

# Genetic diversity of rhizobia associated with common bean (*Phaseolus vulgaris* L.) grown under no-tillage and conventional systems in Southern Brazil

G. Kaschuk<sup>a,b,1</sup>, M. Hungria<sup>a,\*</sup>, D.S. Andrade<sup>c</sup>, R.J. Campo<sup>a</sup>

<sup>a</sup> *Embrapa Soja, Cx. Postal 231, 86001-970, Londrina, PR, Brazil*

<sup>b</sup> *Department of Microbiology, UEL, Londrina, Brazil*

<sup>c</sup> *IAPAR, Cx. Postal 481, 86001-970 Londrina, Brazil*

Received 23 April 2004; accepted 20 June 2005

## Abstract

Brazil is the largest producer and consumer of the common bean (*Phaseolus vulgaris* L.), but yields are often low and may be improved by a higher N supply through symbiosis with rhizobia. One main limitation to the N<sub>2</sub>-fixation process is the susceptibility of the symbiosis to environmental stresses frequent in the tropics, such as high soil temperatures and low soil moisture contents. Among other benefits, the no-tillage (NT) system reduces those stresses resulting in higher N<sub>2</sub> fixation rates and yields; however, the effects of NT on rhizobial diversity are poorly understood. This study evaluated the diversity of rhizobia compatible with common bean in cropping areas under the NT or the conventional tillage (CT) systems in Ponta Grossa, State of Paraná, Southern Brazil. Genetic diversity was assessed by DNA analyses using the methodologies of BOX-PCR and RFLP-PCR of the 16S rDNA region. A high level of diversity was observed among the strains and the DNA profiles from the CT system were quite different from those from the NT system. Twenty-three RFLP-PCR profiles were obtained, indicating that many tropical rhizobial species remain to be described. Strain differentiation was achieved in the BOX-PCR analysis; diversity was slightly higher under the NT when compared with the CT system. Surprisingly, the rhizobial grouping based on cluster analysis of the RFLP-PCR of the 16S rDNA region indicated a higher diversity of species under the CT. It could be that the environmental stability offered by the NT system has led to a decrease in the number of species, with the predominance of the most successful ones, although genetic diversity within each species has increased. The results obtained in this study show that we still understand poorly the relation between microbial diversity and soil sustainability and that the complexity of the ecosystems require the evaluation of several parameters to define and monitor soil quality.

© 2005 Elsevier B.V. All rights reserved.

**Keywords:** Bacterial diversity; Common bean; Nitrogen fixation; Rhizobium; Tillage systems

## 1. Introduction

Common bean (*Phaseolus vulgaris* L.) is a main component in the diet, and often the most important source of protein of over 300 million people in Latin America and South and West Africa. Brazil is the largest producer and consumer of common bean worldwide: 4,286,200 tons were produced in 2003/2004, but with the very low mean yield of only

\* Corresponding author. Tel.: +55 43 33716206; fax: +55 43 33716100.

E-mail addresses: [hungria@cnpso.embrapa.br](mailto:hungria@cnpso.embrapa.br), [hungria@sercom-tel.com.br](mailto:hungria@sercom-tel.com.br) (M. Hungria).

<sup>1</sup> Present Address: Plant Production Systems Group, WUR, PO Box 430, 6700AK, Wageningen, The Netherlands.

699 kg ha<sup>-1</sup>. Common bean is not considered an important cash crop, thus poor technology, cropping in soils with low organic matter content and fertility, especially deficient in N, contribute greatly to the low yields obtained in Brazil.

An increase in the N supply through the symbiotic association with efficient rhizobial strains might play a key role in obtaining agricultural and economic sustainability of the common bean crop in the tropics (Hungria et al., 2000, 2003; Mostasso et al., 2002). However, poor nodulation and low N<sub>2</sub>-fixation rates have been frequently reported in field experiments performed worldwide (e.g., Graham, 1981; Hardarson, 1993). A contributory factor may be the high sensitivity of the symbiosis to environmental stresses. One main limiting factor in the tropics is the low P availability, that can be considered as the most widespread production constraint in many regions, as the Brazilian Cerrados, and the situation is aggravated by the intense fixation of the nutrient in oxisols (Goedert, 1983). Other major limitations in the tropics are the high soil temperature and lack of moisture (Graham, 1981; Hungria et al., 1993; Hungria and Vargas, 2000). Both high temperatures and shortage of water compromise every step of the development and function of the symbiosis, from rhizobial survival, maintenance of the genome, chemotaxis, the root infection process, nodule formation, to activity of enzymes related both to N<sub>2</sub> fixation and N assimilation (Hungria and Vargas, 2000).

The no-tillage (NT) management system has become widely adopted in many countries of South America. In Brazil, the area devoted to NT has increased from 2.02 Mha in 1992/1993 to more than 19 million ha today. In comparison with conventional tillage (CT), NT enhances soil moisture content and helps in the regulation of soil temperature, in addition to protecting the soil against erosion by water, improving soil structure and the stability of the aggregates, and, with time, increases the soil organic matter content, often resulting in higher yields (e.g., Derpsch et al., 1991; Bayer et al., 2002; Castro Filho et al., 2002; Hernández and López-Hernández, 2002). Good productivity has also been reported for the common bean under NT and minimum tillage systems (Deibert, 1995).

When compared with CT, NT also benefits the biological N<sub>2</sub> fixation process, most likely due to the lower soil temperatures and higher soil moisture content. There are reports of increases in rhizobial survival, growth and diversity, in induction activity of nodulation genes, in nodulation, nodule distribution in the soil profile, and in N<sub>2</sub> fixation rates (Voss and Sidiras, 1985; Ferreira et al., 2000; Hungria and Vargas,

2000). However, these studies were performed with soybean, an important cash crop in Brazil and the effects of management system on rhizobial genetic diversity were mentioned in just one study (Ferreira et al., 2000).

Common bean is a promiscuous host. It is nodulated by a variety of rhizobia, and in Brazil there are reports of symbioses with *Rhizobium tropici*, *R. etli*, *R. leguminosarum* and *R. giardinii*, with bacteria belonging to the genera *Mesorhizobium* and *Sinorhizobium*, and with other bacteria that may well represent new species (Mostasso et al., 2002; Grange and Hungria, 2004). The sensitiveness and promiscuity of common bean symbiosis may represent an interesting model for the examination of effects of different soil management systems on rhizobial diversity. Therefore, the aim of this research was to characterize rhizobia nodulating common bean grown under NT and CT in Southern Brazil.

## 2. Material and methods

### 2.1. Field sites and soil sampling

The sites were located at the Experimental Station of the Instituto Agronômico do Paraná (IAPAR), Ponta Grossa, Paraná, Southern Brazil. The area is located at an altitude of 880 m, 25°13'S and 50°1'W. The average rainfall is 1507 mm year<sup>-1</sup>, with 123 rainy days year<sup>-1</sup>; the rainiest month is January (184 mm) and the driest is August (77 mm); according to Koeppen's classification, the climate is type Cfb. The soil, a Dark Red Latosol (Haplorthox) containing (g kg<sup>-1</sup>), 730 sand, 40 silt and 240 clay.

