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Morphological, Physiological and Genetic Characterization of Two New *Bradyrhizobium* Strains Recently Recommended as Brazilian Commercial Inoculants for Soybean

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Abstract

Large scale soybean inoculation in Brazil started in 1960 and, since then, several strains from other countries were introduced into the Brazilian soils. Since 1992, two new strains have been used as Brazilian inoculants, CPAC 7 and CPAC 15, which are natural variants of CB 1809 (received from Australia in 1966) and SEMIA 566 (isolated from a North American inoculant in 1966), respectively, and were obtained in a selection program for higher efficiency of N_2 fixation and competitiveness. Analyses of morphological and physiological parameters, particularly the ones that differentiate the species *B. japonicum* and *B. elkanii*, indicated that CB 1809 and the variant CPAC 7 show the characteristics of *B. japonicum*, while SEMIA 566 and CPAC 15 fit into the species *B. elkanii*. Differences between parental and variant strains included also several profiles in RAPD analysis. Increases in nodulation, nodule occupancy and yield due to inoculation were observed even in a soil containing 2.2×10^5 cells/g of soil. The

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evaluation of nodule occupancy is essential to confirm the benefits of inoculation and, since the newly-recommended strains belong to serogroups already established in most Brazilian soils, the RAPD technique seems to be a suitable technique to differentiate the parental from variant strains.

Keywords: *Bradyrhizobium japonicum, Bradyrhizobium elkanii, Glycine max,* inoculation, nitrogen fixation, nodulation, nodule occupancy, RAPD, serology, soybean

1. Introduction

Soybean [*Glycine max* (L.) Merrill] is the most important leguminous crop in Brazil, being responsible for 16.9% of the total world production. In the 1993/1994 growing season, 11.35 million hectares were planted with soybean in Brazil, with an average yield of 2,156 kg/ha, exporting approximately 120 kg of N/ha by the grains. Since the N content of Brazilian soils is usually low, ranging from 0.05 to 0.30%, the N necessary for plant growth is almost completely supplied by *Bradyrhizobium* bacteria through the symbiotic nitrogen fixation process (Cattelan and Hungria, 1994; Hungria et al., 1994).

Searching for strains with higher efficiency of N_2 fixation and competitiveness is the goal of most laboratories that work with the soybean symbiosis. In Brazil, two new strains with these characteristics, CPAC 7 and CPAC 15, were recently obtained at EMBRAPA-CPAC (Vargas et al., 1992a,b). The strain CPAC 7 was obtained under laboratory conditions from a subculture of CB 1809 (received from CSIRO, Australia, in 1966), using the method proposed by Peres et al. (1984), that selects individual nodules with higher rates of N_2 fixation (C_2H_2 reduction). In contrast to the parental CB 1809, CPAC 7 is also able to nodulate the cultivar IAC-2 (Vargas et al., 1992a,b). Strain CPAC 15 is a natural variant of SEMIA 566 (isolated in 1966 from a North American inoculant), and it was isolated from a Brazilian savanna soil ("Cerrado") several years after the last inoculation. The strain was also selected for higher efficiency of N_2 fixation using the technique of Peres et al. (1984), and higher competitiveness in pots containing an elevated number of naturalized bradyrhizobia cells. In several trials performed over seven years in Cerrado soils inoculated for the first time, CPAC 7 and CPAC 15 were able to increase yield by up to 750 kg/ha, in comparison with the other commercially recommended strains (Vargas et al., 1992a,b).

Although these two new strains have been officially recommended for utilization as Brazilian inoculants since 1992, a few basic studies were performed to compare morphological, physiological and genetic parameters among parental and natural variant strains. Consequently, in this work we

have investigated how these strains fit into the subdivision of *Bradyrhizobium japonicum* or *B. elkanii* (Kuykendall et al., 1992), considering several morphological and physiological parameters. We have also investigated soybean responses to inoculation in soils with established *Bradyrhizobium* populations because, although poor responses to the addition of bacteria are reported in soils with established populations (Thies et al., 1991), there are reports, in Brazil, of responses to inoculation even after several years of inoculation (Hungria et al., 1994). Finally, using the RAPD technique, we have evaluated genetic modifications that occurred in the genome due to the adaptation to Cerrado soils or the selection program and the suitability of this technique to differentiate strains that belong to the same serogroup.

