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Characterization of soybean *Bradyrhizobium* strains adapted to the Brazilian savannas

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Abstract

Brazilian soils are originally free from soybean bradyrhizobia and the first inoculants were brought to the country in this century, but a search for adapted strains started immediately and still continues. A strain selection program was established at Embrapa based on the reisolation of strains after a long period of adaptation to the soils followed by a search for variant genotypes with higher N_2 fixation capacity and competitiveness. A second approach of this program consists of searching for variant colonies of a single strain with higher N_2 fixation rates and competitiveness, following a short period of adaptation to the soil. In this study, using both approaches, strains belonging to three serogroups, CB 1809, 532C and SEMIA 5020, were obtained. In general, the variant strains showed differences in colony morphology (mucoidy) but produced similar protein and lipopolysaccharide profiles. Within serogroup CB 1809, containing variants obtained via the second approach, a low level of DNA polymorphism was detected relative to the parental genotype by ERIC and REP-PCR. However, within the two other serogroups, containing variant strains obtained via the first approach, a high level of polymorphism in ERIC and REP-PCR fingerprints was observed relative to the putative serologically related parental genotypes. These results show that a great variability can be detected following adaptation of *Bradyrhizobium* strains to the soil, although other potential explanations for the DNA polymorphisms observed are discussed. Some of the variant strains obtained by both methodologies were found to have higher rates of N_2 fixation and almost all were more competitive than the parental genotypes, suggesting that it is possible to select variant strains which can contribute to an improved plant N nutrition status.

Keywords: Bacterial community; *Bradyrhizobium*; Competitiveness; Nitrogen fixation; Nodulation; Soybean

1. Introduction

Soybean [*Glycine max* L. (Merrill)] is today the most important export crop in Brazil and, due to its high protein content (40%), also plays an important role in the diet of the population. The success of the crop in the country relies on an efficient symbiosis with N_2-fixing bacteria belonging to the genus *Bradyrhizobium*. To sustain a mean yield of 2500 kg ha^{-1}, the crop needs about 200 kg of N ha^{-1}. Therefore, a supply of 300–400 kg of N ha^{-1} is required to maintain soil fertility levels. Since chemical

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N fertilizers are very expensive in Brazil, the crop would be economically impracticable in the absence of biological N_2 fixation [12,13,28,32].

Soybean was introduced into Brazil in the last century, but large-scale commercial crop production started in the early 1960s. As Brazilian soils are originally free from soybean bradyrhizobia [8,13,15, 16,29,30], during the establishment of the crop, the seeds had to be inoculated with strains. The inoculant strains were initially brought from the USA, but a search for strains adapted to Brazilian conditions started concomitantly with the expansion of commercial soybean crop production [6,8,15,16,18,19]. The selection of adapted strains usually consisted of inoculation of an area, followed, after a short period of time, by reisolation of bacteria from nodules of soybean trap plants. For example, SEMIA 566, a strain used in Brazilian inoculants from 1966 to 1978, was reisolated from an area which had previously received an inoculant distributed by Dixie Inoc., probably an inoculant from Nitragin (Milwaukee, USA) [28]. In the 1960s, soybean was cultivated in the southern region of Brazil, but during the following decade a new agricultural frontier was opened in the central region, the 'Cerrados', an edaphic type of savanna occupying 207 million hectares and representing about 25% of Brazilian land. The Cerrados are quite distinct from other areas of Brazil, especially in relation to soil chemical properties. The absence of nodules in non-inoculated soybean has been reported since the first trials performed in the Cerrados [18,19,29,30] and several studies were then performed to optimize plant cropping, including the selection of soybean bradyrhizobia adapted to the region [18,19,29,30].

Today, the most successful strain selection program is carried out at Embrapa-Cerrados. The program has already identified two strains, CPAC 15 and CPAC 7 (belonging to the serogroups of SEMIA 566 and CB 1809, respectively) [31], which consistently increase nodulation and yield under field conditions [9,17,31,32], leading to their use in commercial inoculants since 1992 [31]. Studies comparing CPAC 7 and CPAC 15 with their respective parental strains indicated several morphological, physiological and genetic modifications [4,10,11, 17].

Brazilian soybean yield has increased from 1500 kg ha^{-1} in the 1960s to 2500 kg ha^{-1} today. Consequently, more efficient and competitive strains adapted to Brazilian soils must continue to be isolated so that the biological N_2 fixation process can keep pace with the increasing N demand of soybean plants. The objective of this study was to identify new, efficient and competitive *Bradyrhizobium* strains belonging to the serogroups CB 1809, 532C and SEMIA 5020, and to detect morphological, physiological and genetic changes occurring during the adaptation of the strains to the soil.

2. Materials and methods

2.1. Bacterial strains

Nomenclature and observations about the strains are shown in Table 1.

2.2. Morphological, serological and physiological characterization

The morphological characterization proceeded as described before [4]. Serological reactions against the antisera of whole cells of 20 strains which had been used as commercial inoculants since the 1960s were examined as described by Somasegaran and Hoben [23].

