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Phenotypic grouping of Brazilian *Bradyrhizobium* strains which nodulate soybean

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Abstract Several years of research have shown that there is a high genetic and physiological variability among *Bradyrhizobium japonicum* strains, culminating in a subdivision into two bacterial genotypes, and the description of the new species *B. elkanii*. In Brazil, large-scale soybean inoculation started in 1960 and today 15 million doses of inoculants are sold per year for an estimated area of 12 million ha. Efforts have been made to find strains able to fix high amounts of N_2 under Brazilian soil conditions, but few laboratories cover basic studies on N_2 fixation, such as strain classification into the two *Bradyrhizobium* species. In this study several characteristics of 40 soybean *Bradyrhizobium* strains, including 4 reference strains of *B. japonicum* (genotype I) species, 3 of *B. elkanii* (genotype II) and 1 of a mixed genotype were evaluated. The parameters analysed in vitro were: colony morphology, serological grouping, intrinsic resistance to antibiotics, synthesis of indole acetic acid, expression of hydrogenase activity and growth in a medium enriched with asparagine. In vivo, analyses performed included the nodulation of Rj_4 soybean cultivar Hill and the detection of symptoms caused by rhizobitoxine. These evaluations allowed a phenotypic grouping which positioned most of the strains utilized in Brazilian inoculants and studies, as well as some new strains isolated from the Cerrado region, within the species *B. elkanii*. However, environmental stresses and adaptation of *Bradyrhizobium* strains to the soil caused a large physiological and genetic variability in some isolates from the Cerrado soils in relation to the putative parental strain introduced 15 years ago, placing these isolates in an intermediate position between the two *Bradyrhizobium* species.

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Introduction

Soybean is a legume having grains with a high protein content and, for this reason, demands considerable amounts of nitrogen (N). To reach yields of 2 500 kg ha^{-1}, the crop needs about 200 kg of N ha^{-1}, with 67–75% being stored in the grain. The capacity of this legume to satisfy its nutritional demand for N via symbiotic nitrogen fixation, performed by the association of bacteria of the genus *Bradyrhizobium* with its roots, is well documented, so that today in Brazil it is recommended that no N fertilizer at all should be applied to this crop (Cattelan and Hungria 1994; Hungria et al. 1994).

The normal practice is to inoculate the soybean seeds at planting with suitable strains of *Bradyrhizobium* spp., but the continuous selection of more efficient and competitive strains is essential to maintain and increase soybean yields. Since the early 1980s, several laboratories have shown that there is a large genetic and physiological variability between *Bradyrhizobium* strains which nodulate soybean (Hollis et al. 1981; Stanley et al. 1985; Kuykendall et al. 1988; Minamisawa 1989, 1990; Minamisawa and Fukai 1991; Minamisawa et al. 1992). For this reason, Kuykendall et al. (1992) suggested the division of these strains into two species, *Bradyrhizobium japonicum* and *B. elkanii*, a nomenclature confirmed later by the International Committee of Taxonomy (Anonymous 1993).

Brazil is today the world's second largest soybean producer, with almost 12 million ha planted. The strains used in the soybean inoculants have to be recommended by the National Committee of Microbiologists and, in the last crop season, about 100% of 1st year crops and 55% of the crops in areas with established populations of soybean bradyrhizobia were inoculated. To supply the demand of rhizobial inoculants, large-scale production started in the early 1960s with North American strains because there

were no native bacteria able to nodulate soybean (Cattelan and Hungria 1994; Hungria et al. 1994). However, the classification of most strains which are or were used in Brazilian studies within the species *B. japonicum* or *B. elkanii* is still to be done.

The objective of the present work was thus to evaluate diverse characteristics of *Bradyrhizobium* spp. strains utilized in inoculation trials or inoculants in Brazil since 1966, as well as of other naturalized strains recently isolated from the Brazilian Cerrado soils after several years of inoculation. Cerrado is an edaphic type of savanna which occupies about 25% of the Brazilian land. The area was originally free of soybean bradyrhizobia and the first inoculants used carried the strain SEMIA 566, which was recommended until 1978. The stressed environmental conditions of Cerrado, characterized by long periods of water stress and high temperatures, cause changes in bacteria (Hungria and Vargas 1996; Hungria et al. 1996; Nishi et al. 1996) which should be studied in more detail, since soybean has been established as the most important grain crop in that region. With the evaluation of parameters such as hydrogenase activity, production of rhizobitoxine and synthesis of indole acetic acid, it was possible to establish phenotypic groupings of the most-studied Brazilian strains and the recent isolates from Cerrado soils.

