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Recovery of soybean inoculant strains from uncropped soils in Brazil

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Abstract

Soybean (*Glycine max* (L.) Merrill) and the corresponding bradyrhizobia were introduced in Brazil several decades ago and have been intensively used since then. However, in the past decade the possibility has been raised of native bradyrhizobia strains able to nodulate soybean. To clarify this, a modern cultivar and six unimproved promiscuous genotypes were inoculated with soil dilutions from 14 diverse uncropped soils bearing native vegetation. Isolates of *Bradyrhizobium* were obtained from seven of the soils, and most proved intolerant of acidity, salinity and high temperature. Thirty-nine of the 40 isolates showed similarity to seven strains that have been, or are, used in commercial inoculants. Characteristics evaluated included: synthesis of indole acetic acid, profiles of protein, lipopolysaccharide and DNA after amplification by PCR with ERIC primer, partial sequence of 16S rRNA and symbiotic properties. From the similarity of these strains to inoculant bradyrhizobia dispersal from soybean-cropped areas seems likely. However, in some strains (as those belonging to serogroup SEMIA 5039), PCR clustering was different from groupings based on serology, profiles of protein and lipopolysaccharide, indicating the presence of indigenous strains with similar properties, or genetic transfer from inoculant strains to indigenous bradyrhizobia, or variability due to the adaptation to different soil conditions. Only one isolate did not fit into the characteristics of known strains and could represent an indigenous soybean *Bradyrhizobium*, but this isolate showed poor symbiotic performance.

Keywords: Bacteria dispersion; Biological nitrogen fixation; *Bradyrhizobium*; Glycine max; Competitiveness; Bradyrhizobia ecology

1. Introduction

Soybean was introduced in Brazil in 1882 as *Soja hispida* (D'utra, 1882), but not until the 1940s were commercial crops of modern genotypes (*Glycine max* (L.) Merrill) grown in the South Region. The crop acquired economical importance from the 1960s onwards (Vargas and Hungria, 1997), and today soybeans are cultivated in several Brazilian states, and occupy 13 Mha. The country is the second worldwide in soybean grain production.

At the time of crop expansion, most experiments in which disinfected seeds were used reported zero or close to zero nodulation and yellow plants in N poor soils, indicating that Brazilian soils were void of rhizobial strains able to establish an effective symbiosis with soybean (Stamford, 1972; Lopes et al., 1976; Peres, 1979; Vargas and Suhet, 1980; Lopes and Giardini, 1981; Suhet et al., 1981; Vargas et al., 1982). However, some studies performed at that time

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reported the presence of nodules in the absence of inoculation (Freire and Vidor, 1981; Yuyama et al., 1981). However, all experiments showed that inoculation was needed, and inoculants were introduced in the 1960s, mainly from USA. A selection program to obtain strains adapted to Brazilian soils was started soon after and still continues (Peres, 1979; Peres and Vidor, 1980; Peres et al., 1993; Vargas et al., 1992; Vargas and Hungria, 1997). Nowadays, more than 90% of the soils cropped to soybean have been inoculated at one time or another, and contain high populations of the strains used in commercial inoculants over the past three decades.

Experiments performed in areas with naturalized populations of soybean bradyrhizobia have recently identified strains showing morphological, physiological, genetic and symbiotic differences with inoculant strains applied to these soils (Hungria et al., 1996; Nishi et al., 1996; Boddey and Hungria, 1997; Hungria et al., 1998; Santos et al., 1999). After some years of soybean cropping, there are also reports of a high percentage of nodules occupied by strains belonging to unknown serogroups (Freire et al., 1983; Vargas et al., 1993; Vargas and Hungria, 1997; Ferreira et al., 2000). Furthermore, the detection of soybean nodules in an Amazonian soil never cropped before with that legume (Yuyama et al., 1981) had to be confirmed. Finally, in a recent survey of rhizobia from a wide range of forest Leguminosae species in Brazil, Moreira (2000) reported the isolation of several *Bradyrhizobium japonicum* strains, although, they were not evaluated for the ability of nodulating soybean.

Four hypotheses can be advanced to explain the variability between parental and naturalized strains, and the presence of unknown serogroups. First, it is possible that native soybean bradyrhizobia exist in Brazilian soils, but that the cultivars used at the time of crop expansion excluded them. Second, that environmental conditions of the tropics, including high temperatures, drought and low soil fertility may lead to variability among naturalized strains (Hungria and Vargas, 2000). Third, as has been shown by the results of Sullivan et al. (1995, 1996) and Sullivan and Ronson (1998), transfer of symbiotic genes from inoculant strains to native non-infective rhizobia could have occurred and non-symbiotic bacteria resembling *B. japonicum* have been isolated from grassland soil in Japan (Saito et al., 1998). The last possibility is the introduction of additional bradyrhizobia as seed contaminants.

The studies described in this paper aimed to clarify the first hypothesis, determining if there are indigenous bradyrhizobia in Brazilian soils able to effectively nodulate soybean. To test this question, a modern and six unimproved promiscuous soybean genotypes were inoculated with soil dilutions from several uncropped soils growing native vegetation. As nodulation was confirmed, isolates were compared with known strains from the Brazilian germplasm bank, to determine if they represented native bradyrhizobia or were strains dispersed from cropped areas.

2. Material and methods

2.1. *Bradyrhizobium japonicum/B. elkanii* reference strains

Seven strains from commercial inoculants account for more than 95% of the bradyrhizobia established in Brazilian soils cropped with soybean. They are: SEMIA 566, SEMIA 586 (=CB 1809), SEMIA 587, SEMIA 5019 (=29 w), SEMIA 5039 (=532C), SEMIA 5079 (=CPAC 15) and SEMIA 5080 (=CPAC 7). Information about the strains and their sources were given before (Boddey and Hungria, 1997; Santos et al., 1999), except for SEMIA 566, that was isolated not from a previously inoculated soil, but from a soybean nodule of cultivar Hardee grown in a Leonard jar that received Nitragin inoculant (J.R.J. Freire, personal communication). Three strains used for short periods in commercial inoculants were also included as reference strains in some analyses: SEMIA 513, SEMIA 527 and SEMIA 535, supplied by FEPAGRO (Fundação Estadual de Pesquisa Agropecuária, Porto Alegre, Rio Grande do Sul, Brazil). Two efficient strains, SEMIA 5061 (=INPA 037), isolated in the State of Amazonas and SEMIA 5020 (=965, =J5033), from Japan, were also used in some assays. Their origin and characteristics have been described elsewhere (Boddey and Hungria, 1997). Finally, strains USDA 6, USDA 31, USDA 73, USDA 76, USDA 94, USDA 110, USDA 122 and USDA 123, obtained from United States Department of Agriculture (USDA, Beltsville, MD), were used for comparison in the DNA analyses.

