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Tillage method and crop rotation effects on the population sizes and diversity of bradyrhizobia nodulating soybean

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Abstract

This study was conducted in an area of Brazil cultivated with soybean since the early 1960's but which for the last 17 yr was under different tillage (no-tillage, NT; conventional tillage, CT) and crop rotation (soybean, S/wheat, W/maize, M; S/W; M/W) systems. The area had not received any inoculant for the last 15 yr and our objective was to investigate the effects of tillage and cropping systems on the bradyrhizobia population. The NT system and crop rotations with soybean resulted in high populations of bradyrhizobia, but even in the treatment where soybean had not been cultivated for 17 yr (M/W) the number of viable cells in the soil was high. A total of 142 bradyrhizobia isolated from the different treatments were characterized based on colony morphology, serological reaction, DNA analysis by RAPD, protein and Nod factors profiles. The analyses resulted in grouping of the isolates into 16 DNA, five protein and three Nod factors profiles. A high proportion (37.5%) of the isolates did not react with any known serogroup. Both NT and crop rotations with soybean resulted in a higher bradyrhizobia diversity, with the lowest number of genomic patterns occurring in the CT with M/W rotation. However, there was no relationship between the treatment combinations and genetic relatedness. The evaluation of symbiotic performance under greenhouse conditions showed that the isolates with higher rates of N_2 fixation were also isolated from NT with S/W or S/W/M crop rotations. Consequently, the use of agronomic practices such as NT and crop rotation with legumes will not only contribute to agricultural sustainability, but also help to maintain bradyrhizobia population and diversity.

Keywords: Bacteria diversity; *Bradyrhizobium*; Crop rotation; Nitrogen fixation; Tillage system

1. Introduction

Sustainable agriculture seeks to provide for the needs of the present without compromising the potential in the future. Therefore, practices which produce suitable yields and economic returns and, at the same time, enhance an maintain soil quality are preferred over those that degrade the soil as a resource base. A component of soil quality maintenance is favoring the action of beneficial soil organisms, one of the most important of which are the root nodule bacteria involved in the biological nitrogen fixation process.

Several combinations of legumes and nonlegumes are used in multiple cropping systems, rotation or intercropping and contribute to the regenerative processes that must operate in a sustainable system (Bohlool et al., 1992). Symbiotic nitrogen fixation plays a key role in these cropping systems and a right combination can bring benefits in N status of both legumes and nonlegumes (Nambiar et al., 1982; Crookston et al., 1991; Hungria et al., 1997a,b).

Different tillage systems also affect soil sustainability. Conventional tillage, with the traditional practices of plowing and disking to prepare land, reduces soil organic matter and increases erosion, especially in the tropics. As an alternative, no-tillage management, sow-

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ing directly through the mulch, protects the soil against erosion by water, improves soil structure stability and moisture content and, with time, also increases soil organic matter content (Blevins et al., 1977; Kemper and Derpsch, 1981; Sidiras et al., 1982; Pavan et al., 1985). Several studies have also indicated that the no-tillage system increases soil biomass and the size of the microbial population (Powlson and Jenkinson, 1981; Staley et al., 1988; Alvarez et al., 1995). For no-tillage systems in the south region of Brazil, differences of about 50% in soil biomass and rhizobial populations, in relation to the conventional tillage, were reported (Hungria and Vargas, 1996; Hungria et al., 1997a). Consequently, there has been an appreciable increase in the adoption of no-tillage management by farmers, and the system currently occupies 4.5×10^6 ha in Brazil, mainly cropped with soybean and maize, representing 30% of the country's cultivated area (Gassen and Gassen, 1996).

Soybean was introduced in Brazil around 100 yr ago, but large-scale commercial cultivation did not begin until the 1960's. Brazilian soils were originally free of soybean bradyrhizobia but inoculation has established populations of some few strains in most of the 12×10^6 ha which are today cultivated with this crop (Vargas and Hungria, 1997). Previous evaluations performed in Brazil have demonstrated that some crop rotations and the no-tillage management system favor bradyrhizobia populations, nodulation, nitrogen fixation rates and yield (Voss and Sidiras, 1985; Hungria and Stacey, 1997; Hungria et al., 1997a). However, the scale of bradyrhizobia diversity in different agronomic systems has yet to be determined. Consequently, we studied an area cultivated with soybeans for more than 30 yr but which was under different tillage and crop rotation systems for the last 17 yr. The area had not been inoculated for the past 15 yr. Our objective was to evaluate tillage and crop effects on the soybean bradyrhizobia population.

2. Materials and methods

2.1. Bacteria count and isolation

Samples were taken in 1995. Seventeen years previously the experiment had been installed in an area cultivated with soybean since the early 1960's in an oxisol at the Experimental Station of Instituto Agronômico do Paraná, district of Londrina, State of Paraná, in the south region of Brazil. The experiment was a 2×3 factorial in a split-plot design, with three replicates for each treatment. The experimental plots measured 8×25 m and plots were separated by 2.0 m. Tillage treatments consisted of the no-tillage, NT, with sowing directly through the mulch and the conventional tillage, CT, with the traditional practices of plowing and disking to prepare land. Tillage systems were localized on the main plots and crop rotations (soybean (*Glycine max*)/wheat (*Triticum aestivum*)/maize (*Zea mays*), S/W/M; soybean/wheat, S/W; maize/wheat, M/M) on the subplots. The crops received fertilizers according to the soil analysis realized before each cropping. The area had not received any inoculant for the last 15 yr and N fertilizer was never applied to the soybean crop.

