

Sampling effects on the assessment of genetic diversity of rhizobia associated with soybean and common bean

Odair Alberton^{a,b,1}, Glaciela Kaschuk^{a,b,2}, Mariangela Hungria^{a,*}

^a *Embrapa Soja, Cx Postal 231, 86001-970 Londrina, Paraná, Brazil*

^b *Department of Microbiology, Universidade Estadual de Londrina, Brazil*

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Abstract

Biological nitrogen fixation plays a key role in agriculture sustainability, and assessment of rhizobial diversity contributes to worldwide knowledge of biodiversity of soil microorganisms, to the usefulness of rhizobial collections and to the establishment of long-term strategies aimed at increasing contributions of legume-fixed N to agriculture. Although in recent decades the use of molecular techniques has contributed greatly to enhancing knowledge of rhizobial diversity, concerns remain over simple issues such as the effects of sampling on estimates of diversity. In this study, rhizobia were isolated from nodules of plants grown under field conditions, in pots containing soil, or in Leonard jars receiving a 10^{-2} or a 10^{-4} serially-diluted soil inoculum, using one exotic (soybean, *Glycine max*) and one indigenous (common bean, *Phaseolus vulgaris*) legume species. The experiments were performed using an oxisol with a high population (10^5 cells g^{-1} soil) of both soybean rhizobia, composed of naturalized strains introduced in inoculants and of indigenous common-bean rhizobia. BOX-PCR was used to evaluate strain diversity, while RFLP-PCR of the ITS (internally transcribed spacer) region with five restriction enzymes aimed at discriminating rhizobial species. In both analyses the genetic diversity of common-bean rhizobia was greater than that of soybean. For the common bean, diversity was greatly enhanced at the 10^{-4} dilution, while for the soybean dilution decreased diversity. Qualitative differences were also observed, as the DNA profiles differed for each treatment in both host plants. Differences obtained can be attributed to dissimilarity in the history of the introduction of both the host plant and the rhizobia (exotic vs. indigenous), to host-plant specificity, rhizobial competitiveness, and population structure, including ease with which some types are released from microcolonies in soil. Therefore, sampling method should be considered both in the interpretation and comparison of the results obtained in different studies, and in the setting of the goals of any study, e.g. selection of competitive strains, or collection of a larger spectrum of rhizobia. Furthermore, effects of sampling should be investigated for each symbiosis.

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1. Introduction

Bacteria collectively known as ‘rhizobia’ are able to establish symbiotic associations with many legumes and a few non-legumes, of which the most important feature is the capability of forming root nodules in which the fixation of atmospheric nitrogen (N_2) takes place. Worldwide, some 44–66 million tons of N_2 are fixed annually, providing nearly

half of all N used in agriculture. The fixation of N_2 by legumes has the potential to contribute greatly to more economically viable and environmentally friendly agriculture. The importance of growing grain and forage legumes is underscored by estimates that they occupy 12–15% of the Earth’s arable land and account for one third of humankind’s dietary protein needs and for up to two thirds under subsistence conditions (Graham and Vance, 2003).

Besides contributing both to worldwide knowledge about soil biodiversity and to the utility of rhizobial collections, the assessment of rhizobial genetic diversity plays an important role in developing long-term strategies to increase the contribution of the biological N_2 fixation to agricultural productivity. Particularly in the last decade, impressive progress has been made in understanding rhizobial diversity with the application of several tools for the analysis of DNA. Examples of useful techniques are the amplification of rhizobial DNA with specific primers such as REP, ERIC,

* Corresponding author. Tel.: +55 43 33716206; fax: +55 43 33716100.
E-mail addresses: hungria@sercomtel.com.br, hungria@cnpso.embrapa.br (M. Hungria).

¹ Present address: Department of Soil Quality, WUR, P.O. Box 8005, 6700 EC Wageningen, The Netherlands.

² Present address: Plant Production Systems Group, WUR, P.O. Box 430, 6700 AK Wageningen, The Netherlands.

BOX (e.g. de Bruijn, 1992; Versalovic et al., 1994; Chen et al., 2000; Ferreira and Hungria, 2002; Mostasso et al., 2002; Grange and Hungria, 2004; Kaschuk et al., 2005), the analysis of ribosomal genes (16S and 23S rRNA) and intergenic regions by RFLP (restriction fragment length polymorphism) and sequencing of DNA (Laguerre et al., 1996; Vinuesa et al., 1998; Chen et al., 2000; Bernal and Graham, 2001; Andrade et al., 2002; Mostasso et al., 2002; Grange and Hungria, 2004; Kaschuk et al., 2005). However, despite these advances and continued refinement of molecular techniques, concerns remain over simple issues such as the effects of sampling methods on the assessment of rhizobial diversity.

Research on rhizobial diversity has employed various sampling methods. In most studies, rhizobia have been obtained either from plants inoculated with serially-diluted soil (e.g. Sessitsch et al., 1997; Mercante et al., 1998; Stralio et al., 1999; Ferreira et al., 2000; Bala et al., 2001; Andrade et al., 2002; Ferreira and Hungria, 2002; Grange and Hungria, 2004) or from field-grown plants (e.g. Amarger et al., 1994; Eardly et al., 1995; Sullivan et al., 1995; Martínez-Romero et al., 1991; Vasquez-Arroyo et al., 1998; Chen et al., 2000; Diouf et al., 2000; Mostasso et al., 2002; Silva et al., 2003). Less commonly, legumes have been grown in pots containing soil (Palmer and Young, 2000) or in tubes containing N-free nutrient solution (Harrison et al., 1987; Frémont et al., 1999). Rhizobia have also been isolated from soil without a host plant, using Petri dishes containing selective media (Bromfield et al., 1994; Louvrier et al., 1996).