2.2. Bradyrhizobial isolates

The 14 field sites used in this study are shown in Fig. 1. Soil samples were collected using soil drills cleaned with alcohol (95%) and flamed between samplings. Each consisted of 20 subsamples randomly collected at a depth of 0–20 cm, and mixed in a sterile bag. The samples were passed through a 4-mm sieve and divided for the determination of moisture content, chemical properties and for bradyrhizobia isolation, with all operations done as quickly as possible. Moisture content was determined after drying the soil at 105 °C for 4 days, and chemical analyses were performed according to Pavan et al. (1992). Soybean seeds of six unimproved genotypes, Pickett-71, Peking (=plant introduction, PI1 7852.B), PI 245331, PI 437654, PI 88788 and PI 90763 and of a modern cultivar (BR-16) were used to trap rhizobia. The seeds were surface-sterilized with alcohol and 10% sodium hypochlorite (Vincent, 1970) and pre-germinated for 2 days.

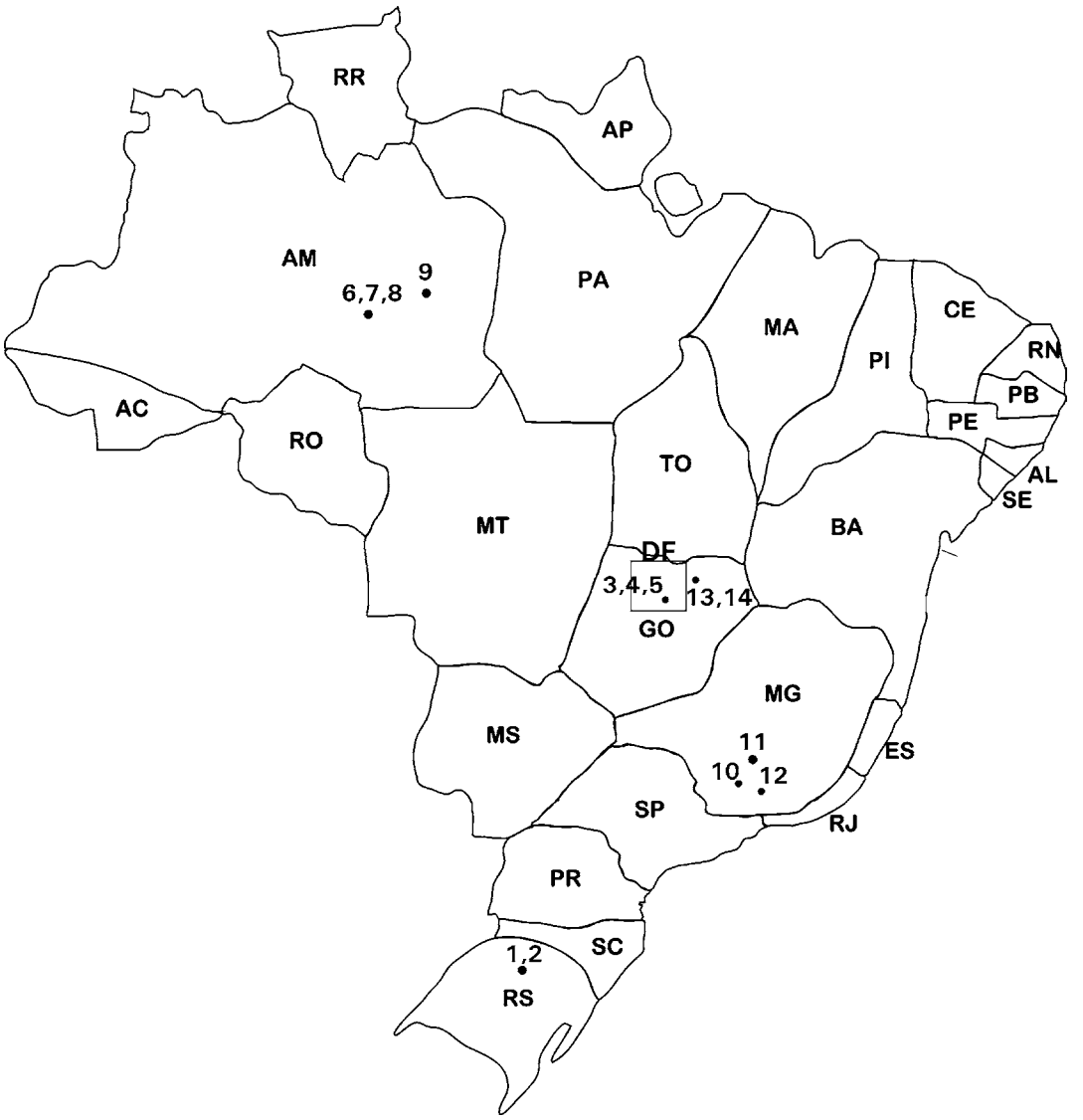

Fig. 1. Map showing the sites where the soil samples used in this study were collected.

Each seedling was inoculated with 500 μl of a 10^{-1} soil dilution (in 0.85% w/v NaCl, agitated with glass beads for 30 min), and inoculation was done in triplicate. Plants were grown in a sterile system using glass jars containing 150 ml of N-free sterile plant nutrient solution (Hungria et al., 1996), and covered with filter paper. Plants were grown under greenhouse conditions, with mean temperatures of 28/22 °C (day/night) for 4 weeks. A total of 40 nodules were obtained and rhizobial strains isolated using standard microbiological methods (Vincent, 1970). Culture purity was confirmed by repeatedly streaking the bacteria on yeast extract-mannitol agar (YMA) medium (Vincent, 1970) with congo red (25 μg ml^{-1}), and by their gram-stain reaction. Bacteria showing a slow growth rate (visible only after 5–7 days) and alkaline reaction on YMA containing 25 μg ml^{-1} bromothymol blue (color change from green to blue) were reinoculated on cultivar BR-16, to confirm their identity and effectiveness. Bacteria grown in YMB (YM broth) were mixed with glycerol (1:1, v:v) and stored at −80 °C. Working cultures were maintained on YMA slants at 4 °C.

