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ORIGINAL PAPER

Soybean [*Glycine max* (L.) Merrill] rhizobial diversity in Brazilian oxisols under various soil, cropping, and inoculation managements

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Abstract In this study, soybean nodules were collected from 12 sites in the State of Mato Grosso, in the Brazilian Cerrados, where both exotic soybean [*Glycine max* (L.) Merrill] and bradyrhizobial strains have been introduced from 1 to 18 years before. All soils were originally devoid of rhizobia capable of effectively nodulating soybean and varied in terms of chemical and physical properties, inoculation procedures, and cropping systems. Rhizobial genetic diversity was assessed on 240 isolates by *rep*-PCR fingerprinting with BOX primer, and indices of diversity (abundance-based coverage estimator and traditional and modified Shannon indices) were applied to the profiles obtained. The genetic diversity was much greater than expected, as after the introduction of a maximum of four strains, up to 13 profiles were identified, some sharing many similar bands with the inoculant strains, but others quite distinct from the putative parental genotypes. The increase in the number of *rep*-PCR profiles could be attributed to genetic variability due to the stressful tropical environmental conditions, but also might indicate that indigenous rhizobia become capable of nodulating the host legume. After the third year of cropping with the host legume, inoculation did not affect rhizobial diversity. A high content of clay decreased diversity in comparison with that seen in a sandy soil, probably due to reduced aeration. Diversity was higher under the no-tillage system when compared to the conventional tillage management, highlighting the importance of maintaining crop residues in tropical soils. Understanding the ecology of exotic rhizobia after being introduced into new cropping areas represents a first step towards the establishment of better strategies of inoculation, which in turn may result in sustainability and higher plant yields.

Keywords Bacterial diversity · *Bradyrhizobium* · *Glycine max* · Nitrogen fixation · Shannon index

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Introduction

Soil microbial biodiversity is a key component of sustainability, playing critical roles in the maintenance of physical and chemical properties of soil and in the supply of nutrients to plants. The importance of global biodiversity is increasingly being recognized, and the Convention on Biological Diversity has identified soil biodiversity as requiring particular attention with regard to agricultural productivity

(CBD 2002, 2005). Therefore, studies aimed at understanding, exploring, and utilizing biodiversity, as well as at increasing the input of nutrients through microbiological processes, are very important especially in countries where new areas and exotic crops are being incorporated into agriculture.

Soybean [*Glycine max* (L.) Merrill] was introduced in Brazil in 1882, but commercial crop expansion began only in the 1960s in the South Region. In the 1970s commercial crop expansion advanced to a new agricultural frontier in the Central-West Region: the "Cerrados," an edaphic type of savannah occupying 207 million hectares and representing 25% of Brazilian land (Goedert 1983; Hungria and Vargas 2000, Hungria et al. 2005a,b, 2006). The Cerrados are quite distinct from other areas of Brazil, especially in relation to soil chemical properties (low pH, high contents of Al and Mn, and low contents of P and N; Goedert 1983). The State of Mato Grosso, the third in area in the country, is now the first in soybean production (34%), and 71% of its agricultural area is cropped with the legume (CONAB 2005).

Historical reports in Brazil indicate that the soils are devoid of rhizobial strains able to establish effective symbioses with the legume; therefore, inoculation is needed to guarantee root nodulation and N_2 fixation in the first year of planting (Ferreira and Hungria 2002; Hungria et al. 2005a, 2006). The use of strains recommended by a committee of rhizobiologists is legislatively mandated, and massive inoculations with relatively few selected strains have taken place over the years; since 1979, only four strains have been recommended. At this point, it is important to assess the diversity of the rhizobial communities established in soils of interest, to aid the delineation of strategies to guarantee adequate supplies of N to the cultivars of increasing productivity that are released every year (Hungria et al. 2005a,b, 2006).

Nothing is known about the diversity of exotic rhizobial populations introduced in inoculants in the soils of Mato Grosso, originally devoid of soybean rhizobia, as well as about their interactions with indigenous rhizobia. Therefore, in this study we evaluated rhizobial populations trapped by soybean plants at 12 sites in the State of Mato Grosso under varied soil, crop, and inoculation managements.

Materials and methods

Field sites and sampling

Plant and soil samples were taken from 12 farms in the State of Mato Grosso, in the Brazilian Cerrados (Table 1). Areas ranged from 50 (site 3) to 3,200 ha (site 11). The minimum distance between farms was 50 km. Each area was divided into ten subareas, spatially distributed to cover the whole property; therefore, each subarea represented one-tenth of the whole property, ranging from 7 to 320 ha. From each subarea, ten soybean plants were randomly collected, totaling 100 plants per site. Twenty nodules were randomly chosen from each sample of 100 plants. In addition, ten subsamples of soil (a layer of 0–10 cm thick) were taken from each of the ten subareas and mixed to represent one sample per field site; soil samples were used for the analysis of chemical and physical properties.

Temperature is almost constant throughout the year in the Cerrados and the total yearly rainfall ranges from 1,000 to 1,800 mm, with 80% falling between November and April corresponding to the summer cropping season (Goedert 1983); due to its length, farmers usually do not grow crops in the winter dry season.