Materials and methods

Rhizobial strains and plant genotypes

The strains used in this study are described in Table 1. The strains considered as representative of each species were: USDA 110, USDA 122, USDA 123, PJ 17-1 and PJ 17 for *B. japonicum*, USDA 73 for mixed genotype and USDA 31, USDA 94 and USDA 76 for *B. elkanii*. Strains USDA 31, 73, 76, 94, 110 and 122 were received from Dr. Peter van Berkum (USDA, Beltsville, Md.), PJ 17 and PJ 17-1 from Dr. Harold J. Evans (University of Corvallis, Oregon) USDA 123, NC 1005, 532C, 29w, SEMIA 587, INPA 37, CB 1809 and SEMIA 566 from Dr. Yara Kolling (FEPAGRO, Porto Alegre, Brazil), R54-a, DF 395, SM_1b, 965 and DF 383 from Rosa M. Pittard (Embrapa-Agrobiologia, Seropédica, Brazil), and CPAC 7 and CPAC 15 from Dr. Milton A. T. Vargas (Embrapa-Cerrados, Planaltina, Brazil).

Strain CPAC 15 and the 17 "S" strains were isolated from the Cerrado region, an edaphic type of savanna localized in the Central Region of Brazil. The Cerrado is a region originally 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 these isolates came had been inoculated exclusively with strain SEMIA 566 about 15 years ago, and has not been inoculated since (Hungria and Vargas 1996; Nishi et al. 1996). Consequently, these isolates seem to be natural variants of SEMIA 566, since they share the same serological properties.

The following soybean [*Glycine max* (L.) Merrill] cultivars were utilized (genealogy in parentheses): BR-16 (D69-B10-M58 × Davis); Hill [(Dunfield × Aberlandt) × (D632-15) × (D-49-2525) × (S-110 × CNS)]; Clark [Lincoln (2) × Richland]. Alfalfa seeds (*Medicago sativa* L.) of cultivar Crioula were utilized for the rhizobitoxine test. The seeds and information about the cultivars were obtained from the germplasm bank of Embrapa-Soja, Londrina, Brazil.

Morphological and serological analyses

The morphological characterization of strains was performed after 5 and 8 days of growth at 28°C on plates with YMA medium (Vincent 1970). The serological characterization of strains was done by the immuno-agglutination procedure (Somasegaran and Hoben 1985), testing against the antisera of SEMIA 566, SEMIA 587, 29w, CB 1809, SEMIA 5025 (from Thailand, = TAL 411, received from FEPAGRO, Porto Alegre, Brazil), 532C, INPA 037, NC 1005, CPAC 7 and CPAC 15.

Evaluation of resistance to antibiotics

Intrinsic resistance to low levels of four different antibiotics, utilized by Sawada et al. (1990), was evaluated by reading the optical density at 600 nm, after growth for 7 days at 28°C in tubes containing liquid YM medium (Vincent 1970) with or without each antibiotic. Strains were considered resistant to the antibiotic when growth was at least 50% of that of the control without the antimicrobial compound. Their resistance to high levels of seven of the antibiotics used by Kuykendall et al. (1988) was evaluated in the same manner. The low levels tested were (in μg ml^{-1}): streptomycin, 40 and 80; rifamycin, 25; spectinomycin, 10; kanamycin, 15. The high levels were (in μg ml^{-1}): tetracycline, 100; chloramphenicol and carbenicillin, 500; erythromycin, 250; nalidixic acid, 50; rifamycin, 500; streptomycin 100. Stock solutions of streptomycin, carbenicillin, kanamycin, spectinomycin and erythromycin were prepared in distilled water, nalidixic acid in 0.35 N NaOH, tetracycline in 70% ethanol and chloramphenicol and rifamycin in methanol. Antibiotics were sterilized by filtration (Millipore, 0.22 μm) and added to the sterilized YM medium.

Accumulation of indole acetic acid

The bacteria were grown in Tris-YMRT medium, enriched with 0.3 mM tryptophan (Owens and Wright 1964; Minamisawa and Fukai 1991) for 7 days at 30°C, in the dark. The concentration of indole acetic acid (IAA) was estimated using the colorimetric procedure of Gordon and Weber (1951), as modified by Minamisawa et al. (1992).

Tolerance to asparagine in vitro

The ability to grow in an asparagine-enriched medium (after Döbereiner et al. 1970) was evaluated by the extent of growth after 7 days in YM medium modified with 0.2 g of yeast extract l^{-1} and supplemented with 3 or 7 mM asparagine. Growth inhibition or stimulus was evaluated by turbidimetry, measuring the optical density (OD) at 600 nm and using the formula $[(OD_{7\ mM} - OD_{3\ mM}): OD_{7\ mM}] \times 100$.

Expression of hydrogenase activity in vitro

For the evaluation of this parameter bacteria were grown in the defined medium of Maier et al. (1978) for the expression of hydrogenase in free-living rhizobia, and the analysis proceeded as described previously (Nishi et al. 1996).

