

4. Characterization of rhizobia nodulating tropical leguminous trees.

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Abstract

Leguminous trees are abundant in tropical regions of Africa, South America and Asia, where they are valued by foresters and farmers, especially in arid or semi-arid zones, as soil improvers and as sources of firewood and aerial forage. Most tropical leguminous trees form nodules and nitrogen is fixed in symbiosis with rhizobia.

Good characterisation of the rhizobia is necessary to inoculate tree nurseries efficiently. Earlier studies indicated that the bacteria able to form nodules on the roots of tropical trees belonged to the genera *Rhizobium* and *Bradyrhizobium*. Modern taxonomic techniques are powerful tools that can reveal the great diversity amongst tree rhizobia in tropical areas. They have shown that rhizobia nodulating *Leucaena leucocephala* and related species belong to different known species (*Rhizobium loti*, *R. tropici*, *R. galegae*, *Sinorhizobium fredii*) and unnamed species (*Rhizobium* spp. and *Bradyrhizobium* spp.). In Senegal we focussed on diverse rhizobia isolated from three Acacia species, *A. albida*, *A. senegal* and *A. tortilis* growing in different ecological regions of Senegal. Our results led to the identification of several *Bradyrhizobium* groups nodulating *A. albida*, three of which are new. Among the fast-growing strains isolated from nodules of diverse Acacia species, we found at least three new groups. One of them represents a new species of the amended genus *Sinorhizobium*, *S. teranga*. Another group of fast growers have been differentiated containing at least two new genomic species. Recent results on a great collection of new isolates confirmed definitely the enormous biodiversity of tree rhizobia.

Introduction

In arid and semi-arid tropical areas where severe erosion occurs, trees are of major importance for maintenance and regeneration of soil fertility, culture yield increase, cattle feeding and production of wood and fruits. In these respects, tropical leguminous trees play major economical and ecological roles, because of their ability to fix atmospheric nitrogen in symbiosis with bacteria belonging to the Rhizobiaceae family. Several authors had early



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demonstrated that nitrogen fixation can be improved by inoculating the seedlings in nurseries with selected bacterial strains, but until recently little was known about the phylogenetical relationships between these bacteria. Over the last 12 years, Rhizobiaceae taxonomy has been extensively revised. The aim of this communication is to summarize the current taxonomy of the rhizobia, especially those isolated from tropical trees, and to examine the status of rhizobia occurring in deep-soil environments.

Taxonomy of tree rhizobia.

For nearly a century, all legume-nodulating bacteria were defined as *Rhizobium*; modern taxonomy using molecular approaches revealed that bacteria inducing nodules on legumes could no longer be put in a single genus. Jordan (1) proposed to separate the fast- and slow-growing rhizobia in two genera, *Rhizobium* and *Bradyrhizobium*. *Rhizobium* was divided in three species (2,3): *R. leguminosarum* (comprising 3 biovars, *phaseoli*, *trifolii* and *viciae*), *R. meliloti* and *R. loti*. Since then two other genera, i.e. *Azorhizobium* and *Sinorhizobium* together with ten species have been proposed (for reviews see 4 and 5). This tendency will increase in the future since heterogeneous groups have been identified already within this classification and more "unknown plants" discovered in the world are investigated for nitrogen-fixing symbiotic bacteria. For example, temperate rhizobia were for a long time the only ones studied but in the last decade several reports on tropical rhizobia have been published (6,7,8,9,10,11,12). Contrary to what was long thought, i.e. tropical soils harboured only wide host-range slow-growing *Bradyrhizobium*, these authors showed the great diversity of both fast- and slow-growing nodule-inducing isolates in tropical zones and demonstrated that these belonged to one of four described genera, *Rhizobium*, *Bradyrhizobium*, *Azorhizobium* and *Sinorhizobium*.

Until now, taxonomical studies on bacteria nodulating tropical leguminous trees focused essentially on rhizobia associated with two major groups of trees: *Leucaena* (*Leucaena leucocephala*) and related species, and acacias (especially sahelian acacias).

Rhizobia associated with *Leucaena* spp.