2.3. Characterization of the isolates

2.3.1. Morphological characterization

For the evaluation of this and other parameters, an initial inoculum of 10^4 cells ml^{-1} was prepared in YMB at pH 6.8. A loop of the initial inoculum was streaked on YMA and colony morphology (color, mucoidy, transparency, diameter, form, borders, elevation) was evaluated after 5 and 7 days of growth, in the dark, at 28 °C, after Vincent (1970).

2.3.2. Serological reaction

Somatic agglutination reactions were performed as described by Boddey and Hungria (1997), using antisera against the seven soybean *Bradyrhizobium* strains used in Brazilian inoculants over the past three decades. Titers for the antisera were equal or higher than 1:800 and for the analyses were diluted with 0.85% of saline solution to 1:30 (v/v). Controls included the strains or isolates without anti-serum.

2.3.3. Tolerance of acidity, alkalinity, salinity and high temperature

One hundred microliter of initial inoculum were transferred to triplicate tubes containing 5 ml of YMB and of pH 3.5 or 9.5. Cultures were incubated with agitation, in the dark, at 28 °C for 7 days. Tolerance of salinity was determined in tubes containing 5 ml of YMB, pH 6.8 and 0.3 and 0.5 M NaCl. Controls for acidity, alkalinity and salinity tolerance tests consisted of bacteria growing in YMB with pH 6.8. To determine high temperature tolerance a loop of inoculum was streaked onto triplicate plates containing YMA and allowed to grow at 28 (control) and 40 °C for 7 days.

2.3.4. Synthesis of indole acetic acid (IAA)

Bacteria were grown in Tris–YMRT medium enriched with 0.3 mM tryptophan and were colorimetrically evaluated for the synthesis of IAA (Boddey and Hungria, 1997).

2.3.5. Protein and lipopolysaccharide fingerprintings

Protein and lipopolysaccharide (LPS) profiles were determined as described by Ferreira et al. (2000) and Hungria et al. (2001b), respectively.

2.3.6. ERIC-PCR genomic fingerprinting

Bacteria DNA was amplified by PCR using enterobacterial repetitive intergeneric consensus (ERIC) primer (de Bruijn, 1992), as described before (Santos et al., 1999). Banding patterns were photographed and analyzed using Bionumerics program (Applied Mathematics, Kortrijk, Belgium). The unweighted pair-group method with arithmetic mean (UPGMA) clustering method and the coefficient of Jaccard (J) were used.

2.3.7. 16S rRNA partial sequence determination

Eighteen isolates, reference Brazilian strains SEMIA 566, SEMIA 586, SEMIA 587, SEMIA 5019, SEMIA 5039, SEMIA 5061, SEMIA 5079 and SEMIA 5080 and strains USDA 31 and USDA 123 were submitted to the direct sequencing of PCR fragments obtained by amplification with primers which amplify the DNA region coding for the 16S rRNA, as described earlier (Chen et al., 2000). Strains USDA 6, USDA 76, USDA 94, and USDA 110 were sequenced as control treatments. The generated rDNA sequences, confirmed forward and backward, were aligned pair-wise and a phylogeny tree inferred using the UPGMA algorithm and the Bionumerics program. The sequences were also submitted to the GenBank

database to seek for significant alignments of 16S rRNA sequences.

2.4. Symbiotic properties

2.4.1. N_2-fixation capacity

Seeds of cultivar BR-37 were individually inoculated with each isolate and with seven reference strains, and plants were grown in Leonard jars under greenhouse conditions. Bacterial growth, seed sterilization, inoculation procedure and plant-growth conditions were as described by Hungria et al. (1998). Non-inoculated controls were included. Plants were harvested 45 days after planting, nodules were counted and, after drying at 65 °C to constant weight, nodule and shoot dry weights were determined. Total N in shoots was evaluated (Hungria et al., 1998). The experiment was performed in a randomized block design with three replicates, and the data were statistically analyzed by Tukey's test ($P \leq 0.05$).

2.4.2. Nodule occupancy

The experiment was performed in Leonard jars as described in Section 2.4.1, except for that each isolate (10^9 cells ml^{-1}) was co-inoculated in a proportion of 1:1 (v:v) with the competitive strain SEMIA 587 (10^9 cells ml^{-1}). Plants were harvested 45 days after planting and 30 nodules were randomly collected per treatment and assayed with SEMIA 587 anti-serum (Hungria et al., 1998). The experiment was performed in a randomized block design with three replicates, and the data were statistically analyzed by Tukey's test ($P \leq 0.05$).

3. Results

3.1. Bradyrhizobial isolates obtained from uncropped areas

Table 1 includes the main chemical properties of the uncropped soils used in this study; all were acidic, most of them saturated with Al and with low levels of N and P. Bradyrhizobia were not recovered from seven of the soils and 40 isolates were obtained from the remaining seven soils, 42.5% of them from plants inoculated with soils from two sites in the State of Amazonas (Table 1). The ability of plant genotypes to trap the isolates decreased as follows: PI 90763 > PI 437654 > BR-16 = PI 245331 = Pickett-71 (Table 2). Peking and PI 88788 were unable to capture bradyrhizobial isolates.