Five days before soybean (S/W; S/W/M) or maize (M/W) harvest, five soil samples were recovered from each subplot (totaling 15 samples per each of the six treatments). Each one of the five soil samples consisted of 10 samples randomly collected within each 8×25 m plot, at the depth of 0 to 15 cm, and mixed in a sterilized bag. Soil samples were collected with a soil drill cleaned with alcohol (95%) and flamed between samplings. Analyses proceeded immediately, the samples were sieved (4 mm) and divided for the determination of moisture content, chemical properties and rhizobia isolation. Chemical analyses were performed according to Pavan et al. (1992) and moisture content after drying the soil at 105°C for 4 d.

For both the evaluation of the established bradyrhizobia population and for strain isolation, 10 g of fresh soil of each subplot were serially diluted (to 10^{-9}), with five replicates per dilution. Soil dilutions (1 ml of each dilution) were used to inoculate surface sterilized (Vincent, 1970) soybean seeds of cultivar BR-16 (cultivar resulting from the crossing of D69-B10-M58 and Davis). Three seeds were placed in each modified Leonard jar (Vincent, 1970) containing sand and vermiculite (1:2, v/v) and thinned to two seedlings per jar 3 d after emergence. Plants were grown under greenhouse conditions, with mean temperatures of 28/22°C (day/night) and received N-free nutrient solution (Andrade and Hamakawa, 1994). Plants were harvested 5 weeks after emergence, divided into shoots and roots and roots were washed to evaluate nodulation. The number of bradyrhizobia cells was evaluated by the most probable number (MPN) counting technique (Vincent, 1970), using the statistical tables of Andrade and Hamakawa (1994). To express the results as g^{-1} soil, the correction was made according to the moisture content. All statistical data processing was performed by using the version 6 of the package SAS (SAS, 1990), considering the split-plot design with three replicates.

From each treatment, 25 nodules were randomly collected from the dilution 10^{-2}, totaling 150 nodules for the whole experiment. Rhizobia were isolated from nodules according to standard methodologies (Vincent, 1970) and reinoculated in soybean cultivar BR-16, to confirm their infectivity.

2.2. Characterization of isolates

2.2.1. Morphological and serological analyses

The morphological and serological characterization of the isolates were performed as described by Boddey and Hungria (1997). The isolates were tested against the antisera of strains SEMIA 527, SEMIA 566 (the same serogroup as CPAC 15, =SEMIA 5079), 532c (=SEMIA 5039), SEMIA 587, 29w (=SEMIA 5019), CB 1809 (=SEMIA 586 and belonging to the same serogroup as CPAC 7, =SEMIA 5080). These strains had been used in Brazilian commercial inoculants for variable periods of time since the early 1960's.

2.2.2. Protein fingerprinting

Protein profiles were determined for all isolates, using 30 ml of cultures grown on TY medium (Beringer, 1974) for 5 d. Cells were centrifuged, washed three times in 0.85% NaCl and once in 10 mM Tris–HCl pH 7.6. Cells were resuspended in 10 mM Tris–HCl pH 7.6 to the concentration of 10^9 cells ml^{-1} and 200 µl of this suspension were mixed with 200 µl of sterilized lysis buffer containing: water, 8 ml, 0.5 M Tris–HCl pH 6.8, 2 ml, glycerol, 1.6 ml, 10% SDS, 3.2 ml, mercaptoethanol, 0.8 ml and 0.05% bromophenol blue, 0.4 ml. Cells were boiled for 5 min and centrifuged at 10,000 rev min^{-1} for 5 min, harvesting the supernatant. Samples were run in an acrylamide gel (29.2 g of acrylamide and 0.8 g of *N*,*N*′-methylenebisacrylamide in 100 ml). The gel for separation was prepared at 12.5% (containing, in 50 ml: water, 16.75 ml; 1.5 M Tris–base pH 8.8, 12.5 ml; 10% SDS (sodium dodecyl sulfate), 500 µl; bis/acrylamide, 20 ml; 10% ammonium persulfate, 250 µl; TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine), 25 µl) and the gel for concentration was prepared at 3.5% (water, 9.26 ml; Tris, 3.75 ml; SDS, 150 µl; bis/acrylamide, 1.75 ml; ammonium persulfate, 75 µl; TEMED, 7.5 µl). The buffer contained, l^{-1}: Tris–base, 15.1 g; glycine, 72.0 g; SDS, 5 g. The buffer for the run consisted of (5×): Tris–base, 15 g; glycine, 72.0 g; 0.5% SDS. The samples were run at 100 V for 3–4 h. Gel staining was as follows: 2 h in 50% methanol: 10% acetic acid; one night in 5% methanol: 7% acetic acid; washings of 5 min for two times with distilled water; staining for 4 h with 0.1% of Coomassie brilliant blue in water, followed by discoloration for 2 h in 50% methanol: 10% acetic acid and finally several washes with 7% acetic acid.