2.3. Protein and lipopolysaccharide fingerprintings

Protein profiles of all isolates were determined in 300-μl cultures of bradyrhizobia grown on TY medium [3] for 5 days. Cells were centrifuged, washed three times in 0.85% NaCl and once in 10 mM Tris-HCl pH 7.6, resuspended in 10 mM Tris-HCl pH 7.6 to a concentration of 10^9 cells ml^{-1}, and 200 μl of that suspension was mixed with 200 μl of sterilized lysis buffer [H_2O, 8 ml; 0.5 M Tris-HCl pH 6.8, 2 ml; glycerol, 1.6 ml; 10% SDS (sodium dodecyl sulfate), 3.2 ml; mercaptoethanol, 0.8 ml and 0.05% bromophenol blue, 0.4 ml]. Cells were boiled for 5 min, centrifuged and the supernatant was harvested. Sample running and staining procedures were as described by Alfenas et al. [1]. A standard protocol was employed to obtain lipopolysaccharide profiles [21].

Table 1
Bacterial strains used in the experiments

Strain	Other designations	Observations
SEMIA 566		Isolated in the State of Rio Grande do Sul, from a previously inoculated soil and recommended commercially from 1966 to 1978
CB 1809	SEMIA 586, USDA 136b, TAL 379	Sent to Brazil by Dr. Norris (CSIRO) in 1966 (originally the strain had been carried from the USA to Australia by Dr. Peter J. Dart, personal communication)
532C	SEMIA 5039	Isolated in the State of Rio Grande do Sul, from an area previously inoculated with a North American inoculant [18], and recommended in commercial inoculants from 1965 to 1966 and in 1976 and 1978
SEMIA 5020	965, BR 95, J5033	Strain received from Japan and first tested for N_2 fixation ability in the State of Rio Grande do Sul [18]. The strain was not recommended in commercial inoculants, but was used in several field experiments performed by Universities and Experimental Stations in Brazil
SEMIA 587		Isolated in 1967 in the State of Rio Grande do Sul, from an area previously inoculated with a North American inoculant and used in commercial inoculants from 1968 to 1975 and since 1979
29w	SEMIA 5019	Strain isolated in the State of Rio de Janeiro, from a soil with a high manganese content (no record of which strains had been previously inoculated in the area), and used in commercial inoculants since 1979
CPAC 7	SEMIA 5080	Natural variant of CB 1809, obtained under laboratory conditions and characterized by a higher competitiveness than the parental strain [31], being recommended for commercial inoculants since 1992
CPAC 15	SEMIA 5079	Strain belonging to serogroup SEMIA 566, and isolated from a soil in the Cerrados region several years after inoculation with that strain [31]. Recommended for commercial inoculants since 1992

2.4. PCR genomic fingerprinting with arbitrary short and specific primers

For the genetic analyses, the DNA from strains was extracted and amplified by PCR with ERIC and REP primers (at a concentration of 50 pmol μl^{-1}) following the methodology of de Bruijn [5]. PCR cycles were slightly modified from the protocol of Dr. de Bruijn's laboratory and the analysis was performed in an MJ Research Inc. PT 100 Thermocycler. For REP1R-1 and REP2I primers, the following cycles were used: 1 cycle at 95°C for 6 min; 35 cycles at 94°C for 1 min, at 45°C for 1 min and at 65°C for 8 min; 1 cycle at 65°C for 16 min; and a final soak at 4°C. For ERIC1R and ERIC2 primers: 1 cycle at 95°C for 7 min; 35 cycles at 94°C for 1 min, at 52°C for 1 min and at 65°C for 8 min; 1 cycle at 68°C for 16 min; and a final soak at 4°C. After separation of amplified fragments by electrophoresis on a 1.5% agarose gel, the presence or absence of bands was transformed in a binary matrix based on presence/absence (1/0). For each gel internal and external standards with defined marker sizes were used and the combined and normalized data were put into the matrix for statistical analysis. Cluster analysis was carried out 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 J (Jaccard) coefficients.

2.5. Nitrogen fixation capacity

Each strain was grown in YM (yeast mannitol) medium [33], for 7 days, at 28°C, and cultures were adjusted to a concentration of 10^9 cells ml^{-1}, according to previous analyses performed with each strain and considering optical density and plate and plant counts after inoculation with serial dilutions. Soybean [*Glycine max* L. (Merrill)] seeds of cultivar BR-16 were surface-sterilized [33] and incubated with the inoculum (1 ml $seed^{-1}$) for 30 min. Four seeds were sown in every modified Leonard jar [33] containing sterile sand and vermiculite (1:2, v/v) and filled with N-free nutrient solution [2]. Plants were

