# Rhizobia associated with neotropical tree *Centrolobium tomentosum* used in riparian restoration

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**ABSTRACT**

*Centrolobium tomentosum* is a tropical legume tree indicated for functional and structural restoration of riparian areas. This study was conducted to characterize the rhizobia isolated from nodules of *C. tomentosum in situ* and to determine their capacity of renodulation, in an experimental area of land rehabilitation in the Rio Doce valley. Nodulation potential to inoculation with 2 selected fast-growing *Rhizobium* strains separately and a mixed inoculum of arbuscular mycorrhizal fungi was evaluated by the use of antibiotics resistance. Flood disturbance were observed not to affect renodulation by fast-growing strains. DNA fingerprinting RAPD (random amplified polymorphic DNA) and lipopolysaccharides (LPS) profiles were used to examine molecular relationships among field isolates, inoculants and reference strains. Maximal renodulation was exhibited by strain BHCBAb1 after 24 months after transplantation. *Centrolobium tomentosum* forms symbiosis with fast- and slow-growing *Rhizobium* strains, and it is suggested that their nursery culture could be improved by inoculation of selected strain under low nitrogen-input conditions.

**Keywords:** *Centrolobium tomentosum*; tree legume; *Rhizobium*; inoculation; renodulation; Doce River

Biological nitrogen fixation technologies have potentiality for ecological rehabilitation of degraded soils. Leguminous trees are used for revegetation because of their ability to form symbiotic associations with rhizobia and mycorrhizal fungi, which guarantee nitrogen supply, increase nutrients absorption and help in plant establishment in stress situations (Herrera et al. 1993). Leguminous trees are highly represented in the Brazilian Atlantic forest, and *Centrolobium tomentosum* Guill. ex Benth. (Araribá) (Fabaceae) is a typical gap species often growing after human disturbance, and showing pioneer characteristics on river margins. *Centrolobium tomentosum*, with relatively fast growth and good quality of wood is indicated for various uses and with high forestry potential in Brazil and is also used as shading tree in agroforestry systems for wood production in Bolivia.

Arbuscular mycorrhizal association (AM) with *C. tomentosum* can improve legume production (Marques et al. 2001), but the selection of compatible AM fungi and rhizobia for the legume is necessary in order to ensure the optimal economic and ecological potential, as reported for *Robinia pseudoacacia* (Tian et al. 2003).

The Parque Estadual do Rio Doce – PERD (36 113.6 ha) is one of the last Atlantic forest sites in the Minas Gerais State. The NW limit is naturally the Piracicaba River and the Doce River in the East. The Park limits with urban centers, agriculture areas and extensive eucalypt cultivation, carried out by the Agroforest Company "Companhia Agrícola Florestal (CAF)".

Tropical legumes trees can form nodules and fix nitrogen with several different rhizobia, fast-growing rhizobia as well slow-growing rhizobia; Moreira et al. (1993) reported one fast-growing isolate for *Centrolobium* sp. and other slow-growing isolate from *C. tomentosum* in Brazil. However, rhizobial symbiosis with this native tree legume has not been elucidated.

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Therefore, the objectives of this study were: (i) to characterize rhizobia associated with the tropical legume *C. tomentosum*, in terms of some physiological features and genetic relationship, and (ii) to monitor the persistence of selected inocula in soil conditions on the basis of growth and phenotypic characteristics. The goal was to obtain data that might be useful in practical production of *C. tomentosum* in forestry.

## MATERIAL AND METHODS

### Description of the study areas

The sampling sites were located in the southeast of the Brazilian State of Minas Gerais, a tropical zone with a yearly mean rainfall of 1200 mm and mean temperature of 20.3°C. The soil is fertile, slightly acid with pH of 5.77. The climate is humid tropical with summer rainfall (Köppen climatic type, Aw). The yearly mean rainfall takes place in the warmest months (October–March). The revegetated area can be found on the margins of the Doce River and was a formerly felled eucalypt site (*Eucalyptus grandis*).

Further samples were obtained in the State Park "Parque Estadual of Rio Doce" (PERD) – Atlantic Forest for comparison purposes. Within the PERD (semidecidual forest – Atlantic Forest), Minas Gerais, the strains used as inoculants and samples were obtained.

With the aim to compare them, other nodules were harvested in the Preserved area (near the Laboratory site) and Nurseries within the Preserved area; furthermore, a nearby site with vegetation dominated by grasses (Sítio Velho) was also sampled.

This study was carried out in a riparian area (0.65 ha) in the proximities of PERD, propriety of CAF – Santa Bárbara Company (Belgo Mineira Company), located in the Doce River margin. An old *Eucalyptus grandis* plantation was harvested. Predominant soil is Oxisol, pH was acid and OM (organic matter) content low (Marques et al. 2001). The study area (19°53'32'' S and 42°33'39'' W), 149 m high, in the Doce River basin presented a mean annual temperature of 20.3°C, the mean monthly maximum temperature was in January (22.9°C), and the minimum one in July (19.7°C) (Meteorologic Station CAF – Santa Bárbara). Field work was carried out from April 1996 to May 1999, when monthly visits were conducted to collect botanical material. The experimental design was a complete randomized block design with 3 replicates of each treatment. The plots (24 × 15 m) contained 40 plants with the spacing of 3 × 3 m among plants and rows; between blocks the space was 3 m. More details can be found in a previous publication (Marques et al. 2001). The treatments in each block were arranged in a completely randomized design and their respective control. The four treatments were: (1) inoculated BHCBAb1, (2) inoculated BHCBAb3, (3) inoculated BHCBAb1 + arbuscular mycorrhiza (AM) and (4) inoculated BHCBAb3 + AM.