Determination of the production of rhizobitoxine through its effect on the roots of alfalfa (*Medicago sativa*)

The test was performed according to the methodology of Minamisawa and Fukai (1991). Glass tubes containing medium with autoclaved bacterial supernatants were prepared as described before (Nishi et al. 1996). Surface-sterilized seeds (Vincent 1970) of alfalfa were placed on the surface of the medium and allowed to germinate and grow for 8 days in a growth chamber under continuous light at 30°C. The control treatment consisted of tubes containing 10 mM Tris and 1.5% agar at pH 7.5. 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.

Table 1 Reference strains of *Bradyrhizobium japonicum* (genotype I), *B. elkanii* (genotype II) and the mixed genotype. Also strains and isolates characterized in this study.

Strain	Origin	Principal characteristics[a]
Reference strains of *B. japonicum*		
USDA 110 (= TAL 102)	USA	Group Ia (1, 2, 3); sTI (4); genotype I-A1 (5)
USDA 122	USA	Genotype I-A2 (5)
USDA 123	USA	Group I (1, 2)
PJ 17	USA	Hup^- mutant (6); genotype I-A2 (5)
PJ 17–1	USA	Isogenic revertant mutant Hup^+ of strain PJ 17 (6); genotype I-A2 (5)
Reference strain of the mixed genotype		
USDA 73	USA	Mixed genotype (7)
Reference strains of *B. elkanii*		
USDA 31	USA	Group II (1, 2); sTII (4), genotype II-B1 (5)
USDA 76	USA	Group II (1, 2); sTII (4); genotype II-B2 (5); *B. elkanii* II (3)
USDA 94	USA	Group II (1; 2); sTII (4); *B. elkanii* II-a (3)
Strains of *Bradyrhizobium* utilized in Brazilian studies and/or inoculants and characterized in this study		
NC 1005	USA	Isolated at the University of North Carolina
532C (= SEMIA 5039)	Brazil	Isolated in the State of Rio Grande do Sul; efficient but with low competitiveness (8)
29w (= SEMIA 5019)	Brazil	Isolated in the State of Rio de Janeiro; high to medium efficiency (9, 10) and highly competitive (8, 10); recommended commercially since 1979
R54-a	Brazil	Isolated in the State of Rio de Janeiro, from a previously inoculated soil with high levels of Mn (11)
SEMIA 587	Brazil	Isolated in 1967, in the State of Rio Grande do Sul; efficient and competitive (8, 10); recommended commercially from 1968 to 1975 and since 1979
INPA 037 (= SEMIA 5061)	Brazil	Isolated in the State of Amazonas
DF 395	Brazil	Isolated in Brasília; forms many nodules, but low efficiency of N_2 fixation (9)
SM_1b	Brazil	Isolated in 1963, in the State of Rio de Janeiro, from a previously inoculated soil; shows medium (11) to high (9) efficiency
965 (= J 5033)	Japan	Shows low (8) to high (9) efficiency
DF 383	Brazil	Isolated in Brasilia; forms few nodules but is very efficient (9)
CB 1809 (= SEMIA 586, USDA 136b, TAL 379)	USA	Sent from USA to Australia and from there to Brazil, in 1966; very efficient (11) despite a low nodule mass (9); also shows low competitiveness (8)
CPAC 7 (= SEMIA 5080)	Brazil	Isolated in Brasília from a subculture of CB 1809 (12); characterized by high competitiveness (10, 12); recommended commercially since 1992.
SEMIA 566	Brazil	Isolated in the State of Rio Grande do Sul, from a previously inoculated soil; recommended commercially from 1966 to 1978
CPAC 15 (= SEMIA 5079)	Brazil	Isolated in Brasília from soils inoculated several years before with strain SEMIA 566 (12); very efficient (10, 12); recommended commercially since 1992.
Isolates from the Cerrado region		
S-127; S-204; S-220; S-273; S-335; S-340; S-370; S-372; S-381; S-406; S-452; S-468; S-478; S-481; S-490; S-506; S-516		Isolated at EMBRAPA-Cerrados, in Brasília

[a] Bibliographic references in parenthesis: (1) Hollis et al. (1981); (2) Kuykendall et al. (1988); (3) Kuykendall et al. (1992); (4) Stanley et al. (1985); (5) Minamisawa (1990); (6) Lepo et al. (1981); (7) Minamisawa (1989); (8) Peres (1979); (9) Neves et al. (1985); (10) Nishi et al. (1996); (11) Döbereiner et al. (1970); (12) Vargas et al. (1992)

Table 2 Characterization of intrinsic resistance to antibiotics of 40 strains of *Bradyrhizobium* which nodulate soybean. The results were confirmed in three replicates (*kan*, kanamycin, *rif*, rifampicin, *spe*, spectinomycin, *str*, streptomycin, *car*, carbenicillin, *chl*, chloramphenicol, *nal*, nalidixic acid, *tet*, tetracycline, + growth equal or greater than 50% of that of the control, – growth less than 50% of that of the control)