In Brazil, rhizobia associated with the two main legume crops have contrasting histories. Soybean (*Glycine max* L. Merrill) is an exotic legume introduced to the country in the last century; it now occupies 21 Mha with a mean yield of 2737 kg ha⁻¹. Brazilian soils were originally void of rhizobia able to effectively nodulate soybean, however, the massive inoculation with a few strains used in commercial inoculants for the last decades has resulted in established populations estimated at 10³ cells g⁻¹ or more in most soils cropped with this legume (Ferreira and Hungria, 2002). Within these naturalized populations, morphological, physiological, genetic and symbiotic variability has been reported and attributed both to adaptation processes and genetic transfer (Santos et al., 1999; Ferreira et al., 2000; Hungria and Vargas, 2000; Ferreira and Hungria, 2002; Galli-Terasawa et al., 2003). On the other hand, despite being first globally as producer and consumer of common bean (*Phaseolus vulgaris* L.), Brazil has one of the lowest average yields, of only 826 kg ha⁻¹. Populations of indigenous rhizobia able to nodulate beans are estimated at 10³–10⁶ cells g⁻¹ soil, with a high level of genetic diversity; they are usually very competitive, but with low N₂-fixation efficiency (Hungria and Vargas, 2000; Hungria et al., 2000; 2003; Andrade et al., 2002).

With the objective of understanding the reasons underpinning reported differences in rhizobial diversity in similar ecosystems the purpose of this study was to evaluate, by using of molecular techniques, the effects of sampling methods on the assessment of genetic diversity of microbial symbionts associated with one exotic and one indigenous legume species.

2. Material and methods

2.1. Experiments

Field plots were established for both common bean (*P. vulgaris*) and soybean (*G. max*) rhizobia on a Dark Red Latosol (*oxisol*) at Embrapa Soja, Londrina, Brazil. Soil for greenhouse experiments was taken from the same field site, at a depth of 0–20 cm, as described before (Andrade and Hamakawa, 1994). Soil chemical analyses showed the following characteristics: pH in CaCl₂, 5.31; C (g dm⁻³), 15.2; no exchangeable Al (cmol_c dm⁻³); cation-exchange capacity (CEC) (cmol_c dm⁻³), 11.4; P, Ca, Mg and K contents (cmol_c dm⁻³), 16.7, 4.91, 1.97, and 0.63, respectively.

Quantitative estimates of the rhizobial populations, by the plant-infection method (Vincent, 1970) have indicated 10⁵ cells g⁻¹ of soil for the soybean cultivar BRS 133 and also 10⁵ cells g⁻¹ soil for the common-bean cultivar IAPAR 81.

The cultivars used to trap rhizobia were BRS 133 for soybean and IAPAR 81 (black seeds) for common bean; the seeds were surface-sterilized (Vincent, 1970). Each study consisted of rhizobia isolated from four treatments: (1) nodules of field-grown plants; (2) nodules of plants grown in pots, each containing 2 kg of soil; (3) nodules of plants grown in Leonard jars and inoculated with 0.5 ml of a 10⁻² dilution of soil or with (4) a 10⁻⁴ soil dilution. Those dilutions were chosen because they are the most frequently used in experiments involving rhizobial diversity; at the 10⁻¹ dilution there are often problems with excess of fungi and in this experiment both populations were estimated at 10⁻⁵, therefore, the 10⁻⁴ dilution would guarantee good nodulation. Average nodule number per plant was of 30 at the 10⁻² and 10 at the 10⁻⁴ dilution.

In treatments 3 and 4, also known as the plant-infection technique, the Leonard jars contained sterilized sand and vermiculite (1:2 v/v) and received N-free nutrient solution with a final pH of 6.5 (Andrade and Hamakawa, 1994). The serially diluted soil started from 10 g of soil dissolved in 90 ml of sterilized 0.85% NaCl, followed by agitation for 30 min at 180 rev min⁻¹, from which 10 ml were transferred to 90 ml of sterilized NaCl that was diluted up to 10⁻⁴. At the field, mean temperatures during the growth period were of 27/22 °C (day/night) (first set of experiments) and 22/19 °C (second set). Plants of treatments 2, 3 and 4 were grown in a greenhouse, with mean temperatures of 28/22 °C (day/night). The experiment was performed twice for each legume and each treatment had four replicates in each experiment.

2.2. Reference strains

Common bean rhizobia reference strains included: *Rhizobium tropici* type A CFN 299 (=USDA 9039, =LMG 9517), type B CIAT 899^T (=UMR 1899, =USDA 9030, =TAL 1797, =HAMB1 1163, =SEMIA 4077, =ATCC 49672), *R. etli* CFN 42^T (=USDA 9032) (received from CFN, Cuernavaca, Mexico). *R. tropici* strain PRF 81 (=SEMIA 4080) came from

the Embrapa Soja germplasm bank, *R. giardinii* bv. *giardinii* strain H152^T and *R. gallicum* bv. *gallicum* strain R602^T were provided by INRA, Dijon, France.

Soybean rhizobia reference strains included the four strains used today in Brazilian commercial inoculants and classified as *B. japonicum*, SEMIA 5079 (=CPAC 15) and SEMIA 5080 (=CPAC 7) and *B. elkanii*, SEMIA 587 and SEMIA 5019 (=29w), as well as *B. japonicum* strain SEMIA 566, used in Brazilian inoculants for many years (Chueire et al., 2003); the five strains came from the Embrapa Soja germplasm bank.

2.3. Rhizobia isolation and morpho-physiological characterization of the isolates

Forty nodules from each treatment and legume species 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 mg kg⁻¹) and a uniform Gram-stain reaction. Colony morphology (color, mucosity, transparency, borders and elevation) and acid/alkaline reaction were evaluated on YMA containing bromothymol blue (0.00125 mg kg⁻¹) as indicator and plants were incubated in the dark at 28 °C. Bean rhizobia growth took 3–5 days and soybean rhizobia 5–7 days. At 3, 5 and 7 days acid/alkaline reaction was verified; acidity was always observed at 3 and alkalinity at 7 days. Each colony was transferred to YM liquid broth (YMB) and after growth was mixed with glycerol (25%) 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 rev min⁻¹.