The seedlings of *Centrolobium tomentosum* (*Papilionoideae*) were inoculated with BHCBAb1 and BHCBAb3 *Rhizobium* strain from Brazil (Marques et al. 2001), separately and with a mixed inoculum of arbuscular mycorrhizal fungi (AMF). Uninoculated plants were fertilized; inoculated plants were fertilized without nitrogen. Fast growing strains of *Rhizobium* sp. BHCBAb1 and BHCBAb3, isolated from the root nodules of *C. tomentosum* collected at PERD, were selected after being previously screened for its effectiveness under greenhouse conditions. The inoculum of *Rhizobium* sp. was grown on yeast extract mannitol broth (YEM; Vincent 1970), and 1 ml ($10^7$ CFU/ml) inoculant per plant was used (Somasegaram and Hoben 1985).

Plants were inoculated with AMF by placing, into each pot, 1 ml of suspension composed by 200 spores/ml of an equal mixture of 3 species: *Gigaspora margarita* Becker and Hall BHCBGi1, *Scutellospora heterogama* Walker and Sanders BHCBSc2 and *Glomus etunicatum* BHCBGE1 isolated from pot cultures with *Brachiaria decumbens* Stapf. The AMF strains used were obtained from the Biological Science Institute, Belo Horizonte (BHCB) culture collection.

### Nodule occupancy, isolates and strains

Nodules (30) from each treatment were collected randomly at 12, 16, 24 and 28 months after transplantation. Nodules were surface sterilized and plated on selective YEM agar medium. Rhizobia were from fresh nodules obtained by a standard method and using YMA medium (YMA; Vincent 1970). Single colonies were picked and checked for purity by repeated streaking. All strains were incubated at 28°C and maintained on YMA slants at –4°C, or in 20% (v/v) glycerol at –20°C. The denomination for each strain was as follows: BHCB (Belo Horizonte, Biological Sciences), Ab (refers

to the vegetal species), followed by a number. In the case of nursery seedlings nodules, the codification was e.g. BHCBAbN, and nodules from the adjacent grassland Sítio Velho were recorded indicating SV. The isolates and reference strains used are listed in Table 1.

A total of 75 rhizobia isolates of *C. tomentosum* roots were obtained from the revegetated riparian area, and isolates of nodules from 3 sites within the Rio Doce Park. All of the isolates were maintained in YMA broth at –4°C. Representative reference strains of *Rhizobium* and *Bradyrhizobium* species were used.

**Growth conditions**

A loopful of the crushed nodule was streaked across the surface of a petri dish containing YMA medium with 0.25 mg/l bromothymol blue (BTB) as pH reaction indicator. In instances where there was more than one type of colony from a single nodule, colonies were re-isolated on fresh plates. Isolates were characterized by colony appearance and color, extracellular polysaccharide (EPS) production, ability to change pH of the media, and confluent growth on YMA plates incubated at 28°C after 3, 5 and 7 day incubation.

The growth categories used were: (a) fast colonies (FG) after 3-day incubation, acid producers; (b) slow colonies (SG) after 7-day incubation, alkali producers.

Some representative isolates and strains, as well as the two inoculated strains, were checked by Gram staining (Vincent 1970), motility, NaCl tolerance, and genetic analysis (RAPD) with the object to differentiate them.

**Phenotypic features**

Standard methods for rhizobial phenotypic characterization (Chen et al. 1995) were used. The NaCl tolerance of some of the strains was tested by growing them in YEM containing 0, 1, 1.5 and 2% NaCl. The tests were done in YEM broth inoculated with 0.1 ml of culture in logarithm growth phase and isolates were grown in an orbital shaker at 150 rpm and 28°C. Tests were done in triplicate. Growth was estimated from the optical densities (OD) recorded at 600 nm every day.

The motility in 0.3% agar of the inoculated strains, 2 isolates and reference strain *Rhizobium tropici* BR322 (CIAT 899) was tested. The diameter of the migration zone of the strains was observed. Tests were performed in triplicate.

For the antibiotic resistance test, stock solutions of streptomycin (SIGMA), kanamycin (SIGMA), chloramphenicol (Sigma), ampicilin (Calbiochem) were filter-sterilized and aliquots of each antibiotic concentration were added aseptically to freshly prepared sterile YMA at 50°C to give the final concentrations of 2, 4, 8, 16, 32, 62, 125, 250 and 500 µg/ml. For rifampicin (SIGMA) the final concentrations were: 1, 2, 4, 9, 18, 37, 75, 150 µg/ml; and for tetracycline (SIGMA): 0.5, 1, 2, 4, 9, 19, 38, 76, 152 µg/ml. Control plates contained no antibiotics. Plates were inoculated with a cotton pad soaked in exponentially growing cultures in YEM, and incubated at 28°C for 3 days before scoring for growth. Selected 63 isolates and the 2 inoculated strains were used. Tests were performed in triplicate.

For a numerical taxonomy analysis a total of 24 characters were included in the analysis of the 22 selected FG isolates and inocula. The distance matrix was calculated by using the Jaccard coefficient and a dendrogram showing the relationships among the bacteria was constructed by using the UPGMA method.

**Lipopolysaccharide profiles**

A total of 28 rhizobial representative isolates were used for the study. LPS extraction was done according to De Maagd et al. (1988), and sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) was used. After electrophoresis, gels were stained for carbohydrates (LPS) with silver staining. Densitometric analysis was performed with GelCompar 4.1. Reference strains were: *Rhizobium tropici* CIAT 899 and *Rhizobium etli* CFN42.

