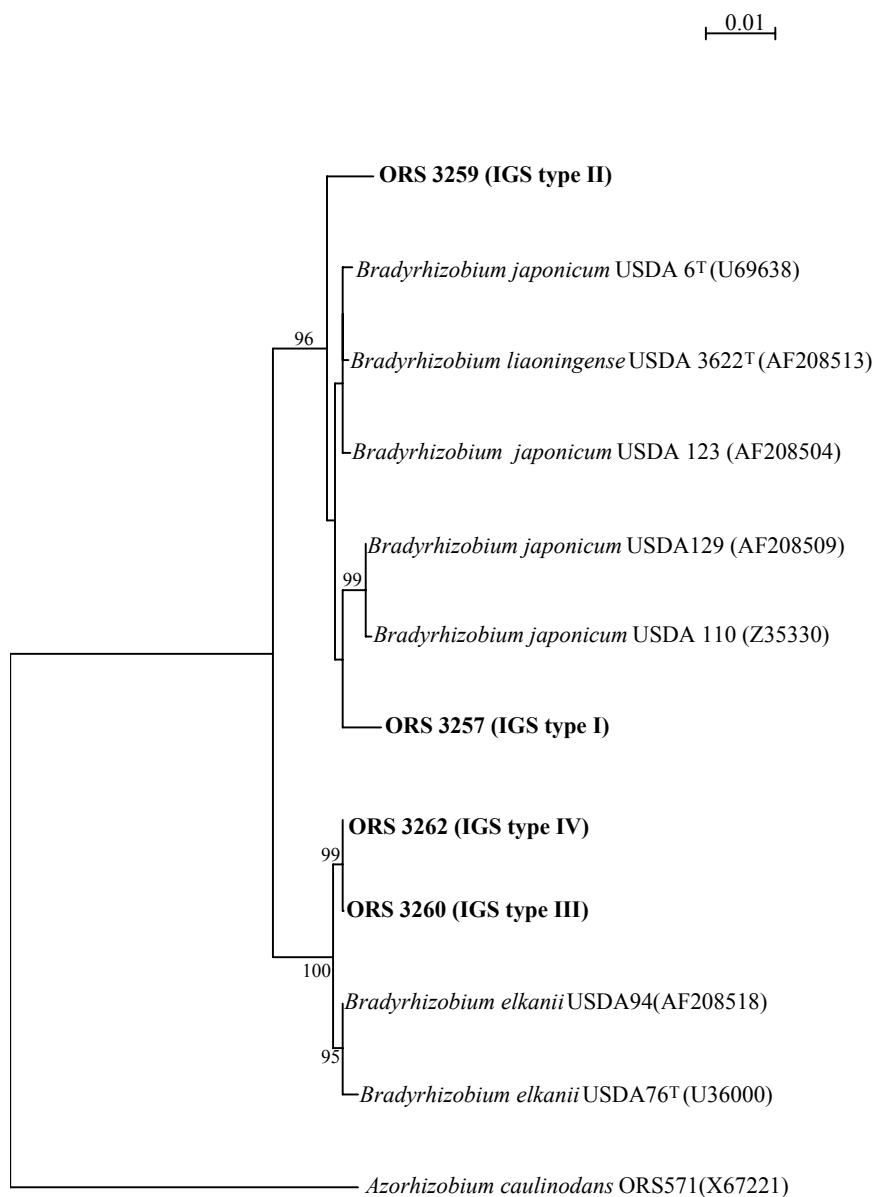


Figure 2. Phylogenetic relationships between *V. unguiculata* bradyrhizobia isolates and reference strains based upon aligned sequences of 16S rDNA genes, constructed as rooted tree using the neighbour-joining method. Sequences accession numbers are listed in parentheses. Bootstrap values expressed as a percentage of 1000 replications are listed at the nodes when 70% or more.