2. Material and Methods

Bacteria

Bradyrhizobium strains representative of the species: *B. japonicum* strain USDA 110 and *B. elkanii* USDA 76 were considered as representatives based on the work of Hollis et al. (1981), Kuykendall et al. (1988, 1992) and Minamisawa (1989, 1990). The strains were received from Dr. Peter van Berkum (Soybean and Alfalfa Research Laboratory, USDA, Beltsville, MD 20705, USA).

"Brazilian" Bradyrhizobium strains: SEMIA 566 was isolated by a Brazilian institution (IPAGRO) in the State of Rio Grande do Sul, Brazil, in 1966, from a North American inoculant received from Nitragin Co., USA. Strain CB 1809 was sent from Australia to Brazil in 1966, where it is also denominated SEMIA 586. CPAC 15 (= SEMIA 5079) is a strain isolated from a savanna soil ("Cerrado") in the Central Region of Brazil, at EMBRAPA-CPAC, several years after the last inoculation with SEMIA 566. Cerrado is a region free of *B. japonicum* and/or *B. elkanii*, confirmed by the total absence of nodules in non-inoculated field plots (Vargas et al., 1982). The area from which CPAC 15 was isolated has been inoculated exclusively with SEMIA 566. CPAC 15 seems to be a natural variant of SEMIA 566, since they share the same serological properties. CPAC 15 was adapted to the Cerrado conditions and is more efficient and competitive than the parental strain (Vargas et al., 1992a,b; Nishi and Hungria, 1993). CPAC 7 (= SEMIA 5080) is a strain selected under laboratory conditions from a subculture of CB 1809, showing higher efficiency of N_2 fixation, according to the method of Peres et al. (1984), which evaluates N accumulated in plants inoculated with individual colonies. In Cerrado soils,

CPAC 7 showed higher competitiveness and increased soybean yield (Vargas et al., 1992a,b).

Plants

The following soybean [*Glycine max* (L.) Merrill] cultivars were used and, between parenthesis, their genealogy: BR-16 (D69-B10-M58 X Davis); IAC-2 (La 41-1219 X Yelnanda); Lee (S-10 X CNS); Hill [(Dunfield X Aberlandt) X (D632-15) X (D-49-2525) X (S-110 X CNS)]; Clark [Lincoln (2) X Richland]. For a toxin test alfalfa seeds (*Medicago sativa* L.) of cultivar Crioula were utilized. The seeds and information about the cultivars were obtained from the germoplasm bank of EMBRAPA-CNPSo.

Analyses: Morphological and physiological parameters evaluated for species subdivision

Parameters evaluated in vitro: Colony morphology – transparency, elevation, borders, diameter, color, pH, form and mucoidy in YMA medium (Vincent 1970) – were verified after five and eight days of growth at 28°C.

Intrinsic resistance to high levels of seven antibiotics (μg/ml): tetracycline, 100; chloramphenicol and carbenicillin, 500; erythromycin, 250; nalidixic acid, 50; rifampicin, 500; streptomycin, 100 (concentrations proposed by Kuykendall et al., 1988) was tested. Stock solutions of streptomycin, carbenicillin and erythromycin were prepared in distilled water, nalidixic acid in 0.35 M NaOH, tetracycline in 70% ethanol and chloramphenicol and rifampicin in methanol. Antibiotics were sterilized by filtration (Millipore, 0.22 μm) and added to sterilized YM medium (Vincent, 1970). Strains were considered resistant to the antibiotic when their growth in tubes with liquid YM medium with antibiotic (evaluated by O.D. at 600 nm), after seven days at 28°C, was at least 50% of that in the tubes with YM without the antimicrobial compound.