**Assessment of genetic diversity**

In order to assess the differences between the two inoculated strains, a DNA fingerprinting AP-PCR (Arbitrarily Primer Polymerase Chain Reaction) using arbitrary primers (RAPD) was used to examine molecular relationships among field isolates, inoculants and reference strains. DNA extraction was according to Sá et al. (1993). Inoculants, two field isolates and 4 reference strains (*Rhizobium tropici* BR322, *Rhizobium etli* CFN42, *Bradyrhizobium japonicum* 29W

Table 1. Isolates and reference strains used in this study

| Strain/isolate | Host plant | Growth | Site/subsite | Geographic origin |
|---|---|---|---|---|
| **Inoculants** | | | | |
| BHCBAb1, BHCBAb3 | *Centrolobium tomentosum* | FG | PERD | Brazil |
| **Other rhizobial isolates** | | | | |
| BHCBAb4 to BHCBAb12 | *Centrolobium tomentosum* | FG, SG | revegetated area (control treatment) | Brazil |
| BHCBAb13 to BHCBAb31 | *Centrolobium tomentosum* | FG, SG | revegetated area (inoculated, BHCBAb1 treatment) | Brazil |
| BHCBAb32 to BHCBAb43 | *Centrolobium tomentosum* | FG, SG | revegetated area (inoculated, treatment BHCBAb3) | Brazil |
| BHCBAb44 to BHCBAb66 | *Centrolobium tomentosum* | FG, SG | revegetated area (inoculated, treatment BHCBAb1 + AM) | Brazil |
| BHCBAb67 to BHCBAb81 | *Centrolobium tomentosum* | FG, SG | revegetated area (inoculated, treatment BHCBAb3 + AM) | Brazil |
| BHCBAbL1 to BHCBAbL8 | *Centrolobium tomentosum* | SG | PERD (near Laboratory site) | Brazil |
| BHCBAbN1 to BHCBAbN25 | *Centrolobium tomentosum* | FG | PERD (Nursery) | Brazil |
| BHCBAbSV1 to BHCBAbSV9 | *Centrolobium tomentosum* | SG | adjacent grassland (Sítio Velho) | Brazil |
| **Reference strains** | | | | |
| *Rhizobium tropici* type B BR322 | *Phaseolus vulgaris* | FG | | Brazil |
| *Rhizobium etli* BR100260 | *Phaseolus vulgaris* | FG | | Brazil |
| *Bradyrhizobium japonicum* 29W | *Glycine max* | SG | | Japan |
| *Bradyrhizobium* sp. Semia 566 | *Glycine max* | SG | | Brazil |

PERD – Parque Estadual do Rio Doce, Brazil; FG – fast grow; SG – slow grow; AM – arbuscular mycorrhiza; isolates and strains are deposited at BHCB – Belo Horizonte Ciências Biológicas Collection

Table 2. Recovery of introduced fast and native slow-growing strains from root nodules of *Centrolobium tomentosum* harvested at 16 months (before Doce River flooding) and 24 months (after flooding)

| Treatment | Before flooding | | After flooding | |
|---|---|---|---|---|
| | N nodules[a] | (%) | N nodules[a] | (%) |
| Control | 60 | 40[b]/60[c] | 62 | 30[b]/70[c] |
| Inoculated BHCBAb1 | 85 | 65/35 | 46 | 37/63 |
| Inoculated BHCBAb3 | 37 | 86/14 | 42 | 50/50 |
| Inoculated BHCBAb1 + AM | 66 | 79/21 | 51 | 49/51 |
| Inoculated BHCBAb3 + AM | 34 | 59/41 | 47 | 17/83 |

[a]number of nodules examined; [b]percentage of fast-growing strains; [c]percentage of slow-growing strains; AM – arbuscular mycorrhiza

and Semia 566, belonging to the Laboratory Microorganism-Plant Interactions – UFMG collection) were used. Amplification was done according to Steindel et al. (1993). Four selected primers (Operon Technologies, Inc., Alameda, CA, USA) were tested: OPC04 (ACGGGACCTG), OPA04 (AGGACTGCTC), OPE07 (GTGTCAGTGG), OPA10 (TGGTCGGGTG). The DNA bands were silver-stained and photographed.

## RESULTS AND DISCUSSION

*Centrolobium tomentosum* nodules were of varied sizes, globoses, smooth and yellow. In this study, dual infection by both FG and SG bacteria was observed.

The total of 28 typical slow-growing (SG) isolates *Bradyrhizobium* sp. (*Centrolobium tomentosum*) and 47 typical fast-growing (FG) isolates *Rhizobium* sp. were obtained from *C. tomentosum* in the revegetated riparian area. Eight SG strains were obtained in the Preserved area, whereas nineteen FG and six SG strains from nurseries. In the cultivated plots FG rhizobia were dominant; however, in other sites within the Park, SG rhizobia dominated.

Slow growth bacteria occupied nodules collected from the Preserved area (PERD), the nodules of plants cultivated in the same area though manipulated in the greenhouse were predominantly occupied by FG ones (Table 1). The bulk of isolates were watery types with moderate to copious extracellular polysaccharide (EPS). Fast-growing isolates were wet translucent, and the majority of them acidified the culture medium (YMA). Slow-growing strains were less translucent and alkalinized the medium. Tested isolates were Gram-negative.

Table 3. Screening of salt-tolerant (NaCl) strains isolated from *Centrolobium tomentosum* nodules at PERD

| Concentrations (µg/ml) | 0.1 | | 1 | | 1.5 | | 2 | | 3 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Source | FG | SG | FG | SG | FG | SG | FG | SG | FG | SG |
| **Study area** | | | | | | | | | | |
| Control | 2[a] | | 2 | | 1 | | – | | – | |
| Inoculated BHCBAb1 | 2 | | 2 | | 1 | | – | | – | |
| Inoculated BHCBAb3 | 3 | | 2 | | 1 | | 1 | | 1 | |
| Inoculated BHCBAb1 + AM | 2 | | 2 | | 2 | | – | | – | |
| Inoculated BHCBAb3 + AM | | 2 | | 1 | | 1 | | 1 | | 1 |
| **Other sites** | | | | | | | | | | |
| Inoculants | + | | + | | + | | – | | – | |
| BHCBAbL1 | | 1 | – | | – | | – | | – | |