The area representing the NT system had been planted to maize (*Zea mays* L.) in summer and wild radish (*Rabanus sativus*) or common oat (*Avena sativa*) in winter for 6 years. Two years before collecting the samples common bean was planted on the wild radish residue. The main chemical characteristics were: pH in CaCl<sub>2</sub>, 4.77; exchangeable Al (cmol<sub>c</sub> dm<sup>-3</sup>) 0.09; cation exchange capacity (CEC) (cmol<sub>c</sub> dm<sup>-3</sup>), 13.66; P (cmol<sub>c</sub> dm<sup>-3</sup>), 2.5. The area representing the CT had been cultivated with common bean in summer for 10 years and left with natural spontaneous vegetation in winter. The main chemical characteristics were: pH in CaCl<sub>2</sub>, 4.82; exchangeable aluminum (cmol<sub>c</sub> dm<sup>-3</sup>), 0.06; CEC (cmol<sub>c</sub> dm<sup>-3</sup>), 13.66; P (cmol<sub>c</sub> dm<sup>-3</sup>), 2.5. Field sites had 1 ha under NT and 1 ha under CT. The system NT requires the cultivation of cover crops, as wild radish and common oat, and unfortunately we have found no area under NT with more than 2 years of bean

cropping. The most important characteristic in common between the two areas was that neither one had history of inoculation of common bean. N-fertilizer was applied at a rate of 20 kg ha<sup>-1</sup> at sowing and of 40 kg ha<sup>-1</sup> after 30 days. Macro and micronutrients were applied according to the chemical soil analysis and diseases and insect predation were controlled when necessary.

## 2.2. References strains

Common bean rhizobia reference strains included: *Rhizobium tropici* IIA CFN 299 (=USDA 9039, =LMG 9517), IIB CIAT 899<sup>T</sup> (=UMR 1899, =USDA 9030, =TAL 1797, =HAMBI 1163, =SEMIA 4077, =ATCC 49672) and *R. etli* CFN 42<sup>T</sup> (=USDA 9032) (received from CIFN, Cuernavaca, Mexico). *R. tropici* strain PRF 81 (=SEMIA 4080) came from the Embrapa Soja germplasm bank, and *R. giardinii* bv. *giardinii* strain H152<sup>T</sup> and *R. gallicum* bv. *gallicum* strain R602<sup>T</sup> were provided by INRA, Dijon, France.

## 2.3. Rhizobial isolation and morpho-physiological characterization of the isolates

Plants were collected on December. Nodules were obtained from plants randomly collected at the field sites, taking 40 subsamples from each area, spatially distributed to cover the whole field area. Previous studies from our group have shown that 25 samples would be representative of an experimental area of about 1 ha (Ferreira et al., 2000; Grange and Hungria, 2004), but we have decided to increase that number to forty samples per treatment. In the laboratory, nodules were randomly chosen and rhizobia were isolated using standard procedures (Vincent, 1970). Purity of the cultures was confirmed by repeatedly streaking the bacteria on yeast extract-mannitol agar (YMA) medium (Vincent, 1970) and verifying a single type of colony morphology, absorption of Congo red (0.00125%) and a uniform Gram-stain reaction. Colony morphology (color, mucosity, transparency, borders, elevation) and acid/alkaline reaction were evaluated after 2 and 3 days of growth on YMA containing bromothymol blue (0.00125%) as indicator, in the dark, at 28 °C. Each colony was transferred to YM liquid broth (YMB) and after growth the broth was mixed with glycerol (1:1, v:v) and stored at -80 °C. Working cultures were maintained on YMA slants at 4 °C. Rhizobia were cultured routinely at 28 °C in YMB on a rotary shaker operating at 65 cycles/min. Forty isolates were randomly selected to represent each tillage system.

## 2.4. Genetic characterization

### 2.4.1. Extraction of DNA of rhizobial isolates

For DNA extraction, bacteria were grown in 15 ml of YMB (modified to contain 5 g l<sup>-1</sup> of mannitol), for 3 days, at 28 °C and were then centrifuged at 10,000 rpm for 10 min. The pellet was transferred to a 1.5 ml Eppendorf tube and washed three times with saline solution (0.85% NaCl) and once in PBS (containing, in 500 ml: NaCl 150 mM, 4.383 g; NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O 2.6 mM, 0.1793 g; Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O 7.6 mM, 1.36 g). The pellet was resuspended in TE 50:20 (Tris-HCl 50 mM pH 8.0; EDTA Na<sub>2</sub> 20 mM, pH 8.0; Tris-HCl 1 M, pH 8.0) at a concentration of 10<sup>9</sup> cells ml<sup>-1</sup> and 1.4 ml were transferred to another Eppendorf tube and centrifuged at 12,000 rpm for 10 min. The supernatant was discarded and the pellet resuspended in 400 µl of TE 50:20 and received 50 µl of SDS (sodium dodecyl sulphate) at 10% in water (w/v); 5 µl proteinase K (20 mg ml<sup>-1</sup>); 10 µl of lysozyme (5 mg ml<sup>-1</sup>); 2 µl of RNase (10 mg ml<sup>-1</sup>, prepared in Tris-HCl 10 mmol<sup>-1</sup> pH 7.5, NaCl 15 mmol l<sup>-1</sup>) and incubated at 37 °C for 1 h. The samples were passed through insulin syringes three times to decrease viscosity, then NaCl and AcONa were added to final concentrations of 250 and 300 mmol l<sup>-1</sup>, respectively. Samples were homogenized, left for 1 h at 4 °C, were centrifuged at 12,000 rpm for 15 min, recovering 300 µl of the supernatant, to which 600 µl of cold 95% ethanol were added and samples were left overnight at -20 °C. The following day, samples were centrifuged at 12,000 rpm for 15 min, the ethanol was discarded, and 400 µl of cold 70% ethanol were added. Samples were centrifuged again at 12,000 rpm for 15 min, the ethanol was discarded and the precipitates left to dry at room temperature for approximately 3 h. Finally, each precipitate was resuspended in 50 µl of TE 10:1. All solutions used for DNA extraction were previously autoclaved. Purity of the DNA was confirmed by electrophoresis in mini-gels (8 cm × 10 cm) of agarose at 1.5%, for 25 min at 80 V, using as a comparison low DNA Mass<sup>TM</sup> Ladder (Invitrogen<sup>TM</sup>, Life Technologies). Gels were stained with ethidium bromide and visualized under UV light.