2.4. Genetic characterization

2.4.1. PCR amplification with specific BOX primer

DNA of isolates was extracted as described by Kaschuk et al. (2005) and amplified by PCR with the primer BOX A1R (5'-CTACGGCAAGGCGACGCTGACG-3', InvitrogenTM, Life Technologies) (Versalovic et al., 1994). The final volume of PCR reaction and cycles for amplifications were also performed as describe before (Kaschuk et al., 2005). Gels were stained with ethidium bromide, visualized under UV radiation and photographed with a Kodak Digital Science 120 apparatus.

2.4.2. Restriction fragment length polymorphism (RFLP) analysis of PCR-amplified internal transcribed space (ITS) 16S-23S rDNA genes

The DNA of bacteria were amplified with primers that codify the ITS region between the 16S and 23S rRNA genes, FGPS 1490 (5'-TGCGGCTGGATCACCTCCTT-3') and FGPS 132' (5'-CCGGGTTTCCCCATTCGG-3') (InvitrogenTM, Life Technologies) (Laguerre et al., 1996). Amplification was achieved with a final volume of 50 µl containing: 29.63 µl of sterile milli-Q water; 1.67 µl of dNTPs (stock at 6 mM of each); 5 µl of buffer 10× (500 mM KCl; 100 mM

tris-HCl, pH of 8.3); 1.5 µl of MgCl₂ (50 mM); 5 µl of each primer—FGPS 1490 e FGPS 132' (stock at 2.5 pmol µl⁻¹); 2 µl of DNA (50 ng µl⁻¹) and 0.2 µl of Taq DNA polymerase (stock at 5 U µl⁻¹). The amplification cycles were: one cycle for 3 min at 95 °C; 35 cycles for denaturation (1 min at 94 °C), for annealing (1 min at 55 °C) and for extension (2 min at 72 °C); one final cycle of 3 min at 72 °C and maintenance at 4 °C. Amplification was verified by looking at fragments in a horizontal electrophoresis agarose gel at 1.5% comparing with Low DNA MassTM Ladder (InvitrogenTM, Life Technologies). For the RFLP, the methodology of Laguerre et al. (1996) was applied to the PCR products, with the following enzymes: *Hha* I (5'-GCG/C-3'; 3'-C/GCG-5'); *Hinf* I (5'-G/ANTC-3'; 3'-CTNA/G-5'); *Msp* I (5'-C/CGG-3'; 3'-GGC/C-5'); *Rsa* I (5'-GT/AC-3'; 3'-CA/TG-5') and *Mbo* I (5'-/GATC-3', 3'-CTAG/-5') (InvitrogenTM, Life Technologies). For each enzyme, a 10-µl mixture was prepared containing: 0.5 µl of enzyme (10 U µl⁻¹); 1 µl of specific buffer for each enzyme (10×); 6 µl of the PCR product and 2.5 µl of sterile milli-Q water. The mixtures were incubated in the water bath at 37 °C according to instructions for each enzyme: *Hha* I, 1 h; *Hinf* I, 3 h; *Msp* I, 2 h; *Mbo* I, 5 h and *Rsa* I, 2 h. The fragments obtained were analysed by horizontal electrophoresis in a gel (20×12 cm) with 3% of agarose and TBE 0.5X, and carried out at 120 V for 4 h.

2.4.3. Cluster analysis

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

2.5. Genetic diversity

Indices of diversity (Shannon and Weaver, 1949), richness (Margalef, 1958) and evenness (Pielou, 1977) were estimated based on the number of isolates belonging to each group of profiles in BOX-PCR and RFLP-PCR, considering a 70% of similarity in the cluster analysis with the UPGMA algorithm and the J coefficient.

3. Results

3.1. Morpho-physiological characterization

Most rhizobial isolates from common bean acidified the YMA medium containing bromothymol blue as pH indicator. When isolated from field-grown plants, 76% of strains showed an acidifying reaction and the others were neutral, whereas when isolated from pot-grown-plants and at dilutions of 10⁻² and 10⁻⁴, 83, 92 and 93% of the strains showed acid reaction, respectively. No rhizobia showed alkaline reaction (data not shown). Most isolates were creamy (>83%), but some were white or orange; they were predominantly convex (>73%) in morphology (data not shown). Mucus production was strong in

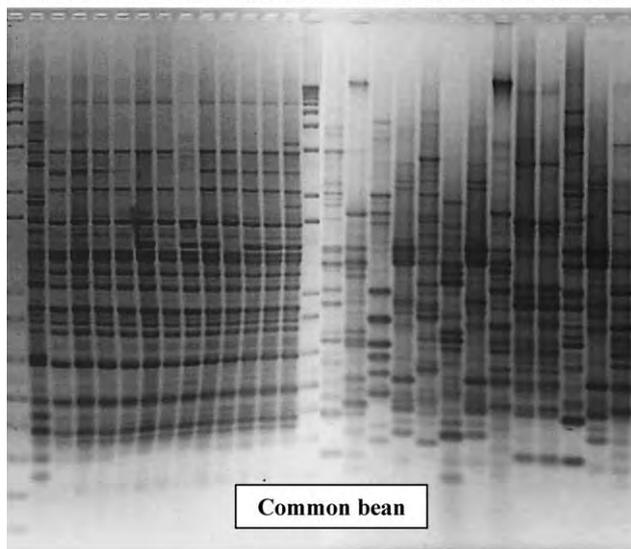
24% of the field isolates and in 18% of those from the pot treatment, increasing to 27 and 47% at the 10^{-2} and 10^{-4} dilutions, respectively (data not shown).

All strains from field- and pot-grown soybean produced an alkaline reaction, in comparison to 80% at dilution 10^{-2} and 73% at 10^{-4} (data not shown). All colonies were cream, convex and with a regular margin (data not shown). A drastic increase in the production of mucus was observed with the soil dilution, with only 5 and 2.5% of the strains showing strong mucus production in the field and pot treatments, respectively, which increased to 95 and 88% at the 10^{-2} and 10^{-4} dilutions, respectively (data not shown).