AM – arbuscular mycorrhiza; PERD – Parque Estadual do Rio Doce; FG – fast growth; SG – slow growth; [a]number of strains tolerant to this concentration; + = observed growth; – = no growth was detected after 7 days

Table 4. Motility of some strains isolated from *Centrolobium tomentosum* nodules at PERD

| Isolates | FG (mm) | SG (mm) |
|---|---|---|
| BHCBAb8 | | 5.25[a] |
| BHCBAbL1 | | 2.5 |
| **Inoculants** | | |
| BHCBAb1 | 25.35 | |
| BHCBAb3 | 23.83 | |
| **References** | | |
| *Rhizobium tropici* CIAT 899 | 31.25 | |

PERD – Parque Estadual do Rio Doce; FG – fast growth; SG – slow growth; [a]colony diameter in YEM (0.3% agar)

Renodulation (recovery of FG isolates) of *C. tomentosum* inoculated with *Rhizobium* is shown in Table 2. At 16 months BHCBAb1 was the more competitive of the two strains, in the dual inoculation treatment with AM, since a 39% increased FG isolates were recovery as compared with single inoculation. It seems that strain BHCBAb1 persisted in nodules after 28 months, though it could be found in the same proportion as slow-growing isolates. When the same strain is inoculated together with AM fungi, the proportion of nodules occupied by fast-growing bacteria is similar to slow-growing bacteria (Table 2).

After flooding, plants inoculated with *Rhizobium* strain BHCBAb3 showed a recovery of 50% of FG isolates and a lower percentage of nodules occupied by FG bacteria (17%) when co-inoculated with AM.

Uninoculated plants of the control treatment showed a higher number of nodules occupied by SG bacteria (60–70%), which are probably dominant in the cultivated land all the period long (Table 2).

After the flood, which took place in January 1997, a reduction in the percentage of FG strains in the plant nodules of all treatments was observed (Table 2). After the flood, the nodules showed a higher rate of rhizobia viability (percentage of viable nodules in relation to the total number), especially in treatments of inoculation with AM fungi (data not showed). The inoculum survival in nodules after the flood seemed to be depressed.

Some of the analyzed isolates showed phenotypic variability to tolerate different NaCl concentrations. The inoculated strains tolerated up to 1% and 1.5% NaCl, being sensitive to levels above 1.5%. However, in the treatment of inoculation with BHICBAb3 an isolate highly tolerant to NaCl was found. Slow-growing isolates also showed variability in the tolerance to NaCl levels related to the site of origin (Table 3). One isolate from PERD and one isolate from treatment 4 (inoculation with BHCBAb3 + AM) showed the lowest tolerance. All the isolates tested were motile in agar (0.3%). Fast-growing isolates and the inoculants showed more mobility than slow-growing isolates (Table 4).

Fast- and slow-growing isolates were different from one another in terms of their sensitivity

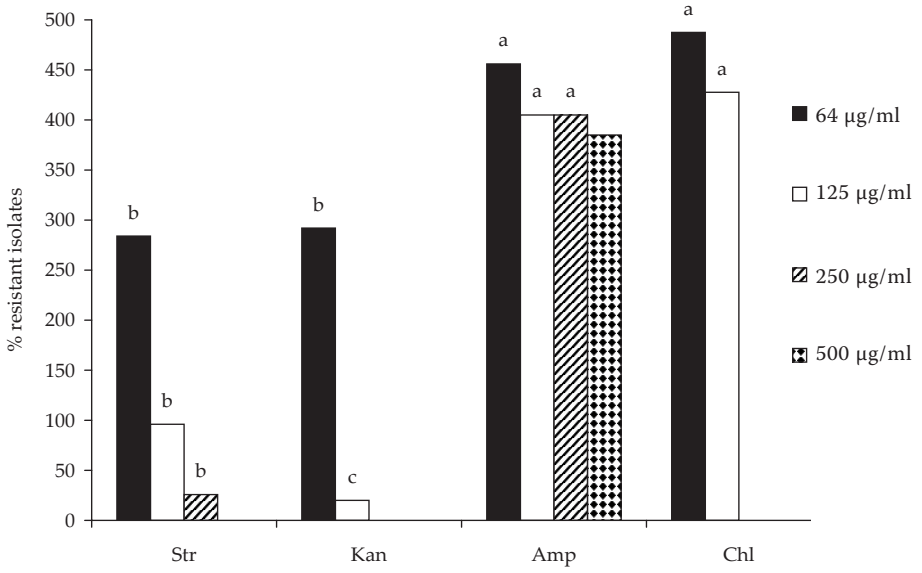


Figure 1. Comparison of intrinsic antibiotic resistance (IAR) of fast-growing rhizobial isolates from native legume tree *Centrolobium tomentosum* in Brazil (treatments with the same letter in both columns are not significantly different at 5%)

to different antibiotics. FG isolates showed little tolerance to tetracycline (Tet) and rifampicin (Rif) and high tolerance to streptomycin (Str), chloramphenicol (Chl), ampicilin (Amp) and kanamycin (Kan).

On the other hand, SG isolates showed little tolerance to Str and Kan (data not showed). The majority of FG isolates showed similar tolerance (64 µg/ml) to Str and Kan than the inoculated strains (Table 5). Some FG isolates resisted to more levels of Str, Kan and Rif. Only the inoculated strain BHICBAb3 and FG isolates in a lower proportion (< 50%) tolerated 4 µg/ml of tetracycline.

In this regard, sensitivity tests to Rif showed that inoculated strains and the FG isolates obtained from treatments co-inoculated with AM fungi were clearly more sensitive than the other isolates.

Inoculated bacteria and all the FG isolates, regardless of the treatment, showed a higher level of tolerance to Chl (Figure 1). On the other hand, the FG native population found in the control treatment tolerated lower Str levels, and were more sensitive to Tet, Amp, and Kan (Table 5). Tetracycline concentration (Table 5) showed the only difference in antibiotic resistance between the inoculated strains.