Table 1
Location, covering native vegetation and chemical properties of the soils used for soybean bradyrhizobial isolation[a]

Soil	District	State	Native vegetation	pH in $CaCl_2$	N (%)	Al[b] (%)	BS[c]	C (%)	P (mg dm^{-3})	Isolates (no.)
1	Passo Fundo	RS	Subtropical forest	4.15	0.54	5.4	46.9	4.87	23.9	2
2	Passo Fundo	RS	Grass field	4.52	0.14	4.8	45.1	2.64	2.7	5
3	Planaltina	DF	Cerrado	4.00	0.13	79.1	2.8	1.95	0.6	0
4	Planaltina	DF	Cerradão	3.85	0.18	56.2	5.9	2.62	1.1	5
5	Planaltina	DF	Cerrado	3.87	0.16	83.0	2.1	2.42	0.6	4
6	Manaus	AM	Tropical forest	3.61	0.69	92.9	1.4	1.29	2.0	5
7	Manaus	AM	Tropical forest	3.58	0.07	95.0	1.1	1.40	1.1	0
8	Manaus	AM	Tropical forest	3.93	0.08	97.8	0.4	0.69	0.6	12
9	Campinara-ma	AM	Tropical forest	3.04	0.04	98.1	0.5	0.73	1.3	0
10	Itutinga	MG	Forest	4.55	0.37	2.3	48.5	4.40	2.1	7
11	Itutinga	MG	Forest	4.32	0.18	57.1	3.0	2.59	0.8	0
12	Lavras	MG	Forest	3.72	0.27	81.2	2.1	3.05	1.7	0
13	Goiânia	GO	Cerrado	4.07	0.13	36.6	11.1	1.46	0.6	0
14	Goiânia	GO	Cerrado	4.36	0.26	5.7	35.6	2.74	0.9	0

RS: Rio Grande do Sul; DF: Distrito Federal; AM: Amazonas; MG: Minas Ge-rais; GO: Goiás.

[a] Also number of isolates obtained from one modern and six unimproved soybean genotypes after inoculation with a 10^{-1} dilution of each soil.

[b] Al (%) = (Al/(Al + K + Ca + Mg)) × 100.

[c] Base saturation = ((K + Ca + Mg)/T_{cec}) × 100, where T_{cec} = K + Ca + Mg + total acidity at pH 7.0 (H + Al).

Table 2
Main characteristics evaluated in vitro and in vivo of the Brazilian soybean bradyrhizobia isolated from uncropped areas and of some reference strains

Strain/ isolate	Soil (state)	Trap host	Serogroup[a]	Tolerance to salinity (NaCl)[b]		Growth at 4 °C[b]	IAA[c]	Protein group[d]	LPS group	DNA group	N_2-fixation[d,e]	Competitiveness[d,f]	16S rRNA[d]
				0.3 M	0.5 M								
SEMIA 566			566	−	−	−	L	I	I	II	L	H	566
SEMIA 5079			566	−	−	−	L	I	I	II	H	H	566
1	1 (RS)	Pickett-71	566	−	−	−	L	I	I	II	L	L	n.e.
2	1 (RS)	Pickett-71	566	−	−	−	L	I	I	II	H	H	566
3	4 (DF)	PI 437654	566	−	−	−	L	I	I	II	L	L	n.e.
15	5 (DF)	PI 437654	566	+	−	+	L	I	I	II	L	H	566
17	8 (AM)	PI 437654	566	+	−	−	L	I	I	II	L	L	n.e.
18	8 (AM)	PI 437654	566	+	−	−	L	I	I	II	L	L	n.e.
19	8 (AM)	PI 437654	566	+	−	++	L	I	I	II	L	L	n.e.
20	8 (AM)	PI 90763	566	+	−	−	L	I	I	II	L	H	n.e.
21	6 (AM)	PI 90763	566	−	−	−	L	I	I	II	L	L	n.e.
22	6 (AM)	PI 90763	566	−	−	−	L	I	I	II	L	L	566
23	6 (AM)	PI 90763	566	−	−	−	L	I	I	II	L	L	n.e.
24	8 (AM)	PI 90763	566	−	−	−	L	I	I	II	L	L	n.e.
25	8 (AM)	PI 90763	566	−	−	−	L	I	I	II	L	L	n.e.
26	8 (AM)	PI 90763	566	−	−	−	L	I	I	II	H	L	n.e.
27	8 (AM)	PI 90763	566	++	++	++	L	I	I	II	L	L	566
28	8 (AM)	PI 245331	566	−	−	−	L	I	I	II	H	L	n.e.
29	8 (AM)	PI 245331	566	−	−	−	L	I	I	II	L	L	n.e.
31	8 (AM)	PI 245331	566	−	−	−	L	I	I	II	L	L	n.e.
SEMIA 5039			5039	−	−	−	L	II	V	III	L	L	5039
6	2 (RS)	PI 437654	5039	−	−	−	L	II	V	I	L	H	5039
7	2 (RS)	PI 437654	5039	−	−	−	L	II	V	I	L	H	n.e.
8	2 (RS)	Pickett-71	5039	−	−	−	L	II	V	I	L	H	n.e.
9	2 (RS)	BR-16	5039	−	−	−	L	II	V	I	L	H	n.e.
10	2 (RS)	BR-16	5039	−	−	−	L	II	V	I	L	H	n.e.
33	6 (AM)	Pickett-71	5039	−	−	−	L	III	V	III	L	L	n.e.
34	10 (MG)	PI 90763	5039	−	−	−	L	II	V	III	L	H	5039
35	10 (MG)	PI 90763	5039	−	−	−	L	II	V	III	L	H	n.e.
36	10 (MG)	PI 90763	5039	−	−	−	L	II	V	III	L	H	n.e.
37	10 (MG)	PI 90763	5039	−	−	+	L	II	V	III	L	H	n.e.
SEMIA 5019			5019	−	−	−	H	I	II	III	H	L	5019
11	4 (DF)	PI 437654	5019	−	−	−	H	V	III	I	H	L	5019
30	8 (AM)	PI 245331	5019	−	−	+	H	I	II	III	H	L	5019
32	8 (AM)	PI 245331	5019	−	−	−	H	I	II	III	H	H	5019
SEMIA 586			586	−	−	−	L	IV	VIII	II	H	L	586
SEMIA 5080			586	−	−	−	L	IV	VIII	II	H	L	586

4	5 (DF)	PI 437654	586	+	+	+	L	IV	VIII	II	H	L	586
SEMIA 587			587	−	−	−	H	VIII	VI	III	H	−	587
40	10 (MG)	BR-16	587	−	−	−	H	VIII	VI	III	H	−	587
38	10 (MG)	BR-16	n.r.	−	−	+	H	VIII	VI	III	H	H	587
39	10 (MG)	BR-16	n.r.	++	++	++	L	VIII	VII	I	H	H	587
12	4 (DF)	PI 245331	n.r.	−	−	−	L	V	IV	I	L	L	5019
13	4 (DF)	Pickett-71	n.r.	−	−	−	H	V	II	I	H	H	5019
14	4 (DF)	Pickett-71	n.r.	−	−	−	H	V	II	I	H	H	5019
5	5 (DF)	BR-16	n.r	−	−	++	M	VI	II	I	H	L	5019
16	5 (DF)	PI 90763	n.r.	++	++	++	L	VII	IX	I	L	L	d.
SEMIA 5061			n.r.				H	I	II	III	n.e.	n.e.	5019
USDA 31			n.r.				H	n.e.	III	III	n.e.	n.e	31
USDA 76			n.r.				H	I	II	III	n.e.	n.e.	76
USDA 94			n.r.				H	n.e.	IV	I	n.e.	n.e.	94

[a] n.r. indicates the isolates that did not react with any of the tested antisera.

