Efficient bean nodulating Rhizobium strains, isolated from different Brazilian cerrado soils, were characterized by RAPD. This study showed great genetic heterogeneity among *R. tropici* and *R. leguminosarum* bv. *phaseoli* strains and allowed the constitution of genetic clusters, besides indicating the most suitable primers for this characterization. The groups of genetically distinct strains can be used in competitiveness studies to select appropriate *Rhizobium* strains for bean inoculation in cerrado soils.

**Key words**: *Rhizobium tropici*, *Rhizobium leguminosarum* bv. *phaseoli*, bean plants, cerrado soils

**INTRODUCTION**

Beans are an important staple crop in the cerrado region of Brazil. In 1990, an area of 801,674 hectares was under cultivation, and the production was 431,628 tons (10). However, the 538 kg/ha average production is considered low. The majority of the bean production in this region takes place on small farms where the use of fertilizers, especially nitrogen, is low. In this context, biological nitrogen fixation assumes an increasing important role. However, inoculating beans with effective strains of *Rhizobium* is not always successful due to several factors, including the competition that occurs between the inoculated strains and those already present in the soil which generally have low effectiveness (6, 9). Competitiveness studies that have been conducted to select bacterial genotypes have been limited due to problems in identifying strains that nodulate this legume. In those organisms, similar antigenic structures (cross-reactivity) are common as seen through serologic tests or classic identification methods (11,14).

However, the development and increased availability of molecular biology techniques have made it possible to obtain information regarding the genomic organization and diversity of rhizobia populations in different soils (1, 3, 7). Genomic DNA fingerprinting using random amplification of polymorphic DNA (RAPD) has been found to be useful in differentiating between very closely related bacteria. The RAPD technique is a polymerase chain reaction (PCR) based assay that was developed to detect polymorphisms in genomic DNA (19, 20). Besides being simpler and cheaper, this method is as effective as the more labor intensive RFLP for establishing genetic relationships and identifying *Rhizobium* strains (3,17).

In this study, effective native *Rhizobium* strains isolated from bean plants cultivated in different cerrado regions of Brazil were characterized using genomic patterns obtained through RAPD with the objective of identifying genetic groupings (clusters) of the strains.
MATERIALS AND METHODS

Culture media, growth conditions and maintenance of Rhizobium strains: The 89 strains of Rhizobium used in this study are listed together with their origin and the chemical characteristics of the soil from which they were obtained (Table 1). These strains were previously phenotypically characterized as Rhizobium tropici and R. leguminosarum bv. phaseoli and selected because of their high level of effectiveness in Leonard jars with their homologue host (9). The strains were grown in liquid manitol medium yeast extract (YM) at 29°C and maintained in the same medium containing agar (YMA) at 4°C (18).

DNA extraction and RAPD analysis: Bacterial DNA genomic was extracted using the method described in Sá et al. (13). DNA amplification reactions of the strains were conducted in a Perkin-Elmer 9600 thermocycler. The amplification reaction was composed of 40 cycles with each cycle having the following steps: denaturation at 94°C for 15 sec., annealing at 35°C for 30 sec. and polymerization at 72°C for one min. An additional cycle for extension was conducted at 72°C for 7 min. Each reaction mixture contained 25µl composed of the following: 2.5 mM of MgCl₂, 10 mM of Tris KCl, 0.01 mM of each DNTP (DATP, DCTP, DGTP, DTTP), 1U of Taq DNA polymerase, 0.4 µM of primer, 11µl of H₂O and 25 ng of DNA. The following 27 primers from the Operon Kit (Operon Technologies Inc.) were tested: OPA-05, OPA-08, OPA-09, OPE-02, OPE-03, OPE-04, OPE-05, OPE-06, OPE-04, OPF-04, OPF-17, OPG-07, OPG-14, OPM-07, OPM-04, OPO-09, OPO-11, OPO-14, OPO-16, OPP-06, OPP-08, OPP-17, OPP-18, OPS-17, OPW-04, OPW-10, OPW-15. Amplification products were separated using electrophoresis in 1.2% agarose gels. The gel was visualized by ethidium bromide staining (ETBR) and then photographed. The ETBR stained bands were considered present (1) or absent (0) and only intense, reproducible bands were considered. This data was used to determine the genetic distance between the strains. Average linkage (UPGMA - unweighted pair group method with averages) dendrograms were generated using the Statistica program (Copy Righ STATSOFT Inc. 1993 - UNMN 06/74). Principal component analysis (PCA) was also performed with the specific combined data between more effective strains of the two species studied (9), in relation to the tested random primers, using the option factor analysis. In this method, 3 independent consecutive factors are extracted and each consecutive factor maximizes the variability not captured by the preceding factor.