The same characteristics of inoculated strains were found in isolates from *C. tomentosum* nodules through phenotypic characteristics of bacteroids and antibiotic resistance under field conditions after 24 months (Table 5).

A cluster analysis was performed (Figure 2), which grouped the inoculants with another FG isolates in a cluster with *R. tropici* BR322 (CIAT899) and other FG isolates with *R. etli* BR100260.

The LPS profiles of rhizobial inoculants (Figure 3) and a sample of LPS profiles obtained (Figure 4) showed that the majority of profiles were typical *Rhizobium* profiles; moreover, some *Bradyrhizobium* profiles were also obtained. The densitometric analyses (data not showed) could differentiate the inoculated strains whereas they present a high similarity.

The RAPD patterns differentiated the two inoculated strains BHCBAb1 and BHCBAb3 (Figure 5). Among these primers, OPE7 showed the highest polymorphism. The SG isolate included in this analysis showed a similar pattern with that observed in *Bradyrhizobium japonicum* 29W and Semia 566, although this isolate showed some bands common with the inoculated strains. The tested isolates originated from nodules of treatment with BHCBAb1 + AM showed high similarity with this inoculated strain (Figure 5).

Table 5. Percentages of resistant fast-growing rhizobial isolates from native legume *Centrolobium tomentosum* and inoculants

| Study area | Streptomycin | | | | Kanamycin | | | Ampicilin | | | Rifampicin | | | | Chloram-phenicol | | Tetracycline | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 32[a] | 64 | 125 | 250 | 32 | 64 | 125 | 64 | 125 | 250 | 500 | 2 | 4 | 9 | 18 | 64 | 125 | ≤ 2 | ≥ 4 |
| Control | 80[b] | 40 | – | – | 75 | 25 | – | 80 | 60 | 60 | 40 | 100 | 66 | – | – | 100 | 75 | 66.6 | 33.3 |
| Inoculated BHCBAb1 | 100 | 60 | 30 | 20 | 90 | 60 | – | 100 | 100 | 100 | 100 | 100 | 66.6 | 33 | 33 | 100 | 77.7 | 83.3 | 16.7 |
| Inoculated BHCBAb3 | 100 | 50 | 25 | – | 100 | 100 | – | 100 | 100 | 100 | 100 | 100 | 50 | 50 | 25 | 100 | 100 | 66.6 | 33.3 |
| Inoculated BHCBAb1 + AM | 59 | 59 | 41 | 5.8 | 47 | 47 | – | 76 | 65 | 65 | 65 | 100 | 7 | 7 | – | 87.5 | 75 | 62.5 | 37.5 |
| Inoculated BHCBAb3 + AM | 75 | 75 | – | – | 60 | 60 | 20 | 100 | 80 | 80 | 80 | 100 | – | – | – | 100 | 100 | 50 | 50 |
| **Inoculants** | | | | | | | | | | | | | | | | | | | |
| BHCBAb1 | + | + | – | – | + | + | – | + | + | + | + | + | – | – | – | + | + | + | – |
| BHCBAb3 | + | + | – | – | + | + | – | + | + | + | + | + | – | – | – | + | + | + | + |

AM – arbuscular mycorrhiza; [a]µg/ml; [b]percentages of fast-growing strains tolerant to the antibiotic concentration; + = observed growth; – = absence of growth

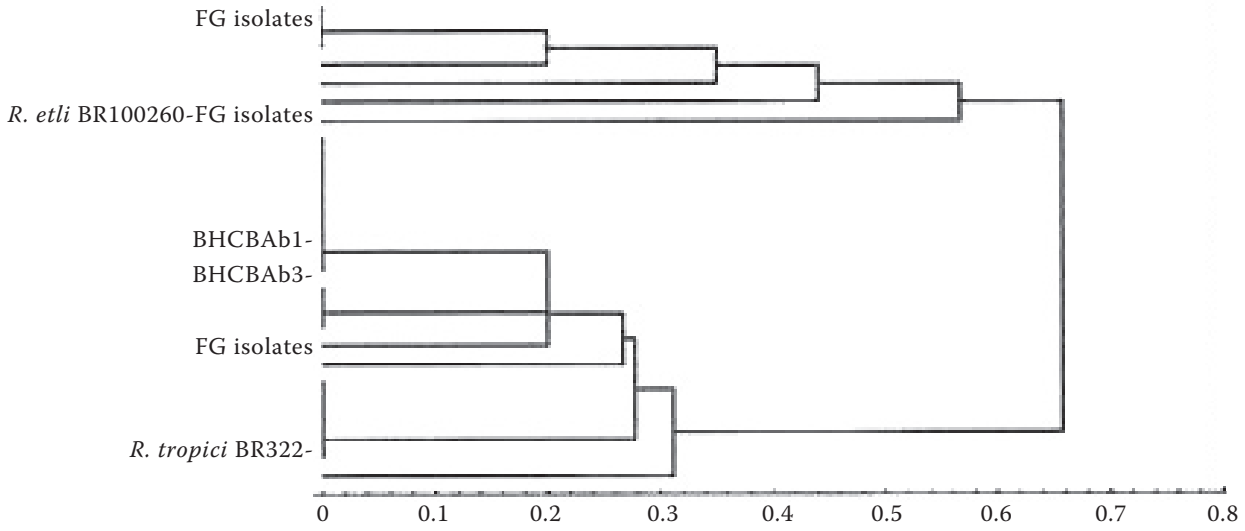


Figure 2. Dendrogram showing the phenotypic similarities among the isolates and strains. The UPGMA method was used for the cluster analysis

In a comparison of rhizobial species across the legume hosts summarized in Table 6, we hypothesized a lower diversity for *C. tomentosum*.