Indole acetic acid (IAA) synthesis in Tris-YMRT medium enriched with filter-sterilized 0.3 mM tryptophan (Owens and Wright, 1964; Minamisawa and Fukai, 1991) was estimated in bacterial cultures grown for seven days at 30°C, in the dark, and values were obtained by the colorimetric procedure of Gordon and Weber (1951), modified by Minamisawa et al. (1992).

Hup phenotype was verified with bacteria grown in tubes containing 7 ml of Maier et al. (1978) liquid medium, for seven days, followed by replacement of 0.5% of the atmosphere by H_2 (99.9% purity), incubation for 18 h and analysis of the H_2 in an analyzer constructed by the instrumentation center of EMBRAPA in São Carlos, São Paulo, Brazil. USDA 110 and USDA 76 were included as positive and negative controls, respectively.

All parameters *in vitro* were evaluated in three replicates.

Parameters evaluated in vivo: Toxicity produced by rhizobial metabolites in alfalfa (cv. Crioula) was evaluated according to the methodology of Minamisawa and Fukai (1991). First bacteria were grown in liquid YM medium for seven days at 28°C and the cells were then centrifuged (9,000 g for 20 min). The pH of the supernatant was adjusted to 7.5, agar added to 1.5% (w/v) and the autoclaved medium distributed in glass tubes. Control treatment consisted of tubes containing 10 mM Tris, 1.5% of agar and pH 7.5. Surface-sterilized (Vincent, 1970) alfalfa seeds were placed on the surface of the medium and allowed to germinate and grow for eight days in a growth chamber under continuous light at 30°C. The strain was classified as a rhizobitoxine producer if the root growth of these plants was inhibited by more than 30% in comparison with the control.

Chlorosis symptoms due to rhizobial toxins in leaves of soybean cv. BR-16 and in the sensitive cultivar Lee (Johnson and Means, 1960) were evaluated in plants grown for four weeks in Leonard jars (Vincent, 1970), containing N-free nutrient solution (Somasegaran and Hoben, 1985), under greenhouse conditions. After 28 days of growth the leaves were examined for toxic symptoms (chlorosis) of rhizobitoxine, receiving the scores 0 (without chlorosis), 1 (mild symptoms, similar to those caused by strain USDA 31) and 2 (strong symptoms, comparable to the chlorosis caused by strain USDA 76). In both soybean and alfalfa these symptoms were claimed to be due to rhizobitoxin (La Favre et al., 1988; Minamisawa and Fukai, 1991) and, although rhizobia can produce other toxins that stunt growth or cause leaf chlorosis, we will call the symptoms here as rhizobitoxine effects.

For the evaluation of nodulation restriction by allele Rj_4, present in soybean cv. Hill (Vest et al., 1972), plants were grown under the same conditions described in the experiments to verify rhizobitoxine effects.

All experiments *in vivo* were performed in a completely randomized design with three replicates.

Response to inoculation in soils with high population of Bradyrhizobium: The experiment was performed in an Oxisol with the following characteristics:

Depth (cm)	pH in $CaCl_2$	N	Al	K	Ca	Mg	H+Al	Al	C	P
		%	meq/100 g soil					%		mg/g soil
0–20	5.20	0.17	0	0.39	5.39	1.87	4.16	0	1.73	12.70
20–40	4.99	0.12	0	0.21	3.76	1.54	4.26	0	1.30	4.93