Table 2
Nodule number (NN, no. plant^{-1}) and dry weight (NDW, mg nodules plant^{-1}), root (RDW, g plant^{-1}) and shoot (SDW, g plant^{-1}) dry weight, total N accumulated in shoots (TNS, mg N shoot^{-1}) and nodule efficiency (NE, mg N in shoots mg^{-1} of nodules) of soybean cultivar BR-16 inoculated with *Bradyrhizobium* strains belonging to serogroups CB 1809, 532C, SEMIA 5020, SEMIA 566, 29w and SEMIA 587

Strain	NN	NDW	RDW	SDW	TNS	NE
Serogroup CB 1809						
CB 1809	123.0 abc[a]	248.3 d–g	0.37 def	2.52 a–d	71.1 bc	0.286 ab
CPAC 7	153.0 abc	298.8 b–e	0.37 def	2.72 abc	73.8 b	0.247 abc
CPAC 390	136.7 abc	460.0 a	0.76 ab	3.47 a	112.5 a	0.244 abc
CPAC 392	84.7 c	286.2 b–f	0.55 a–f	2.20 b–e	59.5 b–e	0.208 a–d
CPAC 393	117.3 abc	321.2 a–e	0.66 a–d	2.74 abc	77.7 b	0.242 abc
CPAC 394	94.7 abc	421.3 ab	0.80 a	2.97 ab	76.4 b	0.181 cd
CPAC 402	122.0 abc	373.6 a–d	0.66 a–d	2.92 abc	80.1 b	0.214 a–d
CPAC 403	170.3 a	423.4 ab	0.71 abc	2.96 ab	73.3 b	0.173 cd
CPAC 404	103.0 abc	388.5 a–d	0.76 ab	3.04 ab	71.2 bc	0.183 cd
CPAC 405	138.3 abc	316.4 a–e	0.64 a–e	2.43 bcd	65.7 bcd	0.208 a–d
Serogroup 532C						
532 C	147.2 abc	145.0 fg	0.33 f	1.12 f	26.2 fg	0.178 cd
CPAC 346	164.7 abc	408.0 abc	0.52 a–f	2.20 b–e	60.2 b–e	0.148 cd
CPAC 351	143.3 abc	217.7 efg	0.33 f	1.52 def	36.6 d–g	0.168 cd
CPAC 357	151.0 abc	198.1 efg	0.33 f	1.37 ef	35.9 d–g	0.181 cd
CPAC 360	111.7 abc	194.4 efg	0.34 ef	1.15 f	26.4 fg	0.136 d
CPAC 361	108.7 abc	144.4 fg	0.32 f	1.03 f	17.9 g	0.124 d
CPAC 471	87.3 bc	132.5 g	0.34 ef	1.11 f	19.9 g	0.150 cd
Serogroup SEMIA 5020						
SEMIA 5020	138.1 abc	145.0 fg	0.33 f	1.37 ef	26.7 fg	0.193 bcd
CPAC 520	137.0 abc	256.9 c–g	0.46 b–f	1.68 def	40.4 c–g	0.157 cd
CPAC 527	131.0 abc	247.4 d–g	0.38 def	1.61 def	33.1 efg	0.134 d
Serogroup SEMIA 566						
SEMIA 566	160.3 abc	245.8 d–g	0.47 b–f	2.35 b–e	60.9 b–e	0.248 abc
CPAC 15	167.0 ab	237.0 d–g	0.42 c–f	2.45 bcd	61.9 b–e	0.300 a
Serogroup 29w						
29w	124.0 abc	274.8 b–g	0.37 def	1.94 c–f	48.8 b–g	0.178 cd
Serogroup SEMIA 587						
SEMIA 587	98.0 abc	277.0 b–g	0.54 a–f	2.51 a–d	54.0 b–f	0.195 bcd

[a]Mean of five replicates. Values followed by the same letter did not show statistical difference (Tukey, $P \leq 0.05$).

grown under greenhouse conditions, with a 12-h photoperiod and temperature of 28/23°C (day/night, with a standard deviation of ±2.8°C) and thinned to two plants per jar 4 days after emergence (DAE). Nutrient solution was replenished every other day and plants were harvested at 45 DAE. The parameters evaluated were nodule number and dry weight, shoot and root dry weight and total N content of shoots. The N content was determined by the indophenol blue colorimetric method of Feije and Anger [7]. The experiment was performed in a randomized block design, with five replicates and statistically analyzed by Tukey's test ($P \leq 0.05$).

2.6. Nodule occupancy

The nodule occupancy experiment was carried out as described in Section 2.5, except that each strain (10^9 cells ml^{-1}) was inoculated in a proportion of 1:1 with strain 29w (10^9 cells ml^{-1}), which is highly competitive and belongs to a different serogroup than all the strains to be tested. In addition to the parameters analyzed in the previous experiment, 60 nodules per treatment were randomly collected and their bradyrhizobia analyzed for serological reactions [23] against the antisera of each inoculated strain and of 29w. The experiment was performed in a random-