### 2.4.2. PCR amplification with specific BOX A1R primer

The DNA of each bacterium was amplified by PCR with primer BOX A1R (5'-CTACGGCAAGGC-GACGCTGACG-3', Invitrogen<sup>TM</sup>, Life Technologies, São Paulo) (Versalovic et al., 1994). The final volume of the PCR reaction was 25 µl and contained: dNTPs

(1.5 mM of each), 5.0  $\mu\text{l}$ ; buffer 10 $\times$  (500 mM KCl; 100 mM Tris–HCl, pH 8.3), 2.5  $\mu\text{l}$ ;  $\text{MgCl}_2$  50 mM, 1.5  $\mu\text{l}$ ; primer (50 pmol  $\mu\text{l}^{-1}$ ), 1  $\mu\text{l}$ ; Taq DNA polymerase (5 U  $\mu\text{l}^{-1}$ ), 0.2  $\mu\text{l}$ ; DNA 50 ng  $\mu\text{l}^{-1}$ , 1  $\mu\text{l}$ ; sterile milli-Q water to complete the volume. The following cycles were used: one cycle of denaturation at 95 °C for 7 min; 35 cycles of denaturation at 94 °C for 1 min, of annealing at 53 °C for 1 min and of extension at 65 °C for 8 min; one cycle of final extension at 65 °C for 16 min; and a final soak at 4 °C. The reactions were carried out in an MJ Research Inc. PTC-100<sup>TM</sup> thermocycler and amplified fragments were separated by horizontal electrophoresis on a 1.5% agarose (low EEO, type I-A) gel (20 cm  $\times$  25 cm), at 120 V, for 6 h. Gels were stained with ethidium bromide, visualized under UV light and photographed with a Kodak Digital Science 120 apparatus.

#### 2.4.3. Restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rDNA genes

The DNA of the bacteria was amplified with primers Y1 (5'-TGGCTCAGAACGAACGCGTGGCGGC-3') (Young et al., 1991) and Y3 (3'-CTGACCCCAACTT-CAGCATTGTTCCAT-5') (J.P.W. Young, unpublished), which amplify almost the full length of the region (1500 bp) corresponding to the 16S rRNA. Five replicates of a mixture with a final volume of 50  $\mu\text{l}$ , containing: dNTPs (1.5 mM of each), 2.0  $\mu\text{l}$ ; 10 $\times$  buffer (200 mM Tris-base, 500 mM KCl, pH 8.4), 5.0  $\mu\text{l}$ ;  $\text{MgCl}_2$  (50 mM), 1.5  $\mu\text{l}$ ; each primer (stock at 10 pmol  $\mu\text{l}^{-1}$ ), 1.0  $\mu\text{l}$ ; Taq DNA polymerase (5 U  $\mu\text{l}^{-1}$ ), 0.2  $\mu\text{l}$ ; DNA (50 ng  $\mu\text{l}^{-1}$ ), 1.0  $\mu\text{l}$ , 38.30  $\mu\text{l}$  of sterile milli-Q water. The amplification cycles were based on Young et al. (1991), modified to increase the annealing temperature, and consisted of: one cycle of denaturation at 93 °C for 5 min; 35 cycles of denaturation at 93 °C for 45 s, of annealing at 64 °C for 45 s and extension at 72 °C for 2 min; one cycle of final extension at 72 °C for 5 min; and a final soak at 4 °C. Each strain produced a single PCR product with the expected MW. For the RFLP, the methodology of Laguerre et al. (1994) was applied to the PCR products, with the enzymes *CfoI* (5'-GCG/C-3'; 3'-C/GCG-5'), *HinfI* (5'-G/ANTC-3'; 3'-CTNA/G-5'), *MspI* (5'-C/CGG-3'; 3'-GGC/C-5'), *RsaI* (5'-GT/AC-3'; 3'-CA/TG-5') and *MboI* (5'-GATC-3'; 3'-CTAG/-5') (Invitrogen<sup>TM</sup>, Life Technologies). For each enzyme, a 10  $\mu\text{l}$  mixture was prepared containing: 6  $\mu\text{l}$  of the PCR product; 1  $\mu\text{l}$  of the specific buffer for each enzyme (10 $\times$ ); 0.5  $\mu\text{l}$  of enzyme (10 U  $\mu\text{l}^{-1}$ ) and 2.5  $\mu\text{l}$  of sterile milli-Q water. The mixtures were incubated in the water bath at 37 °C for the following times: *CfoI*

(1 h); *HinfI* (3 h); *MspI* (2 h); *RsaI* (2 h); *MboI* (5 h). The fragments obtained were analyzed by horizontal electrophoresis in a gel (17 cm  $\times$  11 cm) with 3% of agarose, at 100 V for 4 h. Bands with MW lower than 100 bp were discarded without affecting the analysis, as in other studies (Laguerre et al., 1994; Ulrich and Zaspel, 2000; Odee et al., 2002).

#### 2.4.4. Cluster analysis

Cluster analyses were performed with the BOX A1R-PCR and with the RFLP-PCR products with the Bionumerics program (Applied Mathematics, Kortrijk, Belgium), using the algorithm UPGMA (unweighted pair-group method, with arithmetic mean) and the coefficient of Jaccard (*J*) (Sneath and Sokal, 1973), at a tolerance of 2%.

#### 2.5. Genetic diversity

Indexes of diversity, richness, and evenness were estimated based on the number of isolates belonging to each group of profiles in BOX A1R and of the RFLP-PCR of the 16S rRNA region. Diversity was calculated by using the Shannon index:  $H' = \sum S[(n_1/n) \ln (n_1/n)]$  (Shannon and Weaver, 1949), where  $n_1$  is the number of isolates in each group and  $n$  is the number of isolates in all groups. For richness, the Margalef index was used:  $R_1 = S - 1/\ln(n)$  (Margalef, 1958), where  $S$  is the number of groups and  $n$  is the number of isolates in all groups. The Pielou index was used as a measure of evenness:  $E_1 = H'/\ln(S)$  (Pielou, 1977), where  $H'$  is the Shannon index and  $S$  is the number of groups. Grouping for BOX A1R-PCR was obtained by considering a level of similarity of 70% in the cluster analysis with the UPGMA algorithm and the *J* coefficient. The groups of RFLP-PCR of 16S rRNA region were achieved by combining the different profiles and designating each group of similar profiles with a letter.

### 3. Results

#### 3.1. Morpho-physiological characterization

Forty strains from each tillage method were characterized. Strains varied in relation to morpho-physiological characteristics and 17 different combinations of color, mucosity, transparency, borders, elevation and acid/alkaline reaction were obtained (data not shown). However, a predominant group included 62.5% of the strains showing opaque white color, high production of mucus, regular margin, flat elevation, and producing acid reaction on YMA medium. There