The experimental plot was 3.0 × 2.0 m, with 0.5 m between lines, and plots were separated by 2.0 m and small terraces. Five days before sowing, plots received 300 kg/ha of N-P-K (0-28-20) and 40 kg/ha of micronutrients (containing, in %: Zn, 9.0; B, 1.8; Cu, 0.8; Fe, 3.0; Mn, 2.0; Mo, 0.10). The naturalized bradyrhizobia population, evaluated by the most probable number (MPN) counting technique (Vincent, 1970) in soybean plants, was estimated to be 2.2×10^5 cells/g of soil. Inoculants were prepared to a density of 10^9 cells/ml in semi-solid YM, adding 100 ml of inoculant/kg of seeds. Treatments consisted of strains SEMIA 566, CPAC 15, CB 1809 and CPAC 7 and the controls without inoculation with or without N fertilizer (400 kg of N/ha as urea, split into ten weekly applications). The experiment was performed in a completely randomized block design, with four replicates. At 45 days after planting, 15 plants of each treatment were harvested for nodule number and nodule mass evaluation. Serological analysis of agglutination with thermostable antibodies, of 40 nodules per treatment, was performed according to Somasegaran and Hoben (1985). At the final harvest, yield and N content of grains were evaluated, and values were corrected for 13% moisture after determination of the level of humidity in a grain moisture tester (model Vurroughf 700). N content was determined by the indophenol blue colorimetric method (Feije and Anger, 1972) and oil content by continuous extraction with hexane for six hours in a Soxhlet (Pregnolatto and Pregnolatto, 1985). The results were statistically analyzed by Duncan's test at 0.05% of probability.

Genetic Analysis by RAPD (random amplified polymorphic DNA): Strains CB 1809, CPAC 7, SEMIA 566 and CPAC 15 were grown in YM medium, for five days, centrifuged at 2,800 g for 15 min, washed three times with NaCl (0.85%), resuspended in 0.85% NaCl to 10^6 cells/ml and frozen. Then, 0.5 ml of each strain was sonicated for 4 min, on ice, boiled for 30 s followed immediately on ice, adding 100 µl of TES buffer (10 mM Tris-HCl, 250 mM EDTA, 2.5% SDS, pH 8.0) and incubated for 15 min at 37°C. Samples were then brought to a final concentration of 250 mM NaCl and 300 mM sodium acetate, and left for 15 min at room temperature. Later, samples were centrifuged at 2,800 g for five minutes and the supernatant was mixed with two volumes of cold absolute ethanol, and left overnight at –20°C. Samples were centrifuged at 2,800 g for five minutes, the precipitate dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and the DNA concentration evaluated on a spectrophotometer at 260 nm. DNA amplification was obtained by the RAPD technique (Williams et al., 1990), with 35 short primers. Fifteen primers were synthesized by Dr. Spartaco Astolfi Filho (Laboratório de Biologia Molecular, Universidade de Brasília, Brasília, DF, Brazil): 62 – 5' GGGTAACGCC 3'; 63 – 5' CAGCACCCAC 3'; 64 – 5' TGCCGAGCTG 3'; 130 – 5' CGCGGCCA 3'; 131 – 5' CCTTGACGCA 3'; 132 – 5' CAGGCCCTTC 3'; 133 – 5' TGGTCACTGA 3'; 134 – 5' ACATGCCGTG 3'; 135 – 5'

GATAGATAGATAGATA 3'; 136 – 5' GGAAGTCGCC 3'; 137 – 5' GACAGACAGACAGACA 3'; 138 – 5' AATGGCGCAG 3'; 139 – 5' AGAGGGCACA 3'; 146 – 5' AGGTCACTGA 3'; 147 – 5' GGGGTTGACC 3'.

The other 20 primers used were from kit-S of Operon (Operon Technologies, 1000 Atlantic Ave, Alameda, CA 94501, USA). The amplification reactions were realized by the PCR technique (polymerase chain reaction, Saiki et al., 1988). The final volume of the reaction was 25 µl, containing (µl): stock buffer 10× (500 mM KCl and 100 mM Tris, pH 8.4, at room temperature), 2.5; bovine serum albumin (BSA, Sigma HP type 4), 8 ng/ml, 1; dATP, dCTP, dTTP, dGTP (1.5 mM), 5; DNA (8 ng/µl), 1; primer (30 ng/µl), 1; Taq DNA polymerase (either Amplitaq DNA polymerase, Stoffel fragment, Perkin Elmer Cetus or DNA polymerase synthesized by the Biotechnology Center, CENBIOT, located at the State of Rio Grande do Sul, Brazil) (1.5 U/µl), 1; deionized sterile water, 13.5. Two drops of mineral oil were added to each vial and the samples were submitted to 45 cycles of amplification, in a thermocycler Perkin Elmer Techen MW2. Each cycle consisted of one minute at 94°C, followed by one minute at 40°C and two minutes at 72°C. The amplified fragments were separated by electrophoresis in 1.5% agarose gel and photographed after staining with ethidium bromide (0.5 µg/ml). Cluster analysis was realized with the NTSYS-PC (Numerical Taxonomic and Multivariate Analysis System, version 1.70, Exeter Software, New York) program, using the UPGMA (Unweighted Pair Group Method with Arithmetic averages method) with the Simple Matching (SM) and the Jaccard (J) coefficient. Analysis was also done with the Neighbor-joining (NJ) method.