2.2.3. Genetic analysis by PCR with arbitrary primers (RAPD or AP-PCR)

The DNA of all isolates was extracted according to standard procedures (Sambrook et al., 1989). Amplification was performed with a final volume of 25 µl, containing: water, 11.5 µl; dNTPs (1.5 mM), 5.0 µl; buffer 10 × (500 mM KCl; 100 mM Tris–HCl pH 8.3), 2.5 µl; 20 mM $MgCl_2$, 3.0 µl; primer (30 ng), 1,0 µl; DNA (50 ng), 1.0 µl; Taq polymerase, 1.0 µl (1.2 U). DNA was amplified with 12 10-bp long primers of kit-S of Operon (Operon Technologies, Alameda, CA, USA): 1 (5′ CTACTGCGCT 3′); 3 (5′ CAGAGGTCCC 3′); 5 (5′ TTTGGGGCCT 3′); 6 (5′ GATACCTCGG 3′), 7 (5′ TCCGATGCTG 3′), 9 (5′ TCCTGGTCCC 3′), 10 (5′ ACCGTTCCAG 3′); 12 (5′ CTGGGTGAGT 3′); 16 (5′ AGGGGGTTCC 3′), 17 (5′ TGGGGACCAC 3′); 19 (5′ GAGTCAGCAG 3′) and 20 (5′ TCTGGACGGA 3′). The reaction was carried out in an MJ Research Inc. PT 100 thermocycler using 45 cycles of: at 94°C for 1 min, annealing at 35°C for 1 min and extension at 72°C for 2 min. Each strain was analyzed with each primer at least twice, after DNA extraction of independent liquid cultures. After separation of amplified fragments by horizontal electrophoresis in 1.5% agarose gels (low EEO, type I–A) and staining with ethidium bromide, the gels were photographed under UV radiation and the presence or absence of bands was transformed in a binary matrix of presence/absence (1/0). *B. japonicum* strain USDA 110 (=I1b110, =TAL102, =RCR3427, =61A89), received from Dr P. van Berkum (USDA, Beltsville, MD, USA) was used in these analyses as a control. Cluster analysis was accomplished with the NTSYS-PC program (numerical taxonomic and multivariate analysis system, version 1.70, Exeter Software, New York, USA), using the UPGMA (unweighted pair group arithmetic average clustering) and the SM (simple matching) and the J (Jaccard) coefficients.

2.2.4. Analysis of lipo-chitin Nod signals

Thin layer chromatography analysis (TLC) of lipo-chitin oligosaccharide Nod signals (Nod factors) of 16 isolates representative of the genomic patterns determined by RAPD was performed with ^{14}C-glucosamine as described before (Hungria et al., 1996) after induction with seed exudates of cultivar BR-16.

2.3. Symbiotic performance

2.3.1. Host range

Strain host range was verified with isolates representative of the 16 genomic patterns obtained by RAPD. The experiment was performed in modified Leonard jars containing N-free nutrient solution as described in the bacteria isolation item. Host range was tested using surface disinfected seeds of the following legumes: *Leucaena leucocephala*, *Phaseolus vulgaris* and *Macroptilium atropurpureum*.

2.3.2. N_2 fixation rates

A greenhouse experiment (mean temperature of 28/23°C, day/night) was performed also with 16 isolates

Table 1
Chemical properties of the soils

Tillage system[a]	Crop rotation[b]	pH in $CaCl_2$	Al ($cmol_c\ dm^{-3}$)	K ($cmol_c\ dm^{-3}$)	Ca ($cmol_c\ dm^{-3}$)	Mg ($cmol_c\ dm^{-3}$)	H + Al ($cmol_c\ dm^{-3}$)	Al ($g\ dm^{-3}$)	C ($g\ dm^{-3}$)	P ($mg\ dm^{-3}$)
NT	S/W/M	4.70	0.11	0.61	4.43	2.30	6.69	1.47	1.52	54.1
NT	S/W	4.90	0.04	0.58	5.33	2.34	6.21	0.48	1.64	36.0
NT	M/W	4.90	0.02	0.74	5.18	2.43	5.76	0.23	1.66	33.4
CT	S/W/M	4.60	0.16	0.52	3.85	1.52	6.21	2.64	1.22	18.9
CT	S/W	4.80	0.05	0.55	4.65	1.93	5.76	0.69	1.56	21.6
CT	M/W	4.60	0.07	0.58	4.98	1.77	6.69	0.94	1.52	17.7

[a] NT, no-tillage; CT, conventional tillage.
[b] S, soybean; W, wheat; M, maize.

representative of genomic patterns obtained by RAPD. The experiment used modified Leonard jars filled with N-free nutrient solution, as described in the item of bacteria isolation. Inoculant preparation (10^9 cells ml^{-1}), seed inoculation and plant growth conditions were as previously described (Hungria et al., 1996). Controls included noninoculated plants and plants inoculated with the strains currently recommended for the use in Brazilian commercial inoculants: SEMIA 587, 29w (=SEMIA 5019), CPAC 7 (=SEMIA 5080) and CPAC 15 (=SEMIA 5079). Plants were harvested at 45 d after emergence and measurements of nodule number and dry weight, shoot and root dry weight and plan total N (N-Kjeldahl of shoot N+root N+nodule N−seed N) were recorded. The experiment was performed in a randomized block design with five replicates. The results were submitted to analysis of variance (SAS, 1990) and the treatment means were separated by the Tukey's test at $P \leq 0.05$.