Like other tropical legume trees as *Prosopis* (Jenkins et al. 1987), *Acacia* (Odee et al. 2002), and *Albizia* spp. (Wang et al. 2006), *C. tomentosum* is nodulated by fast- and slow-growing strains. Thus, it appears that *Centrolobium* belongs to the group 2 proposed by Dommergues et al. (1984). This result is consistent with earlier reports (Moreira et al. 1993). Colonies were circular, convex, semi-translucent, and mucilaginous, usually 2–4 mm in diameter with 3 days on YMA.

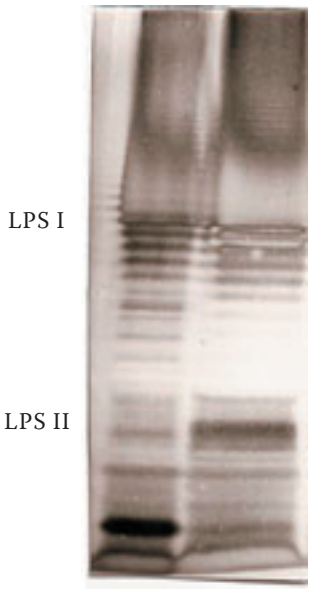


Figure 3. LPS electrophoretic profiles of *Centrolobium tomentosum* rhizobial strains: 1 – BHCBAb1; 2 – BHCBAb3

Acid and alkaline production in YMA medium was used as a tool to indicate the general character of rhizobia. Slow-growing rhizobia can produce alkaline while the fast-growing, acid-producing symbiotic bacteria are classified into *Rhizobium* and *Sinorhizobium.*

Manassila et al. (2007) showed that most of the effective tree legumes rhizobia isolated from trees such as *Acacia auriculaformis*, *A. mangium*, *Milletia leucantha*, *Pterocarpus indicus*, and *Xylia xylocarpa* in Thailand belong to genus *Bradyrhizobium*, which is a common symbiont of most tropical legumes. Wang et al. (2006) also found *Bradyrhizobium* in isolates from root nodules of *Acacia* spp., *Albizia* spp. and *Leucaena leucocephala* grown in the subtropical zones of China, but the isolates have a high diversity (*Agrobacterium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*). They confirmed that the legume species within the genera *Acacia*, *Albizia* and *L. leucocephala* may harbor some common rhizobial species, but they also have different preferences of the microsymbionts.

Jenkins et al. (1987) showed that the surface and phreatic soil environments selected for different rhizobial characteristics in another woody legume *Prosopis glandulosa*, since the low-nutrient phreatic zone with greater constancy in water content and temperature than the surface soil favors SG isolates. In contrast, Dupuy and Dreyfus (1992) reported SG rhizobia in surface and deep soil isolates from *Acacia albida*. Heal and Ineson (1984) also showed that the competition in stable environments selects for SG organisms. In line with those reports, this study found the majority of SG isolates (indigenous

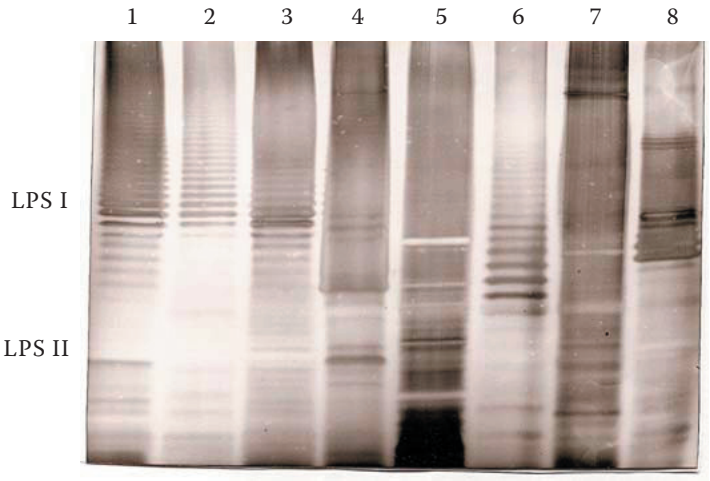


Figure 4. Strains: 1 – BHCBAb1; 2 – BHCBAb9; 3 – BHCBAb31; 4 – BHCBAb10; 5 – BHCBAb50; 6 – BHCBAb67; 7 – BHCBAb68; 8 – BHCBAbN2

rhizobia) in the preserved site (more stable than the revegetated riparian site); it is suggested that in the deep soil of the revegetated area SG isolates dominate, since the number of SG isolates increased after flooding.

On the other hand, the surface environment (more heterogeneous or patchy environment with spatial and temporal variation in soil nutrient availability, water content, population density and activity of the other soil biota) should support both SG and FG organisms, although FG are predicted to have the advantage of being favored in conditions of climate adversity and a diversity of microsites capable of supporting SG as well as FG strains may be available in the surface rooting zone (Jenkins et al. 1987). In this study, fast growing isolates were dominant in revegetated site and nursery seedlings (disturbing soils) reinforcing this hypothesis. The diversity within the rhizobial population could offer them advantages to adapt to different environments for survival and nodulation.

Results suggest that inoculated *Rhizobium* strains renodulated the *Centrolobium tomentosum* plants under field conditions after 24 months, since the same characteristics of inoculated strains were found in isolates from *C. tomentosum* nodules through phenotypic characteristics of bacteroids and antibiotic resistance.