The sites, originally covered with natural vegetation of the "Cerrados," had been cleared from 2 to 20 years before. The traditional cropping history of the State of Mato Grosso includes: (1) clearance by removal of valuable wood and burning the remaining vegetation; (2) growing rice (*Oryza sativa*) in the first year, supported by residual soil N; (3) establishing pasture (*Brachiaria brizantha*) of low protein content for beef production [usually not more than 0.5 AU (animal unit) ha^{-1} in the dry season and 1.0 AU ha^{-1} in the raining season]; (4) after several years of pasture, addition of fertilizers, correction of pH, and introduction of inoculated soybean. This management was used in properties 1, 2, and 12, and all steps but growing rice were used in properties 4, 5, 8, 9, and 11; management in the remaining properties did not include *Brachiaria* (Table 1).

Both soybean and exotic *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* were introduced in the properties from 1 to 18 years ago. Some farmers had grown soybean for only a year (at sites 4, 8, 9, 10, 11) and others for much longer periods (15 years, site 1; 18 years, site 7; Table 1). Four exotic strains were introduced into the areas: *B. elkanii* strains SEMIA 587 and SEMIA 5019 (=29W; used in commercial inoculants since 1979) and *B. japonicum* strains SEMIA 5079 and SEMIA 5080 (= CPAC 15 and CPAC 7, respectively; used in commercial inoculants since 1992). Brazilian commercial inoculants carry two strains, usually in two combinations, SEMIA 587+5019 or SEMIA 5079+5080. Inoculation history in each property is shown in Table 1.

Conventional tillage (CT) was used on one farm, with the traditional practices of plowing and disking, and nine had adopted the no-tillage (NT) system, with seeds sown directly through the residues of the previous crop. Furthermore, one farmer (at site 8) used NT with scarification every 3 years, and another one (at site 6) cropped soybean under CT and in the second year introduced NT and organic management without chemical inputs (Table 1).

Table 1 Information about the sites where soybean nodules and soils were sampled in the State of Mato Grosso, in the Brazilian Cerrados

No.	Site	Area (ha)	Soil management[a]	Inoculation[b]		Soybean (years)	Cropping history[c]
				At sowing	Previously		
1	Primavera do Leste/Paranatinga	825	NT	No	Yes	15	Rice (1st year), soybean (summer, 15 years), and in some years, maize or millet (winter)
2	Jaciara/Sto. Antonio Leverger	400	NT	No	Yes	3	Rice (1st year), *Brachiaria* (10 years), soybean
3	Jaciara/Cabeça do Rio Prata	50	NT	No	Yes	2	Rice (1st year), soybean
4	Primavera do Leste	500	NT	Yes	No	1	*Brachiaria* (10 year), soybean
5	Jaciara/Celma	100	NT	Yes	Yes	3	*Brachiaria* (10 yr), soybean (summer) and millet (winter)
6	Sapezal	235	Organic	Yes	Yes	2	Soybean under CT (1st year), organic soybean
7	Guiratinga	70	CT	Yes	Yes	18	Rice (1st year), soybean (summer) in rotation with maize (summer)
8	Pedra Preta	950	NT	Yes	No	1	*Brachiaria*
9	Jaciara/Celma	200	NT	Yes	No	1	*Brachiaria* (10 years), millet (1 year),
10	Campo Verde	1,000	NT	Yes	No	1	natural vegetation
11	Rondonópolis	3,200	NT	Yes	No	1	*Brachiaria* (12 year)
12	Jaciara	1,500	NT	No	Yes	3	Rice (1st year), soybean (8 year), *Brachiaria* (10 year), soybean

[a] *NT* No tillage, *CT* conventional tillage; organic, without chemical application; scarification every 3 years
[b] Previous inoculation when soybean was grown in these sites.
[c] Species: *Brachiaria* (*Brachiaria brizantha*), maize (*Zea mays*), millet (*Pennisetum glaucum*), and rice (*Oryza sativa*).

All soils were typical oxisols of the Cerrados region and chemical properties, as well as physical contents of sand, clay, and silt were evaluated after Pavan et al. (1992) and are shown in Table 2.

Reference strains of *B. japonicum* and *B. elkanii*

Rhizobium reference strains included strains that had been used in commercial inoculants in the late 1970s: *B.*