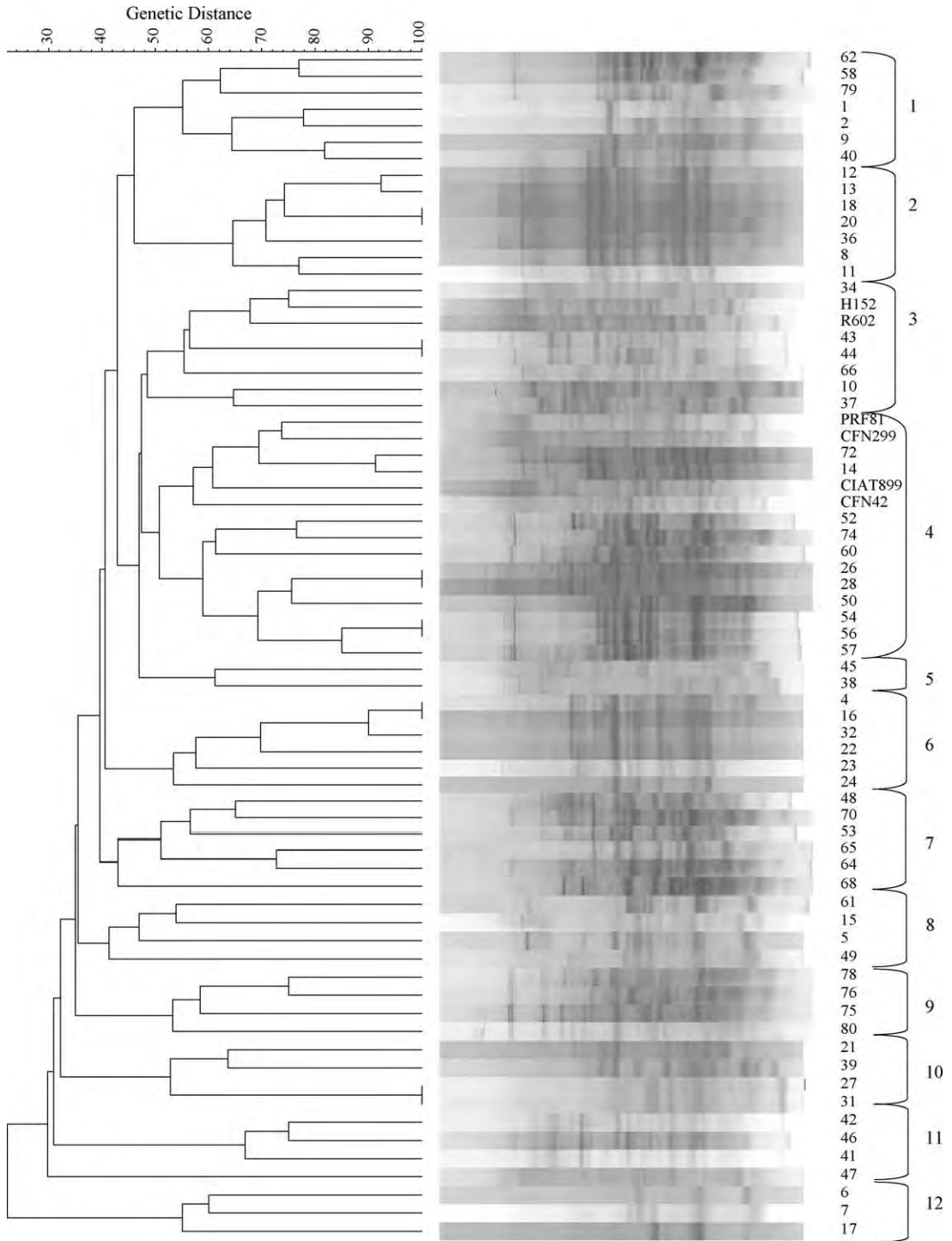


Fig. 1. Dendrogram based on cluster analysis of BOX A1R-PCR products using the UPGMA algorithm and the Jaccard coefficient, of rhizobial strains isolated from nodules of common bean plants grown in field sites under a conventional (CT) or no-tillage (NT) system, in Ponta Grossa, PR. Strains 1–40 are from the CT and 41–80 from the NT system.

was no recognizable effect of the tillage method on the morpho-physiological characteristics.

### 3.2. BOX AIR-PCR genomic fingerprinting

Based on morpho-physiological characterization, 35 strains were randomly chosen from each tillage method and submitted to amplification by BOX AIR-PCR. Successful amplification was achieved with 33 CT and 31 NT strains. A high level of genetic diversity was observed in both groups of strains. Considering a similarity of 70% in the clustering analysis with the UPGMA algorithm and the coefficient of Jaccard, it was possible to distinguish 20 different profiles among the strains from the CT system, which were grouped at a

final level of similarity of only of 26% (data not shown). Only four pairs of strains showed identical profiles. Reference strains of *R. tropici* and *R. etli* were joined at a 56% level of similarity, but none of the strains from this study was positioned in the same cluster, while strain 34 was joined to *R. giardinii* with a similarity of 75%. The profiles of the other strains were quite different from those of the reference strains (data not shown). A high level of genetic diversity was also observed among the strains isolated from the NT, and considering a 70% level of similarity 22 different profiles were distinguished (data not shown). The strains from the NT were also clustered at a low final level of similarity (32%), and only two pairs of strains showed identical profiles. Again, the profiles of the

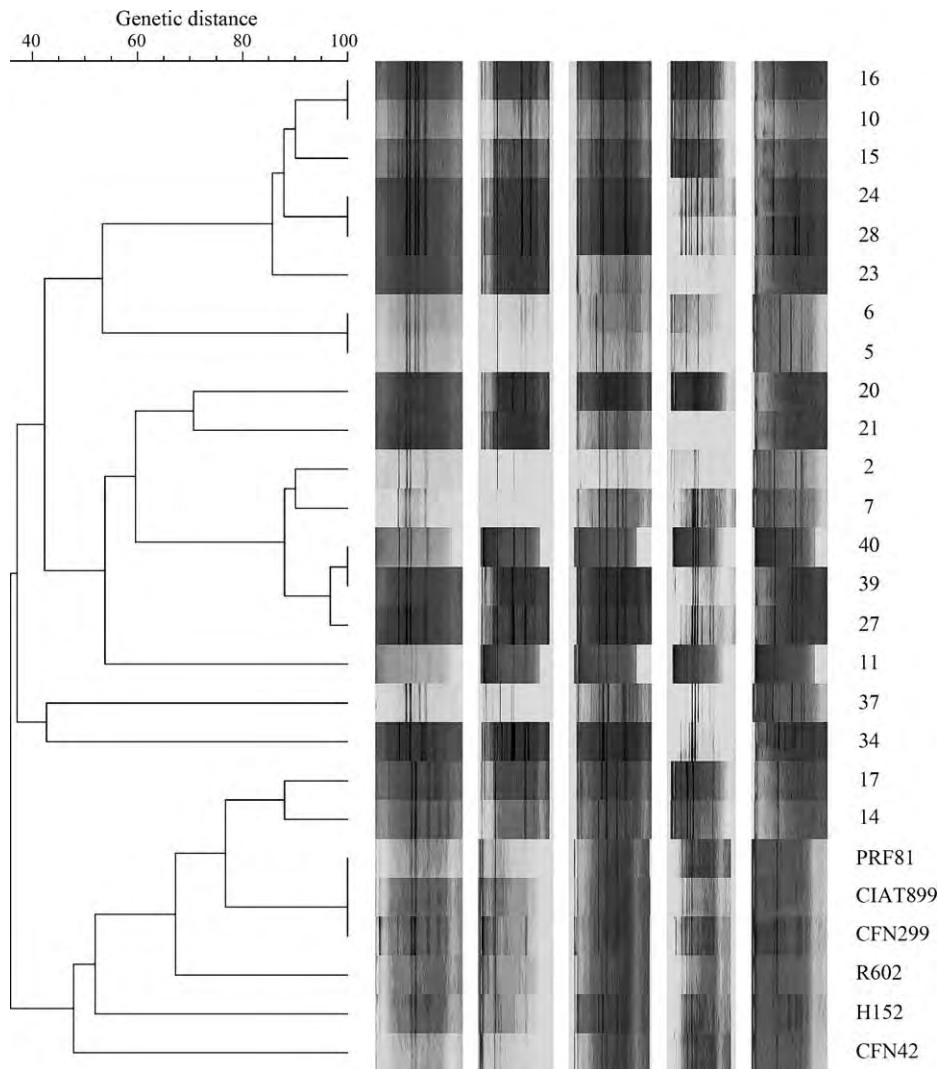


Fig. 2. Dendrogram based on cluster analysis of RFLP PCR of the 16S rDNA region, with the UPGMA algorithm and the Jaccard coefficient, of rhizobial strains isolated from nodules of common bean plants grown in a field site under the conventional tillage system, in Ponta Grossa, PR.

strains differed from those of reference strains, and just one strain, 72, was clustered with *R. tropici* and *R. etli*, with a similarity of 54%, while two strains with identical profiles, 43 and 44, were joined to *R. giardinii* at a 67% level of similarity (data not shown).