Strain	Low levels[a, c]					High levels[b, c]						
	kan	rif	spe	str		car	chl	ery	nal	rif	str	tet
	15	25	10	40	80	500	500	250	50	500	100	100
Reference strains of Bradyrhizobium japonicum												
USDA 110	–	+	+	–	–	–	–	–	–	–	–	–
USDA 122	–	+	+	+	+	–	–	–	–	–	–	–
USDA 123	–	–	–	–	–	–	–	–	–	–	–	–
PJ 17	+	–	+	+	+	+	–	+	–	–	–	–
PJ 17–1	+	–	–	+	+	+	–	+	–	–	–	–
Reference strain of the mixed genotype												
USDA 73	–	+	–	+	+	+	–	+	+	+	+	+
Reference strains of Bradyrhizobium elkanii												
USDA 31	+	+	+	+	+	+	+	+	+	+	+	+
USDA 76	+	+	+	+	+	+	+	+	+	+	+	+
USDA 94	+	+	+	+	+	+	+	+	+	+	+	+
Strains utilized in Brazilian studies and/or inoculants												
NC 1005	–	+	–	–	–	+	+	–	+	+	–	–
532C	+	+	–	–	–	+	–	+	–	+	–	+
29w	+	+	+	+	+	+	+	+	+	+	+	+
R 54-a	+	+	+	–	–	+	–	+	+	+	–	+
SEMIA 587	+	+	+	+	+	+	+	+	+	+	+	+
INPA 037	–	+	+	+	+	+	+	+	+	+	+	+
DF 395	–	+	+	–	–	+	+	+	+	+	–	–
SM_1b	–	+	+	+	+	+	+	+	+	+	+	–
965	+	+	–	–	–	+	–	+	+	+	–	–
DF 383	–	+	–	+	+	+	+	+	+	+	+	+
CB 1809	+	+	–	–	–	–	–	–	+	+	–	+
CPAC 7	+	+	–	–	–	–	–	–	+	+	–	+
SEMIA 566	+	+	–	+	+	+	–	+	+	+	+	+
CPAC 15	+	+	–	+	+	+	–	+	+	+	+	+
Isolates from the Cerrado region												
17 isolates (% of +)	76	29	29	35	18	94	12	53	100	88	35	47

[a] According to Sawada et al. (1990).
[b] According to Kuykendall et al. (1988).
[c] Concentrations in μg ml^{-1}

Determination of the production of rhizobitoxine by symptoms of chlorosis in the leaves of two soybean cultivars

Soybean seeds of cultivar Lee, sensitive to rhizobitoxine (Johnson and Means 1960), were inoculated with a suspension of cells of each of the strains and planted in Leonard jars (Vincent 1970) containing N-free nutrient solution (Somasegaran and Hoben 1985). After 28 days, the leaves were examined for toxic symptoms (chlorosis) of rhizobitoxine, receiving the score 0 (no chlorosis), 1 (mild symptoms, similar to those caused by strain USDA 31) and 2 (severe symptoms, comparable to the chlorosis caused by strain USDA 76). The experiment was repeated with soybean Brazilian cultivar BR-16.

Repression of nodulation by the Rj_4 allele

The Hill cultivar of soybean, which contains the Rj_4 allele (Vest and Caldwell 1972), was grown in the greenhouse in Leonard jars and N-free nutrient solution, as described in the previous section, and inoculated with each of the 40 *Bradyrhizobium* strains. Nodulation of the plants was evaluated 21 days after plant emergence.

Parameters analysed as positive or negative were confirmed in at least three replicates and, for quantification, experiments were performed in a completely randomized design with three replicates.

Cluster analysis

The data obtained for each of the characteristics were transformed into a binary code, of 0 and 1, in the form of a matrix, and submitted to the statistical analyses of grouping. A total of 32 parameters were considered: diameter of colonies (≤1.0 mm, 0; ≥1.0 mm, 1); colour (white, 0; cream, 1); shape (circular, 0; punctiform, 1); mucoidy (low or intermediate, 0; high, 1); for each of the serogroups SEMIA 566, SEMIA 587, 29w, CB 1809, SEMIA 5025, 532C, INPA 037 and NC 1005 (negative reaction, 0; positive reaction, 1); resistance to the each of the levels of antibiotics tested, in a total of 12 treatments (no, 0; yes, 1); synthesis of IAA, divided into three levels, 0 to ≤10 μM, >10 to ≤20 μM and >20 μM (no, 0; yes, 1); growth in asparagine-enriched medium (stimulus, 0; inhibition, 1); hydrogenase activity (no, 0; yes, 1); rhizobitoxine in alfalfa, in soybean cv. Lee and in soybean cv. BR-16 (absence of symptoms, 0; presence of symptoms, 1). The phenogram was constructed on the basis of all the characteristics evaluated in this study and using the coefficient of similarity of Jaccard and the unweighted pair-group method with arithmetic mean (UPGMA) procedure, with the statistics program NTSYS (Numerical Taxonomic and Multivariate Analysis System, version 1.70, Exeter Software, New York).