GenBank database revealed that the four IGS types are very similar to some *Bradyrhizobium* strains isolated from nodules of *F. albida* collected in Senegal (Dupuy and Dreyfus, 1992). Tree topology confirmed that the strains harboring IGS types I and II are related to *B. japonicum* and *B. liaoningense* reference strains, whereas the strains IGS types III and IV can be grouped with *B. elkanii* reference strain (Figure 3). The strain ORS 3259 (IGS type II) is grouped with *F. albida* strains belonging to genospecies IV of Willems et al. (2001). IGS sequences of strains ORS 3260 (IGS type III) and ORS 3262 (IGS type IV) were identical to those of some strains of genospecies VII (Willems et al., 2001). The strain ORS 3257 appears to be more different from all the described strains and it formed a separated branch.

DISCUSSION

In this study we characterized an indigenous population of *Bradyrhizobium* nodulating three cowpea cultivars under favorable and water-deficient conditions. Several authors have demonstrated the discriminating power of PCR-RFLP analysis of 16S-23S IGS regions for grouping genetically related strains (Doignon-Bourcier et al., 2000; Gürtler and Stanisich, 1996; Jensen et al., 1993; Laguerre et al., 1996; Leblond-Bourget and Decaris, 1996; Normand et al., 1996; Vinuesa et al., 1998). Using this technique, we resolved the cowpea bradyrhizobial diversity in 85 nodules as four genotypes.

Sequence analysis of 16S rRNA gene have also been used frequently for microbial taxonomy, and it is a