3. Results and discussion

Although the soil chemical analysis was unreplicated, the aluminum content for the crop rotation S/W/M was higher, while under the NT both magnesium and phosphorus contents were higher (Table 1). Although the sampling was made in a long-term experiment, few data were published through the years, but soon after the plot establishment it was demonstrated that NT improved soil structure stability, moisture content, reduced soil temperature and increased soil organic matter content (Kemper and Derpsch, 1981; Sidiras et al., 1982; Pavan et al., 1985). In the samples taken during this experiment, carbon content was slightly higher (about 10%) under the no-till (Table 1), but an important feature was that the mean soil temperature during the 12 months before the collection was 6°C lower for the NT, with maximum temperature under the CT during the summer frequently reaching 42–44°C (data not shown).

The number of soybean bradyrhizobial cells was higher under the no-tillage system and under the crop rotations including soybean (Table 2), confirming results obtained in Brazil (Hungria and Stacey, 1997; Hungria et al., 1997a). Increases in population due to cropping with the homologous host legume were also reported for other rhizobial species, e.g. cowpea bradyrhizobia (Thies et al., 1995). Of 150 bacteria isolated from soybean nodules randomly collected from plants inoculated with soil dilutions from the six different agronomic systems, 142 have shown slow growth and alkali production in YM medium and eight were characterized by a faster growth and acid production. This study has characterized the 142 *Bradyrhizobium* isolates.

Of the four methodologies we employed, i.e., morphological characterization, serological reaction, protein profile and DNA fingerprinting after PCR reaction with arbitrary short primers (RAPD or AP-PCR), the last one was the most discriminating to characterize the isolates. Using the RAPD, the 142 isolates have shown 16 distinct genomic patterns and the presence of each of these profiles in the different agronomic systems is shown on Table 2. A higher *Bradyrhizobium* diversity was observed in soils under NT with the crop rotations with S/W/M, while the lowest occurred in soils under CT and the M/W rotation. The presence of soybean in the crop rotation significantly affected bradyrhizobia diversity, increasing the number of genomic patterns (Table 2).

The majority of the isolates were amplified by the PCR reaction with the 10-bp long primers used in this study and, when one isolate was not amplified by one primer, amplification was obtained by the others. For example, the isolate with the genomic pattern 11 was not amplified by primer 7, but amplification was obtained with primer 20 and a contrasting situation occurred with isolate 2 (lines 11 and 2, Figs. 1A and B). Differences were detected also among primers in relation to the grade of polymorphism obtained. For example, the use of primer 7 resulted in a lower level of polymorphism, with isolates 3, 4, 5, 7 and 12 showing similar fingerprintings (Fig. 1A). However, other primers were able to detect polymorphic bands among these same isolates, e.g. primer 20 (Fig. 1B).

The cluster analysis using the PCR products obtained by the amplification with the 12 short and arbitrary primers has indicated a high degree of genetic diversity among the 16 genomic patterns obtained. Using both the SM (Fig. 2) and the Jaccard (Fig. 3) coefficients, several clusters were formed. With the SM coefficient, a major cluster included isolates 1, 2, 13, 17, 15, 16 and 12, which linked to another cluster containing isolates 3, 4, 5, 7 and 6 at the level of 0.738. Finally, isolates 8 and 11 were linked to these two groups at a level of 0.669, followed by isolates 14, 9 and 10, this last one linked at a level of 0.649 (Fig. 2). With the Jaccard coefficient, which is more discriminating, producing discrete clusters at lower levels of similarity (Goodfellow et al., 1985), a higher number of clusters was obtained, with *B. japonicum* strain USDA 110 occupying a position isolated from all other isolates (Fig. 3). However, there was no relationship between genetic relatedness and tillage/cropping systems. For example, isolates 4, 12, 13 and 15, detected in the agronomic system with lower bradyrhizobia diversity (CT with the M/W rotation), were far distant from each other on the cluster analysis. Also the isolates which appeared more frequently, 1, 9, 11 and 12, were not genetically related.

Since the advent of the polymerase chain reaction (PCR) (Saiki et al., 1988), this method has been used in an increasing number of studies and the same has happened with the RAPD (Random Amplified Polymorphic DNA), a variation of PCR which utilizes primers with arbitrary sequences to begin DNA copy and amplification (Welsh and McClelland, 1990; Williams et al., 1990). PCR analysis with arbitrary or specific primers (particularly the repetitive sequences ERIC, REP and BOX) has also proven to be a useful technique for identification and characterization of rhizobia strains, being less laborious than other methods (de Bruijn, 1992; Harrison et al., 1992; Versalovic et al., 1994; Richardson et al., 1995; Selenska-Pobell et al., 1995; Hungria et al., 1998). However, in a bean (*Phaseolus vulgaris*) rhizobia study the RAPD detected more genetic diversity than the ERIC and REP-PCR

Table 2
Population size of soybean bradyrhizobia and genomic patterns identified by PCR using arbitrary short primers of isolates from soils under different tillage and crop rotation systems for the last 17 yr. The area had been cultivated with soybean since the early 1960's and has not received any inoculant for the last 15 yr

Tillage system[a]	Crop rotation[b]	*Bradyrhizobium* (No. cells g^{-1} soil)	Genomic pattern[c]	No. of genomic patterns[c]
NT	S/W/M	4.47×10^3 a[d]	1, 2, 3, 5, 7, 8, 9, 11, 13, 14, 16	11 a
NT	S/W	5.01×10^3 a	1, 3, 4, 6, 9, 10, 11, 12, 14, 15	10 ab
NT	M/W	5.24×10^2 bc	4, 7, 9, 11, 12, 16	6 bc
CT	S/W/M	1.51×10^3 ab	1, 2, 3, 4, 6, 8, 9,10, 12	9 ab
CT	S/W	1.58×10^3 ab	1, 5, 6, 9, 11, 13, 15, 16	8 abc
CT	M/W	2.88×10^2 c	4, 12, 13, 15	4 c
dms for tillage system		1.51×10^2		n.s.
dms for crop rotation		1.15×10^2		3.7