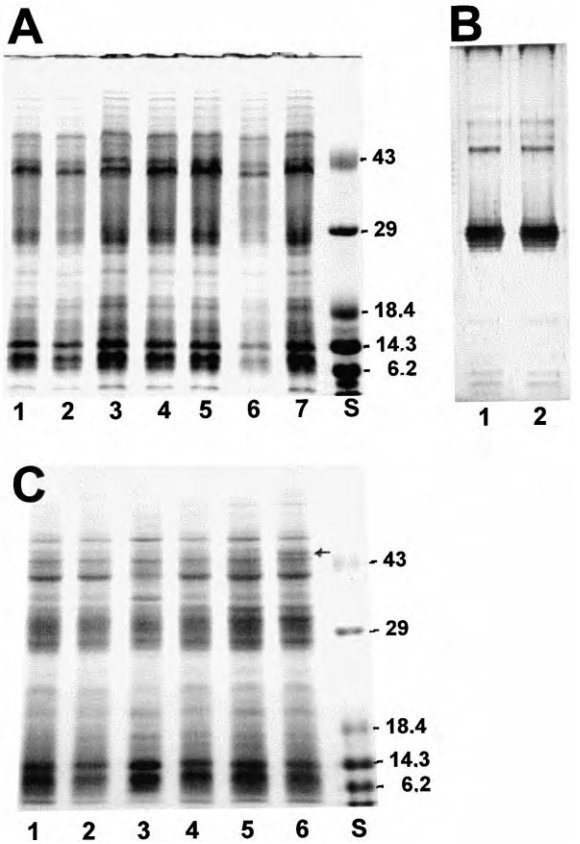

Fig. 1. A: Protein profiles of strains belonging to serogroup of CB 1809: 1, CPAC 390; 2, CPAC 392; 3, CPAC 393; 4, CPAC 394; 5, CPAC 402; 6, CPAC 404; 7, the parental CB 1809. B: Lipopolysaccharide profiles of strains 1, CPAC 390; 2, CB 1809. C: Protein profiles of strains belonging to serogroup 532C: 1, CPAC 346; 2, CPAC 351; 3, CPAC 357; 4, CPAC 360; 5, CPAC 361; 6, the putative parental 532C. The arrow on the right side indicates a band which differs among the putative parental and the variant strains. S indicates the protein standard with the size markers in kDa on the right side.

ized block design, with five replicates, the plants were collected at 45 DAE and the results were statistically analyzed by Tukey's test ($P \leq 0.05$).

3. Results

3.1. Strains selected for this study

3.1.1. Strains belonging to serogroup CB 1809

Strain CB 1809 was classified as very efficient in terms of nitrogen fixation [6], and was commercially recommended in 1977, but showed low competitiveness and was unable to nodulate an important commercial cultivar of the 1970s, IAC-2 [18]. Therefore the main objective within this serogroup was to obtain variant strains with a higher competitiveness and adapted to the soil conditions of the Cerrados region. The methodology employed to obtain the strains was based on the method of Peres et al. [20] and had been used before to obtain strain CPAC 7 [9,31]. Several individual colonies from a pure culture were tested in Leonard jars for competitiveness, with nodule occupancy evaluated by serology [23]. Promising strains were used in field experiments, in Cerrados soils which had not been previously inoculated and where the soybean control treatment showed zero nodulation. Bacteria were reisolated from soybean nodules of the inoculated treatments characterized by the best symbiotic performance (nodulation) and yield. As the strains were obtained under laboratory controlled conditions, CB 1809 is here called the parental genotype. Eight isolates from serogroup CB 1809 were selected and used in this study: CPAC 390, CPAC 392, CPAC 393, CPAC 394, CPAC 402, CPAC 403, CPAC 404 and CPAC 405. The strain grouping is shown in Table 2.

3.1.2. Strains belonging to serogroups 532C and SEMIA 5020

The methodology used to select strains belonging to these serogroups was also based on Peres et al. [20] and had been used before to obtain CPAC 15 [9,31]. First, areas of the Experimental Station of Embrapa-Cerrados that two decades before had shown zero nodulation and were at that time planted with soybean seeds inoculated with either 532C or SEMIA 5020 strains were identified. Soybean seeds were surface sterilized, the control without inoculation had zero nodulation and inoculants were prepared under laboratory conditions with pure cultures. For this study bacteria were reisolated from these areas, from field-grown soybean nodules showing a diameter of 2–3 mm and internal pink color. Nodules were grouped according to their serological reactions [23] using antisera of all strains which had been officially used in Brazilian commercial inoculants or in field trials performed at the Experimental Station. The strains were then tested individually for