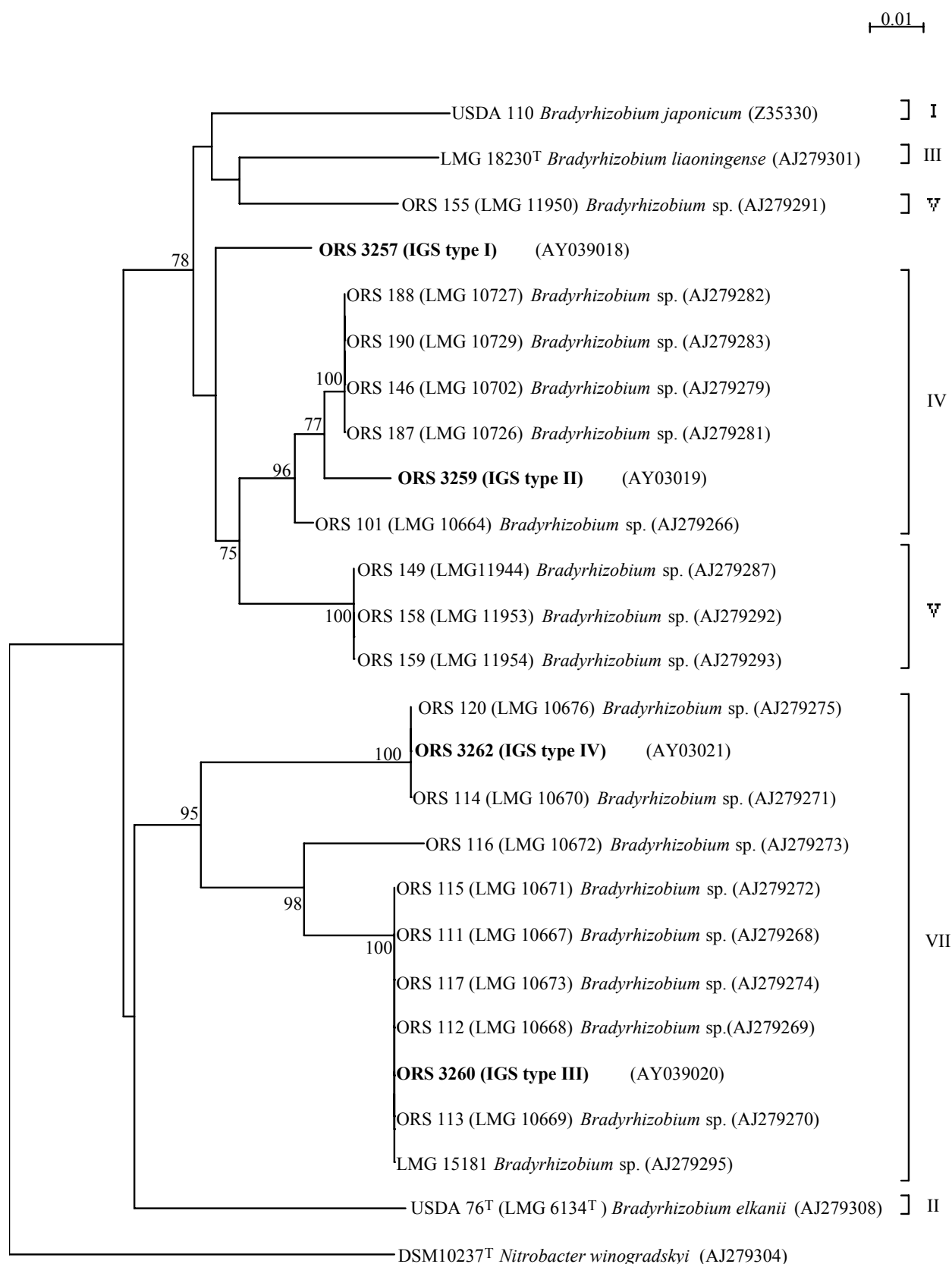


Figure 3. Phylogenetic relationships between *V. unguiculata* bradyrhizobia isolates, reference strains and some *A. albidia* isolates based upon aligned 16S-23S rDNA IGS regions sequences constructed as rooted tree using the neighbour-joining method. Sequences accession numbers are listed in parentheses. Bootstrap values expressed as a percentage of 1000 replications are listed at the nodes when 70% or more. Roman numerals on the right-hand side refer to previously described genospecies by Willems et al., 2001.

powerful and accurate method for determining inter- and intra-specific relationships. On the basis of 16S rDNA sequences, the strains we isolated from cowpea are closely related to *B. japonicum* (ORS 3257 and ORS 3259, representatives of IGS types I and II, respectively) and *B. elkanii* (ORS 3260 and ORS 3262, IGS types III and IV) species. However, investigation of the evolutionary relationships among *Bradyrhizobium* species is problematic, because of limited sequence divergence of their 16S rDNA genes. The evolutionary relationships are more resolved when reconstructions are made using sequence divergence of the spacer region between the 16S and 23S rDNA (Mehta and Rosato, 2001; van Berkum and Fuhrmann, 2000; Willems et al., 2001; Zhang et al., 2001). Using DNA-DNA hybridizations and IGS sequence analysis of 62 bradyrhizobia strains, Willems et al. (2001) showed, that the *Bradyrhizobium* is comprised at least seven genospecies. The alignments of IGS sequences of the cowpea isolates with related IGS sequences found in GenBank database revealed that they are very closely related some strains isolated from nodules of *F. albida* collected in Senegal (Dupuy and Dreyfus, 1992). In *Bradyrhizobium* genus, strains with at least 96% spacer sequence similarity are considered to belong to the same genospecies (Willems et al., 2001). Strain ORS 3259, belonging to IGS type II showed at least 98% of spacer sequence similarity within *Bradyrhizobium* genospecies IV of Willems et al. (2001) and can consequently belong to this genospecies. Each of the strains representatives of the IGS types III and IV revealed more than 96% of similarity with genospecies VII. The strain ORS 3257 (IGS type I), could correspond to a new genospecies due to its lower IGS similarity.

The test of cross nodulation showed that the strains isolated from cowpea nodules nodulated *F. albida* (Table 3). Conversely, all the strains isolated from *F. albida* nodules nodulated the cowpea. Only those grouped with cowpea strains on the basis of IGS sequences were used. This suggests that in addition to similarity of IGS regions, they probably share nodulation genes.