Fig. 1 shows the dendrogram obtained with the BOX A1R-PCR products of all strains from this study, which were clustered at a final level of similarity of only 22%. One cluster included two strains, 72 and 14, and reference strains of *R. tropici* and *R. etli* with a

similarity of 57%, and nine other strains, most from NT, were joined to this group at a 52% level of similarity (cluster 4). Six other strains from CT and NT were positioned in cluster 3 with *R. gallicum* and *R. giardinii*, with a similarity of 48%. Fig. 1 shows also that strains from CT were quite different from those from the NT, and only two strains, 14 (CT) and 72 (NT) (cluster 4) shared high similarity of BOX A1R profiles (92%). In relation to the other clusters shown on Fig. 1, just two, 1 and 8, included strains from both systems, while the

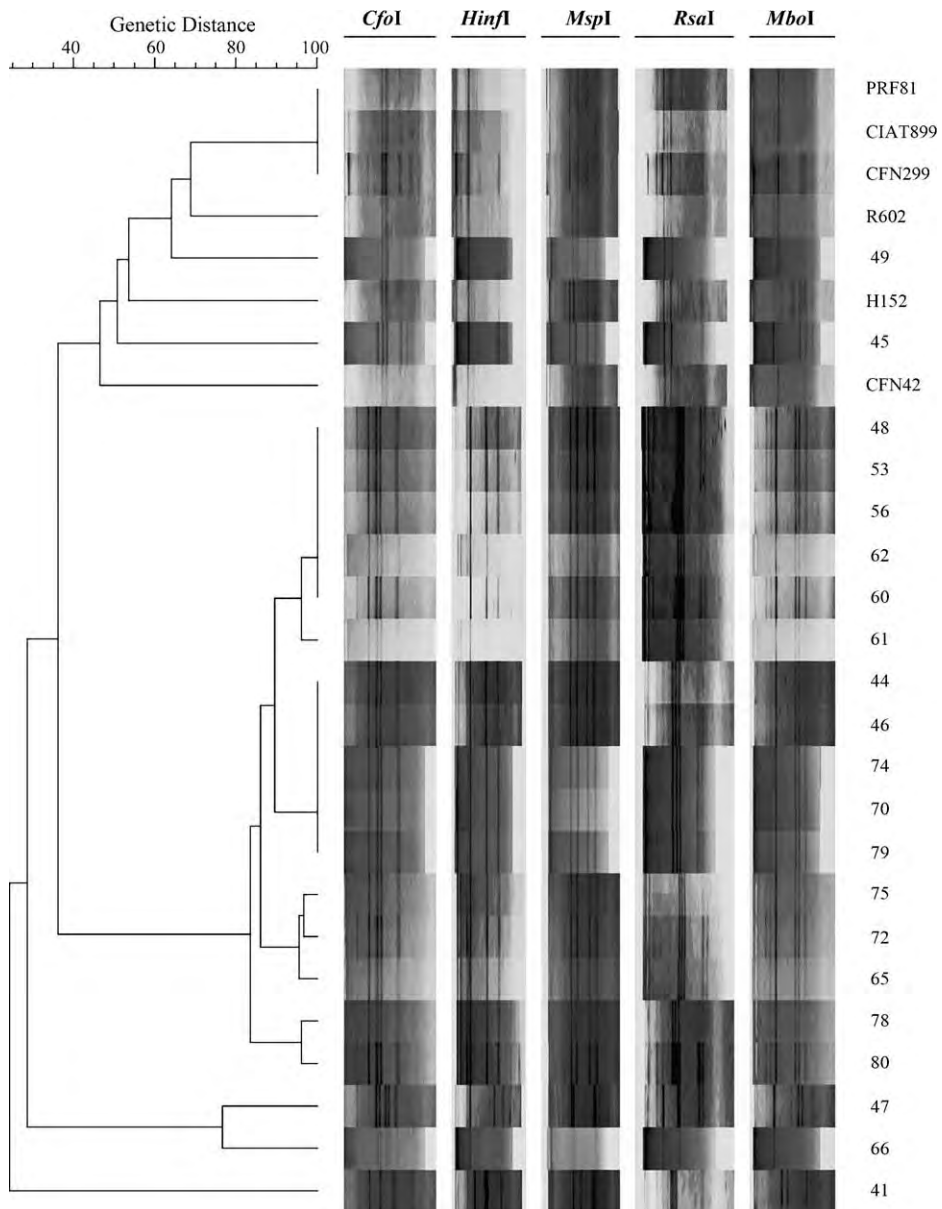


Fig. 3. Dendrogram based on cluster analysis of RFLP PCR of 16S rDNA region, with the UPGMA algorithm and the Jaccard coefficient, of rhizobial strains isolated from nodules of common bean plants grown in a field site under a no-tillage system, in Ponta Grossa, PR.

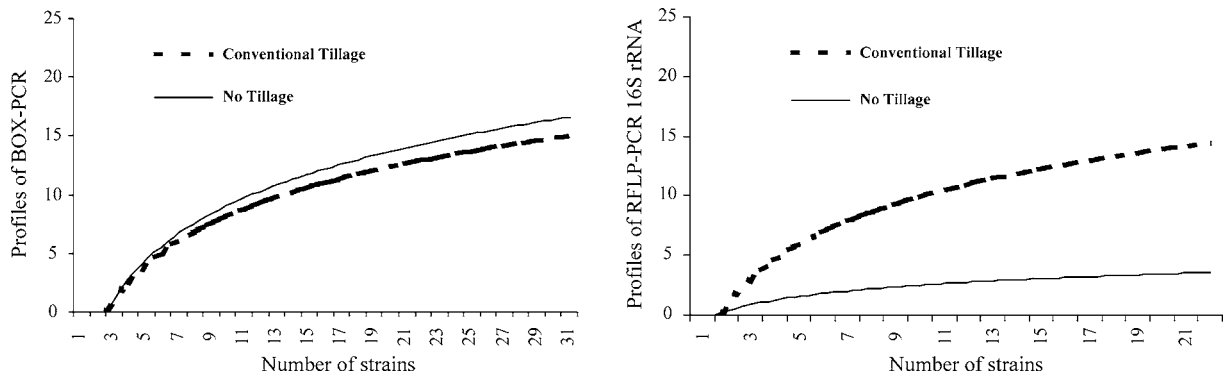


Fig. 4. Cumulative diversity in function of the number of common bean rhizobial strains and of the profiles obtained in the DNA analysis by BOX A1R-PCR or RFLP-PCR of the 16S rDNA region, in field sites under a conventional (CT) or no-tillage (NT) system, in Ponta Grossa, PR.

others clustered strains either from the CT (2, 6, 10, and 12) or from the NT (5, 7, 9, 11) system.