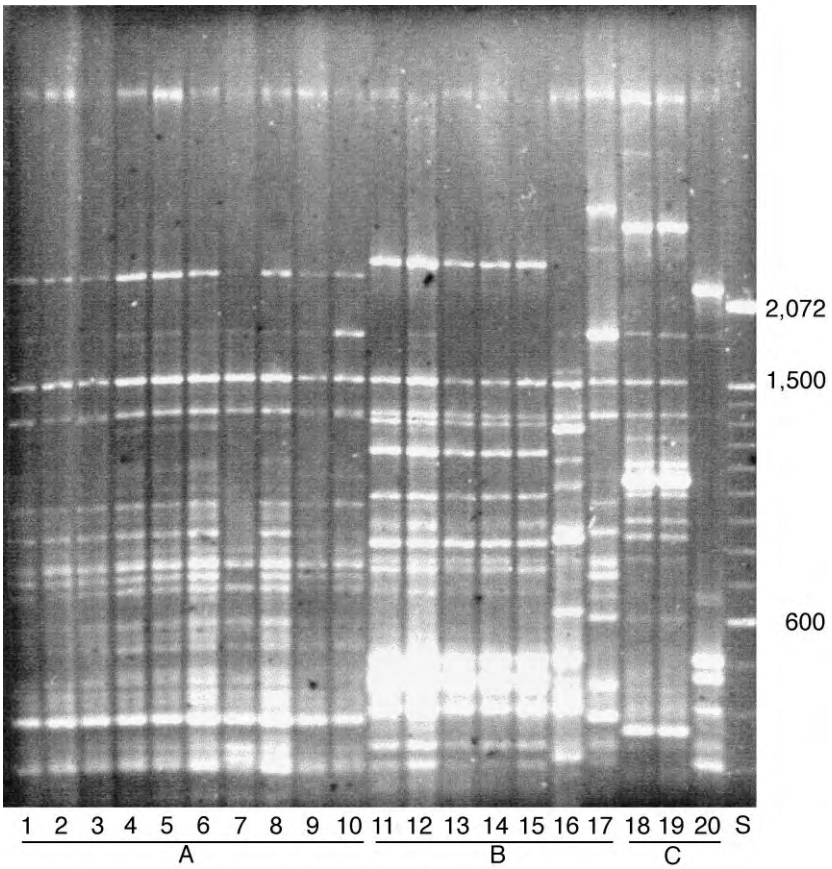

Fig. 2. ERIC-PCR fingerprint patterns obtained for the following strains. A: Serogroup CB 1809: lane 1, CPAC 390; 2, CPAC 392; 3, CPAC 393; 4, CPAC 394; 5, CPAC 402; 6, CPAC 403; 7, CPAC 404; 8, CPAC 405; 9, CPAC 7; 10, CB 1809. B: Serogroup 532C: lanes 11, CPAC 346; 12, CPAC 351; 13, CPAC 357; 14, CPAC 360; 15, CPAC 361; 16, CPAC 471; 17, 532C. C: Serogroup SEMIA 5020: lanes 18, CPAC 520; 19, CPAC 527; 20, SEMIA 5020 (from Embrapa-Cerrados). The last lane shows the DNA molecular mass standard (S) and the size markers are indicated in bp on the right side.

N_2 fixation capacity under greenhouse conditions. Strains 532C and SEMIA 5020 will be called here 'putative' parental strains, since the variant strains were reisolated two decades after inoculation with these strains and reacted exclusively against the antiserum of 532C or SEMIA 5020. Promising strains belonging to serogroups 532C and SEMIA 5020 were used in field experiments, also in areas free of soybean bradyrhizobia. Bacteria were reisolated from nodules of the treatments with the best symbiotic performance (nodulation) and yield. Six strains belonging to serogroup 532C (CPAC 346, CPAC 351, CPAC 357, CPAC 360, CPAC 361 and CPAC 471) and two belonging to serogroup SEMIA 5020 (CPAC 520 and CPAC 527) were selected and used in this study. The strain grouping is shown in Table 2.

The serogroups of CB 1809, 532C and SEMIA 5020 were confirmed by the serological analysis of agglutination with antibodies of whole cells, and with antibodies of bacterial cellular wall lipopolysaccharides, both visually and using ELISA.

3.2. *Morphological, physiological and genetic characterization of the strains*

The main difference in colony morphology found between parental strain CB 1809 and the variant strains belonging to this serogroup was that the variant strains produced more mucus. Protein and lip-