Using the nitrogen-15 isotope dilution technique, Ndiaye et al., (2000) sorted 16 senegalese cowpea cultivars according to their capacity to fix nitrogen in optimal conditions and showed that cultivar effect is responsible for nitrogen-fixing efficiency. Their results also showed that B-21 was the worse nitrogen fixing cultivar, while Mouride cultivar showed a medium nitrogen fixing potentials. However, cultivar-strain interactions on cowpea are not well known, and cowpea can nodulated by different rhizobial strains (Allen and Allen, 1981; Eaglesham et al., 1987; Thies et al., 1991; Trinick and Hadobas, 1989). However, cultivar effects on nodule occupancy have been already observed on other plant species including soybean and bean (Balatti and Pueppke, 1990; Douka et al., 1986; George et al., 1987; Josephson et al., 1991; Keyser and Cregan, 1987; Sadowsky et al., 1987; Streeter, 1994). It appears that the

distribution of the IGS types among cowpea cultivars revealed a specificity of nodulation. The competitive ability of a strain to nodulate a plant host could be modified upon certain environmental conditions. For Mouride cultivar, for example, the IGS type I become less dominant in water-limited conditions, and this provides an opportunity to for IGS types III and IV strains to nodulate.

Diversity of strains within nodules of cowpea appears to be greater in water-limited conditions than in well-irrigated conditions. More than 80% of the evaluated nodules presented the IGS type I and only a small percent exhibited the IGS type II, III or IV. Eleven out of 41 investigated nodules showed IGS types II, III and IV under water-limited conditions, whereas in favorable environment these IGS types were found only in 5 out of 44 nodules. In another investigation, the rare IGS types II, III and IV was found in nodules sampled on 41 sites covering 600 km from the North to the Southeast of Senegal (Wade et al., 2002). In this study, the water limitation was applied 32 days after seedling. In the field, however, the first nodules appear about ten days after seedling (data not shown). This means that by the time the water limitation started, nodules were already being formed. The abundant nodulation by the IGS type I strain could be explained by its ability to infect in favourable water conditions. Field observation showed that the process of nodulation goes on for at least the first 35 days. Therefore, the water-limited conditions would influence the formation of new forming nodules. In fact, the nodules harboring the IGS types II and III strains were formed mainly in water-limited conditions.

Using the same experimental materials in this study, Sarr et al. (2001) compared the degree of sensitivity to water deficit and found that the water stress actually occurred on all three cultivars. According to their results and our data, the respective water sensitivity and drought resistance of these cultivars could be due to vegetative properties towards drought, but also to the identity of nodulating strains. B-21 cultivar, exclusively nodulated by the strain IGS type I in water-limited condition, is most sensitive of the cultivars. Conversely, Mouride showing a good drought resistance, harbored the greater rhizobial diversity.

In conclusion, it appears that in a single restricted area, cowpea can be nodulated by at least four different *Bradyrhizobium* strains belonging to three genospecies. The distribution of different IGS types according to water conditions suggests that different behaviours exist among the cowpea rhizobia, and nodulating strains appeared more diverse in water-limited conditions.

ACKNOWLEDGEMENTS

T. K.-W. is grateful to IRD for personnel grants. Part of this work was supported by AUF-UREF. We thank Eric Giraud and Philippe Jourand (LSTM) for excellent technical advice.