Table 2 Chemical and physical properties of the soils

Site	Chemical						Physical			
	pH $CaCl_2$	Al ($cmol_c\ dm^{-3}$)	CEC ($cmol_c\ dm^{-3}$)	BS ($cmol_c\ dm^{-3}$)	C ($g\ dm^{-3}$)	P ($g\ dm^{-3}$)	N (%)	Clay (%)	Silt (%)	Sand (%)
Primavera do Leste/ Paranatinga	4.93	0.04	6.28	3.72	17.9	18.8	0.08	34.0	3.6	62.4
Jaciara/Sto. Antonio Leverger	4.67	0.19	5.59	2.60	21.0	7.4	0.09	42.2	5.6	52.2
Jaciara/Cabeça do Rio Prata	4.68	0.20	4.15	1.77	10.6	4.0	0.04	15.3	1.8	82.9
Primavera do Leste	4.69	0.22	4.56	1.80	18.2	9.3	0.05	19.2	3.1	77.7
Jaciara/Celma	5.14	0.00	7.03	4.49	25.0	28.4	0.07	50.1	6.2	43.7
Sapezal	4.90	0.04	6.51	3.49	25.5	13.0	0.11	52.7	5.7	41.6
Guiratinga	5.04	0.00	5.72	3.18	19.2	7.4	0.08	32.7	2.4	64.9
Pedra Preta	6.33	0.00	8.24	6.35	21.7	18.1	0.06	22.4	4.9	72.7
Jaciara/Celma	4.88	0.06	6.21	3.26	22.1	8.8	0.09	27.3	5.5	67.2
Campo Verde	4.43	0.39	4.57	1.69	18.2	7.3	0.06	22.7	2.4	74.9
Rondonópolis	5.11	0.00	7.00	4.79	23.4	30.7	0.06	20.7	3.6	75.7
Jaciara	4.50	0.27	6.08	3.04	26.9	6.4	0.14	63.8	7.8	28.4

CEC Cation exchange capacity; *BS* base saturation $=((K + Ca + Mg/T_{cec})) \times 100$, where $T_{cec} = K + Ca + Mg +$ total acidity at pH 7.0 (H + Al)

japonicum SEMIA 566 (belongs to the same serogroup as USDA 123, is very competitive and was used in commercial inoculants from 1966 to 1978); SEMIA 5079 (a natural variant of SEMIA 566, selected after adaptation to the Cerrados soils, used since 1992); and SEMIA 5080 (a natural variant of CB 1809—a strain originally from USA but came to Brazil from Australia—also selected for adaptability to Cerrados soils, is very efficient in the process of N_2 fixation but shows low competitiveness; used since 1992). The Brazilian strain selection program that searches for natural variants of *B. japonicum* and *B. elkanii* adapted to the environmental conditions of the country has been detailed described elsewhere (Hungria and Vargas 2000; Hungria et al. 2006). Two *B. elkanii* strains were included: SEMIA 587 (used in commercial inoculants from 1968–1975 and since 1979) and SEMIA 5019 (used since 1979). The strains have been described in detail elsewhere (Nishi et al. 1996; Santos et al. 1999; Hungria and Vargas 2000; Hungria et al. 2006), and they represent the majority of the strains applied to the soybean-cropped area of the Cerrados (Hungria et al. 2006).

Rhizobial isolation

Twenty nodules from each field site 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, uniform absorption of Congo red and uniform Gram-stain reaction. Single colonies were individually transferred to YM liquid broth (YMB, Vincent 1970) and after growth the broth 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 cycles per minute.

Extraction of DNA and *rep*-PCR fingerprinting with BOX primer

DNA was extracted from reference strains and rhizobial isolates as described before (Kaschuk et al. 2006b). To obtain clean DNA, the extraction procedure included, for each 400 μl of bacteria resuspended in TE 50/20, the addition of 50 μl of 10% sodium dodecyl sulfate, 5 μl of proteinase K (20 mg ml^{-1}), 10 μl of lysozyme (5 mg ml^{-1}), and 2 μl of RNAse (10 mg ml^{-1}). After two steps of purification with ethanol at 99.5 and 70%, the pellet was resuspended in 50 μl of TE 10/1 to estimate the concentration of the DNA. Samples were then diluted to 20 ng of DNA μl^{-1} and were kept at −20°C. PCR amplification of repetitive regions of the DNA (*rep*-PCR) was carried out with BOX-A1R primer (5′-CTACGG CAAGGCGACGCTGACG-3′, Invitrogen™; Versalovic et al. 1994). Volumes and amplification cycles were performed as described before (Kaschuk et al. 2006b) and the reaction was performed in an MJ Research PT 200 thermocycler. The amplified fragments were separated by electrophoresis on 1.5% agarose (low EEO, type I-A) gels (20×25 cm) at 120 V for 7 h. The 1-kb Plus DNA Ladder (Invitrogen™) was used as a molecular marker at the right, left, and central lanes of each gel. Gels were stained with ethidium bromide, visualized under UV radiation, and photographed. As in previous studies by our group (Germano et al. 2006), the profiles obtained were confirmed in triplicate.

Genetic diversity

Cluster analyses of the BOX−PCR products from each strain from each area and also considering all areas together were performed with the Bionumerics program (Applied Mathematics, Kortrijk, Belgium, version 1.50). First, the sizes of the fragments in each analysis were normalized according to the molecular weight of the DNA markers. The fingerprintings obtained were then analyzed using the unweighted pair-grouping method with arithmetic mean (UPGMA) algorithm (Sneath and Sokal 1973) with the coefficient of Jaccard (1912), with a tolerance of 5%.