[b] (−): absence of growth, (+): growth of 20–50%, and (++): growth >50% in relation to the growth in YMB (or on YMA) medium at 28 °C.

[c] Synthesis in tryptophan enriched (0.3 mM) medium: L, low, 4.6–14 μM; M, medium, 43 μM; H, high, 127–246 μM.

[d] n.e. indicates not evaluated, d. indicates different from all other strains.

[e] H and L indicates total N accumulated in tissues higher or lower than 51.2 mg of N per plant, respectively.

[f] H and L indicate nodule occupancy equal or higher than 50% or lower than 50%, respectively.

3.2. Morphological and serological characterization

Isolates grouped into five known serological groups, with seven strains not reacting with any of the tested antisera (Table 2). Colony morphology (data not shown), serological reaction and site of isolation (Table 2) were often related. For example, isolates 6–10, from soil 2 (RS), showed diameter of colonies ≤0.5 mm, white color and produced little mucus while isolates 34–37, from soil 10, showed cream colonies ≤0.5 mm and low mucoidy (data not shown).

3.3. Tolerance to acidity, alkalinity, salinity and high temperature

Although, isolated from acid soils, the 14 isolates, and all seven reference strains were able to grow in YMB at pH 6.5 and 9.0 (data not shown). However, only one isolate from serogroup SEMIA 566 (27) was able to grow at pH 3.5 (data not shown). Tolerance of the isolates to salt is shown in Table 2, with nine isolates able to grow in 0.3 M NaCl and four of them at 0.5 M NaCl. Ten isolates grew at 40 °C (Table 2). Isolates 39 and 16, with unknown serological reaction, were tolerant of salinity and high temperature and isolate 27 was tolerant of salinity, high temperature and acidity.

3.4. Synthesis of indole acetic acid

Eighty percent of the isolates accumulated 4.6–14.4 μM of IAA. They included isolates belonging to serogroups SEMIA 566, SEMIA 5039 and SEMIA 586, and three isolates with unknown serological reaction (Table 2). Isolate 5, from DF and with unknown serological reaction, accumulated 43 μM of IAA. Isolates and reference strains belonging to serogroups SEMIA 5019 and SEMIA 587, as well as three isolates with unknown serological reaction (38, 13 and 14) accumulated 127–246 μM (Table 2).

3.5. Protein and lipopolysaccharide fingerprintings

It was easier to group the strains by the LPS than by the protein profile. Isolates and reference strains grouped into eight protein (PP) and nine lipopolysaccharide (LPS) profiles (Table 2). SEMIA 566, SEMIA 5079 and all 18 isolates belonging to this serogroup clustered in PP-I and LPS-I. In contrast, isolates 39 and 16 showed unique LPS banding, and isolates 33, 5 and 16 unique protein banding. Isolates 11–14 showed similar protein bandings, but different from reference strains (Table 2).

3.6. ERIC-PCR genomic fingerprinting

The DNAs amplified by PCR with the specific primer ERIC produced an average of 15 bands per isolate. PCR products were combined to produce a dendrogram, divided in three main groups that were then subdivided in eight subgroups (Fig. 2). Six of the seven isolates with unknown serological reaction, as well as reference strain USDA 94 clustered in one group. Group II clustered, at a 30% level of similarity, all isolates belonging to serogroups SEMIA 566 (same serogroup as SEMIA 5079) and SEMIA 586 (profile not shown) (same serogroup as SEMIA 5080) and synthesizing low amounts of IAA, as well as reference strains SEMIA 513 and SEMIA 527. Group III included several reference strains as well as isolates reacting with serogroups SEMIA 5039, SEMIA 587, SEMIA 5019 and isolate 38, with unknown reaction (Fig. 2).

3.7. 16S rRNA partial sequence analysis

Four isolates from serogroup SEMIA 566 (2, 15, 22 and 27) were randomly chosen with the analysis of 560 bp showing a 100% identity with strains SEMIA 566 and SEMIA 5079. The sequence for commercial strain SEMIA 5079 showed 99% similarity (645 of 646 bp with one gap) with *B. japonicum* strains USDA 6 (accession number U69638.2), LMG 6138 (X66024), IAM 2608 (D12781) and USDA 123 (AF236087), but also with *B. lupini* strain DMS 30140 (X87273). Two isolates chosen from serogroup SEMIA 5039 showed 100% identity with that strain in 520 bp. Strains SEMIA 586, SEMIA 5080 and isolate 4 were also identical in 550 bp. The sequence obtained for commercial strain SEMIA 5080 had a 99% similarity (569/572 bp) with *B. japonicum* strains DMS 30131 T (X87272) and USDA 110 (L23330). Strain SEMIA 5019 and isolates 30 and 32 were also identical in the partial 16S rRNA sequence. Identical sequences with SEMIA 5019 were found also in isolates 11, belonging to serogroup SEMIA 5019, and isolates 12–14 and 5, with unknown