3. Results

Morphological and physiological parameters evaluated for species subdivision

In relation to colony morphology, all strains were opaque, convex (elevated), with smooth borders and produced alkali in the medium after five to eight days of incubation. Diameter, color and form did not seem to be related with grouping of the bacteria. However, differences were verified in mucus production, which was higher with the two new "Brazilian" strains, CPAC 7 and CPAC 15 (Table 1).

B. japonicum strain USDA 110 did not show antibiotic resistance to any of the antibiotics at the tested concentrations, while *B. elkanii* USDA 76 was resistant to all of them. In relation to the "Brazilian" strains, CB 1809 and CPAC 7 were resistant to the concentrations of tetracycline, nalidix acid, rifampicin and streptomycin tested, while SEMIA 566 and CPAC 15 were

resistant to all antibiotics except for chloramphenicol, showing a pattern closer to the *B. elkanii* strain (Table 2).

Table 1. Morphological characterization of representative strains of *Bradyrhizobium* and the "Brazilian" strains CB 1809, CPAC 7, SEMIA 566 and CPAC 15, that nodulate soybean. Parameters evaluated in YMA medium (Vincent, 1970), eight days after growth at 28°C.

Strains	Diameter (mm)	Color	Form	Mucus
Representative strains				
B. japonicum USDA 110	1.0	cream	punctiform	little
B. elkanii USDA 76	1.5–2.5	white	punctiform	little
"Brazilian" strains				
CB 1809	0.5	white	circular	intermediate
CPAC 7	0.5–2.0	white	circular	much
SEMIA 566	≤0.5	white	punctiform	little
CPAC 15	0.5–1.0	white	circular	much

Table 2. Antibiotic resistance of representative strains of species *B. japonicum* and *B. elkanii* and the "Brazilian" strains CB 1809, CPAC 7, SEMIA 566 and CPAC 15, that nodulate soybean. The antibiotics tested were (μg/ml): Tetracycline (100), chloramphenicol (500), carbenicillin (500), erythromycin (250), nalidixic acid (50), rifampicin (500), streptomycin (100).

Strains	Antibiotics						
	Tet	Chl	Car	Ery	Nal	Rif	Str
Reference strains							
B. japonicum USDA 110	–*	–	–	–	–	–	–
B. elkanii USDA 76	+	+	+	+	+	+	+
"Brazilian" strains							
CB 1809	+	–	–	–	+	+	–
CPAC 7	+	–	–	–	+	+	–
SEMIA 566	+	–	+	+	+	+	+
CPAC 15	+	–	+	+	+	+	+

*Positive indicates that after seven days at 28°C growth in liquid YM medium with antibiotics was at least 50%-of that in tubes without the antimicrobial compound.

For the other physiological parameters evaluated *in vitro* and *in vivo*, such as IAA synthesis, Hup phenotype, toxicity by rhizobitoxine and nodulation of a cultivar with the Rj_4 allele, the strains CB 1809 and CPAC 7 behaved similar to the representative strain of *B. japonicum*, while the two other strains, SEMIA 566 and CPAC 15, expressed greater similarity with *B. elkanii* strain USDA 76 (Table 3).