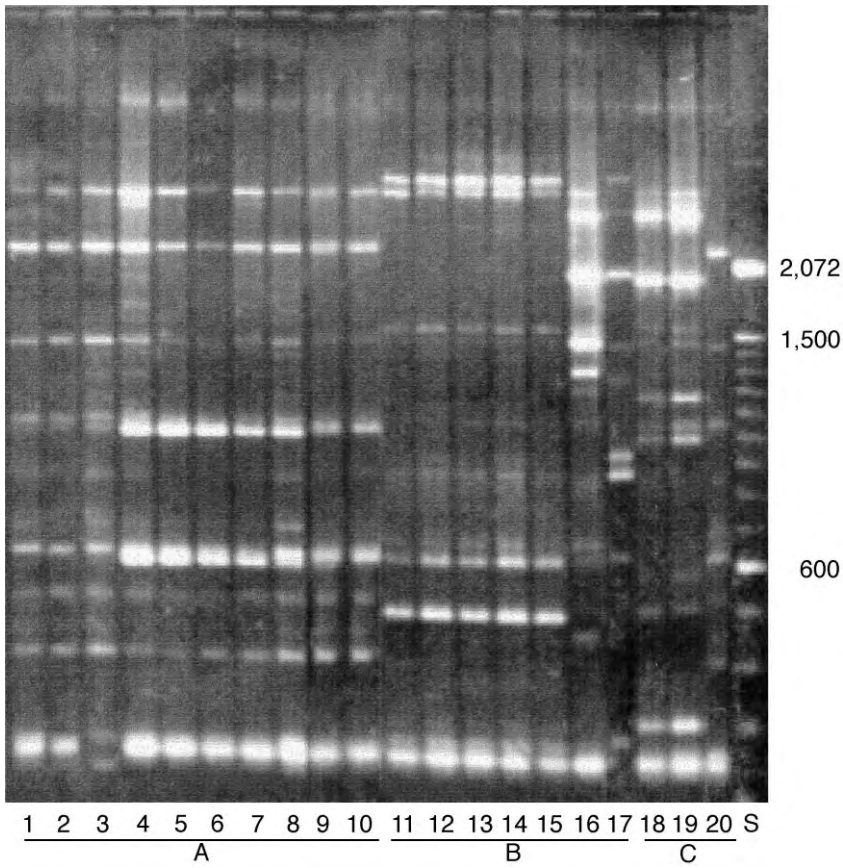

Fig. 3. REP-PCR fingerprint patterns obtained for soybean *Bradyrhizobium* strains. The order of the lanes is the same as in Fig. 2.

opolysaccharide profiles of variant strains belonging to this serogroup were similar to that of the parental CB 1809. The protein profiles of six of the eight variant strains used in this study are shown in Fig. 1A, and the lipopolysaccharide profiles of variant strain CPAC 390 and the parental CB 1809 in Fig. 1B.

Putative parental strains 532C and SEMIA 5020 also produced less mucus than the adapted variant strains belonging their respective serogroups. The protein profiles obtained with adapted strains belonging to serogroup 532C were similar to that found with the putative parental strain. Fig. 1C displays the results obtained with five of the six variant strains as well as the putative parental genotype. A slight difference was detected in all variant strains, which did not show a band above 43 kDa detected in the parental 532C (Fig. 1C) (protein profiles confirmed four times). The lipopolysaccharide profiles of variant strains and the putative parental 532C were identical (data not shown). Identical protein and lipopolysaccharide profiles were obtained for the putative parental strain SEMIA 5020 and the two adapted variant strains belonging to this serogroup (data not shown).

Using ERIC-PCR genomic fingerprintings, a very limited level of polymorphism, especially in the region between 600 and 1000 bp, was detected between CB 1809 (Fig. 2, lane 10) and the variant strains belonging to this serogroup obtained under laboratory conditions, and exposed to a short period of adaptation in soil (Fig. 2, lanes 1–9). Similar genomic fingerprinting profiles within this serogroup were also obtained by REP-PCR, with only a few polymorphisms being detected between the parental and the variant strains, such as a missing band in

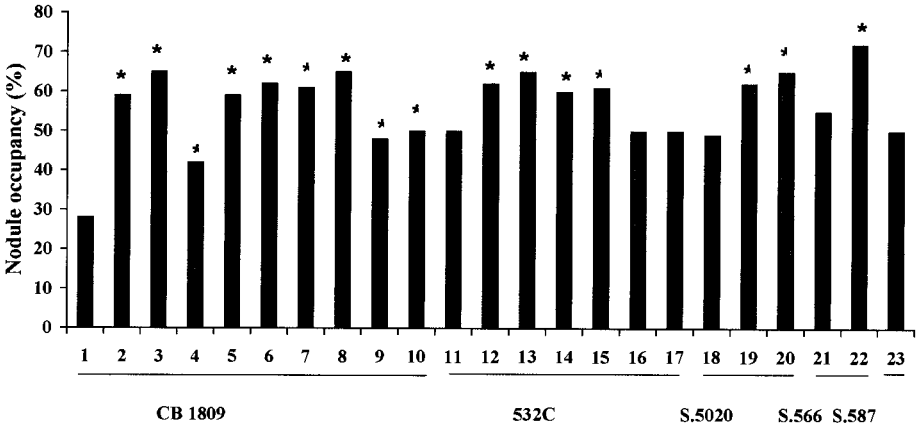

Fig. 4. Nodule occupancy (% of nodules occupied by each strain) by variant and parental strains when mixed in a ratio of 1:1 with strain 29w. Occupancy was evaluated by the serological analysis of 60 nodules, from five replicates, at 45 days after emergence. Strains are represented by the following numbers: Serogroup CB 1809: 1, parental CB 1809; 2, CPAC 7; 3, CPAC 390; 4, CPAC 392; 5, CPAC 393; 6, CPAC 394; 7, CPAC 402; 8; CPAC 403; 9, CPAC 404; 10, CPAC 405; Serogroup 532C: 11, putative parental 532C; 12, CPAC 346; 13, CPAC 351; 14, CPAC 357; 15, CPAC 360; 16, CPAC 361; 17, CPAC 471; Serogroup SEMIA 5020: 18, putative parental SEMIA 5020; 19, CPAC 520; 20, CPAC 527; Serogroup SEMIA 566: 21, parental SEMIA 566; 22, CPAC 15; Serogroup SEMIA 587: 23, SEMIA 587. Within the same serogroup, columns with * indicate that nodule occupancy by the variant strain was statistically different from the parental genotype (Tukey, $P \leq 0.05$).

strains CPAC 390, CPAC 394 and CPAC 402 in the region between 500 and 600 bp (Fig. 3, lanes 1, 4 and 5).