REFERENCES

- Allen ON, Allen EK (1981). The Leguminosae: A source book of characteristics, uses and nodulation. The University of Wisconsin press, USA.
- Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25:3389-3402.
- Aurag J, Brhada F (1995). Dynamique des populations de *Rhizobium* introduites dans le sol: influence du stress hydrique, de l'acidité et de la texture du sol. In: INRA (eds) Facteurs limitant la fixation symbiotique de l'azote dans le Bassin Méditerranéen. Paris, pp 149-158.
- Balatti PA, Pueppke SG (1990). Cultivar-specific interactions of soybean with *Rhizobium fredii* are regulated by the genotype of the root. *Plant Physiol.* 94:1907-1909.
- Barrera LL, Trujillo ME, Goodfellow M, Garcia FJ, Hernandez-Lucas I, Davila G, van Berkum P, Martinez-Romero E (1997). Biodiversity of bradyrhizobia nodulating *Lupinus* spp. *Int. J. Syst. Bacteriol.* 47:1086-1091.
- Date RA (1982). Collection, isolation, characterization and conservation of *Rhizobium*. In: JM. Vencent (eds) Nitrogen fixation in legumes. Academic Press Publishers, pp 95-109.
- Doignon-Bourcier F, Sy A, Willems A, Torck U, Dreyfus B, Gillis M, de Lajudie P (1999). Diversity of bradyrhizobia from 7 tropical *Leguminosae* species native of Senegal. *Syst. Appl. Microbiol.* 22:647-661.
- Doignon-Bourcier F, Willems A, Coopman R, Laguerre G, Gillis M, de Lajudie P (2000). Genotypic characterization of *Bradyrhizobium* strains nodulating small senegalese legumes by 16S-23S rRNA Intergenic Gene Spacers and Amplified Fragment Length Polymorphism Fingerprint Analyses. *Appl. Environ. Microbiol.* 66:3987-3997.
- Douka CE, Nychas AE, Xenoulis AC (1986). Nitrogen fixation in soybeans as influenced by cultivar and *Rhizobium* strain differences. *Biol. Fertil. Soils* 2:113-118.
- Dupuy NC, Dreyfus BL (1992). *Bradyrhizobium* population occur in deep soil under the leguminous tree *Acacia albida*. *Appl. Environ. Microbiol.* 58:2415-2419.
- Dupuy N, Willems A, Pot B, Dewettinck D, Vandebroune I, Maestroujan G, Dreyfus B, Kersters K, Collins MD, Gillis M (1994). Phenotypic and genotypic characterization of bradyrhizobia nodulating the leguminous tree *Acacia albida*. *Int. J. Syst. Bacteriol.* 44:461-473.
- Eaglesham ARJ, Stowers MD, Maina ML, Goldman BJ, Singlair MJ, Ayanaba A (1987). Physiological and biochemical aspects of diversity of *Bradyrhizobium* sp. (*Vigna*) from three west african soils. *Soil Biol. Biochem.* 19:575-581.
- Felsenstein J (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783-791.
- Figueiredo MVB, Vilar JJ, Burity HA, França FP (1999). Alleviation of water stress effects in cowpea by *Bradyrhizobium* spp. inoculation. *Plant Soil* 207:67-75.
- George T, Ben Bohlool B, Singleton PW (1987). *Bradyrhizobium japonicum*-environment interaction: nodulation and interstrain competition in soils along an elevational transect. *Appl. Environ. Microbiol.* 53:1113-1117.
- Gürtler V, Stanisich A (1996). New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. *Microbiology* 142:3-16.
- Hollis AB, Kloos WE, Elkan GH (1981). DNA:DNA hybridization studies of *Rhizobium japonicum* and related *Rhizobiaceae*. *J. Gen. Microbiol.* 123:215-222.
- Hunt PG, Matheny TA, Wolum AG (1988). Yield and N accumulation responses of late-season determinate soybean to irrigation and inoculation with various strains of *Bradyrhizobium japonicum*. *Soil Sci. Plant Anal.* 19:1601-1612.
- Jensen MA, Webster JA, Straus N (1993). Rapid identification of bacteria on the basis of Polymerase Chain Reaction-Amplified ribosomal DNA spacer polymorphism. *Appl. Environ. Microbiol.* 59:945-952.
- Jordan DC (1982). Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov. a genus of slow-growing, root nodules bacteria from leguminous plants. *Int. J. Syst. Bacteriol.* 32: 136-139.