In previous studies, we have shown that a level of similarity of 70% of the *rep*-PCR profiles analyzed in the Bionumerics program with a tolerance of 5% is adequate for the use in studies about rhizobial diversity in Brazilian soils (Grange and Hungria 2004; Alberton et al. 2006; Kaschuk et al. 2006a,b; Batista et al. 2006); therefore, profiles showing similarity higher than 70% were also considered to be similar in this study.

Abundances of profiles in each area were analyzed using the SPADE (Species Prediction And Diversity Estimation; Chao and Shen 2005) program. Richness was calculated with ACE (Abundance-based Coverage Estimator), a nonparametric estimator proposed by Chao and Lee (1992), based on the separation of observed species into rare or abundant groups with only the rare groups used to estimate the number of missing species. In this analysis, the estimated coefficient of variation (CV) is defined as the parameter used to characterize the degree of heterogeneity among species. The conventional Shannon index was calculated with the equation proposed by Shannon and Weaver (1949) and a nonparametric estimation of the Shannon index of diversity was based on the sample-coverage method and on unseen species (Chao and Shen 2003). The difference between these parameters is that the traditional Shannon index (MLE-Maximum Likehood Estimator) ignores missing species, whereas in the method

proposed by Chao and Shen (2003), missing individuals are considered on the basis of rare species, that is, those with lower numbers of individuals than k (cutoff of the model). In this study we considered k=4. Similarity between communities was assessed by the abundance-based index of Jaccard (adjusted to consider unseen species; Chao et al. 2005), and prediction of new species in a further survey was performed as recommended by Shen et al. (2003).

Results and discussion

In this study rhizobial isolates were obtained from soybean nodules of plants growing on oxisols of 12 sites in the State of Mato Grosso, Brazil. The soils were initially devoid of indigenous soybean rhizobia and both the legume and four soybean inoculant strains of *B. japonicum* and *B. elkanii* have been introduced from 1 to 18 years before. Rhizobial strains were isolated from 20 nodules randomly chosen from 100 plants collected from ten subplots that covered each field site; therefore, 240 rhizobial isolates were used in this study. Collecting nodules directly from fields was considered the best method for assessing soybean rhizobial diversity, as in a recent study, more genotypes were trapped from field-grown soybean than from plants inoculated with soil dilutions under greenhouse conditions (Alberton et al. 2006). However, it is noteworthy to comment that the choice of the sampling method varies with the host legume history, as for common bean (*Phaseolus vulgaris* L.), also in Brazil, higher diversity was obtained when nodules were sampled from plants inoculated with soil dilutions (Grange and Hungria 2004; Alberton et al. 2006) and de Oliveira et al. (2006) have demonstrated the feasibility of using direct PCR-denaturing gradients gel electrophoresis methods based on soil DNA.

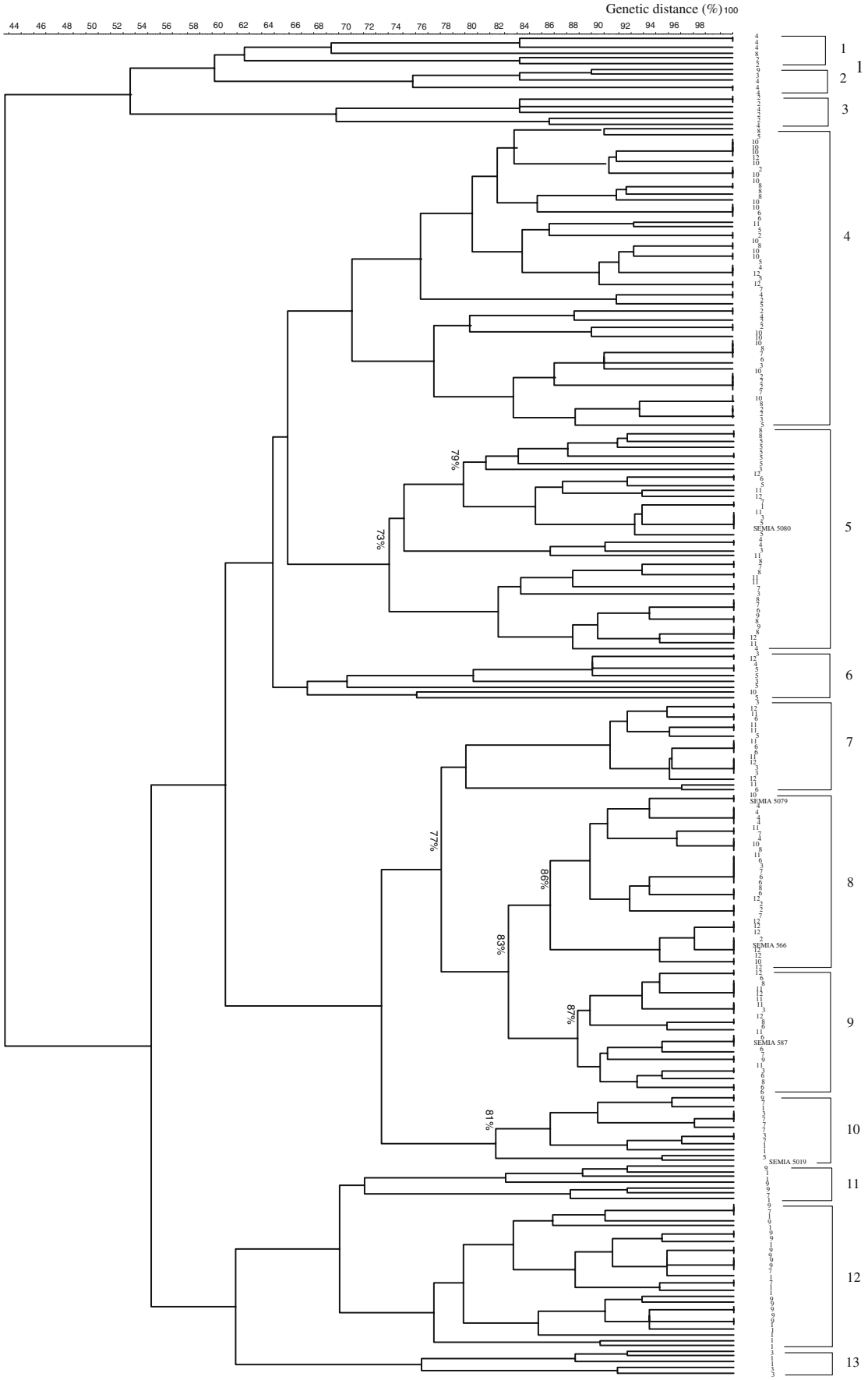
After isolation, genetic diversity was assessed by amplifying the DNA with primers for repetitive and conserved regions of the DNA (Versalovic et al. 1994). The choice of this methodology was based on previous studies from our laboratory, including repetitive primers ERIC, REP (de Bruijn 1992), and BOX (Versalovic et al. 1994), which have shown high sensitivity in detecting the diversity of tropical rhizobia at the strain level, although it does not cluster strains at the species or genus level (e.g., Chen et al. 2000; Ferreira and Hungria 2002; Galli-Terasawa et al. 2003; Grange and Hungria 2004; Kaschuk et al. 2006a,b). Farms were chosen to represent the whole variety of agricultural conditions found in the State of Mato Grosso and sampling represented 9,030 ha (Table 1). Although the soils had been cropped at least once and thus lime and fertilizers had been used, soils were generally acid, except for site 8, with some Al toxicity, low P, and N contents. Nine soils had high percentage of sand (up to 83%), and three others had high clay contents (up to 64%; Table 2).