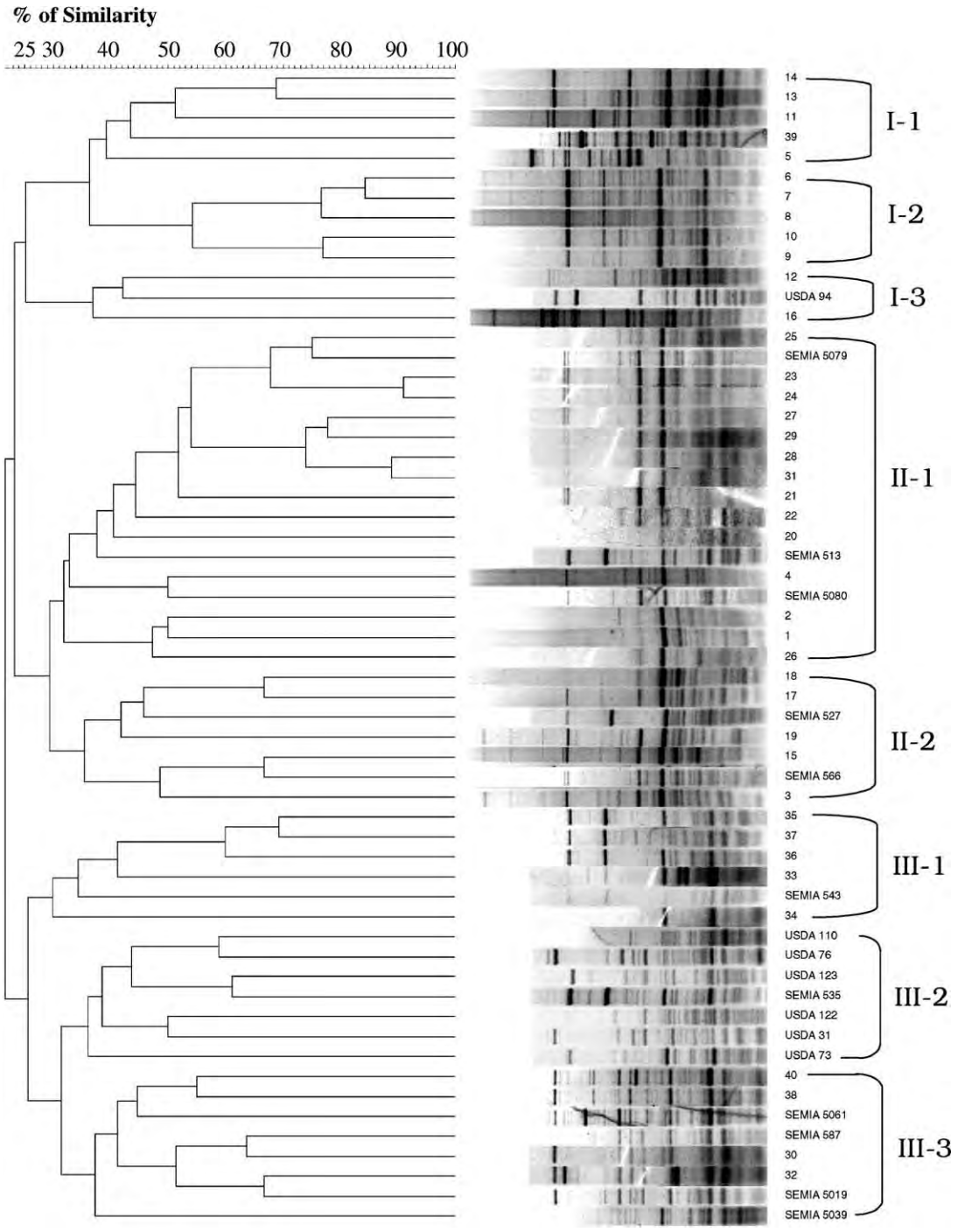

Fig. 2. Genetic dendrogram showing the soybean bradyrhizobial isolates obtained from Brazilian uncropped soils and some reference strains used in this study after cluster analysis of ERIC-PCR products using the UPGMA method and Jaccard coefficient.

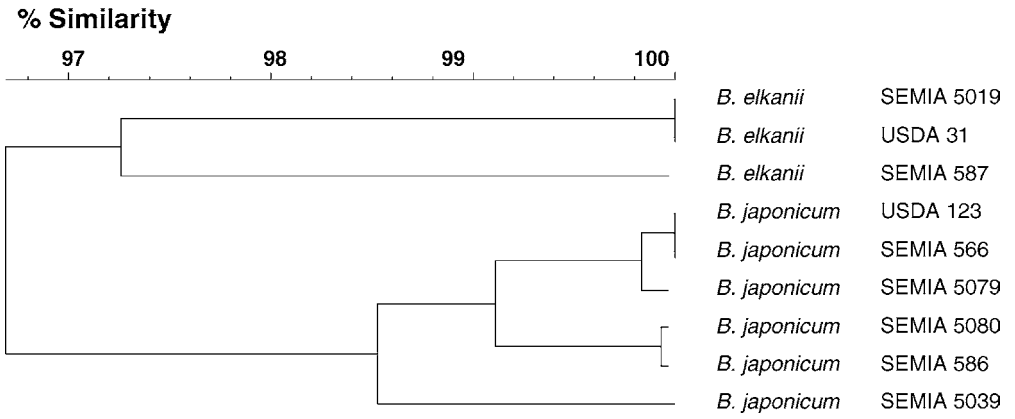

Fig. 3. Dendrogram built by the use of the UPGMA algorithm with the aligned partial 16S rRNA sequences of the main serogroups found in Brazilian soils and of reference strains of *B. japonicum* and *B. elkanii*. GenBank accession numbers are given in the text.

serological reaction. Commercial strain SEMIA 5019 showed a 99% similarity with *B. elkanii* USDA 31 (M55487) and *Bradyrhizobium* sp. strain LMG 9520, isolated from *Acacia albida* (X70402). The partial sequence of SEMIA 5061 was equal to that of SEMIA 5019, confirming the similarities detected in protein, LPS and ERIC-PCR profiles. Finally, strain SEMIA 587 and isolates 40, 38 and 39 were identical in 420 bp of the 16S rRNA. Strain SEMIA 587 had a 100% identity (469/469 bp) with *Bradyrhizobium* sp. LMG 9520 (X70402) and a 99% (468/469 bp) identity with *B. elkanii* USDA 76 (U35000). The partial sequence of one isolate, 16, differed from all described species of rhizobia and, therefore, the isolate is being studied in more detail.