[a] NT, no-tillage; CT, conventional tillage.
[b] S, soybean; W, wheat, M, maize.
[c] Genomic patterns obtained after the analysis of 25 nodules from each treatment. For the number of genomic patterns, values followed by the same letter did not show statistical difference (Tukey, $P \leq 0.05$).
[d] Means of 15 values (five soil samples from each of three replicates) followed by the same letter did not show statistical difference (Tukey, $P \leq 0.05$). The dms ($P \leq 0.05$) for individual effects of tillage system and crop rotation is also shown, n.s. means not significant.

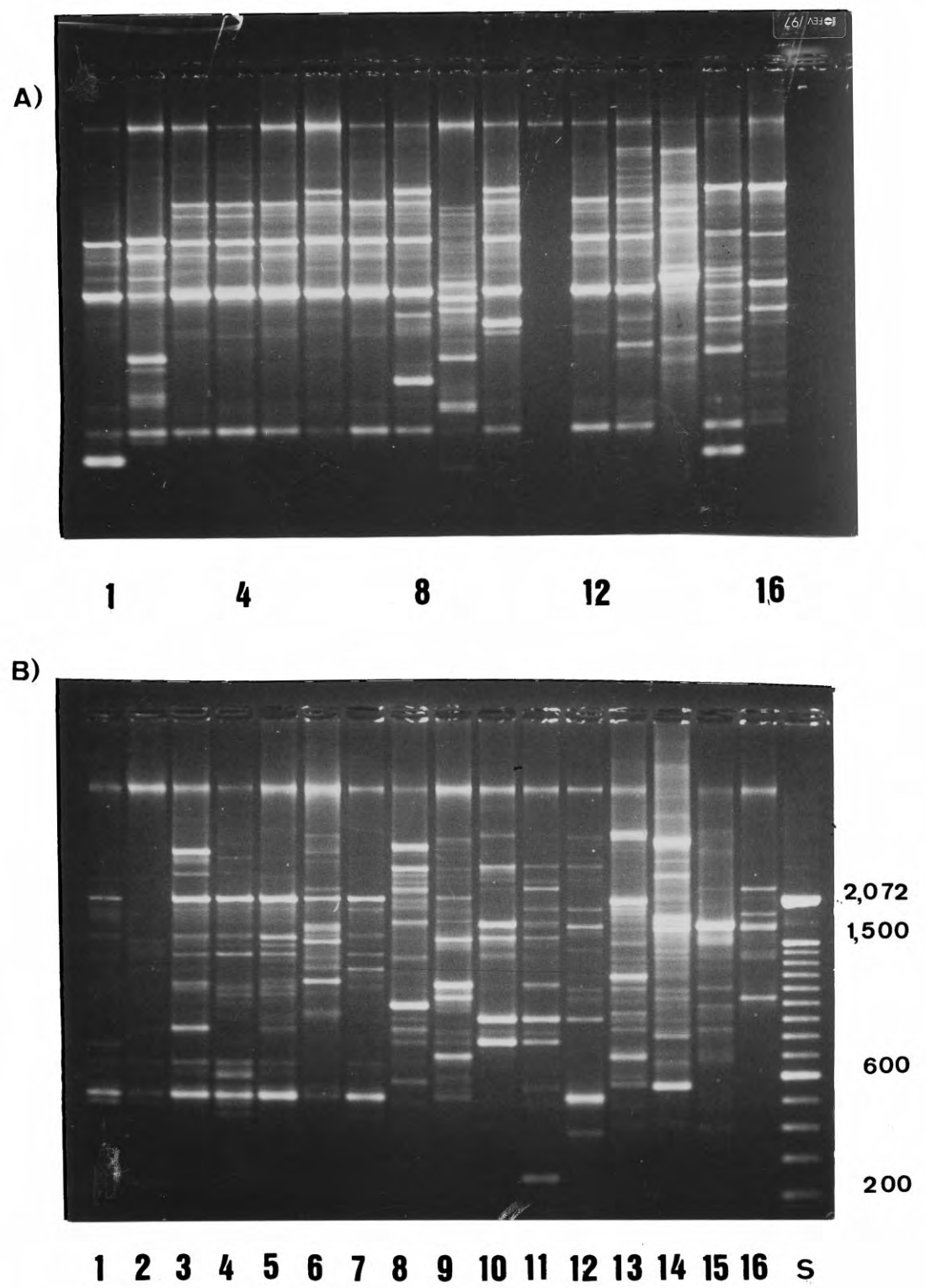

Fig. 1. DNA fragments obtained by PCR amplification using arbitrary primers, of 16 soybean bradyrhizobia isolates representative of the genomic patterns obtained under different tillage and crop rotation systems. Amplification was obtained with primers OPS-07 (A) and OPS-20 (B) of Operon. The last lane indicates the DNA standard (S) with the size markers in bp in the right side.