3.2. BOX-PCR genomic fingerprinting

For common bean, 40 strains were obtained from each treatment; however, after being stored for 6 months, some ceased to grow. Therefore, the DNA of 40, 37, 40 and 31 strains from the field, pot, 10^{-2} and 10^{-4} treatments were analyzed, respectively, and considering a level of similarity of 70%, resulted in 7, 8, 9 and 21 distinct BOX-PCR profiles, respectively. Hence, dilution of the soil allowed the host legume to trap a greater genetic diversity. The most contrasting treatments were field and 10^{-4} dilution, and the BOX-PCR profiles of some of the strains from both treatments are displayed on Fig. 1. Cluster analysis of BOX-PCR profiles of field isolates showed that 21 out of 40 strains grouped at a 75% similarity (data not shown), whereas at the 10^{-4} dilution several clusters were observed, each with few strains, joined at final level of similarity of only 40%; interestingly, none of the reference strains was in the same cluster as any of the indigenous strains (Fig. 2).

P 115 116 117 118 119 120 121 122 123 124 125 126 127 P 518 519 520 521 522 523 524 525 526 527 528 530 531



Field

10^{-4} dilution

Fig. 1. Profiles of BOX-PCR obtained with common-bean rhizobia isolated from field-grown plants, or from plants grown in Leonard jars under greenhouse conditions and inoculated with a 10^{-4} soil dilution. 'P' indicates a DNA pattern of known molecular weight.

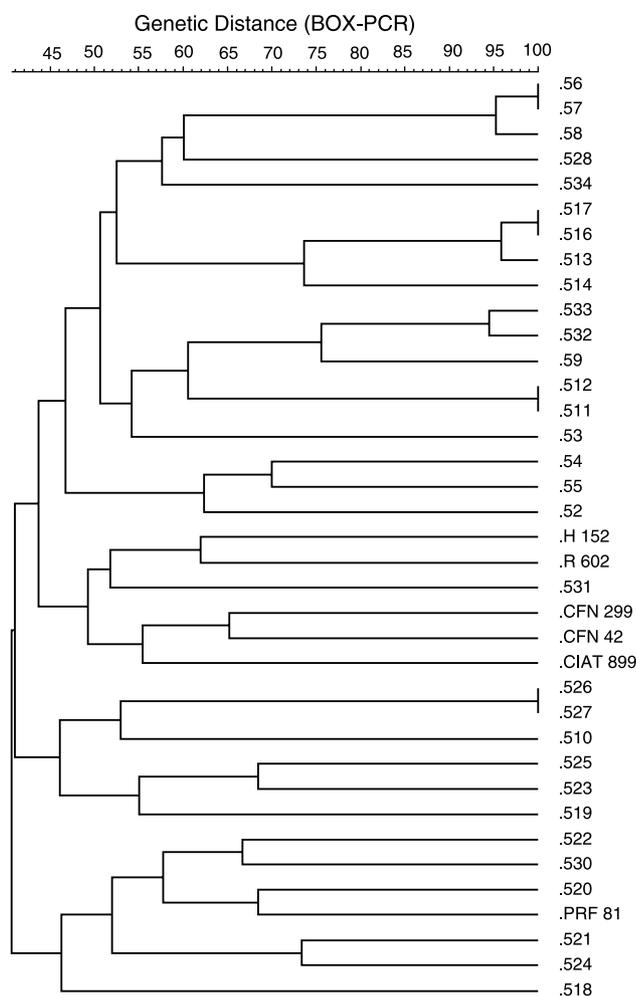
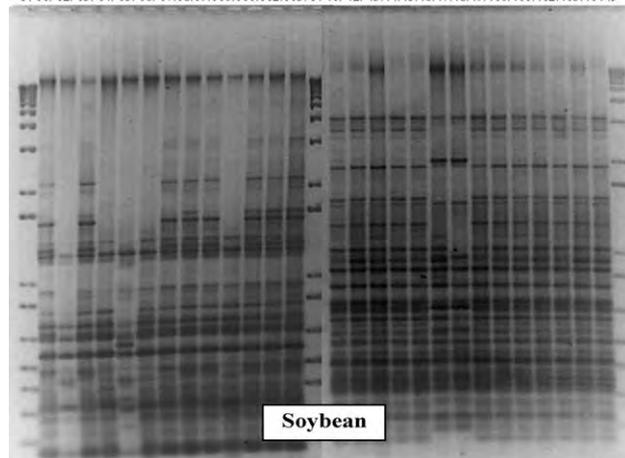


Fig. 2. Dendrogram showing common-bean rhizobial strains isolated from nodules of plants inoculated with a 10^{-4} soil dilution, after cluster analysis of BOX-PCR products using the UPGMA algorithm and the Jaccard coefficient.

P 11 12 13 14 15 16 17 18 19 110 111 112 113 P 41 42 43 44 45 46 47 48 49 410 411 412 413 414 P



Field

10^{-4} dilution

Fig. 3. Profiles of BOX-PCR obtained with soybean rhizobia isolated from field-grown plants, or from plants grown in Leonard jars under greenhouse conditions and inoculated with a 10^{-4} soil dilution. 'P' indicates de DNA pattern of known molecular weight.