A high level of genetic diversity was observed in the cluster analysis with all strains isolated from the 12 sites (240 strains), and in a global analysis of the BOX-PCR products the strains were distributed in several clusters with a very low final level of similarity of only 43% (Fig. 1). Many strains positioned in the same cluster shared similar bands with the inoculant strains (data not shown); however, several strains were highly dissimilar from the putative parental genotypes, fitting into distinct clusters. Furthermore, many of the clusters did not include any of the inoculant strains (Fig. 1).

A detailed analysis of each area was then performed, also considering the *rep*-PCR profiles. In previous studies from our group, we have considered that strains with a similarity lower than 70% in the *rep*-PCR analysis were distinct from the putative parental genotypes (Grange and Hungria 2004; Kaschuk et al. 2006a,b; Alberton et al. 2006). Using the same criteria for each one of the areas, only 44% of the strains (105 strains) from this study have shown a similarity higher than 70% with one of the four inoculant strains (Table 3).

When the analysis of each area was considered, 20 strains clustered with strain SEMIA 566 at a similarity of 90%, and 21strains were clustered with SEMIA 5079 also at a similarity of 90% (Table 3). SEMIA 566 is the putative parental strain of SEMIA 5079 (Hungria and Vargas 2000; Hungria et al. 2006), yet the strains show differences in several morphological, physiological, symbiotic and genetic properties, including DNA profiles with specific or random primers (Boddey and Hungria 1997; Hungria et al. 1996, 1998, 2006; Nishi et al. 1996; Santos et al. 1999; Ferreira et al. 2000; Ferreira and Hungria 2002). Together, those two very competitive strains, which belong to the same serogroup as USDA 123 (Mendes et al. 2004; Hungria et al. 2006), represented 18% of all the nodules sampled (Table 3). The competitiveness of this serogroup was highlighted in the study of Mendes et al. (2004) in which, after 4 to 6 years of introduction of four strains in an oxisol of the Cerrados, dispersal and competitiveness resulted in more than 50% of the nodules being occupied by strains of the SEMIA 566 serogroup. In addition, dispersal of bacteria belonging to serogroup SEMIA 566 to places far from cropped areas was also reported in Brazil (Ferreira and Hungria 2002).