Table 3. Characterization *in vivo* and *in vitro* of representative strains of species *B. japonicum* and *B. elkanii* and the "Brazilian" strains that nodulate soybean, CB 1809, CPAC 7, SEMIA 566 and CPAC 15. Parameters evaluated were indole acetic acid synthesis in tryptophan-enriched medium after seven days of growth (IAA); Hup phenotype (Hup); toxicity caused by rhizobitoxine (RT) and evaluated by root elongation of alfalfa, cv. Crioula, or by chlorosis in leaves of soybean cv. Lee and cv. BR-16; and nodulation of soybean cv. Hill, which contains the allele Rj_4. Results were confirmed in three replicates.

Strains	Parameters					
			RT			Rj_4
	IAA (μM)	Hup	Alfalfa Crioula	Soybean Lee	Soybean BR-16	Hill
Reference strains						
B. japonicum USDA 110	6.85	+*	−**	−***	−***	+
B. elkanii USDA 76	44.31	−	+	+	+	−
"Brazilian" strains						
CB 1809	17.16	+	−	−	−	+
CPAC 7	13.12	+	−	−	−	+
SEMIA 566	24.66	−	+	+	+	−
CPAC 15	31.46	−	+	+	+	−

*Hup^+ strains oxidized 1.60 nmoles H_2/h/tube, while Hup^- strains did not show oxidation of H_2. **(+) if the toxin inhibited root growth by more than 30% in relation to the control. ***(+) if the strain produced mild symptoms, similar to those caused by USDA 31, or strong symptoms, comparable to the chlorosis caused by strain USDA 76.

Response to inoculation in soils with high population of Bradyrhizobium

The naturalized population in the Oxisol, estimated at 2.2×10^5 cells/g of soil, was very high, but the two serogroups studied were distributed, in the control treatment without inoculation, in similar percentages, of 20.6% for

serogroup CB 1809 and of 21.2% for serogroup SEMIA 566 (Table 4). All inoculated strains were able to establish in the rhizosphere, allowing increases in nodule number, nodule mass and nodule occupancy, evaluated at 45 days after emergence (Table 4). On average, strains CB 1809, CPAC 7, SEMIA 566 and CPAC 15 increased nodule occupancy by more than 100%, occupying in average 50% of the nodules in the inoculated plots. Although statistical differences were not found in yield due to inoculation, higher values were achieved by the inoculation with CPAC 7, that produced 128 kg/ha more than the non-inoculated control and 156 kg/ha more than the non-inoculated control receiving N-fertilizer. In this field experiment, CPAC 7 and CPAC 15 did not show a better performance than the parental strains in relation to total N or oil content in the grains (Table 4).

Table 4. Effects of inoculation of soybean, cv. BR-16, with strains CB 1809, CPAC 7, SEMIA 566 and CPAC 15 on an Oxisol containing 2.2×10^5 cells/g of soil, on nodulation parameters and occupancy by inoculated strains, at 45 days after emergence. At the final harvest, grain yield, total N and oil content were evaluated.

Treatment	Nodulation*				Grains		
	Number (n/pl)	Mass (mg/pl)	Occupancy (%) 1809	566	Yield kg/ha)	Total N (kg N/ha)	Oil (kg oil/ha)
CB 1809	15.4 b**	41.2 b	51.2 a	–***	4043 a	272 a	782 a
CPAC 7	20.8 a	63.5 a	43.8 a	–	4137 a	263 ab	766 ab
SEMIA 566	18.1 ab	45.8 b	–	53.8 a	4109 a	275 a	839 a
CPAC 15	15.4 b	43.8 b	–	53.8 a	3892 a	245 b	736 b
without inoc.	9.5 c	23.9 c	20.6 b	21.2b	4009 a	266 ab	795 ab
without inoc. + N-fertiliz.****	15.6 b	42.7 b	20.3 b	21.0 b	3981 a	251 bc	795 ab
CV (%)	25.1	32.7	18.0		9.9	5.8	6.7

*Evaluated at 45 days after emergence. **Means of four replicates and values followed by the same letter did not show statistical difference ($P \leq 0.05$, Duncan's test). ***Not determined. ****400 kg of N/ha as urea, split ten times.