### 3.3. Profiles of RFLP-PCR of the 16S rDNA region

The level of similarity of 70% in the BOX A1R-PCR analysis was considered as reference to choose the strains for the PCR-RFLP analysis of the 16S rDNA region, thus the profiles of 20 strains from the CT and of 21 (one strain did not amplify with the primer) from the NT were obtained. Again, a high polymorphism was achieved. Four defined clusters were observed for the strains from the CT system, which were joined at a final level of similarity of only 35%, and genetic diversity was high even within each cluster (Fig. 2). Only two strains, 14 and 17, were positioned in the same cluster as the reference strains and the level of diversity detected for the other clusters might well indicate at least three other species (Fig. 2). In contrast, 16 out of the 21 strains from the NT fit into the same cluster, with a similarity of 85%, which joined the cluster containing the reference strains at a 35% level of similarity (Fig. 3). Strains from the NT system were grouped at a final level of similarity of 25%. Considering all strains, 23 different profiles of RFLP-PCR were obtained, besides those of the reference strains.

### 3.4. Genetic diversity

Considering the analysis with the BOX A1R-PCR products, the indices of Shannon ( $H'$ ) for diversity, Margalef ( $R_1$ ) for richness and Pielou ( $E_1$ ) for evenness were slightly higher in the NT (2.98, 6.17 and 0.97, respectively) than in CT (2.82, 5.43 and 0.94, respectively) system. However, when the RFLP-PCR products were considered, both  $H'$  and  $R_1$  were considerably lower

in the NT (1.41 and 1.91, respectively) than in the CT (2.72 and 5.01, respectively) system. In addition, genetic diversity was also evaluated by a curve of cumulative profiles in relation to the number of strains analyzed. The genetic diversity by BOX A1R-PCR was slightly higher for the strains isolated from the NT system, but those strains showed lower diversity when the RFLP-PCR of the 16S rDNA region was considered (Fig. 4).

## 4. Discussion

Neither area considered in this study had a history of inoculation with rhizobia, thus the strains trapped by the common bean plants probably were indigenous. However, it is possible that some strains were introduced through the years on seeds, since common bean seeds usually carry many viable rhizobial cells (Andrade and Hungria, 2002). The strains analyzed in this study were characterized by a high level of morphophysiological and genetic diversity and, added to previous reports on populations of common bean rhizobia in Brazil (Straliotto et al., 1999; Andrade et al., 2002; Mostasso et al., 2002; Grange and Hungria, 2004), confirm both the promiscuity of the host plant and the high level of diversity that remains poorly characterized in tropical soils. Indeed, in relation to the promiscuity of common bean, Michiels et al. (1998) have shown that the legume is able to perceive signals for nodulation from many rhizobia although most of the interactions are not effective.

Consensus sequences such as REP, ERIC and BOX, related to repetitive and conservative elements diffused in DNA have been extensively used in ecology, genetic and taxonomic studies, as well as for rhizobial strain identification (e.g., de Bruijn, 1992; Judd et al., 1993; Versalovic et al., 1994; Laguerre et al., 1997) and the



technique has also proven to be valuable in studies of rhizobia isolated from tropical soils (e.g., Chen et al., 2000; Chueire et al., 2000; Mostasso et al., 2002; Fernandes et al., 2003; Grange and Hungria, 2004). Indeed, also in this study, the genetic characterization using the BOX A1R-PCR technique was clearly very effective for differentiating the strains. Very few strains did not amplify with this primer, but failure of amplification with consensus primers has been reported before for strains both from soybean (Judd et al., 1993) and common bean (Mostasso et al., 2002). It is noteworthy that the profiles of the strains from the CT were quite different from those from the NT system and all strains were clustered at the very low level of similarity (22%). As the number of strains analyzed from each system differed, the indexes of diversity proposed by Kennedy and Smith (1995) and Kennedy (1999) were used, since they have been successfully applied in analyses using electrophoretic profiles of plasmids and obtained by PCR and RFLP (Palmer and Young, 2000; Bernal and Graham, 2001; Andrade et al., 2002). Both indexes  $H'$  and  $R_1$ , indicative of genetic richness, as well as the index  $E_1$ , representing evenness, were slightly higher in the NT when compared to the CT system, which was also demonstrated in the curve of cumulative profiles. In a previous study with soybean rhizobia, genetic diversity analyzed with random primers was also higher in NT when compared with a CT system (Ferreira et al., 2000).

The analysis by the RFLP-PCR products of the 16S rDNA region has often been used for rhizobial species designation, since it shows good agreement with the 16S rRNA genes (e.g., Laguerre et al., 1994, 1997; Terefework et al., 1998), although some species closely related cannot be distinguished (Laguerre et al., 1997). The high diversity of rhizobial species trapped by common bean in this study was evident when one considers that 23 profiles of RFLP-PCR were obtained. A previous study performed in Brazil with common bean has also identified 20 different RFLP-PCR profiles of the 16S rDNA in the analysis of 101 strains (Grange and Hungria, 2004), thus, certainly, many tropical rhizobial species remain to be described. However, also confirming previous observations by Laguerre et al. (1997), Chueire et al. (2000), Mostasso et al. (2002) and Grange and Hungria (2004), analyses with consensus sequences as BOX A1R-PCR and the RFLP-PCR of the 16S rDNA region were poorly related, as the former methodology is useful for strain identification, but not for phylogenetic characterization.

In this study, the profiles and indexes related to the RFLP-PCR analysis showed greater genetic diversity of

rhizobial species in the CT system, and the prevalence of one profile in the NT. It is important to note that the higher genetic diversity observed at the strain level (BOX A1R-PCR) under the NT system is not contradictory to the lower genetic diversity at the species level (RFLP-PCR), as the DNA regions analyzed are different.

Unfortunately, we did not find areas with similar cropping histories, and the soil under CT was cropped with the legume for 10 years, while the area under NT was cropped only for 2 years. It is then possible that, although common bean indigenous rhizobial population in Brazilian soils is always very high (Andrade and Hungria, 2002; Hungria et al., 2003), different cropping histories might affect diversity. Increases in rhizobial population due to cropping with the homologous host legume have been reported (e.g., Thies et al., 1995; Ferreira et al., 2000), and may as well increase diversity, mostly due to rhizosphere effects (Peña-Cabriaes and Alexander, 1983; Ferreira et al., 2000). However, Grange and Hungria (2004) surveyed the genetic diversity of common bean rhizobia from 14 Brazilian soils with different cropping histories, and apparently the presence of the host legume had no effect on rhizobial diversity.