The highest percentage of occurrence of a single strain group was obtained with SEMIA 5019 (11%); however, its presence was restricted to three sites (Table 3). SEMIA 5019 has been used in Brazilian commercial inoculants together with SEMIA 587 since 1979. Therefore, isolation of the strain in the field sites growing soybean for longer periods (sites 1 and 7, cropped with soybean for 15 and 18 years, respectively) is expected, as well as in site 9, inoculated just once with a commercial inoculant containing SEMIA 587+


Genetic distance (%)
44 46 48 50 52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96 98 100
1
2
3
4
5
6
7
8
9
10
11
12
13
79%
72%
77%
86%
83%
87%
81%
SEMIA 5080
SEMIA 5079
SEMIA 566
SEMIA 587
SEMIA 5019

◀ **Fig. 1** Dendrogram based on cluster analysis of BOX-PCR products (with the UPGMA algorithm, the Jaccard coefficient, and a tolerance of 5%) of rhizobial strains isolated from nodules of field-grown soybean in twelve sites of the State of Mato Grosso, in the Brazilian Cerrados. *Numbers* correspond to the sites described in Table 1

5019. The high competitiveness of SEMIA 5019 in comparison to SEMIA 587 was observed at all three sites, especially in site 1, with no record of recent inoculation. SEMIA 5019 was isolated from an acid soil with high Mn content and was selected in the 1970s for improved nodulation and superior N_2-fixation capacity, during agricultural expansion in the Cerrados region, while SEMIA 587 was selected in the late 1960s, in Rio Grande do Sul, South Region, for use in subtropical conditions (Hungria and Vargas 2000; Hungria et al. 2006). The results of this study confirm previous surveys showing that saprophytic capacity and competitiveness of SEMIA 5019 in the Cerrados are often greater than those of SEMIA 587, while the contrary has been observed in the South Region (Hungria et al. 2006).

Strain CB 1809, originally from the USA, was taken to Australia and from there to Brazil in 1966. Although viewed as very efficient, it has shown poor competitiveness in Brazilian soils; thus, strain SEMIA 5080, derived from CB 1809, was selected on the basis of the greater competitiveness (Hungria and Vargas 2000; Hungria et al. 2006). The results obtained in this study confirm that, under the conditions of the Cerrados, SEMIA 5080 might be as competitive as SEMIA 566, SEMIA 5079, and more than SEMIA 587 (Table 3).

Table 3 Grouping of rhizobia isolated from soybean nodules in relation to the reference strains established in Brazilian Cerrados soils

Site	Reference strains (SEMIA)					Other clusters
	566	5079	587	5019	5080	
Primavera do Leste/ Paranatinga	0	0	0	10	1	9
Jaciara/Sto. Antonio Leverger	3	0	0	0	0	17
Jaciara/Cabeça do Rio Prata	1	1	1	0	0	17
Primavera do Leste	0	5	0	0	0	15
Jaciara/Celma	0	0	0	0	4	16
Sapezal	1	4	6	0	2	7
Guiratinga	1	2	1	3	3	10
Pedra Preta	1	3	3	0	6	7
Jaciara/Celma	0	0	2	13	1	4
Campo Verde	1	2	0	0	0	17
Rondonópolis	3	2	2	0	3	10
Jaciara	9	2	1	0	2	6

Strains are considered to be in the same cluster when showing a similarity higher than 70% in the BOX-PCR analysis using the UPGMA method and the Jaccard coefficient with 5% of tolerance, for each of the 12 sites.

Studies performed in Brazil during soybean expansion reported a lack of nodulation in areas cropped for the first time (Ferreira and Hungria 2002; Hungria et al. 2006). However, dispersal of inoculant strains did occur (Ferreira and Hungria 2002; Mendes et al. 2004) and Andrade and Hungria (2002) demonstrated that viable cells on the soybean seed coat can be transported to new cropping areas. After introduction through inoculation and some years of adaptation to the soil conditions, a high level of diversity in morphological, physiological, genetic, and symbiotic properties has been reported especially under the stressful conditions of the Cerrados (Hungria et al. 1996, 1998; Nishi et al. 1996; Boddey and Hungria 1997; Santos et al. 1999; Hungria and Vargas 2000; Galli-Terasawa et al. 2003; Batista et al. 2006). In the USA, Streeter (1994) has also noted substantial "genetic adjustment" during the period of adaptation, leading to wide genetic diversity in field populations.

Strains fitting into the category of other clusters (Table 3) represented from 20% of all isolates from each area in Jaciara (site 9, a farm that has received inoculant just once) to 85% at sites 2 and 3 (grown with soybean and inoculated at least twice), and at site 10 (which received inoculant just once). After some years of soybean cropping, a high percentage of strains belonging to unknown serogroups or showing highly dissimilar DNA profiles have been detected in Brazil (Santos et al. 1999; Ferreira et al. 2000; Galli-Terasawa et al. 2003; Hungria et al. 2006; Batista et al. 2006). For example, in the South Region, Ferreira et al. (2000) reported that 38% of the strains belonged to unknown serogroups after 17 years of soybean cropping, and in the Cerrados that percentage was about 50% after some years of cropping (Galli-Terasawa et al. 2003; Mendes et al. 2004). Soils of the Cerrados are very inhospitable (Goedert 1983), and environmental stressful conditions may cause a strong selective pressure generating rhizobial variability (Hungria and Vargas 2000; Sprent 1994). In addition, horizontal gene transfer between one inoculant strain of *Mesorhizobium loti* and indigenous strains of *Mesorhizobium* has been demonstrated under field conditions in New Zealand (Sullivan and Ronson 1995; Sullivan et al. 1995, 1996). Increasing evidences of high rates of horizontal gene transfer in the soils of the Cerrados have also been reported (Galli-Terasawa et al. 2003; Batista et al. 2006) and might help to explain the high genetic diversity observed in several isolates of this study that were positioned in clusters showing very low genetic similarity in comparison to the inoculant strains (Fig. 1). Overall, the diverse population of rhizobia in this study is striking evidence for the rapid evolution of these organisms under the harsh environmental conditions in the Brazilian Cerrados, probably both by genetic adaptation and horizontal gene transfer.