In this study we showed that dual inoculation increased the renodulation (recovery of the inoculated strains) in *C. tomentosum*, contrary to the reports with *Acacia nilotica* (50% decrease in reno-

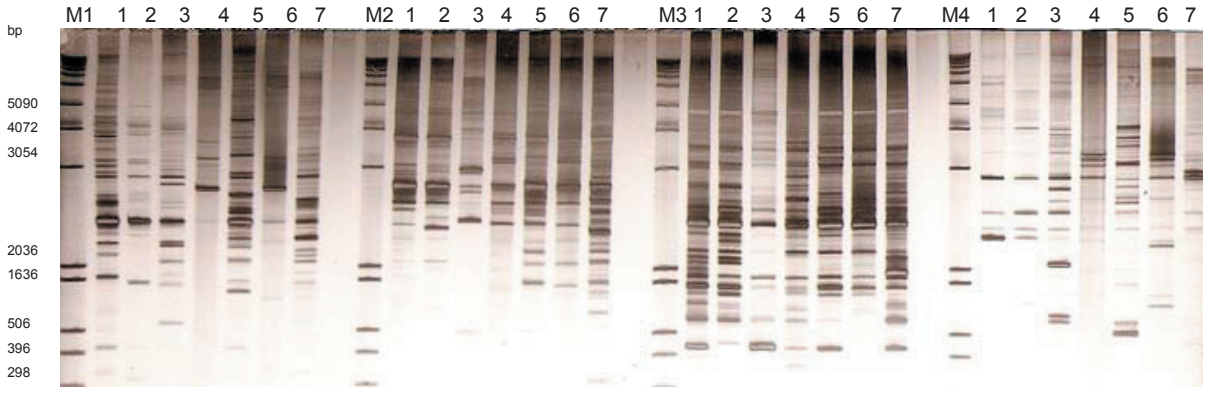


Figure 5. Example of RAPD banding patterns obtained for the inoculated strains, two *Centrolobium tomentosum* isolates, reference *Rhizobium* sp. and soybean nodulating strains. M: DNA molecular marker (1 μg). Strains: 1 – *Bradyrhizobium* sp. Semia 566 (soybean); 2 – *Bradyrhizobium japonicum* 29W (soybean); 3 – *Rhizobium tropici* BR322 (CIAT899) (bean and *L. leucocephala*); 4 – BHCBAb1 (*C. tomentosum*); 5 – BHCBAb3 (*C. tomentosum*); 6 – BHCBAb65 (*C. tomentosum*); 7 – BHCBAbL2 (*C. tomentosum*). Primers used: OPC4, OPA4, OPE7 and OPA10, respectively

Table 6. Distribution of rhizobial species associated with tree legume genera

| Legume genus | Strain number | Rhizobial species |
|---|---|---|
| *Acacia*[a] | 16 | 3 |
| *Albizia*[a] | 31 | 8 |
| *Centrolobium*[b] | 75 | 2 |
| *Leucaena*[a] | 13 | 6 |
| Total | 140 | 19 |

[a]Wang et al. (2006); [b]preliminary results (this study)

dulation) (Lal and Khanna 1993). When a legume having a compatible indigenous *Rhizobium* population in soil is inoculated, the inoculated bacteria have to compete with them, since nitrogen-fixing efficiency and competitive ability of a strain are not necessarily correlated (Tas et al. 1996).

High salinity is one of the common stresses faced by soil bacteria in tropical and subtropical regions. Salt-tolerant rhizobial strains can be utilized for reclamation of saline and alkaline lands and for inoculant formulation in saline soils (Mnasri et al. 2007). The majority of the isolates showed lower growth in 1.5% NaCl, except for one isolate, but rhizobia exhibit a large range of sensitivities to salinity. In this sense, the inoculated strains could not be differentiated.

Resistance to antibiotics (this study) and the serology test (Marques et al. 2001) were in agreement with conclusions based on the persistence of the inoculated strains in the revegetated area. All the isolates showed Amp tolerance. Except for Amp, isolates were sensitive to most antibiotics tested although there was some variability in antibiotic resistance. Slow-growing strains exhibited higher levels of resistance to Tet and Rif than the fast-growing strains (Kremer and Peterson 1982) and results obtained in this study are in accordance.

The *C. tomentosum* isolates tolerate high levels of Chl and Amp and lower levels of Str than other isolates from legumes. Based on antibiotic resistance patterns, the majority (> 75%) of FG isolates from treatments 3 and 4 showed the same tolerance (Rif at 2 µg/ml, Chl at 125 µg/ml) than the inoculated strains and no other isolates were resistant to the same high concentration. Fast growing isolates from treatment 1 showed > 60% of recovery of the inoculated strain (BHCBAb1), whereas a > 50% recovery of inoculated strain BHCBAb3 was showed from isolates of treatment 2. It seems that strain BHCBAb3 recovered less frequently than the other strain, except for Chl results.

In tree legumes, renodulation over long periods has proved to be poor. Makatiani and Odee (2007) showed that it is better to inoculate with effective indigenous than exogenous rhizobia *Sesbania sesban* plants.

Even with a large indigenous population of compatible rhizobia, it is still possible to obtain responses to inoculation provided the inoculated strains are competitive and highly effective.

This study shows the ability of introduced BHCBAb1 *Rhizobium* to survive and renodulate in natural conditions. Strain BHICBAb1 seems to persist in the field trial since the majority of the tested isolates originated from nodules of treatment with BHICBAb1 showed high similarity with this inoculated strain and were grouped in the same cluster, presenting high similarity in LPS patterns.

In this paper, *C. tomentosum* show the renodulation potential of introduced native FG strains after 2 years. These results indicate the importance of selecting strains for forestry production since indigenous microbes are more suitable for this (Rao and Tak 2001). However, many practical problems such as time of inoculation and the AM species colonizing *C. tomentosum* roots need to be investigated.

All these observations suggest that *C. tomentosum* must be inoculated with FG strains for revegetation purposes. Thus, further studies are needed to clarify the diversity of rhizobia associated with *Centrolobium* species, as well as to confirm the taxonomic position of the isolates. Potential novel species could be found in the nodule isolates originated from *Centrolobium*. Also, the use of AM compatible species to select rhizobial strains for this legume is indicated.

## REFERENCES


Chen W.X., Wang E., Wang S., Li Y., Chen X., Li Y. (1995): Characteristics of *Rhizobium tianshanense* sp. nov., a moderately and slowly growing root nodule bacterium isolated from an arid saline environment in Xinjiang, P.R. China. Int. J. Syst. Bacteriol., *45*: 153–159.