Table 3 Accumulation of indole acetic acid (IAA), expression of the enzyme hydrogenase (Hup) and stimulation of inhibition of growth by asparagine, in vitro, by 40 *Bradyrhizobium* strains and isolates which nodulate soybean

Strain	Serogroup[a]	IAA[b] (μM)	Hup[c]	Growth with aspargine[d]
Reference strains of *B. japonicum*				
USDA 110	n.r.[e]	6.85[f]	+[e]	+6.57[f]
USDA 122	n.r.	4.88	+	+5.57
USDA 123	n.r.	4.88	+	–37.68
PJ 17	SEMIA 5025	5.23	–	+24.31
PJ 17–1	SEMIA 5025	7.08	+	+16.06
Reference strain of mixed genotype				
USDA 73	n.r.	12.20	–	–22.05
Reference strains of *B. elkanii*				
USDA 31	n.r.	44.36	–	–11.00
USDA 76	n.r.	44.31	–	–27.00
USDA 94	n.r.	37.31	–	–15.64
Strains used in Brazilian studies and/or inoculants				
NC 1005	NC 1005	30.55	–	–10.55
532C	532C	34.54	–	–7.95
29w	29w	32.20	–	+37.74
R 54-a	n.r.	30.73	–	+23.23
SEMIA 587	SEMIA 587	36.60	–	+28.02
INPA 037	INPA 037	26.52	–	–5.29
DF 395	SEMIA 587	34.21	–	+10.71
SM_1b	n.r.	44.68	–	+33.74
965	n.r.	30.18	–	–7.82
DF 383	SEMIA 587	22.63	–	–15.07
CB 1809	CB 1809/CPAC 7	17.17	+	–62.89
CPAC 7	CB 1809/CPAC 7	13.12	+	–12.73
SEMIA 566	SEMIA 566/CPAC 15	24.66	–	–26.42
CPAC 15	SEMIA 566/CPAC 15	31.46	–	–36.76
Isolates from the Cerrado region				
17 isolates (% of the isolates with the characteristic	SEMIA 566/CPAC15 (100%)	≤12.20 (65%) 12.20–30.00 (35%)	– (100%)	–(0–15, 29%) –(20–35, 53%) –(55–73, 18%)

[a] Tested against the antiserum of strains SEMIA 566, SEMIA 587, 29w, CB 1809, SEMIA 5025, 532C, INPA 037, NC 1005, CPAC 7 and CPAC 15. The serogroup was confirmed in three replicate tests; (*n.r.* no reaction)
[b] Evaluated after 10 days of growth, in the dark, on the medium of Minamisawa and Fukai (1991), enriched with 0.3 m*M* of tryptophan
[c] Evaluated after 7 days of growth on the medium of Maier et al. (1978) (+, oxidation of H_2 between 1.60 and 2.04 nmol of H_2 $tube^{-1}$ h^{-1} and –, no oxidation of H_2).
[d] Percentage of growth in medium enriched with 7 m*M* of aspargine in relation to the medium with 3 m*M* (+ % of stimulation, – % of inhibition)
[e] Data were confirmed in three replicates
[f] Data represent the means of three replicates

Results

The morphological characteristics of the *Bradyrhizobium* spp. strains evaluated in this study were very similar, there being no marked differences between the reference strains of the two different species and the mixed genotype within the parameters evaluated. Parental strain SEMIA 566 produced little mucus, while 77% of that adapted to the Cerrado region, as well as CPAC 15, produced more mucus than the putative parental strain (data not shown). Furthermore, 59% of these isolates also showed a colony diameter larger than the parental strain (data not shown).

The resistance to low and high levels of antibiotics showed no relationship to the serogroups, but confirmed that strains belonging to the species *B. japonicum* are less resistant than those belonging to *B. elkanii* (Table 2). The strains used in Brazilian studies and/or inoculants showed a pattern closer to the representative strains of the species *B. elkanii*, with all levels of the antibiotics tested. The isolates from the Cerrado region varied largely in relation to the resistance to low and high levels of antibiotics and differed considerably from the putative parental strain SEMIA 566 (Table 2). Only one isolate, S-340, showed the same pattern as SEMIA 566 in resistance to the low levels of antibiotics, and two (S-204 and S-335) were able to grow in the presence of all antibiotics. The other isolates showed a lower resistance to the low levels tested, with 53% of the isolates being able to grow in just one or none of the antibiotics tested (data not shown). When the high levels of antibiotics were tested, both the "Brazilian" strains and the isolates from Cerrado showed a pattern closer to *B. elkanii* strains, and the main exception detected was in relation to the resistance to chloramphenicol. Of the "Brazilian" strains studied, CB 1809 and CPAC 7 were the least resistant to the antibiotics tested (Table 2).