Genetic Analysis by RAPD

Twenty one of the 35 primers tested complimented the DNA of the strains tested: 62, 63, 64, 131, 132, 134, 136, 138, 139, 147, and OPS-1, 3, 5, 7, 9, 11, 16, 17, 18, 19 and 20. The amplification with these primers resulted in 156 bands, 80 of

which differed between the parental SEMIA 566 and its natural adapted isolate CPAC 15, and 53 differentiated the parental CB 1809 from its natural variant CPAC 7 and Fig. 1 displays the results with six of these primers. When the SM coefficient was utilized with the pair of strains SEMIA 566 and CPAC 15, the level of similarity obtained was 0.449, while the use of Jaccard coefficient showed a level of 0.302. For the pair of strains CB 1809 and SEMIA 5080, the values were, respectively, 0.629 and 0.393. With the Neighbor-joining analysis, both pairs were distant at a level of 2.0. The qualitative dendrogram obtained for the two pairs of strains with the 156 bands and the Jaccard coefficient are shown in Fig. 2.

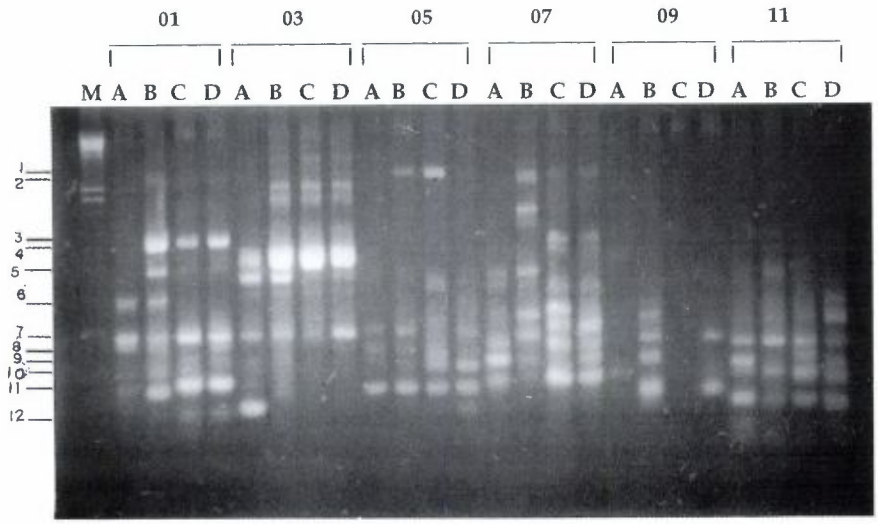

Figure 1. Comparison of fragments of DNA of strains (A) CB 1809 and (B) SEMIA 566 and their respective variants (C) CPAC 7 and (D) CPAC 15, with primers OPS 1, 3, 5, 7, 9 and 11. M is Lambda-Hind III, MW = 23.13, 9.42, 4.36, 2.32, 2.03, 0.56, and 0.12 kbp.

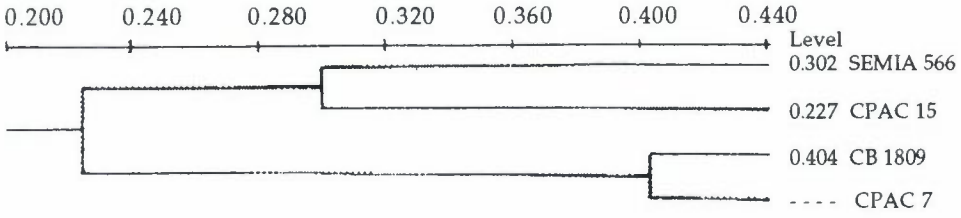

Figure 2. Dendrogram showing the pair of strains SEMIA 566 (parental strain) X CPAC 15 (natural variant) and CB 1809 (parental strain) X CPAC 7 (natural variant) considering 156 bands obtained from the RAPD profiles with 21 primers that amplified the DNA.