A different result was found with strains isolated after a long period of adaptation to the soil. Within serogroup 532C, the ERIC-PCR profiles of five of the six strains (Fig. 2, lanes 11–15) were similar, but the genomic fingerprinting of strain CPAC 471 (Fig. 2, lane 16) differed considerably from the other variants. Furthermore, all variants were quite distinct from the putative parental strain 532C (Fig. 2, lane 17). An even higher level of dissimilarity, in relation to the putative parental strain, was observed within serogroup SEMIA 5020, since the two variant strains belonging to this serogroup showed similar profiles (Fig. 2, lanes 18 and 19), while a completely different profile was obtained with the putative parental genotype (Fig. 2, lane 20). The profiles obtained with REP primers confirmed these differences reported for serogroups 532C and SEMIA 5020 (Fig. 3). To rule out the possibility of mislabeling parental strains 532C and SEMIA 5020, they were recovered from the Brazilian germ plasm bank at FEPAGRO (RS), where they were originally deposited in the 1960s. They were also recovered from the rhizobium bank at Embrapa-Cerrados, which received these strains 20 years ago; these represent the strain source for the inoculant produced at that time. Strains 532C and SEMIA 5020 from both laboratories were not identical, but showed a high level of similarity with both ERIC and REP primers (data not shown). Antibodies of whole cells and antibodies of bacterial cellular wall lipopolysaccharides of 532C and SEMIA 5020 from both laboratories were prepared and the adapted strains reacted positively with both of them and with none of the 20 other antibodies of strains which had been used as commercial inoculants in Brazil. When the bradyrhizobium collections from Embrapa-Cerrados, Embrapa-Soja and FEPAGRO were analyzed, including strains which were or were not used in commercial inoculants, none of the strains showed similar ERIC and REP-PCR profiles to 532C and SEMIA 5020.

3.3. Symbiotic performance

Regarding the N_2 fixation capacity, evaluated under greenhouse controlled conditions, the best symbiotic performance in terms of nodule mass, shoot dry weight and N accumulation in shoots was achieved by inoculation with variant strains belonging to serogroup CB 1809 (Table 2). The highest

rate of N_2 fixation resulted from inoculation with strain CPAC 390, which allowed an increase of 58% of N accumulated in soybean shoots in relation to the plants inoculated with the parental CB 1809; these increases were 58%, 130% and 108% in relation to three other strains used today in commercial inoculants, CPAC 15, 29w and SEMIA 587, respectively. The good symbiotic performance of CPAC 7, which belongs to this serogroup and is also recommended in Brazilian inoculants, was confirmed in this experiment. In relation to the other strains obtained in this work, only CPAC 346, belonging to serogroup 532C, showed a good symbiotic performance (Table 2).

The great majority of variant strains obtained in this study showed higher nodule occupancy than their respective parental genotypes (Fig. 4). Within the serogroup of CB 1809, increases of up to 132% (CPAC 390 and CPAC 403) in nodule occupancy were obtained in relation to the parental strain. Increases in nodule occupancy were also verified even when variant strains were obtained from more competitive parental genotypes, such as 532C and SEMIA 5020 (Fig. 4).

4. Discussion

Brazilian soils are usually very poor in N and are originally free of soybean bradyrhizobia [8,13,15,16,29,30]. Therefore, with the introduction of the soybean crop to the country, inoculants containing foreign strains were used. However, with the crop's expansion a selection program for strains adapted to Brazilian soils was started. Efficient and competitive strains were obtained over the years, allowing an adequate supply of N in response to the increasing demands of more productive cultivars. The country today has specific legislation for quality control of commercial inoculants, which can only contain strains recommended by a committee of microbiologists. Four approved strains exist for soybean, SEMIA 587, 29w, CPAC 7 and CPAC 15, all obtained by reisolation from previously inoculated soils after a long or short period of adaptation [8,12,13,28,32].