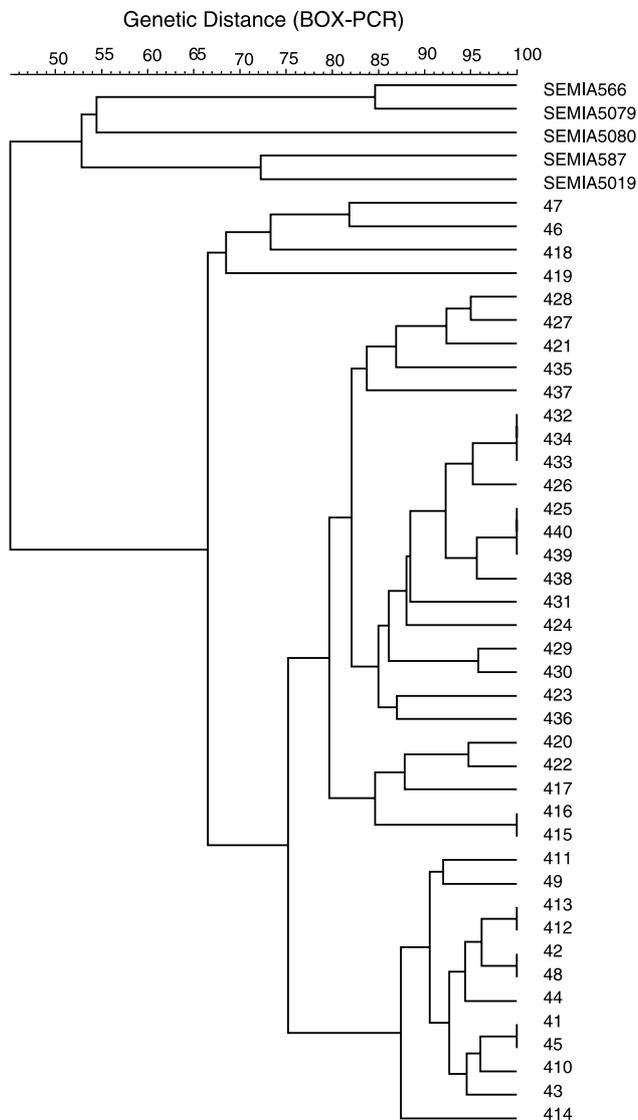


Fig. 4. Dendrogram showing the soybean rhizobial strains isolated from nodules of plants inoculated with a 10^{-4} soil dilution, after cluster analysis of BOX-PCR products using the UPGMA algorithm and the Jaccard coefficient.

Rhizobial diversity was lower when using soybean as a trapping host, when compared to common bean. However, in contrast to that observed with the common bean, diversity with soybean decreased with the soil dilution. Considering a level of 70% similarity in the cluster analysis, of the 40 strains from each treatment, 10 different BOX-PCR profiles were obtained for the field isolates, 11 for the pot, 8 for the 10^{-2} treatment and only 3 for the 10^{-4} ; one example of the two most contrasting treatments is shown in Fig. 3. In the analysis of BOX-PCR, strains from the field clustered at a final level of similarity of 40% (data not shown), whereas at the 10^{-4} dilution the final level of similarity of the isolates was of 65% (Fig. 4). Interestingly, most strains showed profiles different from those of strains used massively in inoculants and established in the majority of the Brazilian soils cropped with soybean (e.g. Fig. 4 for the 10^{-4} dilution).

Experiments were repeated with both crops and the results were confirmed (data not shown).

3.3. Profiles of RFLP-PCR of the internal transcribed space (ITS) between the 16S and the 23S rDNA genes

The DNAs of the soybean and common bean strains from the two most contrasting treatments, the field and 10^{-4} dilution, were submitted to RFLP-PCR analysis of the ITS region with five restriction enzymes. For the common bean, two more strains stop growing, therefore, 39 from the field and thirty from the 10^{-4} dilution were analysed. When amplified with primers for the ITS region, PCR-products obtained for the common bean strains ranged from 800 to 1900 bp and two strains showed two bands, while for the soybean rhizobia the products ranged from 1000 to 1200 bp.

Again, diversity was much greater within the common-bean group than within the soybean. For the common bean isolates from the field, 35 were joined in one cluster at 84% similarity and the final grouping showed a similarity of 22%. On the basis of a 70% similarity, five different profiles were obtained (Fig. 5(A)). In contrast, at the 10^{-4} dilution treatment, at 70% similarity, 25 different profiles were obtained, and the strains were joined at a final level of similarity of only 18% (Fig. 5 (B)). Similarly to the observations of the BOX-PCR analysis, the RFLP-PCR products of the five type species of common-bean rhizobia were distinct and positioned in different clusters from those of the indigenous strains (Fig. 5 (A) and (B)).

Also considering a 70% level of similarity, for the soybean rhizobia isolated from field-grown plants, 25 strains showed the same profile with the five restriction enzymes and 11 others differed from the first group only for the profile with enzyme *Rsa* I (Table 1). When compared with the profiles obtained for the reference strains, rhizobia from the field showed higher similarities with SEMIA 566 and SEMIA 5079 (Table 1). At the dilution of 10^{-4} , 38 of the 40 strains of soybean showed the same profile; strains from this treatment also showed higher similarity with those of the reference strains SEMIA 566 and SEMIA 5079 (Table 1).

3.4. Genetic diversity

The indices of Shannon (H') for diversity, Margalef (RI) for richness and Pielou (EI) for evenness were employed to examine for the BOX-PCR profiles of the strains from both common bean and soybean, on the basis of a 70% similarity. Fig. 6(A) shows that, for common bean strains, H' , RI and EI , the indices increased with dilution, whereas for the soybean strains (Fig. 6 (B)) the indices decreased. For instance, in common bean, H' was 1.21 in field, 1.50 in pot, 1.92 in 10^{-2} and 2.91 in 10^{-4} ($R^2=0.92$). Alternatively, in soybean, H' was 1.79 in field, 2.01 in pot, 1.5 in 10^{-2} and 0.38 in 10^{-4} ($R^2=-0.71$). It was also observed that for the common bean rhizobia, RI showed a remarkable increase in the 10^{-4} treatment, whereas in soybean it decreased considerably when compared to the field strains; furthermore, higher richness was observed in common bean than in soybean. Similar results were observed with RFLP-PCR (Fig. 6 (A) and (B)).