It is well known that common bean rhizobial species differ considerably in relation to several physiological properties, such as tolerance to temperature, acidity, antibiotics, and heavy metals. The environment offered by the NT is far more homogenous than that found under the CT system, including less oscillation in soil temperature and moisture content and increased availability of C and N to the soil microorganisms (Derpsch et al., 1991; Hungria and Vargas, 2000; Bayer et al., 2002; Castro Filho et al., 2002; Hernández and Lopéz-Hernández, 2002). There are reports showing that the favorable environmental conditions under NT benefits rhizobial population, increasing cell survival, genetic diversity of strains, induction of nodulation genes, nodulation and  $N_2$  fixation rates (Hungria and Stacey, 1997; Ferreira et al., 2000). However, there is a possibility that the environmental stability offered by the NT management system leads to predominance of relatively few successful rhizobial species, very effective in fixing  $N_2$ , as has been shown for soybean microsymbionts (Ferreira et al., 2000). In contrast, daily oscillations in soil temperature and moisture and lower availability of organic matter in the CT system, might promote diversity of rhizobial species able to survive in a broad range of environmental conditions, but with lower capacity of  $N_2$  fixation. Indeed, in a previous study, when the capacity for utilization of C and N

among rhizobial strains trapped by promiscuous soybean genotypes was analyzed, strains from cropped areas were positioned in different clusters from those from undisturbed areas. However, the strains from CT areas were clustered together, as they were able to use almost all C and N sources tested, whereas the strains from NT areas were positioned in another cluster, since they used more specific metabolic pathways, as the availability of C and N sources is greater under NT (Hungria et al., 2001). The authors suggested that to survive under the conditions of the CT system, with lower organic matter content, the bacteria needed to metabolize a broader range of C and N compounds, while the strains from NT were able to develop a higher capacity of fixing N<sub>2</sub> (Hungria et al., 2001).

It is well known that different soil and crop management practices affect the equilibrium between the soil and the indigenous organisms, which, in turn, affect soil sustainability. In this context, the NT system has been shown to be crucial for sustainability in the tropics, where soils are frequently exposed to stressful environmental conditions and to erosion factors. As a consequence of a better environment for the soil microorganisms, several studies have reported increased soil biomass under the NT in comparison with the CT system (e.g., Alvarez et al., 1995; Cattelan et al., 1997; Balota et al., 1998). However, despite showing higher microbial-C in relation to the total organic-C, Balota et al. (1998) and Franchini et al. (2002) pointed out that the metabolic coefficient ( $qCO_2$ ) is lower under the NT system, indicating more efficient microbial metabolic routes that contribute to the accumulation of C in the soil with time. When those concepts are applied to our study, it might be that the NT system has led to the selection of a few common bean rhizobial species, which could show higher metabolic effectiveness, among other characteristics. However, at the same time, the better soil environmental conditions allowed diversification within each species, at the strain level.

The importance of biodiversity may rely on a buffering capacity of the soil (Loreau et al., 2001), therefore it has been often suggested that soil microbial diversity must be related to soil health and quality and to agricultural sustainability. In this context, there is a need to define parameters that could be used as bioindicators of soil quality. Nevertheless, the results obtained in this study show that we still poorly understand the relation between microbial diversity and soil sustainability. Most likely, the complexity of the ecosystems will require

the evaluation of several parameters to define and monitor soil quality.

## Acknowledgements

The research was partially funded by Fundação Araucaria (046/2003) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico – 301241/2004-0 and PRONEX). M. Hungria acknowledges a research fellowship from CNPq (301241/2004-0). The authors thank Ligia M.O. Chueire for technical help and Dr. Allan R.J. Eaglesham for helpful suggestions on the manuscript.

## References

- Alvarez, R., Díaz, R.A., Barbero, N., Santanoglia, O.J., Blotta, L., 1995. Soil organic carbon, microbial biomass and CO<sub>2</sub>-C production from three tillage systems. *Soil Till. Res.* 33, 17–28.
- Andrade, D.S., Hungria, M., 2002. Maximizing the contribution of biological nitrogen fixation in tropical legume crops. In: Finan, T.M., O'Brian, M.R., Layzell, D.B., Vessey, J.K., Newton, W. (Eds.), *Nitrogen Fixation, Global Perspectives*. CABI Publishing, Wallingford, UK, pp. 341–345.
- Andrade, D.S., Murphy, P.J., Giller, K.E., 2002. The diversity of *Phaseolus*-nodulating rhizobial populations is altered by liming of acid soils planted with *Phaseolus vulgaris* L. in Brazil. *Appl. Environ. Microbiol.* 68, 4025–4034.
- Balota, E.L., Colozzi-Filho, A., Andrade, D.S., Hungria, M., 1998. Biomassa microbiana e sua atividade em solos sob diferentes sistemas de preparo e sucessão de culturas. *Rev. Bras. Ci. Solo* 22, 641–649.
- Bayer, C., Mielniczuk, J., Martin-Neto, L., Ernani, P.R., 2002. Stocks and humification degree of organic matter fractions as affected by no-tillage on a subtropical soil. *Plant Soil* 238, 133–140.
- Bernal, G., Graham, P.H., 2001. Diversity in the rhizobia associated with *Phaseolus vulgaris* L. in Ecuador, and comparisons with Mexican bean rhizobia. *Can. J. Microbiol.* 47, 526–534.
- Castro Filho, C., Lourenço, A., Guimaraes, M.F., Fonseca, I.C.B., 2002. Aggregate stability under different soil management systems in a red latosol in the state of Parana. Brazil. *Soil Till. Res.* 65, 45–51.
- Cattelan, A.J., Gaudêncio, C.A., Silva, T.A., 1997. Sistemas de culturas em plantio direto e os microrganismos do solo, na cultura da soja, em Londrina. *R. Bras. Ci. Solo* 21, 293–301.
- Chen, L.S., Figueredo, A., Pedrosa, F.O., Hungria, M., 2000. Genetic characterization of soybean rhizobia in Paraguay. *Appl. Environ. Microbiol.* 66, 5099–5103.
- Chueire, L.M.O., Nishi, C.Y.M., Loureiro, M.F., Hungria, M., 2000. Identificação das estirpes de *Bradyrhizobium* e *Rhizobium* utilizadas em inoculantes comerciais para as culturas da soja e do feijoeiro pela técnica de PCR com “primers” aleatórios ou específicos. *Agric. Trop.* 4, 80–95.
- de Bruijn, F.J., 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Appl. Environ. Microbiol.* 58, 2180–2187.