4. Discussion

Morphological and physiological parameters evaluated in the pair of strains CB 1809 X CPAC 7 and SEMIA 566 X CPAC 15 show a similarity of the first pair to *B. japonicum*, and of the second pair to *B. elkanii* species. These results were confirmed by more detailed analyses, that included eight representative strains of *B. japonicum*, *B. elkanii* and mixed genotype and 32 strains that were or are used in Brazilian studies or inoculants (Boddey and Hungria, 1994a). As morphological, physiological and genetic analyses have shown that the two other strains recommended for Brazilian inoculants, SEMIA 587 and 29w, belong to the *B. elkanii* species (Rumjanek et al., 1993; Boddey and Hungria, 1994a), we now show that the only *B. japonicum* strain utilized in commercial Brazilian inoculants today is CPAC 7. The ecological and economic implications of the utilization of each species are still not clear, since the results of experiments evaluating the efficiency of N_2 fixation and competitiveness of the two species have shown that *B. japonicum* is more efficient than *B. elkanii*, but that a mixed genotype between the two species included the most competitive strains (Boddey and Hungria, 1994b). One strategy to improve strain performance could be either to select more competitive variants from *B. japonicum* or to select more efficient variants of *B. elkanii* or, preferentially, of the mixed genotype. As proposed by Minamisawa et al. (1992), we have also found that the IAA synthesis in tryptophan-enriched medium is an easy assay that seems to be highly related with the subdivision of the species. However, more effort should be put on experiments to define the relation of *Bradyrhizobium* species with efficiency of N_2 fixation and competitiveness, to speed up the selection program.

The field inoculation trial results confirmed some others recently compiled by Hungria et al. (1994), showing that responses to inoculation in Brazil can be obtained even in soils with high populations of *B. japonicum* and *B. elkanii*. In this experiment, although statistical differences in yield due to inoculation were not observed, the treatment with CPAC 7 showed higher yield than the non-inoculated control. The application of a high dose of N fertilizer, 400 kg N/ha, split in ten times, was experimentally determined as the level required for maximum plant yield in the complete absence of N_2 fixation (C. M. Borkert, unpublished data). In this experiment, the high level of N fertilizer did not bring any benefit to N plant nutrition or yield, showing that even the established populations of rhizobia were more efficient than the fertilizer. Furthermore, nodule occupancy by the inoculated strains increased from an average of 20% to 50%. Results obtained in the United States of America show that a population as low as 20 to 50 cells/g of soil inhibits the response to inoculation (Singleton and Tavares, 1986; Thies et al., 1991). It is possible that

the differences observed between trials performed in USA and Brazil reside in the higher competitiveness of strains USDA 110 and USDA 123, established in North American soils. However, although several experiments have demonstrated that the variants CPAC 7 and CPAC 15 are more efficient in the process of N_2 fixation and more competitive (Vargas et al., 1992a,b) than the parental ones, in this work this was not observed. Since this experiment was performed in the Southern part of the country, it is possible that the variants show a better performance in the Cerrado region, from where they were selected and tested.

The serological analysis has been traditionally used in field trials to identify the strains introduced via inoculants. However, when strains such as CPAC 7 and CPAC 15, that belong to serogroups already present on the soils, are inoculated, it is only possible to infer that the increase in nodule occupancy is due to the introduced strains. After inoculation for one year, it will be impossible to distinguish between the parental and the variant strains. Characterization of *Rhizobium* and *Bradyrhizobium* isolates by the RAPD technique has been reported by several authors, such as Harrison et al. (1992) and Lunge (1993) and, in this work, we observed that the RAPD technique allowed an easy identification of strains that show the same serological reaction. These results open new perspectives to study the ecology and establishment of bradyrhizobial strains that belong to the same serogroup, which is particularly important in the case of CPAC 7 and CPAC 15, that have been widely used in Brazilian inoculants since 1992. The high heterogeneity observed in DNA profiles suggests great genetic variability between parental strains and their natural variants, indicating that environmental effects (CPAC 15) or pressure of selection (CPAC 7) can easily cause genetic modification. At the same time, these results open perspectives to identify genetic alterations related to higher efficiency of N_2 fixation and competitiveness.

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