Table 4 Restriction of nodulation of the soybean cultivar Hill (with *Rj4* allele) and expression of symptoms of the production of rhizobitoxine, evaluated by the inhibition of the elongation of alfalfa roots or by the chlorosis on soybean leaves inoculated with 40 strains of *Bradyrhizobium* which nodulate soybean

Strain	Nodulation of soybean cv. Hill[a]	Rhizobitoxine		
		Alfalfa roots[b]	Soybean leaves[c]	
			BR-16	cv. Lee
Reference strains of *B. japonicum*				
USDA 110	+[d]	+[d]	0[d]	0[d]
USDA 122	+	+	0	0
USDA 123	+	+	0	0
PJ 17	+	+	0	0
PJ 17–1	+	+	0	0
Reference strain of mixed genotype				
USDA 73	–	+	0	0
Reference strains of *B. elkanii*				
USDA 31	–	–	1	1
USDA 76	–	–	2	1
USDA 94	–	–	2	1
Strains utilized in Brazilian studies and/or inoculants				
NC 1005	+	–	1	0
532C	+	–	1	0
29w	+	–	1	1
R 54-a	+	–	1	1
SEMIA 587	+	–	0	0
INPA 037	+	–	0	0
DF 395	+	–	1	1
SM_1b	–	–	1	1
965	+	–	1	1
DF 383	–	–	1	1
CB 1809	+	+	0	0
CPAC 7	+	+	0	0
SEMIA 566	–	–	1	1
CPAC 15	–	–	1	1
Isolates from the Cerrado region				
17 isolates	+ (70%)	– (100%)	0 (18%) 1 (82%)	0 (65%) 1 (35%)

[a] Plants harvested at 21 days after emergence; (+ normal nodulation and – restriction of nodulation)
[b] Alfalfa, cv. Crioula, grown for 10 days with continuous illumination; (+ normal growth and – inhibiton of root growth by at least 30% in relation to the control)
[c] Symptoms of chlorosis: *0* no chlorosis; *1* mild chlorosis; *2* severe chlorosis
[d] Data confirmed in three replicates

The serological tests showed that all isolates from the Cerrado region were members of the SEMIA 566 serogroup. Other strains showed reaction with their own serogroups and, in some cases, showed cross-reactions with other groups (Table 3).

B. japonicum reference strains accumulated between 4.88 and 7.08 μM of IAA, while with *B. elkanii* strains the concentration reached 44.36 μM (Table 3). The "Brazilian" strains studied accumulated concentrations of IAA similar to the representative strains of *B. elkanii*, except again for CB 1809 and CPAC 7. It was found that 65% of the strains isolated from the Cerrado region accumulated only low levels of IAA, showing a similar behaviour to the mixed genotype strain USDA 73 (Table 3). All isolates from the Cerrado showed the phenotype Hup^- in vitro and the same was observed with the other "Brazilian" strains, with the exception, once more, of CB 1809 and CPAC 7 (Table 3).

B. japonicum reference strains were stimulated by the presence of asparagine in the culture medium, except for USDA 123. In relation to the "Brazilian" strains, 64% were inhibited by the asparagine, as well as all the reference strains of *B. elkanii* and the mixed genotype USDA 73, and 100% of the isolates of Cerrado were inhibited by the presence of asparagine, although to different degrees (Table 3).

In contrast to *B. elkanii* reference strains, *B. japonicum* was able to nodulate the soybean cultivar Hill. Most of the "Brazilian" strains and 70% of the Cerrado strains also showed normal nodulation in the presence of the *Rj4* allele, although the putative parental strain SEMIA 566 was not able to nodulate this soybean cultivar (Table 4).

Finally, the production of rhizobitoxine, evaluated by the observation of detrimental effects on root growth of alfalfa, or the chlorosis produced in two cultivars of soybean, Lee and BR-16, showed that the isolates from the Cerrado were able to cause mild symptoms of chlorosis in young leaves of soybean plants, but that all these strains were able to inhibit the growth of alfalfa roots (Table 4). Among the other strains studied, only CB 1809 and CPAC