- Deibert, E.J., 1995. Dry bean production with various tillage and residue management systems. *Soil Till. Res.* 36, 97–109.
- Derpsch, R., Roth, C.H., Sidiras, N., Kopke, U., 1991. Controle da Erosão no Paraná, Brasil: Sistemas de Cobertura do Solo, Plantio Direto e Preparo Conservacionista do Solo. GTZ-IAPAR, Eschborn, Germany-Londrina, Brazil, pp. 272.
- Fernandes, M.F., Fernandes, R.P.M., Hungria, M., 2003. Caracterização genética de rizóbios nativos dos tabuleiros costeiros eficientes para as culturas do guandu e caupi. *Pesq. Agropec. Bras.* 38, 911–920.
- Ferreira, M.C., Andrade, D.S., Chueire, L.M.O., Takemura, S.M., Hungria, M., 2000. Effects of tillage method and crop rotation on the population sizes and diversity of bradyrhizobia nodulating soybean. *Soil Biol. Biochem.* 32, 627–637.
- Franchini, J.C., Torres, E., Souza, R.A., Crispino, C.C., Souza, L.J., Hungria, M., 2002. Efeito de diferentes sistemas de preparo do solo e sistemas de cultivo na microbiota do solo. In: Resultados de Pesquisa da Embrapa Soja 2001 – Microbiologia de Solos. Embrapa Soja, Londrina, pp. 9–10. (Embrapa Soja. Documentos, 197).
- Goedert, W.J., 1983. Management of the Cerrado soils of Brazil: a review. *J. Soil Sci.* 34, 405–428.
- Graham, P.H., 1981. Some problems of nodulation and symbiotic nitrogen fixation in *Phaseolus vulgaris* L.: a review. *Field Crops Res.* 4, 93–112.
- Grange, L., Hungria, M., 2004. Genetic diversity of indigenous common bean (*Phaseolus vulgaris*) rhizobia in two Brazilian ecosystems. *Soil Biol. Biochem.* 36, 1389–1398.
- Hardarson, G., 1993. Methods for enhancing symbiotic nitrogen fixation. *Plant Soil* 152, 1–17.
- Hernández-Hernández, R.M., López-Hernández, D., 2002. Microbial biomass, mineral nitrogen and carbon content in savanna soil aggregates under conventional and no-tillage. *Soil Biol. Biochem.* 34, 1563–1570.
- Hungria, M., Franco, A.A., Sprent, J.I., 1993. New sources of high-temperature tolerant rhizobia for *Phaseolus vulgaris* L. *Plant Soil* 149, 95–102.
- Hungria, M., Stacey, G., 1997. Molecular signals exchanged between host plants and rhizobia: basic aspects and potential application in agriculture. *Soil Biol. Biochem.* 29, 819–830.
- Hungria, M., Andrade, D.S., Chueire, L.M.O., Probanza, A., Guttierrez-Mañero, F.J., Megías, M., 2000. Isolation and characterization of new efficient and competitive bean (*Phaseolus vulgaris* L.) rhizobia from Brazil. *Soil Biol. Biochem.* 32, 1515–1528.
- Hungria, M., Vargas, M.A.T., 2000. Environmental factors affecting N<sub>2</sub> fixation in grain legumes in the tropics, with an emphasis on Brazil. *Field Crops Res.* 65, 151–164.
- Hungria, M., Chueire, L.M.O., Coca, R.G., Megías, M., 2001. Preliminary characterization of fast growing strains isolated from soybean nodules in Brazil. *Soil Biol. Biochem.* 33, 1349–1361.
- Hungria, M., Campo, R.J., Mendes, I.C., 2003. Benefits of inoculation of the common bean (*Phaseolus vulgaris*) crop with efficient and competitive *Rhizobium tropici* strains. *Biol. Fertil. Soils* 39, 51–61.
- Judd, A.K., Schneider, M., Sadowsky, M.J., de Bruijn, F.J., 1993. Use of repetitive sequences and the polymerase technique to classify genetically related *Bradyrhizobium japonicum* serocluster 123 strains. *Appl. Environ. Microbiol.* 59, 1702–1708.
- Kennedy, A.C., 1999. Bacterial diversity in agroecosystems. *Agric. Ecosyst. Environ.* 74, 65–76.
- Kennedy, A.C., Smith, K.L., 1995. Soil bacterial diversity and ecosystem functioning. *Plant Soil* 170, 75–86.
- Laguerre, G., Allard, M.-R., Revoy, F., Amarger, N., 1994. Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. *Appl. Environ. Microbiol.* 60, 56–63.
- Laguerre, G., van Berkum, P., Amarger, N., Prevost, D., 1997. Genetic diversity of rhizobial symbionts isolated from legume species within the genera *Astragalus*, *Oxytropis*, and *Onobrychis*. *Appl. Environ. Microbiol.* 63, 4748–4758.
- Loreau, M., Naeem, S., Inchausti, P., Bengtsson, J., Grime, J.P., Hector, A., Hooper, D.U., Huston, M.A., Raffaelli, D., Schmid, B., Tilman, D., Wardle, D.A., 2001. Biodiversity and ecosystem functioning: current knowledge and future challenges. *Science* 294, 804–808.
- Margalef, D.R., 1958. Information theory in ecology. *Gen. Sys.* 3, 36–71.
- Michiels, J., Dombrecht, B., Vermeiren, N., Xi, C., Luyten, E., Vanderleyden, J., 1998. *Phaseolus vulgaris* is a non-selective host for nodulation. *FEMS Microbiol. Ecol.* 26, 193–205.
- Mostasso, L., Mostasso, F.L., Vargas, M.A.T., Hungria, M., 2002. Selection of bean (*Phaseolus vulgaris*) rhizobial strains for the Brazilian Cerrados. *Field Crops Res.* 73, 121–132.
- Odee, D.W., Hauka, 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.
- Palmer, K.M., Young, J.P.W., 2000. Higher diversity of *Rhizobium leguminosarum* biovar *viciae* populations in arable soils than in grass soils. *Appl. Environ. Microbiol.* 66, 2445–2450.
- Peña-Cabriales, J.J., Alexander, M., 1983. Growth of *Rhizobium* in unamended soil. *Soil Sci. Soc. Amer. J.* 47, 81–84.
- Pielou, E.C., 1977. *Mathematical Ecology*. Wiley, New York, 385.
- Shannon, C.E., Weaver, W., 1949. *The Mathematical Theory of Communication*. University Illinois Press, Urbana, 117.
- Sneath, P.H.A., Sokal, R.R., 1973. *Numerical Taxonomy*. Freeman, San Francisco, p. 573.
- Straliotto, R., Cunha, C.O., Mercante, F.M., Franco, A.A., Rumjanek, N.G., 1999. Diversity of rhizobia nodulating common beans (*Phaseolus vulgaris* L.) isolated from Brazilian tropical soils. *An. Acad. Bras. Ciênc.* 71, 531–543.
- Terefework, Z., Nick, G., Suomalaine, S., Paulin, L., Lindström, K., 1998. Phylogeny of *Rhizobium galegae* with respect to other rhizobia and agrobacteria. *Int. J. Syst. Bacteriol.* 48, 349–356.
- Thies, J.E., Woome, P.L., Singleton, P.W., 1995. Enrichment of Bradyrhizobium spp. populations in soil due to cropping of the homologous host legume. *Soil Biol. Biochem.* 27, 633–636.
- Ulrich, A., Zaspel, I., 2000. Phylogenetic diversity of rhizobia strains nodulating *Robinia pseudoacacia* L. *Microbiology* 146, 2997–3005.
- Versalovic, J., Schneider, M., de Bruijn, F., Lupski, J.R., 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Meth. Mol. Cell Biol.* 5, 25–40.
- Vincent, J.M., 1970. *A Manual for the Practical Study of Root-Nodule Bacteria*. Blackwell Scientific, Oxford, pp. 164. IBP Handbook, no. 15.
- Voss, M., Sidiras, N., 1985. Nodulação da soja em plantio direto em comparação com plantio convencional. *Pesq. Agropec. Bras.* 20, 775–782.
- Young, J.P.W., Downer, H.L., Eardly, B.D., 1991. Phylogeny of the phototrophic *Rhizobium* strain BTAi1 by polymerase chain reaction-based sequencing of a 16S rRNA gene segment. *J. Bacteriol.* 173, 2271–2277.