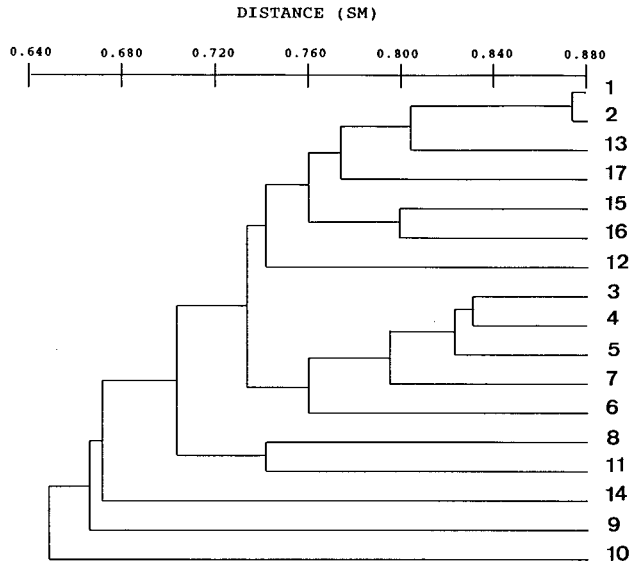

Fig. 2. Dendrogram showing the 16 soybean bradyrhizobia isolates representative of the genomic patterns obtained under different tillage and crop rotation systems. The cluster analysis considered the PCR products obtained with 12 short primers and the Simple Matching (SM) coefficient; strain 17 is *Bradyrhizobium japonicum* USDA 110.

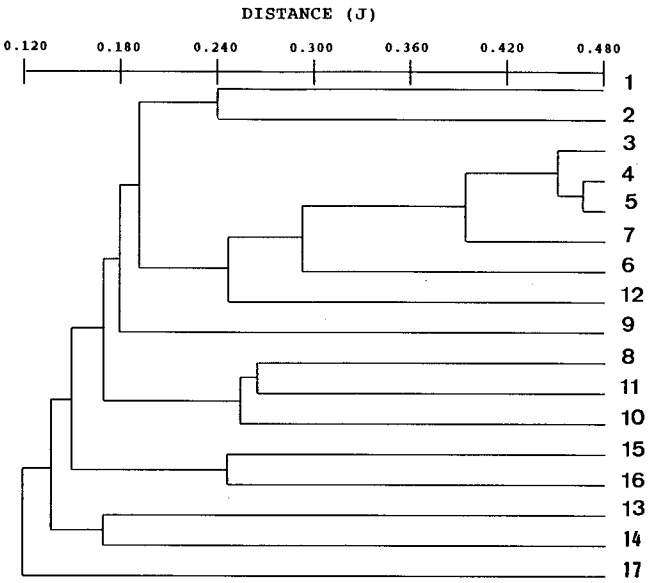

Fig. 3. The same as Fig. 3, but using the Jaccard (J) coefficient.

with specific primers (Hungria et al., 1999). In this study, the PCR with arbitrary primers has also confirmed to be a reliable technique, since the patterns were reproducible in several reactions and with DNA extracted from independent liquid cultures. Furthermore, RAPD is a simple and inexpensive technique and was able to detect a high degree of diversity within the soybean bradyrhizobia.

The 142 isolates were morphologically characterized, but their grouping according to the colony characteristics was difficult. Table 3 shows the morphological

Table 3
Morphological and serological characterization of 16 soybean *Bradyrhizobium* isolates representative of the genomic patterns identified in soils under different tillage and crop rotation systems

Isolate	Diameter (mm)	Color[a]	Elevation	Shape	Mucus	Margin	Serogroup[b]	System[c]	
								Tillage	Rotation
1	< 1.0	cream	convex	circular	+ +	regular	n.r.	CT	M/W
2	1.0	white	convex	circular	+ + +	regular	n.r.	CT	S/W/M
3	1.0	white	flat	circular	+ +	regular	29w	NT	S/W/M
4	1.0	cream	convex	circular	+ +	regular	29w	NT	M/W
5	1.0	cream	convex	circular	+ +	regular	29w	CT	S/W
6	1.0	white	convex	circular	+ +	regular	532c	CT	S/W/M
7	< 1.0	white	flat	circular	+ + +	regular	n.r	NT	M/W
8	1.0	white	convex	circular	+ +	regular	n.r	NT	S/W/M
9	< 1.0	white	convex	circular	+ +	regular	29w	CT	S/W
10	1.0	white	convex	circular	+ +	regular	532c	CT	S/W/M
11	1.0	white	flat	circular	+ + +	regular	566	NT	S/W
12	1.0	white	flat	punctiform	+	irregular	n.r	CT	S/W/M
13	1.0	white	convex	circular	+ +	regular	566	CT	M/W
14	1.0	white	convex	circular	+ +	regular	n.r	NT	S/W/M
15	1.0	white	convex	circular	+ +	regular	532c	CT	M/W
16	1.0	cream	convex	circular	+ +	regular	532c	CT	S/W

[a] In relation to the transparency, the isolates were all opaque.

[b] The isolates were tested against the antisera of isolates SEMIA 527, SEMIA 566 (same serogroup as CPAC 15), 532c, SEMIA 587, 29w and CB 1809 (same serogroup as CPAC 7), which had been used in commercial inoculants for variable periods of time since the early 1960's; n.r. means no reaction with any of the tested serogroups.

[c] CT, conventional tillage, NT, no-tillage, S, soybean; W, wheat; M, maize.

characterization of 16 isolates representative of the genomic patterns, as well as the source of each strain used for this characterization. Differences among the isolates were verified on several properties, e.g. color and mucosity. However, there was no evident relationship between the colony morphology and the presence or absence of the isolates in each agronomic system.