De Maagd R.A., Van Rossum C., Lugtenberg B.J.J. (1988): Recognition of individual strains of fast-growing rhizobia by using profiles of membrane proteins and lipopolysaccharides. J. Bacteriol., *170*: 3782–3785.

Dommergues Y.R., Diem H.G., Gauthier D.L., Dreyfus B.L., Cornet F. (1984): Nitrogen-fixing trees in the tropics: potentialities and limitations. In: Veeger C., Newton W.E. (eds.): Advances in Nitrogen Fixation

Research. Martinus Nijhoff/Dr. W. Junk Publishers, Wageningen: 164–167.

Dupuy N.C., Dreyfus B.L. (1992): *Bradyrhizobium* populations occur in deep soil under the leguminous tree *Acacia albida*. Appl. Environ. Microbiol., *58*: 2415–2419.

Heal O.W., Ineson P. (1984): Carbon and energy flow in terrestrial ecosystems: relevance to microflora. In: Klug M.J., Reddy C.A. (eds.): Current Perspectives in Microbial Ecology. American Society for Microbiology, Washington D.C.: 394–404.

Herrera M.A., Salamanca C.P., Barea J.M. (1993): Inoculation of woody legumes with selected arbuscular mycorrhizal fungi and rhizobia to recover desertified Mediterranean ecosystems. Appl. Environ. Microbiol., *59*: 129–133.

Jenkins M.B., Virginia R.A., Jarrell W.M. (1987): Rhizobial ecology of the woody legume mesquite (*Prosopis glandulosa*) in the Sonoran Desert. Appl. Environ. Microbiol., *53*: 36–40.

Kremer R.J., Peterson H.L. (1982): Nodulation efficiency of legume inoculation as determined by intrinsic antibiotic resistance. Appl. Environ. Microbiol., *43*: 636–642.

Lal B., Khanna S. (1993): Renodulation and nitrogen fixing potential of *Acacia nilotica* inoculated with *Rhizobium* isolates. Can. J. Microbiol., *39*: 87–91.

Makatiani E., Odee D.W. (2007): Response of *Sesbania sesban* (L.) Merr. to rhizobial inoculation in an N-deficient soil containing low numbers of effective indigenous rhizobia. Agrofor. Syst., *70*: 211–216.

Manassila M., Nuntagij A., Kotepong S., Boonkerd N., Teaumroong N. (2007): Characterization and monitoring of selected rhizobial strains isolated from tree legumes in Thailand. Afr. J. Biotechnol., *6*: 1393–1402.

Marques M.S., Pagano M.C., Scotti M.R.M. (2001): Dual inoculation of woody legume (*Centrolobium tomentosum*) with rhizobia and mycorrhizal fungi in southeastern Brazil. Agrofor. Syst., *52*: 107–117.

Mnasri B., Mrabet M., Laguerre G., Elarbi M., Mhamdi A.R. (2007): Salt-tolerant rhizobia isolated from a Tunisian oasis that are highly effective for symbiotic $N_2$-fixation with *Phaseolus vulgaris* constitute a novel biovar (bv. *mediterranense*) of *Sinorhizobium meliloti*. Arch. Microbiol., *187*: 79–85.

Moreira F.M.S., Gillis M., Pot B., Kersters K., Franco A.A. (1993): Characterization of rhizobia isolated from different divergence groups of tropical *Leguminosae* by comparative polyacrylamide gel electrophoresis of their total proteins. Syst. Appl. Microbiol., *16*: 135–146.

Odee D.W., Haukka K., McInroy S.G., Sprent J.I., Sutherland J.M., Young J.P.W. (2002): Genetic and symbiotic characterization of rhizobia isolated from tree and herbaceous legumes grown in soils from ecologically diverse sites in Kenya. Soil Biol. Biochem., *34*: 801–811.

Rao A.V., Tak R. (2001): Influence of mycorrhizal fungi on the growth of different tree species and their nutrient uptake in gypsum mine spoil in India. Appl. Soil Ecol., *17*: 279–284.

Sá N.M.H., Scotti M.R.M., Paiva E., Franco A.A., Döbereiner J. (1993): Selection and characterization of *Rhizobium* strains stable and efficient in the fixation of nitrogen in bean (*Phaseolus vulgaris* L.). Rev. Microbiol., *24*: 38–48.

Somasegaram P., Hoben H.J. (1985): Methods in Legume-*Rhizobium* Technology. NIFTAL, Hawai.

Steindel M., Dias Neto E., Menezes C.L.P., Romanha A.J., Simpson A.J.G. (1993): Random amplified polymorphic DNA analysis of *Trypanosoma cruzi* strains. Mol. Biochem. Parasitol., *60*: 71–80.

Tas E., Leinonen P., Saano A., Rasanen L.A., Kaijalainen S., Piippola S., Hakola S., Lindstrom K. (1996): Assessment of competitiveness of rhizobia infecting *Galega orientalis* on the basis of plant yield, nodulation, and strain identification by antibiotic resistance and PCR. Appl. Environ. Microbiol., *65*: 529–535.

Tian C., He X., Zhong Y., Chen J. (2003): Effect of inoculation with ecto- and arbuscular mycorrhizae and *Rhizobium* on the growth and nitrogen fixation by black locust, *Robinia pseudoacacia*. New For., *25*: 125–131.

Vincent J.M. (1970): A Manual for the Practical Study of the Root Nodule Bacteria. Blackwell Scientific Publications, Oxford.

Wang F.Q., Wang E.T., Zhang Y.F., Chen W.X. (2006): Characterization of rhizobia isolated from *Albizia* spp. in comparison with microsymbionts of *Acacia* spp. and *Leucaena leucocephala* grown in China. Syst. Appl. Microbiol., *29*: 502–517.

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