RESULTS AND DISCUSSION

Random primer genomic DNA amplification of Rhizobium strains associated with beans produced patterns with high levels of polymorphism. Fingerprints were strain-specific with certain primers as can be seen in Figs. 1 and 2. Data processing generated dendrograms (Figs. 3 and 4) that suggested 6 or more major divisions based on the genetic relationship between the Rhizobium strains of the evaluated species. High degrees of

variation were observed in the *R. tropici* (6-42%) (Fig. 3) as well as in the *R. leguminosarum* bv. *phaseoli* strains (5-40%) (Fig. 4). The constituted groups formed preferentially with strains from the same location (Table 1), indicating the selection of genetically related sub-populations in relation to the soil characteristics of each sample site. Also within each site, specially Brasilia and Janaúba, high levels of genetic variation were observed. Using the random primer OPF-04, as shown in Fig. 1, DNA amplification of *R. tropici* strains from Brasilia had intensely polymorphic patterns even though the strains had common bands. A dendrogram generated (data not shown) from the results showed genetic variation levels of 6 to 44% between


**Figure 3.** Dendrograms showing genomic relationship between 52 strains of *R. tropici* isolated from cerrado soil as determined by RAPD analysis with 13 different arbitrary primers. (1 and #) – Brasilia cerrado soil B, (2) – Sete Lagoas cerrado soil (A, B and C), (3) – Brasilia cerrado soil A, • commercial strains from different origins.

**Figure 4.** Dendrograms showing genomic relationship between 37 strains of *R. leguminosarum* bv. *phaseoli* isolated from cerrado soil as determined by RAPD analysis with 13 different arbitrary primers. (4) – Sete Lagoas cerrado soil (A, B and C), (5) – Janaúba cerrado soil, • commercial strains from different origins.
Table 1. Identification, origin of 52 effective strains of *R. tropici* and 37 *R. leguminosarum* bv. *phaseoli* and the chemical soil analysis of the isolation sites.

<table>
<thead>
<tr>
<th>Rhizobium strains</th>
<th>Specie</th>
<th>Site/sub-site</th>
<th>Chemical soil analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>(26)FJ1.2, (27)FJ1.22, (28)FJ2.1, (29)FJ2.2, (30)FJ2.4, (31)FJ2.12, (32)FJ2.15, (33)FJ2.21, (34)FJ1.22</td>
<td><em>R. tropici</em></td>
<td>Brasilia (B)</td>
<td>4.7 1.02 0.69 26 1.1</td>
</tr>
<tr>
<td>(69)SLA1.2, (71)SLA1.5, (72)SLA1.6, (73)SLA2.2, (74)SLA2.3, (75)SLA3.2,</td>
<td><em>R. tropici</em></td>
<td>Sete Lagoas (A)</td>
<td>5.6 0.05 3.10 32 7.0</td>
</tr>
<tr>
<td>(68)SLA1.1, (70)SLA1.4, (76)SLA3.13</td>
<td><em>R. leguminosarum</em> bv. <em>phaseoli</em></td>
<td>Sete Lagoas (B)</td>
<td>5.8 0.00 3.74 112 12</td>
</tr>
<tr>
<td>(65)SLBR2.1, (66)SLBR3.12, (67)SLBR3.13</td>
<td><em>R. tropici</em></td>
<td>Sete Lagoas (B)</td>
<td>5.8 0.00 3.74 112 12</td>
</tr>
<tr>
<td>(60)SLB3.3, (61)SLB3.12, (62)SLB3.13, (63)SLB7.15, (64)SLB10.6</td>
<td><em>R. leguminosarum</em> bv. <em>phaseoli</em></td>
<td>Sete Lagoas (C)</td>
<td>6.0 0.05 5.84 42 11</td>
</tr>
<tr>
<td>(81)SLP2.13, (82)SLP3.3, (85)SLP4.9, (87)SLP5.9</td>
<td><em>R. tropici</em></td>
<td>Sete Lagoas (C)</td>
<td>6.0 0.05 5.84 42 11</td>
</tr>
<tr>
<td>(77)SLP1.3, (78)SLP1.6, (79)SLP2.2, (80)SLP2.10, (83)SLP4.4, (84)SLP4.7, (86)SLP5.8, (88)SLP19.7, (89)SLP24.1</td>
<td><em>R. leguminosarum</em> bv. <em>phaseoli</em></td>
<td>Janaúba</td>
<td>6.2 0.00 4.82 1.83 13</td>
</tr>
<tr>
<td>(8)SEMIAV23, (48)CIAT899, (55)BR814, (56)BR818, (57)BR855, (58)BR860</td>
<td><em>R. tropici</em></td>
<td>different origin</td>
<td></td>
</tr>
<tr>
<td>(49)BR365, (50)BR376 (52)BR10.026, (53)BR10.028, (54)BR576, (59)SEMI4077</td>
<td><em>R. leguminosarum</em> bv. <em>phaseoli</em></td>
<td>different origin</td>
<td></td>
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</table>