The genus *Leucaena* Benth. consists of 50 species: the lectotype is *Leucaena leucocephala* (Lam.) de Wit, which is a tree originating from central America, and is widely used in tropical forestry because of its high nitrogen-fixing potential. Isolates from nodules of *L. leucocephala* have early been reported as fast-growing rhizobia (13) and to be not specific to *Leucaena* and other trees but able to nodulate other leguminous species such as *Vigna unguiculata* ssp. *sesquipedalis* (snakebean) (14). Rhizobia from *Leucaena leucocephala* were included within the new species *R. loti* (2); it was also demonstrated (15) that various bacteria capable of nodulating and fixing nitrogen with *Leucaena* form different DNA homology groups, most probably representing different species. More recently, studying *R. leguminosarum* biovar *phaseoli* type II strains, Martinez-Romero *et al.* (7) proposed (on the basis of a polyphasic approach using multilocus enzyme electrophoresis, DNA-DNA hybridization, ribosomal DNA organization, 16S rDNA sequence analysis and phenotypic characteristics) that strains nodulating both *Leucaena leucocephala* sp trees and *Phaseolus vulgaris* beans, constitute a novel species, *R. tropici*. This heterogeneity among strains that nodulate *Leucaena* species has been recently enlarged by reports of Zhang *et al.* (8) from Sudan, and Moreira *et al.* (9) from Brazil. *Leucaena* isolated from Sudan belong to four phenotypic clusters and do not group with reference strains (8). Strains isolated from *Leucaena* species from Amazonian forest and sub-humid tropical forest of the south East of Brazil showed a large heterogeneity in their polyacrylamide gel electrophoresis profiles of total cellular proteins (9): some strains group with a *R. loti* strain, three of them with *R. fredii* representative strains, one with *R. galegae*, and the other form at least four independent clusters. Furthermore, Gao *et al.* (10) recently confirmed that isolates from *Leucaena* spp. from China can constitute a new group, different from known species.

Rhizobia associated with sahelian acacias.

The genus *Acacia* Mill is composed of numerous species, i.e. 800-900 species (16), many consisting of trees or shrubs, widespread in savannas and arid regions of Australia, Africa, America and India.

Dreyfus and Dommergues (17), in Senegal, classified 13 *Acacia* species into three groups according to their effective nodulation response patterns with fast- and slow-growing rhizobial strains: the first group (including *A. albida*) nodulated effectively with slow-growing strains, the second (including *A. tortilis* and *A. senegal*) with fast-growing strains, the third (including *A. seyal*) with both slow- and fast-growing strains. However, the exact taxonomical position of those strains remained to be established.

Zhang *et al.* (8), by numerical analysis of 115 phenotypical characters, showed that 97 tree isolates (including 42 strains isolated from *A. senegal* in Sudan) can be separated into 19 different clusters.

In Senegal we characterized strains isolated from different *acacia* species, especially some of particular interest for the sahelian area, like *A. tortilis*, very tolerant to drought, *A. senegal*, which produces arabic gum of economic importance, and *A. albida*, which is traditionally used in agroforestry. Preliminary phenotypic studies (6) confirmed that fast-growing isolates from *Acacia* species belonged to *Rhizobium* and appeared to be closely related to *R. meliloti*. In order to define precisely these relationships, we adopted a strategy of polyphasic taxonomy to characterize more isolates from *Acacia* species. We collected nodules and soil samples in the vicinity of these leguminous trees, in different geographical and ecological zones of the country. As a first screening, the analysis of whole cell protein profiles by SDS-PAGE demonstrated the great diversity of fast- and slow-growing nodule inducing isolates in Senegal (18,19,20). Molecular taxonomy together with classical phenotypic methods were then used to characterize this diversity further.

Eighty senegalese isolates from fast-growing *Rhizobium* nodulating acacias (*A. tortilis* ssp. *raddiana* and *A. senegal*) belong to two main distantly related electrophoretic clusters. Auxanography, using commercial API galleries, revealed homogeneity of carbon source (147 different substrates) utilizations within each group and determinative traits between them both and amongst already described species. Representative strains from each cluster were used in genotypic and phylogenetic studies. DNA-rRNA hybridization and 12S rRNA gene sequencing revealed that the first cluster is phylogenetically related to *R. meliloti*-*R. fredii* subbranch and the second cluster to the *R. loti*, *R. huakuii*, *R. ciceri* subbranch. DNA-DNA cross-hybridizations values

between representative strains of the two clusters and of the described *Rhizobium* species lead to the conclusion that each of the two clusters observe a status of separate species. One of them was proposed as a new species of the emended genus *Sinorhizobium*, *S. teranga* (12). Isolates from acacias originating from North Africa grouped also in *S.teranga*. As the second cluster belonged to a large heterogenous one closely related with cluster 2 of Moreira *et al.* (9) and with atypical *R. loti* strains, we postponed nomenclature decision until more DNA:DNA hybridizations are done. This work is in progress.

All the slow-growing rhizobia characterized so far around the world have been placed in the genus *Bradyrhizobium* which appears now as enormous and heterogenous, comprising many clusters, the species status of which are not determined. Only two species have been proposed to date : *B. japonicum* (1), and *B. elkanii* (21). Links between all the clusters described remain to be done. Eighty-four slow-growing isolates from *A.albida* from different regions of Senegal, and from diferent ecotypes and depth in soil, were characterized by SDS-PAGE whole-cell proteins analysis (11). All those isolates belonged to the *Bradrhizobium* group and they exhibited a great deal of diversity: 90% of the isolates belonged to 6 gelectrophoretic clusters, 3 of which contain reference *Bradyrhizobium* strains, *B. japonicum* and *B. elkanii*, the other clusters containing only senegalese isolates. The remaining isolates (10%) either group in 4 small clusters or have separate positions in the dendrogram (5 strains). DNA:mRNA hybridizations using 16S or 23S-RNA from *B. japonicum* as probe confirmed that all senegalese *A. albida* isolates belong to the *Bradyrhizobium* - *Rhodospseudomonas* RNA branch. First results on 16S-rRNA gene sequencing of representatives of 5 gelectrophoretic clusters indicate that 4 of them constitute a branch as distantly separated from *B. japonicum* than from *Afipia*, *Blastobacter* and *Rhodopseudomonas*.