Considering the equation proposed by Shen et al. (2003) for estimating new species, a further survey with 1,000 individuals might detect 36 and 18 new genotypes in the soils at sites 2 and 4, respectively, and a maximum of five new genotypes at the other sites (Table 4). In addition, in most areas, the CV for rare species was zero, revealing that rare and missing species had equal abundances in the community (low dominance of a few groups) and the same discovery probabilities (Table 4). One main conclusion from these results is that the sampling procedures and the use of 20 strains per site seem adequate to reveal the diversity of rhizobia in the Cerrados.

Both the traditional and the modified Shannon indices delineated three major groups, of high, intermediate, and low diversity (Fig. 2). Although the correlations between these indices and cropping and inoculation histories were low, comparisons of areas most similar in cropping history indicated some effects on rhizobial diversity. At sites 1 and 7, soybean had been grown for long periods of time and, thus, had established populations of bradyrhizobia (Table 1); both areas showed similar percentages (around 50%) of DNA profiles different from reference strains (Table 3). In addition, chemical properties were similar in both areas, including C and N contents and physical composition (about 60% sand; Table 2). The major difference between these areas was the tillage management, and the no-till system (site 1) appeared to result in higher ACE (14.7, Table 4), traditional Shannon (2.702, Fig. 2a) and modified Shannon (2.293, Fig. 2b) indices than with conventional till (site 7) with corresponding values of 11.4, 1.874, and 1.591, respectively. In southern Brazil, after some years of establishment, NT results in higher organic matter contents (Bayer et al. 2002; Hungria et al. 2005a; Franchini et al. 2006) and soil microbial biomass (Balota et al. 2004; Franchini et al. 2006). NT also favors nodulation and N_2 fixation in soybean and common bean (Hungria and Vargas 2000; Hungria et al. 2005a), mainly due to the lower soil temperatures and higher moisture contents under NT and differences in rhizobial diversity (Ferreira et al. 2000; de Oliveira et al. 2006; Kaschuk et al. 2006a,b). In this study, even after 18 years of NT, no differences were observed in the C and N contents of the soils from sites 1 and 7 (Table 2), probably due to the rapid decomposition of the residues of the summer crop and the very low or zero production of plant biomass in the dry season from May to October. Despite not varying in C content, the greater soybean rhizobial diversity observed under NT in this study probably related to lower soil temperatures and higher soil moisture content, which is more favorable to the rhizobial population, as has been noticed before for the soybean symbiosis in the southern region of Brazil (Ferreira et al. 2000).

In Jaciara, two other sites (2 and 12) had similar cropping and inoculation histories (rice, *Brachiaria*, and soybean) for the last 3 years, without inoculation at the most recent soybean sowing and both with NT (Table 1); furthermore, climate conditions are similar at both sites. However, diversity indices were greater for site 2 than for site 12 (Table 4, Fig. 2). The soil at site 2 had the highest clay percentage, associated with higher C and N contents. Heavy soils with high contents of clay often show problems of compaction and poor aeration, especially under NT, this being probably the reason for the decreased diversity of rhizobia, which are aerobic. In relation to the use of inoculants, after the third year of soybean cropping, inoculation history apparently did not affect diversity (Table 1, Table 4).

Table 4 Soybean rhizobial diversity in areas of the State of Mato Grosso in the Brazilian Cerrados