- Josephson KL, Bourque DP, Bliss FA, Pepper IL (1991). Competitiveness of KIM 5 and VIKING 1 bean rhizobia: strain by cultivar interaction. *Soil Biol. Biochem.* 23: 249-253.
- Keyser HH and Cregan PB (1987). Nodulation and competition for nodulation of selected soybean genotypes among *Bradyrhizobium japonicum* serogroup 123 isolates. *Appl. Environ. Microbiol.* 53:2631-2635.
- Kimura M (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16:111-120.
- Kuykendall LD, Saxena B (1991). Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. *Can. J. Microbiol.* 38:501-505.
- Laguerre G, Mavingui M-R, Allard M-P, Charnay M-P, Louvrier P, Mazurier S-I, Rigottier-Gois L, Amarger N (1996). Typing of rhizobia by PCR DNA fingerprinting and PCR-Restriction Fragment Length Polymorphism analysis of chromosomal and symbiotic gene regions: application to *Rhizobium leguminosarum* and its different biovars. *Appl. Environ. Microbiol.* 62:2029-2036.
- Leblond-Bourget N, Decaris B (1996). La séquence de l'Espace Intergénique 16S-23S: méthode rapide et fiable d'identification des souches bactériennes. *Bull. Soc. Fr. Microbiol.* 11. (1):7-10.
- Mehta A, Rosato YB (2001). Phylogenetic relationships of *Xylolla fastidiosa* strains from different hosts, based on 16S rDNA and 16S-23S intergenic spacer sequences. *Int. J. Syst. Evol. Microbiol.* 51:311-318.
- Molouba F, Lorquin J, Willems A, Hoste B, Giraud E, Dreyfus B, Gillis M, de Lajudie P, Masson-Boivin C (1999). Photosynthetic bradyrhizobia from *Aeschynomene* spp. are specific to stem-nodulated species and form a separate 16S ribosomal DNA Restriction Fragment Length Polymorphism group. *Appl. Environ. Microbiol.* 65:3084-3094.
- Mpeperekki S, Makonese F, Wollum AG (1997). Physiological characterization of indigenous rhizobia nodulation *Vigna unguiculata* in zimbabwean soils. *Symbiosis* 22:275-292.
- Ndiaye MAF, Spencer MM, Gueye M (2000). Genetic variability in dinitrogen fixation between cowpea (*Vigna unguiculata* (L.) Walp.) cultivars determined using the nitrogen-15 isotope dilution technique. *Biol. Fertil Soils* 32:318-320.
- Normand P, Ponsonnet C, Nesme X, Neyra M, Simonet P (1996). ITS analysis of prokaryotes. *Molec. Microbial Ecology Manual.* 3. 4. 5:1-12.
- Obaton M (1999). Facteurs pédo-climatiques limitant la fixation biologique de l'azote chez les légumineuses. In: Mulongoy K., Gueye M. and Spencer DSC (eds) Biological nitrogen fixation and sustainability of tropical agriculture. IITA, pp 57-66.
- Perriere G, Gouy M (1996). WWW-query: an on-line retrieval system for biological sequence banks. *Biochimie* 78:364-369.
- Ponsonnet C, Nesme X (1994). Identification of *Agrobacterium* strains by PCR-RFLP analysis of pTi and chromosomal regions. *Arch. Microbiol.* 161:300-309.
- Sadowsky MJ, Tully RE, Cregan PB, Keyser HH (1987). Genetic diversity in *Bradyrhizobium japonicum* serogroup 123 and its relation to genotype-specific nodulation of soybean. *Appl. Environ. Microbiol.* 53:2624-2630.
- Saitou RR, Nei M (1987). A neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 44:406-425.
- Sarr B, Diouf O, Diouf M, Roy-Macauley H, Brou C (2001). Utilisation de paramètres agronomiques comme critères de résistance à la sécheresse chez trois variétés de niébé cultivées au Sénégal et au Niger. *Secheresse* 12(4):259-266.
- Sprent JI (1976). Nitrogen fixation by legumes subjected to water and light stresses. In: PS Nutman (eds) Symbiotic nitrogen fixation in plants. pp 405-420.
- Streeter JG (1994). Failure of inoculant rhizobia to overcome the dominance of indigenous strains for nodule formation. *Can. J. Microbiol.* 40:513-522.
- Thies JE, Bohlool BB, Singleton PW (1991). Subgroups of cowpea miscellany: symbiotic specificity within *Bradyrhizobium* spp. for *Vigna*