A high percentage (37.5%) of the isolates did not react with any known Brazilian serogroup, so this technique was also not adequate to group the isolates (Table 3). The only isolates belonging to the same serogroup which were grouped in the same cluster by RAPD were 3, 4 and 5 and the isolates 15 and 16. The majority of the isolates (62.5%) reacted with three serogroups belonging to strains established in many Brazilian soils cropped with soybean (Vargas and Hungria, 1997). Those three strains, as well as most of the strains established in Brazilian soils, have been classified as *Bradyrhizobium elkanii* (Boddey and Hungria, 1997), justifying the genetic distance observed between *B. japonicum* USDA 110 and the 16 isolates (Fig. 3). In relation to the isolates without a known serological reaction, in a survey of 28 Brazilian areas traditionally cultivated with soybeans, the presence of isolates which did not react with any known serogroup was frequent and represented up to one-third of the bradyrhizobia population (Vargas and Hungria, 1997). Consequently, more detailed genetic studies would be necessary to clarify if those isolates have had modifications in their serological properties or if they were symbionts of native legumes or nonsymbiotic bradyrhizobia which have acquired the symbiotic genes from an inoculant strain upon the introduction of the host legume, as describe for *Mesorhizobium loti* in New Zealand (Sullivan et al., 1995, 1996).

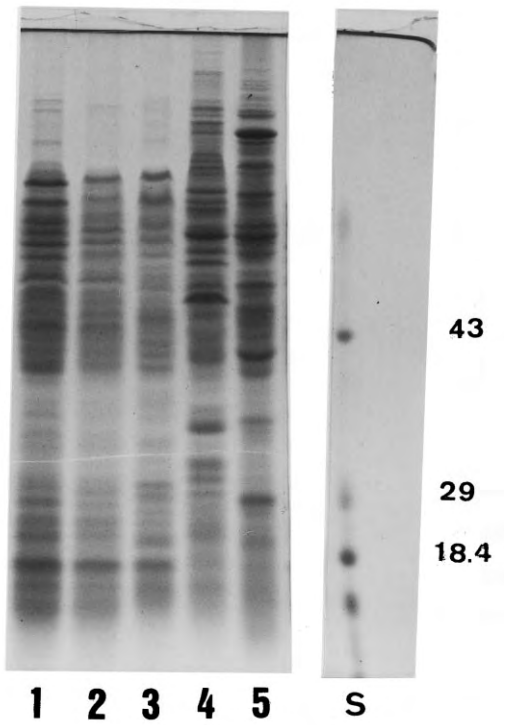

Fig. 4. Protein profiles of soybean bradyrhizobia isolated from soils under different tillage and crop rotation systems. The area had not received any inoculant for the last 15 yr. Line 1 corresponds to isolates with the genomic patterns 1, 2, 3, 4, 5, 6, 7, 9 and 10; line 2 corresponds to isolates 8 and 12; line 3 to strain 14; line 4 is representative of genomic patterns 11, 15 and 16 and line 5 of strain 13. The last lane indicates the standard (S) with the size markers in kDa in the right side.

The analyses of the protein profiles detected a lower variability among the isolates in comparison to the RAPD. The 16 DNA genotypes have been grouped into five protein profiles, with most of the isolates in group 1 (1, 2, 3, 4, 5, 6, 7, 9 and 10). Profile 2 was obtained for isolates 8 and 12; profile 3 characterized isolate 14, and these three first profiles had several bands in common; profile 4 included isolates 11, 15 and 16 and profile 5 was detected exclusively on isolate 13 (Fig. 4). The protein profiles were also not related to the serogroups.

Nod factors were investigated in the 16 representative isolates, trying to better understand the relationships between rhizobia molecular signals, biodiversity and nodulation. Three profiles were identified: Nod factor profile 1 was similar to that obtained for SEMIA 566 (Hungria et al., 1996); this profile was detected in the majority of the isolates (2, 4, 5, 6, 7, 9, 13 and 16). Nod factor profile 2 was detected after *nod* gene induction of isolates 8, 10, 11, 12, 14 and 15. This profile was similar to that shown by strain CPAC 7 (belonging to serogroup CB 1809) (Hungria et al., 1996), a strain usually absent or present in just a very low percentage in Brazilian soils (Vargas and Hungria, 1997) and which did not react serologically with any of the isolates in this study. Finally, profile 3 was detected on isolates 1 and 3 and was very similar to profile 2, except for one band below that observed in profile 2 (data not shown). It has been reported that certain soil management practices such as no-tillage and crop rotation or intercropping with legumes may allow a higher accumulation in the soil of compounds acting as *nod* gene inducers, resulting in higher nodulation and N_2 fixation rates (Hungria and Stacey, 1997; Hungria et al., 1997b). It could well be that those flavonoids accumulated in the soil would also stimulate the survival of bradyrhizobia strains producing different Nod factors, since the three profiles identified in this study were detected under no-tillage and crop rotation with legumes.