The strains. It was possible to detect at least 4 clusters among the analyzed strains from Brasilia and in sub-sites within the region the genetic variation levels were from 9-40% (subsite A) and 7-52% (subsite B). DNA amplification profiles of *R. leguminosarum* bv. *phaseoli* strains from Janaúba showed that in addition to common bands, these strains had bands that allowed for the differentiation of individual strains. The dendrogram resulting from the use of different primers indicated at least 4 distinct clusters and genetic variation of 6-46%. Using OPA-02 primer, *R. tropici* (Fig. 2; lines 1-6 and 18-21) and *R. leguminosarum* bv. *phaseoli* strains (Fig. 2; lines 7-17 and 22-30) from Sete Lagoas produced patterns with high polymorphism, reflected in a dendrogram which showed genetic variation of 6-37%. In samples from the Sete Lagoas region, clusters formed initially by subsite and within them, by *Rhizobium* species. Subsites of this region had similar levels of genetic variation: subsite A 9.5-46.5%; subsite B 16-48%; subsite C 4.5-40%.

<table>
<thead>
<tr>
<th>Chemical soil analysis</th>
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<tbody>
<tr>
<td>pH</td>
</tr>
<tr>
<td>H2O mE/100</td>
</tr>
<tr>
<td>5.2 0.18 3.32 84 7.5</td>
</tr>
</tbody>
</table>
Based on the PCA diagrams (Figs. 5a and b), 2 groups can be distinguished among the most effective strains according to origin of the two species studied (R. tropici and R. leguminosarum bv. phaseoli), which confirms the trend observed in the dendrogram analysis. PCA also verified that the R. leguminosarum bv. phaseoli strains had more variation among themselves than the R. tropici did, probably due to the former’s higher genetic plasticity (12, 13). In addition, this analysis identified adequate primers for strain identification.

Result of the present study confirm the high degree of genetic diversity found using a variety of methods in bean nodulating Rhizobium strains from Mexican (8) and Brazilian soils (6, 13). In general, strains within the Rhizobium genus are fast growing, such as there studied here that nodulate beans. They appear to be more taxonomically diverse and ecologically adaptable than the slower growing strains from other genera like Azorhizobium and Bradyrhizobium These differences probable occur because in the slow growing strains the genetic information, in general, is contained in the chromosomal DNA, while in fast growing strains many genes, including those related to biological nitrogen fixation are located on plasmids which confers dynamic qualities to the genome (5, 12). These characteristics favor recombination between genotypes of the Rhizobium population present in soils (14). Even though new bean nodulating Rhizobium species have been defined recently (4, 15), additional species should be defined to reflect on the taxonomic level, the large diversity of these bacteria. For now, this study established groups of genetically distinct strains that can be used in competitiveness studies to select appropriate strains for inoculation in cerrado region bean plants.

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**REFERENCES**