- unguiculata*, *Phaseolus lunatus*, *Arachis hypogaea*, and *Macroptilium atropurpureum*. Appl. Environ. Microbiol. 57:1540-1545.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25:4876-4882.
- Trinick MJ, Hadobas PA (1989). Effectiveness and competition for nodulation of *Vigna unguiculata* and *Macroptilium atropurpureum* with *Bradyrhizobium* from *Parasponia*. Can. J. Microbiol. 35:1156-1163.
- van Berkum P, Fuhrmann JJ (2000). Evolutionary relationships among the soybean bradyrhizobia reconstructed from 16S rRNA gene and internally transcribed spacer region sequence divergence. Int. J. Syst. Evol. Bacteriol. 50:2165-2172.
- Vincent JM (1970). A manual for the practical study of root nodule bacteria. In: International Biological Programme Handbook no. 5. Oxford, Blackwell Scientific Publication Ltd, pp 73-97.
- Vinuesa P, Rademaker JLW, de Bruijn FJ, Werner D (1998). Genotypic characterization of *Bradyrhizobium* strains nodulating endemic woody legumes of the canary islands by PCR-Restriction Length Polymorphism analysis of genes encoding 16S rRNA (16S rDNA) and 16S-23S rDNA Intergenic Spacers, Repetitive Extragenic Palindromic PCR genomic fingerprinting, and partial 16S rDNA sequencing. Appl. Environ. Microbiol. 64:2096-2104.
- Wade T, N'Doye IN, Braconnier S, Neyra M (2002). Diversity of *Bradyrhizobium* nodulating the cowpea (*Vigna unguiculata* (L.) Walp.) in Senegal (West Africa). In Finan TM, O'Brian MR, Layzell DB, Vessey JK, Newton W (eds) Nitrogen Fixation: Global Perspectives: Proceeding of the 13th International Congress on Nitrogen Fixation, Hamilton, Ontario, Canada. UK: CAB International, p 509.
- Weisburg WG, Barns SM, Pelletier QA, Lane DJ. (1991) 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173:697-703.
- Willems A, Coopman R, Gillis M (2001). Comparison of sequence analysis of 16S-23S rDNA spacer regions, AFLP analysis and DNA-DNA hybridations in *Bradyrhizobium*. Int. J. Syst. Evol. Microbiol. 51:623-632.
- Xu LM, Ge C, Cui Z, Li J, Fan H (1995). *Bradyrhizobium liaoningense* sp. nov., isolated from the root nodules of soybeans. Inter. J. Syst. Bacteriol. 45:706-711.
- Zablotowicz RM, Focht DD, Cannell GH (1981). Nodulation and N fixation of field-grown california cowpeas as influenced by well-irrigated and droughted conditions. Agron. J. 73:9-12.
- Zhang X, Nick G, Kaijalainen S, Terefework Z, Paulin L, Tighe SW, Graham PH, Lindström K (1999). Phylogeny and diversity of *Bradyrhizobium* strains isolated from the root nodules of peanut (*Arachis hypogaea*) in Sichuan, China. Syst. Appl. Microbiol. 22:378-386.
- Zhang Z, Kudo T, Nakajima Y, Wang J (2001). Clarification of the relationship between the members of the family *Thermomonosporaceae* on the basis of 16S rDNA, 16S-23S rRNA internal transcribed spacer and 23S rDNA sequences and chemotaxonomic analysis. Int. J. Syst. Evol. Microbiol. 51:373-383.