In relation to the symbiotic evaluations, none of the isolates was able to nodulate *Phaseolus vulgaris* or *Leucaena leucocephala*, but all nodulated *Macroptilium atropurpureum*. The greenhouse experiment with the 16 isolates representative of genomic patterns has shown

Table 4
Nodule number, nodule dry weight and total N accumulated by soybean plants of cultivar BR-16 inoculated with 16 *Bradyrizobium* isolates representative of the genomic patterns found in soils under different tillage and crop systems. Plants harvested at 45 d after emergence

Isolate	Nodulation		Total N in plants[a] (mg N plant^{-1})
	Number (No. plant^{-1})	Dry weight (mg plant^{-1})	
1	86.7 ab[b]	276.2 a	119.4 a
2	61.7 a–g	249.4 abc	79.7 bcd
3	65.0 a–f	260.0 ab	118.8 a
4	22.3 h	42.1 f	20.3 h
5	62.0 a–g	258.3 abc	88.3 bc
6	53.7 c–h	205.2 cd	71.3 b–e
7	43.5 d–h	160.3 e	52.6 efg
8	75.1 a–d	268.2 a	92.6 ab
9	45.2 d–h	150.5 e	50.3 efg
10	54.6 b–g	210.6 bcd	68.2 b–f
11	52.0 c–h	195.4 de	65.3 b–g
12	31.2 gh	81.3 f	38.4 gh
13	33.8 fgh	90.5 f	40.1 fgh
14	51.6 c–h	190.2 de	60.2 c–g
15	41.8 e–h	161.2 e	55.3 efg
16	51.7 c–h	180.7 de	55.7 d–g
29w	78.3 abc	270.4 a	121.1 a
SEMIA 587	70.6 a–e	265.0 ab	94.8 ab
CPAC 7	88.2 a	292.6 a	118.6 a
CPAC 15	85.1 a	290.5 a	120.3 a

[a] N shoot + N root + N nodules − N seed.
[b] Means of five replicates followed by the same letter did not show statistical difference (Tukey, $P < 0.05$).

that some of them were as effective as the four strains which are currently in use in Brazilian commercial inoculants (Table 4). The most efficient isolates were found in the NT and crop rotation systems including soybean at least every other year and some of them, such as isolates 1 and 8, did not react with any known serogroups.

Our results have shown that the type of tillage and crop rotation system may quantitatively and qualitatively affect soybean bradyrhizobia populations. Besides soybean (Hungria and Stacey, 1997; Hungria et al., 1997a), effects of no-tillage or cropping with the legume host plant resulting in an increased rhizobia population have also been reported for bean rhizobia (Hungria et al., 1997b) and *Sinorhizobium meliloti* (Triplett et al., 1993). Furthermore, in our study high numbers of bradyrhizobia cells were detected even in the absence of the host plant for 17 yr (M/W rotation), supporting earlier suggestions that soybean bradyrizobia populations are quite persistent (Triplett et al., 1993). In relation to the qualitative differences, a higher bean rhizobia diversity was also reported under no-tillage and in the presence of the host plant in monoculture or intercropping, when compared with the monoculture with maize (Hungria and Vargas, 1996; Hungria et al., 1997b), The positive effects of no-tillage, especially under tropical conditions, could be attributed to several factors, such as a lower soil temperature, higher soil moisture content, preservation of soil aggregates and a higher carbon content, favoring soil biomass and many classes of microorganisms, including rhizobia (Powlson and Jenkinson, 1981; Staley et al., 1988; Alvarez et al., 1995; Hungria and Vargas, 1996; Hungria et al., 1997b). Besides that, the host legume would also favor rhizobia population and diversity, mostly due to rhizosphere effects (Peña-Cabriales and Alexander, 1983). Finally, nodulation of the legume host could also represent a rhizobial survival mechanism. In our study, an inadequate soil management, crop rotation exclusively with grasses, reduced bradyrhizobia diversity, and a lower diversity of bean rhizobia was also reported as an effect of addition of N fertilizers (Caballero-Mellado and Martínez-Romero, 1999).

In previous studies we have shown that the adaptation to environmentally stressful conditions which frequently occur in the tropics will result in a high degree of morphological, physiological and genetic changes of soybean bradyrhizobia (Hungria et al., 1996; Nishi et al., 1996; Boddey and Hungria, 1997; Hungria et al., 1998). However, it is possible to take advantage of this diversity and soil adaptation to select more efficient and competitive strains. Indeed, three of the four strains which are currently recommended in Brazilian commercial inoculants, SEMIA 587, 29w and CPAC 15, were selected after a period of adaptation to the soils (Vargas et al., 1992; Vargas and Hungria, 1997). In this study it was also shown that within the higher diversity detected under the no-tillage and crop rotations with soybean it is possible to select more efficient strains. An intriguing question that arises is why there is usually no field response to soybean inoculation in some countries, such as the USA, even in soils with few bradyrhizobia cells (Dunigan et al., 1984; Ellis et al., 1984; Thies et al., 1991), while in Brazil field responses can be obtained for both soybeans (Vargas et al., 1992; Nishi et al., 1996; Hungria and Vargas, 1996; Vargas and Hungria, 1997; Hungria et al., 1998) and beans (Hungria et al., 1999). It could be that the competitiveness of established or naturalized strains is different in both countries. It could also be that due to the stressful tropical conditions there would be an increase in nodulation due to the inoculation with more efficient bacteria in an appropriate physiological state. Lastly, it is possible that the selection program in Brazil has paid more attention to the search of more efficient, competitive and adapted strains.

In conclusion, the results reported here have highlighted the potential benefits of agronomic practices,

such as the no-tillage system and crop rotations with legumes, in agricultural sustainability and maintenance of rhizobia populations and diversity. Furthermore, within this diversity it is possible to select adapted strains with high rates of N_2 fixation.

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