The selection program for soybean bradyrhizobia strains, performed at Embrapa, is today based on the identification of variant strains adapted to the soils showing a higher N_2 fixation capacity and higher competitiveness than the parental genotypes. Two strains obtained in this program, CPAC 7 and CPAC 15, are today recommended for commercial inoculants, since they have proved to increase nodulation, N_2 fixation and yield significantly [12,13,17,28,31,32]. The variant strains, CPAC 7 and CPAC 15, differ from the parental genotypes in other parameters, such as mucus production, synthesis of IAA, Hai phenotype, Nod factor profile and DNA fingerprints with arbitrary or specific primers [4,10,11,17]. Other variant adapted strains, belonging to serogroup SEMIA 566, were also superior to the parental genotype with respect to N_2 fixation rates, competitiveness and agronomic traits [11]. The efficiency of the selection program was confirmed in this paper, since some of the variant strains have shown an outstanding performance in terms of N_2 fixation rates and especially in relation to competitiveness.

In the USA, where soybean is also an important crop, there are frequent reports of a lack of response to inoculation in soils with few cells of established bradyrhizobia [22,27]. Contrarily, in Brazil field responses to inoculation can be obtained even in soils with up to 10^5 cells of soybean bradyrhizobia g^{-1} of soil [9,10,12,13,17,28,32]. Possible explanations for this could reside in a higher competitiveness of bradyrhizobium strains established in North American soils, or in the necessity of adding bacteria in a proper physiological state under the environmentally stressful conditions present in Brazil. However, responses to inoculation and also a higher contribution of the N_2 fixation process reported in Brazil may result from an intense search for more efficient and competitive strains.

In this study, when the variant strains belonging to serogroups CB 1809 were compared with the parental genotype, and those belonging to serogroups 532C and SEMIA 5020 were compared with their respective putative parental genotypes, differences were detected in colony morphology and on ERIC and REP-PCR profiles. For serogroup CB 1809, with variant strains selected under laboratory conditions followed by a short period of adaptation to the soil, the magnitude of the differences in ERIC and REP-PCR profiles was similar to that observed in

the USA for serogroup USDA 123 [14]. However, quite distinct DNA profiles were obtained between variant and putative parental strains within serogroups 532C and SEMIA 5020, although the soybean seeds used 20 years ago were surface sterilized, the control treatment without inoculation at that time had zero nodulation and inoculants were prepared under laboratory conditions with pure cultures of strains 532C and SEMIA 5020.

Until now, the identification of strains by the Brazilian laboratories has been based exclusively on the serological properties and well defined groups with different serological reactions have been established. It is possible that foreign inoculants used in Brazil during the first years of the crop's introduction carried contaminant strains, but for years the serological analysis of nodules collected in the field showed that the known strains occupied most of the nodules [8,10–13,16–20,29–32]. To be able to establish in the soil in such high proportions, these contaminant strains should be extremely competitive and dissemination from other producing areas would have occurred.

The DNA fingerprinting analysis has raised an intriguing question in relation to strains belonging to serogroups 532C and SEMIA 5020. The putative parental genotypes were genetically very distant from the adapted strains. However, the variant strains reacted exclusively with the serogroup corresponding to their respective parental genotypes. Three hypotheses could be proposed to explain the genetic diversity observed in this study. First, contaminant strains, without records in the Brazilian germ plasm bank and belonging to the same serogroups, were carried in the first inoculants and disseminated to the experimental area, miles away from the first grain-producing sites. The second hypothesis is that the stressful conditions of the Cerrados, characterized by environmentally inhospitable conditions, especially subjection to long periods of water stress and high temperatures ($>40°C$), as well as soil fertility problems, such as low pH (<5.0) and aluminum toxicity, caused considerable genomic rearrangements in the parental genotypes, resulting in an enormous genetic variation in the variant strains. The third hypothesis would be raised in light of the results obtained in New Zealand soils [25,26] with *Lotus corniculatus* and *Mesorhizobium loti*, showing that non-symbiotic rhizobia persist in soils in the absence of host legume, acquiring the symbiotic genes from an inoculant strain upon the introduction of the host legume. Therefore, diverse strains arose by transfer of chromosomal symbiotic genes from the inoculant *M. loti* strain ICMP3153 [25,26]. The transfer of the chromosomal symbiotic genes, later termed symbiosis island, to non-symbiotic mesorhizobia was confirmed under laboratory conditions [24]. Consequently, the variant strains belonging to serogroups 532C and SEMIA 5020 could well be resident bacteria (saprophytic), unrelated to the putative parental strains which became good nodulators and N_2 fixers, possibly via transfer of nodulation, nitrogen fixation, and other competition-related genes from the inoculum strain. The following step of this study will be to investigate this third hypothesis, using the approach of Sullivan et al. [26].

The results obtained here indicate the necessity of obtaining DNA fingerprints to classify and identify rhizobium strains in the germ plasm banks worldwide; these fingerprints should be related to the characteristics used before to classify the strains, usually serology. It is possible that other laboratories will also find similar differences between putative parental genotypes and their serologically related variant strains. Studies will continue to clarify the nature of the genetic diversity found for strains reisolated from soils after a long period. When variant strains were obtained under laboratory conditions and subjected to a short-term exposure to the soils, genetic variability was lower. However, in both cases, within the variability detected it was possible to select variant strains with higher N_2 fixation capacity and competitiveness, contributing to a better plant N nutrition status.

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