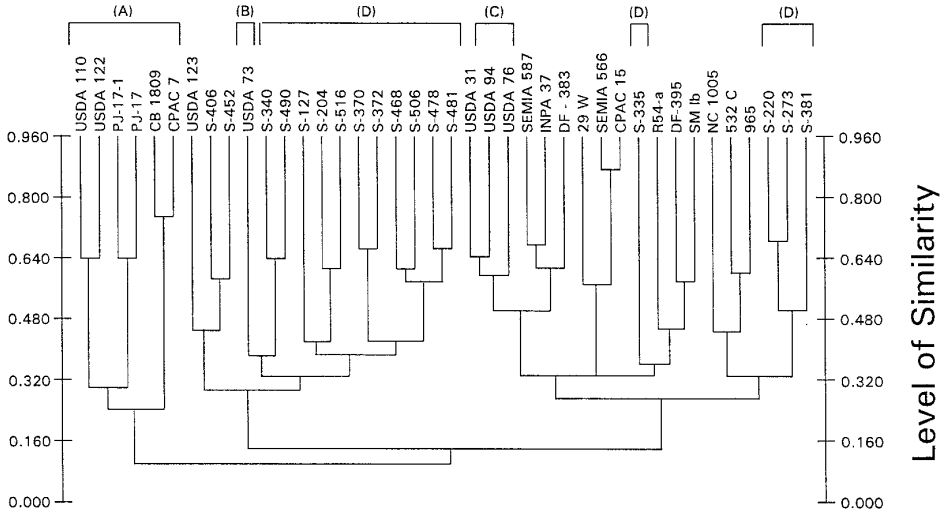

Fig. 1 Phenogram showing the subdivision of soybean *Bradyrhizobium* strains according to 32 parameters evaluated in this study (program NTSYS, UPGMA method and coefficient of Jaccard). *Letters* indicate the reference strains of *B. japonicum* species (*A*), mixed genotype (*B*) and *B. elkanii* species (*C*). (*D* Isolates from the Brazilian Cerrado region and other strains used in Brazilian studies and/or commercial inoculants)

7 did not show symptoms of toxicity on either the roots of alfalfa or the leaves of the two soybean cultivars (Table 4).

The phenogram obtained with the 32 phenotypic variables showed that the majority of the strains isolated from the Cerrado fell into an intermediate group between the species *B. japonicum* and *B. elkanii*. The other strains used in Brazilian inoculants or in other studies were classified as *B. elkanii*. Only the strains CB 1809 and CPAC 7 were classified as *B. japonicum* (Fig. 1).

Discussion

In recent years, several workers have reported substantial differences among *B. japonicum* strains. Genetic differences were reported in 1981, when Hollis et al. (1981) suggested the division into two main groups, I and II, based on studies of DNA homology, including a subdivision of group I in Ia and Ib. A few years later, Stanley et al. (1985) also observed, in studies using hybridization with *nifDH* and homologous sequences of *nodD*, that two groups, sTI and sTII, showed distinct evolutionary lines. Sequencing of genes *nifDK* and *nifE* (Minamisawa 1990; Minamisawa et al. 1992), hybridization with the *hup* gene (Minamisawa 1990; van Berkum 1990; Minamisawa et al. 1992), and the restriction pattern with common *nod* genes (*nodYABC*) of strain USDA 110 (Rumjanek et al. 1993a) and with sequences of 16S rRNA (Young et al. 1991; Rumjanek et al. 1993b) and with RS_{α} (Minamisawa et al. 1992) also confirmed differences between the two groups.

Besides genetic studies, several morphological, physiological and biochemical characteristics were analysed, in attempts to correlate them to the two distinct groups that began to be called genotypes I and II (or GTI and GTII). The characteristics included differences in the resistance to high levels of some antibiotics (Kuykendall et al. 1988), composition of extracellular polysaccharides (Huber et al. 1984; Minamisawa 1989), composition of fatty acids (Kuykendall et al. 1988), expression of dinitrogenase in vitro (Huber et al. 1984), Hup^{+} phenotype (Minamisawa 1990; Minamisawa et al. 1992), colony morphology (Fuhrmann 1990), synthesis of rhizobitoxine (Minamisawa 1989, 1990), synthesis of indole acetic acid (Minamisawa and Fukai 1991; Minamisawa et al. 1992), nodulation of soybean containing genes Rj_4 or rj_1rj_1 (Devine et al. 1983, 1990), isoenzymes of N assimilation in vitro (Rumjanek et al. 1993a). All these results led Kuykendall et al. (1992) to propose the division of strains into two species, creating the *B. elkanii* species, which was later confirmed by taxonomists (Anonymous 1993). Differences among the species, such as induction of outer cortical root swelling, continued to be reported (Yuhashi et al. 1995) but the analyses of several of these parameters in the Brazilian strains have still to be done, although inoculation is largely employed and several studies to isolate more efficient and competitive strains have been performed in Brazil.