Site	Observed species		Sample coverage		Coefficient of variation		New species in a further survey with 1,000 samples		Richness	
	Total	Rare	Total	Rare	Total	Rare	Estimate (SE)	95% C.I.[a]	Estimate (SE)	95% C.I.
Primavera do Leste/Paranatinga	11	11	0.750	0.750	0.000	0.000	3.7 (2.3)	(−0.9, 8.2)	14.7 (3.1)	(11.9, 26.7)
Jaciara/Sto. Antonio Leverger	13	12	0.500	0.375	0.801	0.775	36.0 (33.6)	(−29.9, 101.9)	38.8 (22.5)	(18.9, 125.7)
Jaciara/Cabeça do Rio Prata	10	9	0.750	0.643	0.688	0.000	5.0 (3.6)	(−2, 12)	16.5 (6.2)	(11.3, 41.4)
Primavera do Leste	13	12	0.550	0.400	0.786	0.000	18.0 (12.7)	(−6.9, 42.9)	33.7 (16.8)	(18.2, 96.4)
Jaciara/Celma	10	10	0.850	0.850	0.000	0.000	1.8 (1.2)	(−0.7, 4.2)	11.8 (1.9)	(10.3, 19.8)
Sapezal	7	5	0.850	0.625	0.719	0.378	3.7 (4.6)	(−5.3, 12.7)	10.1 (3.8)	(7.5, 27.2)
Guiratinga	7	5	0.850	0.571	0.952	0.000	3.8 ((3.6)	(−3.4, 10.9)	11.4 (5.0)	(7.7, 33.3)
Pedra Preta	7	4	0.850	0.400	0.622	0.000	6.0 (7.3)	(−8.3, 20.3)	9.6 (3.4)	(7.4, 25.2)
Jaciara/Celma	7	5	0.850	0.571	0.857	0.000	3.8 (3.6)	(−3.4, 10.9)	10.8 (4.5)	(7.6, 30.7)
Campo Verde	7	6	0.850	0.727	0.929	0.224	2.5 (2.9)	(−3.2, 8.2)	11.3 (4.8)	(7.7, 32.4)
Rondonópolis	5	4	0.900	0.667	1.311	0.000	2.0 (2.1)	(−2.1, 6.1)	7.0 (2.6)	(5.3, 19.4)
Jaciara	4	2	0.900	0.000	1.029	0.000	0.0 (−1)	(−1, −1)	8 (7.2)	(4.4, 45.8)

CI Confidence interval

Diversity of microbiological and symbiotic characteristics may represent a source of variability to improve agricultural sustainability. From the plant's point of view, a lower diversity with a higher abundance of more specific, efficient, and competitive rhizobial strains might be desirable (Mendes et al. 2004). However, increased diversity may also be regarded as a positive indicator of soil health and productivity (Ferreira et al. 2000, Kaschuk et al. 2006a,b) or might guarantee a buffering effect, fostering biological N_2 fixation even under variable and stressful environmental conditions (Kennedy and Smith 1995; Kennedy 1999; Loreau et al. 2001).

Predicting the effects of soil management, cropping history, or inoculation procedures on rhizobial diversity in Brazilian soils is a hard task. First, the harsh environmental conditions seem to favor genetic processes that lead to high variability (Hungria et al. 1998; Santos et al. 1999; Hungria and Vargas 2000; Galli-Terasawa et al. 2003; Batista et al. 2006). Second, diversity would be increased with genetic exchanges between exotic *Bradyrhizobium* strains and indigenous strains that nodulate native legumes. Horizontal gene transfer among rhizobia under field conditions has been first demonstrated in New Zealand (Sullivan and Ronson 1995; Sullivan et al. 1995, 1996) and also seems to play a major role in the Brazilian Cerrados (Galli-Terasawa et al. 2003; Batista et al. 2006).

The results reported here contribute to the still poor knowledge of the diversity of soybean rhizobia in the State of Mato Grosso, where the legume occupies today 6.1 million hectares. A major issue is that the often inhospitable environmental found in the Cerrados seem to accelerate diversity change beyond what occurs in subtropical or temperature regions. The priority is now to understand how the rhizobial diversity may be used to ensure high sustainable yields.

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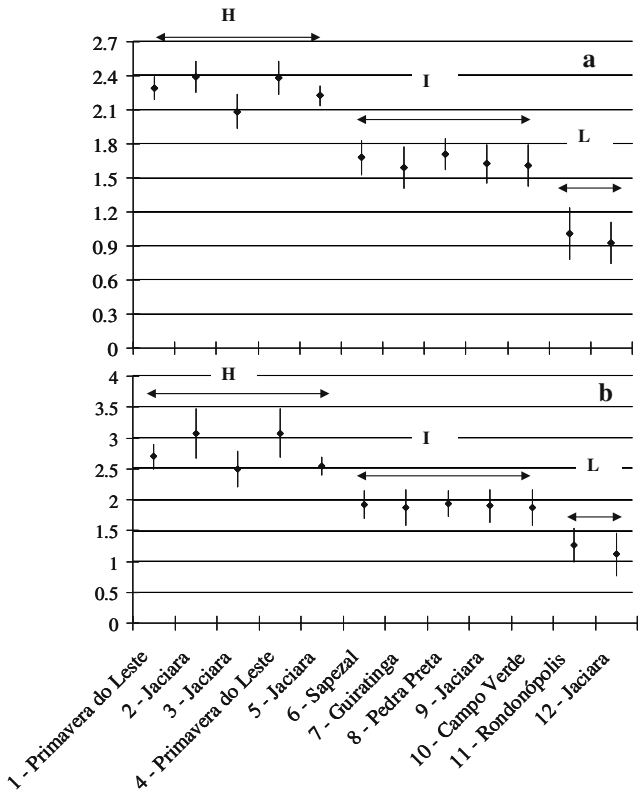

Fig. 2 **a** Traditional Shannon index of diversity, the maximum likelihood estimator (Shannon and Weaver 1949), and **b** Shannon index of diversity based on the Horvitz–Thompson estimator for unseen species and sample coverage method (Chao and Shen 2003), considering the rhizobial BOX-PCR profiles of soybean rhizobia from the State of Mato Grosso, in the Brazilian Cerrados

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