Other tree rhizobia

Rhizobial strains isolated from different genera of tropical trees (*Prosopis*, *Albizzia*, Australian acacias) are often included in characterization studies (6,8,9,12,14,22,23,24,25); these isolates group either within *Rhizobium* or within *Bradyrhizobium*, but their exact taxonomical status is rarely known.

The taxonomic position of different strains nodulating leucaena, sahelian acacias and other trees shown on Table 1.

Table 1: Taxonomic position of rhizobial strains nodulating tropical trees.

Bacterial species	<i>Leucaena</i> spp.	<i>Acacia</i> spp.	other trees
<i>Rhizobium leguminosarum</i>			
<i>Rhizobium loti</i>	+a,e	(+)a	
<i>Rhizobium tropici</i>	+c		
<i>Rhizobium etli</i>			
<i>Rhizobium galegae</i>	+e		
<i>Rhizobium huakuii</i>			
<i>Rhizobium ciceri</i>			
<i>Rhizobium</i> spp.	+a,e,h	+a,g	+e
<i>Sinorhizobium meliloti</i>			
<i>Sinorhizobium fredii</i>	-b/+e		
<i>Sinorhizobium teranga</i>		+g	+g
<i>Sinorhizobium saheli</i>			+g
<i>Bradrhizobium japonicum</i>		+f	
<i>Bradyrhizobium elkanii</i>		f	
<i>Bradyrhizobium</i> spp.	+e	+e,f	+e
<i>Azorhizobium caulinodans</i>			

- + = *nodulating strains*;
- (+) = *ineffective nodulating strains*;
- = *no nodulation*
- a = *Jarvis et al., (2) (polyphasic analysis)*;
- b = *Scholla and Elkan, (29) (polyphasic analysis)*;
- c = *Martinez-Romero et al., (7) (polyphasic analysis)*;
- d = *Zhang et al., (8) (phenotypic analysis)*;
- e = *Moreira et al., (9) (SDS-PAGE analysis)*;
- f = *Dupuy et al., (11) (SDS-PAGE analysis)*;
- g = *de Lajudie et al., (12) (polyphasic analysis)*;
- h = *Gao et al., (10) (phenotypic and DNA relatedness analysis)*.

II. Relationships between diversity and spatial distribution.

Desert and arid zones woody legumes are known to develop deep-phreatic roots systems. Significant rhizobial populations exist at the water-level (in the range of 5.5 to 13 m) under *Prosopis glandulosa* (mesquite) in the Sonoran desert (26,27). We observed similar profiles, at a greater depth (34 m), in Senegal, under *Acacia albida* (28), *A.tortilis* and *A.senegal* (unpublished results).

In Senegal, grouping of isolates from *A.albida* based on SDS-PAGE analysis of the whole-cell proteins was found correlated to geographical origin but independant of depth in soil (11). On the other hand, in Sonoran desert Waldon et l. (23), by numerical taxonomic analysis, and Thomas *et al.* (25) by ndvB hybridization of PstI-digested genomic DNA, suggested that surface and deep strains of mesquite rhizobia were clonally related, but that groups of strains from each soil depth could be distinguished. They hypothesized that the development of distinct rhizobial populations resulted from spatial and temporal separation of the surface and deep soil that formed a dry intermedial soil layer. The differences observed between these two field studies may be due to differences in the discriminatory power of the techniques used, or the various soil conditions. Here again, more studies are necessary to understand the ecology of tree rhizobia.

Conclusion

Taxonomic studies underline that there is a great diversity among rhizobia that nodulate tropical legume, and that these bacteria belong to different genera and species. Furthermore it appears that there is no specific "tree-rhizobia". The various studies carried out in different parts of the world predict that we are at the beginning of the story. Only a few symbionts of a few number of tree species have been investigated so far. Collaborations between groups working on this tropical rhizobia diversity is crucial for the future, in order to evidence relationships between organisms isolated here and there in the world, and to propose coherent classification and taxonomy. More investigations are in progress. We have already found in Senegal even more diversity by studying nodulation of other nitrogen fixing trees like *Prosopis africa*, *P. juliflora*, *Pterocarpus erinaceus* and *P. lucens* and also small legumes with potentialities for soil fertility maintenance or regeneration.

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