In relation to the morphological characteristics analysed in this work, Fuhrmann (1990) found differences in colony morphology between the two species, and colonies with irregular borders were restricted to genotype II. In this study, we did not confirm this relationship. The serological properties of the strains were also not related to their grouping and, as reported before for other *Bradyrhizobium* strains (Sawada et al. 1990), the resistance to low levels of antibiotics showed no relationship to the serogroups. The tests of resistance to high levels of antibiotics confirmed the observations of Kuykendall et al. (1988), which showed that *B. japonicum* strains are less resistant than those of *B. elkanii*. However, the two "Brazilian" groups utilized in the study, including the isolates from the Cerrado region, did not show a pattern as clear as the reference strains, although they were closer to *B. elkanii*.

Minamisawa and Fukai (1991) and Minamisawa et al. (1992) stated that the strains of GTII synthesized 20 μM or more IAA, while GTI was unable to accumulate the hormone even in tryptophan-enriched medium. In this work it was found that 65% of the strains isolated from the Cerrado region accumulated only low levels of IAA,

showing a similar behaviour to the mixed genotype reference strain USDA 73. The other "Brazilian" strains studied accumulated concentrations of IAA which were closer to the reference strains of *B. elkanii*, except for CB 1809 and its natural variant CPAC 7. As suggested before (Minamisawa et al. 1992), the detection of IAA represents an easy and low-cost analysis, which shows a good relationship with species subdivision and may be used for an initial classification of soybean bradyrhizobia.

Minamisawa (1989) observed that the phenotype Hup^+ occurred exclusively in genotype I, and that the expression of phenotype was consistent with the expression of *hup* genes (Minamisawa 1990; Minamisawa et al. 1992). However, not all strains from group I are Hup^+ (Minamisawa et al. 1992), which led Minamisawa (1990) to suggest that the presence of *hup* genes would result from horizontal genetic exchange among strains from GTI. Although the great majority of the isolates from the Cerrado showed intermediate characteristics among the two species, such as in resistance to high levels of antibiotics and particularly IAA synthesis, all were Hup^-. The same was observed with the other "Brazilian" strains except, again, for CB 1809 and CPAC 7.

In a study performed with several strains, which included some of this work, the inhibition of growth in asparagine-enriched medium was proposed as a parameter to identify more efficient bacterial genotypes (Döbereiner et al. 1970). Since, in theory, *B. japonicum* species could be more efficient than *B. elkanii*, due to Hup^+ and rhizobitoxine$^-$ phenotypes, the inhibition of growth in vitro by asparagine was also evaluated in this study. A good relationship was found between this parameter and the subdivision of the reference strains into the two species, but contrary to expectations, *B. japonicum* was stimulated by the amino acid, with the exception of USDA 123, while *B. elkanii* and 71% of the strains used in Brazilian inoculants or studies had their growth inhibited.

Another typical characteristic reported for *B. elkanii* was the restriction of nodulation of cultivars with the Rj_4 allele, such as Hill (Devine et al. 1990; Kuykendall et al. 1992). However, Sadowsky and Cregan (1992) observed that restriction to nodulation could also occur within *B. japonicum* species and, indeed, a great variability in this parameter was detected during the analyses of the Brazilian strains and isolates.

The production of rhizobitoxine evaluated by the observation of detrimental effects on the root growth of alfalfa, or the chlorosis produced in two cultivars of soybean [one already described as susceptible by Owens and Wright (1964)] showed that the isolates from the Cerrado were able to provoke mild symptoms of chlorosis in young leaves of soybean plants, but that all these strains were able to inhibit the growth of alfalfa roots. Once again, only CB 1809 and CPAC 7 did not show symptoms of toxicity on either the roots of alfalfa or the leaves of the two soybean cultivars.

When all parameters were transformed into a binary code and submitted to the statistical analysis of grouping, resulting in a phenogram, two strains recommended in Brazilian inoculants, 29w and SEMIA 587, showed characteristics of *B. elkanii*, confirming some other parameters evaluated before (Rumjanek et al. 1993a). All strains utilized in Brazilian inoculants or studies were also classified as *B. elkanii*, including the third strain commercially recommended, CPAC 15. Only CB 1809 and its natural variant CPAC 7, this last one the fourth strain recommended in Brazil for commercial inoculants, fitted into the characteristics of the species *B. japonicum.*

The majority of the strains isolated from the Cerrado fitted into an intermediate group between the species *B. japonicum* and *B. elkanii*. In previous studies comparing the putative parental strain SEMIA 566 with the natural variant CPAC 15, genetic differences in the RAPD profile were detected (Nishi et al. 1996) as well as in the ability to increase numbers of root hairs (Hungria et al. 1996). Consequently, environmental stresses and the adaptation of rhizobial strains to the soil caused a large physiological and genetic variability in a short time, which placed the strains from the Cerrado region in an intermediate position between the two *Bradyrhizobium* species when some parameters used for species differentiation were evaluated.

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