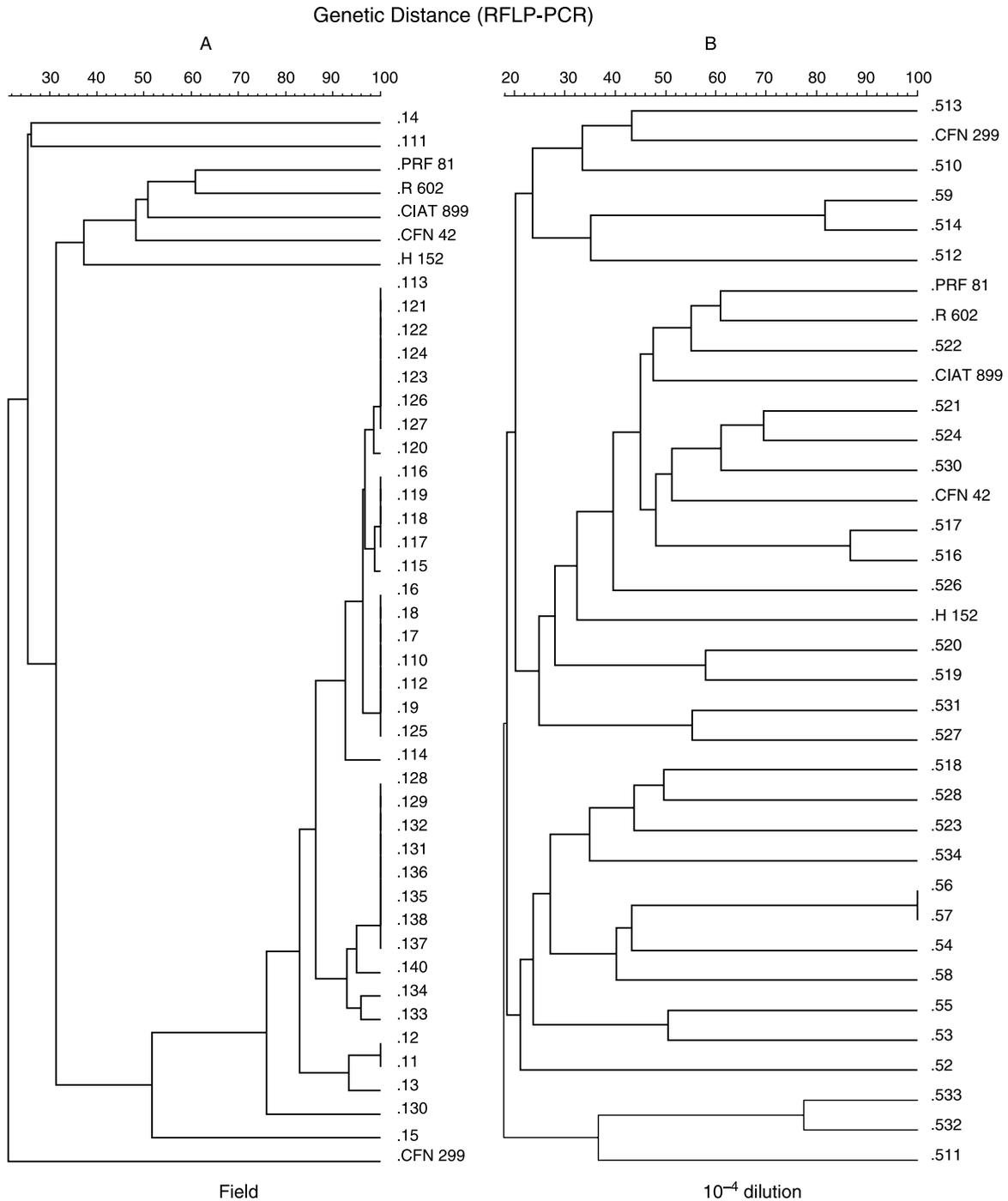


Fig. 5. Analysis of common-bean rhizobial strains isolated from field-grown plants or from plants inoculated with a 10^{-4} soil dilution, considering the RFLP-PCR products of the internal transcribed space (ITS) region between the 16S and 23S ribosomal RNA genes with five restriction enzymes. Clustering used the UPGMA algorithm and the Jaccard coefficient.

4. Discussion

Assessing rhizobial diversity has been a goal of several studies, which seems particularly important in tropical soils, in which probably there are many more varieties of rhizobia than in subtropical regions (Oyaizu et al., 1992; Vinuesa et al., 1998). Particularly in recent decades, molecular tools have been applied in studies of genetic diversity of rhizobia, e.g. DNA amplification with consensus sequences, such as REP,

ERIC and BOX (de Bruijn, 1992; Versalovic et al., 1994), which has proven to be valuable for the detection of diversity at the strain level (e.g. Chen et al., 2000; Bernal and Graham, 2001; Mostasso et al., 2002; Fernandes et al., 2003; Grange and Hungria, 2004). RFLP-PCR associated with ribosomal genes is another useful technique that has been used for phylogenetic studies, as it can discriminate rhizobial species and generally shows good agreement with partial or complete gene sequencing (Laguerre et al., 1996; Vinuesa et al., 1998;

Table 1
Profiles of RFLP-PCR of the ITS region with five restriction enzymes of 40 strains isolated from soybean plants grown at the field and 40 from plants grown in Leonard jars receiving a 10⁻⁴ soil dilution

Treatment	Group	Restriction enzyme					No. strains
		<i>Hha</i> I	<i>Hinf</i> I	<i>Msp</i> I	<i>Mbo</i> I	<i>Rsa</i> I	
Field	1	a	a	a	a	a	25
	2	a	a	a	a	b	11
	3	a	a	b	b	c	1
	4	a	a	c	c	d	1
	5	a	b	b	a	b	1
	6	a	c	a	a	b	1
10 ⁻⁴ dilution	1	a	a	a	a	a	38
	2	a	a	a	a	b	1
	7	b	e	d	e	g	1
SEMIA 566	8	a	a	a	a	c	
SEMIA 5079	8	a	a	a	a	c	
SEMIA 5080	9	a	a	b	a	a	
SEMIA 5019	10	a	a	e	c	d	
SEMIA 587	10	a	a	e	c	d	

Diouf et al., 2000; Doignon-Bourcier et al., 2000; Andrade et al., 2002; Mostasso et al., 2002; Fernandes et al., 2003; Grange and Hungria, 2004). However, contradictory results on rhizobial diversity have been reported, e.g. absence of *R. tropici* in Argentina (Aguilar et al., 1998) and presence in high

proportion in southern Brazil (Hungria et al., 1997, 2000; Andrade et al., 2002); predominance of *R. tropici* (Hungria et al., 1997; Mercante et al., 1998), *R. tropici*/*R. leguminosarum* (Andrade et al., 2002), or *R. etli* (Grange and Hungria, 2004) in southern Brazil; predominance of *R. tropici* (Mercante et al.,