The sequences of the four strains used in current Brazilian commercial inoculants, SEMIA 587, SEMIA 5019, SEMIA 5079 and SEMIA 5080 were submitted to the GenBank database, and received the accession numbers AF234890, AF237422, AF234888 and AF234889, respectively. The sequences of strains SEMIA 566, SEMIA 586, USDA 123 and USDA 31 were also submitted to the GenBank database and received the accession numbers AF236086–AF236089. The phylogenetic tree obtained with the partial 16S rRNA sequences showed that strains USDA 123, SEMIA 566, SEMIA 5079, SEMIA 586, SEMIA 5080 and SEMIA 5039 grouped as *B. japonicum*, while SEMIA 5019 and SEMIA 587 with *B. elkanii* strain USDA 31. SEMIA 587 showed lower similarity with the other strains (Fig. 3).

3.8. N_2-fixation capacity

Statistically significant differences among the 14 isolates and seven commercial strains were verified in terms of nodule number and dry weight at 45 days (Table 3). Nodule number varied 4.5-fold, from isolate 12 (34 nodules per plant) to 38 (152 nodules per plant). Best symbiotic performances (nodule mass, dry weight and total N accumulated in shoots) were obtained with isolates belonging to serogroups SEMIA 5019, SEMIA 586 and SEMIA 587. Some isolates (38, 39, 13, 14 and 5) with unknown serological reaction, but with partial 16S rRNA sequences similar to SEMIA 587 and SEMIA 5019 showed good symbiotic performance, while others (12 and 16) were poor symbionts (Table 3).

3.9. Nodule occupancy

When co-inoculated with each isolate or reference strain, SEMIA 587 occupied 3–100% of the nodules formed (Table 3). Some variability in competitiveness was also found among isolates within individual serogroups (Table 3).

3.10. Summary of the characteristics in vivo and in vitro

Table 2 summarizes the main characteristics of each isolate. For N_2-fixation, values less than 51 mg of N per plant (Table 2) were considered low symbiotic

Table 3
Symbiotic performance of 40 soybean bradyrhizobial isolates and reference strains when single inoculated in cultivar BR-37

Strain/isolate	Single inoculation				Double Inoculation Nodule occupancy by SEMIA 587
	Nodulation		Shoot		
	Number (no. per plant)	Dry weight (mg per plant)	Dry weight (g per plant)	Total N (mg N per plant)	(%)
SEMIA 566	90 a–e	280 a–m	2.50 c–n	45.7 e–l	40
SEMIA 5079	106 a–e	290 a–m	2.85 a–I	70.2 a–i	50
1	101 a–e	334 a–j	1.72 c–l	47.4 e–l	66
2	90 a–e	313 a–m	1.77 b–l	51.6 b–l	26
3	104 a–e	221 e–n	1.23 i–l	33.9 j–l	73
15	65 a–e	139 nm	1.18 j–l	26.1 kl	3
17	87 a–e	270 b–n	1.44 g–l	40.5 g–l	73
18	80 a–e	224 e–n	1.16 j–l	35.2 i–l	76
19	90 a–e	217 h–n	1.18 j–l	32.8 j–l	83
20	103 a–e	298 a–m	1.65 l	48.9 c–l	43
21	124 a–d	318 a–l	1.74 b–l	48.2 d–l	73
22	106 a–e	283 a–m	1.63 e–l	42.8 g–l	76
23	91 a–e	292 a–m	1.55 f–l	45.1 f–l	56
24	98 a–e	305 a–m	1.67 d–l	45.7 e–l	100
25	45 a–e	281 a–n	1.52 f–l	44.4 f–l	93
26	111 a–e	322 a–k	1.82 b–l	52.4 b–l	80
27	98 a–e	291 a–m	1.51 f–l	45.4 e–l	100
28	110 a–e	366 a–h	1.95 b–l	61.1 a–k	90
29	99 a–e	340 a–j	1.52 f–l	44.9 e–l	80
31	94 a–e	257 c–n	1.43 g–l	37.8 h–l	90
SEMIA 5039	80 a–e	190 i–n	0.94 l	27.7 kl	55
6	72 a–e	189 i–n	0.99 kl	27.0 kl	50
7	63 a–e	174 j–n	0.95 l	26.6 kl	50
8	82 a–e	206 i–n	1.00 kl	27.3 kl	23
9	81 a–e	155 k–n	0.94 l	27.2 kl	36
10	77 a–e	203 h–n	1.05 kl	28.4 kl	16
33	105 a–e	229 e–n	1.25 i–l	40.2 g–l	63
34	97 a–e	287 a–m	1.52 f–l	46.3 e–l	25
35	113 a–e	245 c–n	1.31 h–l	36.5 h–l	3
36	82 a–e	229 d–n	1.21 j–l	36.6 h–l	3
37	98 a–e	224 e–n	1.20 j–l	37.7 h–l	23
SEMIA 5019	81 a–e	373 a–k	2.46 a–h	74.5 a–g	55
11	94 a–e	406 a–d	3.20 a	95.2 a	100
30	138 a–c	382 a–g	2.15 a–k	57.3 b–l	56
32	144 ab	380 a–g	2.46 a–h	65.4 a–j	6
SEMIA 586	113 a–e	309 a–m	2.40 a–i	80.2 a–e	80
SEMIA 5080	91 a–e	324 a–k	2.65 a–f	86.0 ab	60
4	107 a–e	383 a–f	2.90 ab	96.2 a	93
SEMIA 587	93 a–e	325 a–k	2.30 a–j	75.2 a–g	100
40	142 ab	450 a	2.86 a–c	84.2 a–d	100
38	152 a	441 ab	2.83 a–d	79.6 a–f	33
39	142 ab	419 a–c	2.65 a–f	70.3 a–i	3
12	34 e	143 k–n	1.08 kl	28.6 kl	90
13	108 a–e	360 a–h	2.42 a–h	70.2 a–h	10
14	118 a–e	366 a–h	2.53 a–g	73.2 a–g	26
5	103 a–e	397 a–e	2.78 a–e	85.1 a–c	100
16	55 a–e	104 n	0.87 l	25.4 l	100

Also nodule occupancy by SEMIA 587 when co-inoculated (1:1, v:v, 10^9 cells ml^{-1}) with each isolate. Plants grown under greenhouse and N-free conditions and harvested at 45 days after planting. Means of three replicates, and when followed by the same letter, within the same column, did not show statistical difference (Tukey's test, $P \leq 0.05$).

performance, those above that figure high (Table 3). For strain competitiveness, this was considered high when SEMIA 587 occupied more than 50% of the nodules (Tables 2 and 3).