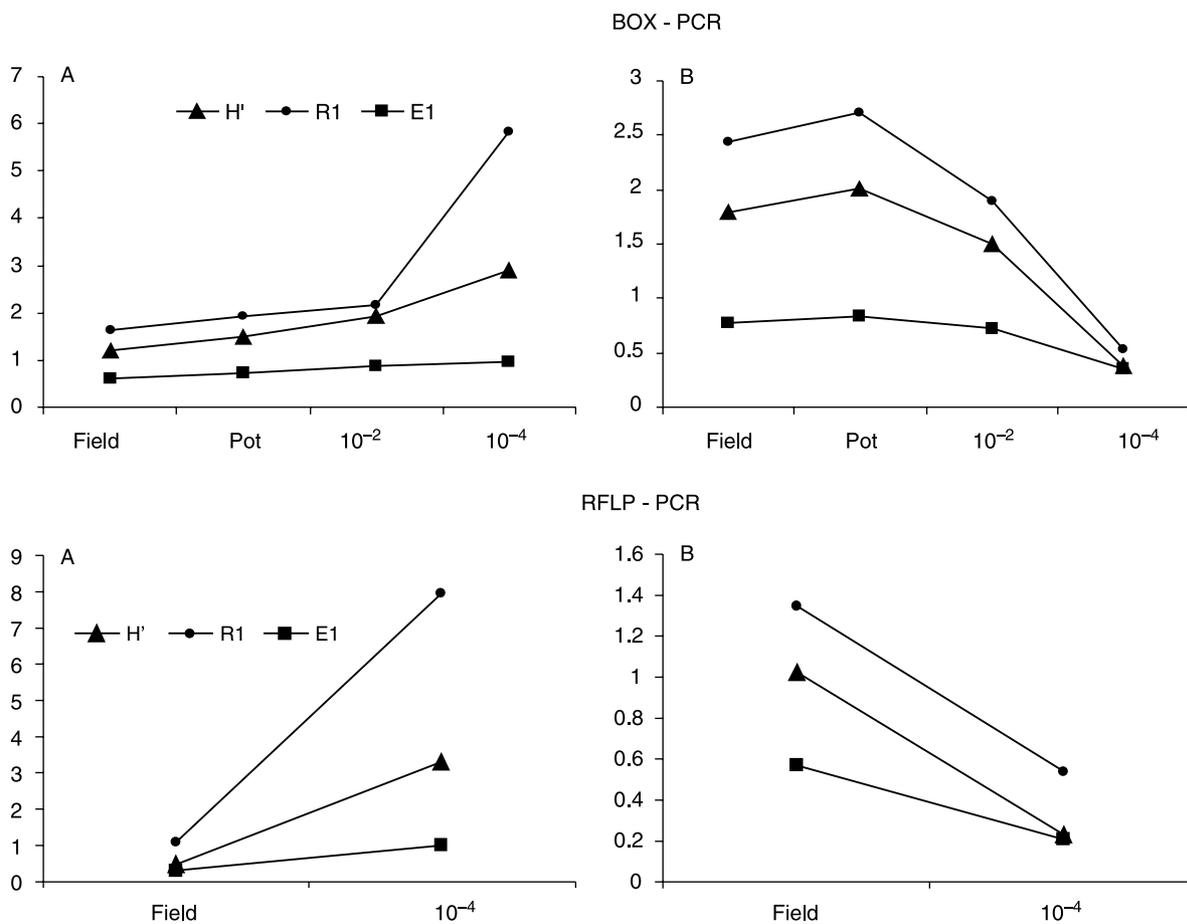


Fig. 6. Indices of diversity (*H'*), richness (*R1*) and evenness (*E1*) of rhizobial strains isolated from nodules of common bean (A) and soybean (B) grown in the field, in pots or inoculated with serially soil dilutions at 10⁻² and 10⁻⁴. Indices calculated considering the BOX-PCR products and the RFLP-PCR products, both discriminated at 70% of similarity by the UPGMA algorithm and the Jaccard coefficient.

1998; Mostasso et al., 2002) or *R. leguminosarum/R. etli* (Straliotto et al., 1999; Grange and Hungria, 2004) in other Brazilian soils. The objective of this study was to verify if assessment of diversity of the rhizobia is affected by the sampling method, plant-growth conditions, and/or history of the introduction of the legume in the soil.

4.1. Differences on rhizobial diversity due to the host plant

Genetic diversity of common-bean rhizobia was consistently greater than that of soybean. Mesoamerica and Andean South America are considered to be the centers of origin of common bean; domestication has been in progress for perhaps 5000 years (Gepts, 1990; Bernal and Graham, 2001); the legume has also been widely cropped in Brazil for thousands of years. The promiscuous nature of common-bean genotypes used in Brazil is revealed by the wide range of rhizobia that have been isolated from it, including *R. leguminosarum*, *R. tropici*, *R. etli* (Martínez-Romero et al., 1991; Mercante et al., 1998; Straliotto et al., 1999; Andrade et al., 2002; Mostasso et al., 2002; Martínez-Romero, 2003; Grange and Hungria, 2004), *R. giardinii* (Mostasso et al., 2002), *Sinorhizobium spp.*, *Mesorhizobium spp.* (Straliotto et al., 1999; Grange and Hungria, 2004), and *Bradyrhizobium* (Hungria et al., 1993), as well as other strains that may well represent new species (Mostasso et al., 2002; Grange and Hungria, 2004). In contrast, soybean is an exotic species introduced to Brazil in 1882 at which time the soils were devoid of rhizobia able to effectively nodulate it; only a few strains have been introduced in inoculants since expansion in planting began in the 1960s (Ferreira and Hungria, 2002). However, within the now-naturalized population, morphological, physiological, genetic and symbiotic variability has been reported and attributed both to adaptation processes and genetic transfer (Santos et al., 1999; Ferreira et al., 2000; Hungria and Vargas, 2000; Ferreira and Hungria, 2002; Galli-Terasawa et al., 2003). Furthermore, it has been reported in Paraguay (Chen et al., 2000) and Brazil (Hungria et al., 2001) that some fast-growing rhizobia genetically close to *R. tropici* may also nodulate soybean.