4. Discussion

Soybean is an exotic species in Brazil and, as in other countries to which it was introduced, early experiments showed that the soils lacked bradyrhizobial strains able to establish effective symbioses (Stanford, 1972; Lopes et al., 1976; Peres, 1979; Vargas and Suhet, 1980; Lopes and Giardini, 1981; Suhet et al., 1981; Vargas et al., 1982). There are, however, reports of soybean nodulation in early experiments performed in areas not cropped before with this legume and in the absence of inoculants (Freire and Vidor, 1981; Yuyama et al., 1981), although, the microbiological methods used in those experiments are not well described. Morphological, physiological and genetic modifications in relation to putative parental strains have also been reported (Hungria et al., 1996; Nishi et al., 1996; Boddey and Hungria, 1997; Hungria et al., 1998; Santos et al., 1999), as well as the detection of a high percentage of isolates with unknown serological reaction after some years of soybean cropping (Freire et al., 1983; Vargas et al., 1993; Vargas and Hungria, 1997; Ferreira et al., 2000).

Recently, strains characterized by a fast-growth rate and acidic reaction in YM were isolated from soybean nodules obtained from the same soils of this study (Hungria et al., 2001b), and the genetic characterization has shown higher similarity with *Rhizobium tropici* (M. Hungria et al., unpublished). Thus indigenous *Rhizobium* strains were able to nodulate soybean, though, they were all poor competitors in the presence of *B. japonicum* and *B. elkanii* (Hungria et al., 2001a). This paper examined the occurrence of indigenous *Bradyrhizobium* strains able to nodulate soybean and indeed, 40 isolates were obtained from soils located in distant sites, on which native vegetation was growing. Soybean is not cropped in the Amazon, where most of the isolates originated, but the State borders with soybean producing areas and, as mentioned earlier, bradyrhizobial strains have already been isolated from that State (Yuyama et al., 1981; Moreira, 2000).

Most of the isolates obtained in this study were not tolerant of acidity, salinity or high temperature in vitro and LPS profile was the easiest method with which to delineate the isolates. In general, N_2-fixation capacity, and, to a lesser extent, competitiveness, were similar among strains of a specific serogroup. Eighteen isolates were similar to strain SEMIA 566 in serological reaction, synthesis of IAA, protein, LPS and ERIC-PCR profiles and partial 16S rRNA sequence, but variability was observed in physiological and symbiotic properties. Strain SEMIA 566 was obtained from a plant grown in a Leonard jar that had received a North American inoculant (J.R.J. Freire, personal communication), therefore, it is not a native bradyrhizobia. That strain was used in commercial inoculants from 1966 to 1978, mainly in the South Region of the country. Since 1992, SEMIA 5079, a variant strain of SEMIA 566 (Vargas et al., 1992; Hungria et al., 1996; Nishi et al., 1996), has been intensively used in inoculants especially in the Central Region of Brazil. SEMIA 566 has been reported to have limited saprophytic competence in the first 2 years, but became established thereafter (Freire et al., 1983; Vargas and Hungria, 1997). Strains from this serogroup were recovered from three distant sites, RS, DF and AM, supporting evidence to the saprophytic competence of this organism and possibly to the transport of strains by wind currents.

A high level of similarity, including complete identity in 520 bp of the 16S rRNA region, was also observed among isolates reacting with serogroup SEMIA 5039, although two ERIC-PCR groups were found, one clustering the isolates from Rio Grande do Sul, where the strain was used in commercial inoculants from 1965 to 1978, and the other one including the isolates from Minas Gerais, where soybean cropping was initiated much later.

Previous studies have shown substantial variation among isolates belonging to serogroups SEMIA 566, SEMIA 5020 and SEMIA 5039 that were recovered from soybean-cropped areas (Boddey and Hungria, 1997; Hungria et al., 1998; Santos et al., 1999). Furthermore, the transfer of symbiotic genes to non-symbiotic native bradyrhizobia, as demonstrated for *Mesorhizobium loti* (Sullivan et al., 1995, 1996; Sullivan and Ronson, 1998) should be investigated in Brazilian soils cropped with soybean, since it could

help to explain the variability among isolates observed in this study.

SEMIA 587, a strain isolated in 1967 from a soybean plant in Santa Rosa, Rio Grande do Sul (J.R.J. Freire, personal communication), is very competitive (e.g. Freire et al., 1983; Vargas and Hungria, 1997) and has been used for many years in commercial inoculants, but just one isolate (40) proved similar to this strain, with two other isolates, 38 and 39, showing identical 16S rRNA partial sequences, but differing in some of the evaluated characteristics. Just one isolate fit into the serogroup of strains SEMIA 586 and SEMIA 5080, used in commercial inoculants for a shorter period of time.

Relatively high variability in all parameters evaluated was obtained among isolates showing similar partial 16S rRNA sequences to SEMIA 5019, classified as *B. elkanii*. That strain was isolated in 1979 from a nodule of soybean line IAC-70-559, in a search for strains with a higher ability to nodulate cultivars that were being used in the Central Region of Brazil, the ''Cerrados'' (Peres, 1979). The plants had been inoculated, but there is no information on the inoculant used (J.R.R. Peres, personal communication). It was also interesting to observe that strain SEMIA 5061, isolated from an area in the State of Amazon in which inoculants had never been used (Yuyama et al., 1981), showed identical properties to SEMIA 5019.

Brazilian soils are probably void of native bradyrhizobial strains able to effectively nodulate soybean, since 39 of the 40 isolates fit, at least partially, into the characteristics of seven strains that were, or are, used in Brazilian commercial inoculants. However, differences among isolates within a serogroup were verified in this study, e.g. two different groups of ERIC-PCR within serogroup SEMIA 5039, although, all strains showed similar protein and LPS profiles. The similarity of most of the isolates with the strains used in commercial inoculants probably indicates a high level of dispersion of bacteria from cropped areas. Just one isolate, 16, showed properties of *Bradyrhizobium* but did not fit into the characteristics of any of the reference strains of *B. japonicum* and *B. elkanii*, and therefore could represent an indigenous *Bradyrhizobium*. However, isolate 16 showed poor N_2-fixation capacity and competitiveness.

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