4.2. Effects of pH

Changes in soil pH may be responsible for differences in the diversity of the rhizobia trapped. For example in Kenya, at pH of 4.5 *R. tropici* was the dominant species nodulating common bean, whereas it changed to *R. etli* at pH of 6.8 (Anyango et al., 1995). In our study, under field conditions and in pots containing soil the pH was of 5.3, while in Leonard jars the pH was of 6.5. However, for the common bean the number of BOX-PCR profiles under field or pot conditions (7 and 8, respectively) did not differ from the number obtained in the dilution of 10^{-2} (9 profiles), and a similar picture was obtained for soybean (10, 11 and 8 profiles, respectively); therefore, pH of the substrate did not affect strain diversity. In contrast, a strong effect was attributed to soil dilution, as at 10^{-4} the number of BOX-PCR profiles was greatly increased for

common bean (21 profiles) and decreased for soybean (3 profiles).

4.3. Effects of soil dilution for both common beans and soybean

Diversity variability due to soil dilution was observed in all analyses. In relation to the morpho-physiological properties, for the common-bean rhizobia, both the percentages of strains with an acid reaction and mucus production evaluated in vitro increased from the survey in the field to the 10^{-4} dilution. For soybean, alkaline reaction decreased and mucus production increased at the 10^{-4} soil dilution. As reported in the previous item, variability of common-bean strains evaluated by BOX-PCR was greatly enhanced from the field to the 10^{-4} dilution, while the opposite was observed for soybean, and similar results were obtained for species diversity, evaluated by the RFLP-PCR analysis. Therefore, for the common bean, five profiles were obtained in the analyses of 39 strains from the field, increasing to 26 profiles in 30 strains from the 10^{-4} dilution. In contrast, for 40 soybean rhizobia, six profiles were observed in the field treatment, decreasing to three at the 10^{-4} dilution. Indices of diversity (H'), richness (RI) and evenness (EI) have been utilized in several studies (Kennedy and Smith, 1995; Kennedy, 1999; Andrade et al., 2002) and, when applied to the profiles obtained in both BOX-PCR and RFLP-PCR analyses, they confirmed that the 10^{-4} dilution resulted in higher diversity for the common bean and lower for the soybean rhizobia. The values reported for the common bean were in the same range as those reported in another study performed in soils of the Paraná State (Andrade et al., 2002).

The promiscuous nature of the common bean is probably associated with an ability to perceive signals for nodulation from many types of rhizobia; however, often there are reports of very low or even ineffective symbioses with indigenous rhizobia (Michiels et al., 1998; Hungria et al., 2000, 2003; Martínez-Romero, 2003). Indeed, several reports show that indigenous common bean strains in South America and Africa are very competitive in nodulating the host, but that the effectiveness of the N_2 -fixation process is usually low (Graham, 1981; Hungria et al., 2000, 2003). The promiscuous nature of the host, associated with the high competitiveness of the rhizobia could explain the results obtained in this study. It is likely that the most competitive strains colonized the common bean under field conditions, resulting in the detection of lower genetic diversity. On the other hand, dilution of the soil resulted in a decreased proportion of the most competitive strains, enabling the less competitive strains to nodulate the host. This could also help to explain why, in searches for more efficient and competitive rhizobia for common bean, using field-grown plants, there was predominance of *R. tropici* (Martínez-Romero et al., 1991; Hungria et al., 2000, 2003; Mostasso et al., 2002), known for tolerating acidity and being very competitive in acid soils (Martínez-Romero et al., 1991; Graham et al., 1994; Hungria and Vargas, 2000; Hungria et al., 2000), whereas in other experiments where serially diluted soil was used as inoculum, *R. etli* and *R. leguminosarum* were

the predominant species (Straliotto et al., 1999; Grange and Hungria, 2004). In one study with white clover (*Trifolium repens* L.), Harrison et al. (1987) did not find an effect of dilution on the genetic structure of *R. leguminosarum* bv. *trifolii*; however, this could be attributed to the high specificity of the host species. Bala et al. (2001), using RFLP-PCR of the ITS, also verified less diversity in higher soil dilutions used as inocula for *Calliandra calothyrsus* Meissn., *Gliciridia sepium* (Jacq.) and *Leucaena leucocephala* (Lam.), and suggested that more competitive but less abundant strains usually occupy the nodules, but with soil dilution, less competitive but more numerous strains would be sampled.

In Brazilian soils, the naturalized population of soybean bradyrhizobia consists of few strains that have been introduced as inoculants in the last 50 years. Therefore, the population descends from these few strains and, after some years, an equilibrium between the main serogroups has often been observed (e.g. Nishi and Hungria, 1996). However, some of the established strains may be more competitive than others, e.g. those belonging to serogroup SEMIA 566 (same serogroup as SEMIA 5079) (Mendes et al., 2004), therefore with soil dilution, added to the more specific nature of the host plant, the most competitive strains will occupy the majority of the nodules. In addition, it is also possible that few genotypes, genetically limited, are more abundant in the rhizobial community and have greater probability of being trapped under higher dilutions. Finally, another important feature is that soil dilution also resulted in qualitative differences, as different profiles were obtained in each treatment, for both host species.

4.4. Conclusion

The parameters utilized in this study confirmed that sampling methods affect the assessment of rhizobia diversity, both quantitatively and qualitatively. The differences reported here could be attributed to dissimilarity in the history of the introduction of both the host plant and the rhizobia (exotic vs. indigenous), to host-plant specificity, rhizobial competitiveness, and population structure, including ease with which some types are released from microcolonies in soil. Therefore, depending on the goal, the sampling should be designed accordingly and the interpretation and comparison of the results obtained in studies of rhizobial diversity should take the sampling method into account. Furthermore, sampling should also be considered in accordance with the purpose of the study, whether for strain selection, for ecology, or for assessing a larger spectrum of rhizobia.

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