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Preliminary characterization of fast growing rhizobial strains isolated from soyabean nodules in Brazil

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Abstract

A survey of soyabean rhizobia was carried out with six Asian and one modern soyabean genotypes as trap hosts. Soyabean seedlings were inoculated with soils from 22 Brazilian field sites, including undisturbed areas or areas traditionally cropped with this legume. A total of 30 fast growing strains, able to establish an effective symbiosis with both types of genotypes, were isolated from 12 of the 22 soils, representing 17% of the population in cropped areas and 24% in undisturbed soils. The bacterial mean generation time varied from 85 to 225 min and after 4 days of growth in YM medium the final pH ranged from 3.7 to 6.9. Although isolated from acid soils, only 37% of the strains were able to grow in TY or YM media at pH 4.0, while 60% were alkaline tolerant (pH 9.5). Most strains produced abundant extracellular polysaccharides (73%), were tolerant to 0.5 M NaCl (60%) and a temperature of 40°C (77%), grew in LB medium (67%) and synthesized melanin (53%). The strains differed in the use of C compounds supplied as sole C sources. The majority of the strains showed an intrinsic resistance to the antibiotics ($\mu g\ ml^{-1}$) chloramphenicol (10), erythromycin (50), gentamicin (20), kanamicin (30), rifampicin (20) and tetracycline (10) and to the heavy metals cobalt chloride (0.5 mM) and potassium chromate (0.25 mM). A cluster analysis with 81 morphological and physiological parameters placed the strains from undisturbed soils in the central part of the dendrogram, even when isolated from distant areas, indicating that they had a common background. The relatedness decreased with the use of the soils for agriculture. Most of the strains isolated from cropped soils under conventional tillage were characterised by a high tolerance to stressful conditions and the ability to grow with several C sources, contrary to most of the strains isolated from soils under no-tillage management system. The strains differed from *S. fredii* in several characteristics and the protein and lipopolysaccharide profiles showed that each strain was unique. Therefore, although soyabean is an exotic plant in Brazil, several indigenous rhizobial strains may also establish an effective symbiosis with this legume. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Bacterial evolution; Nitrogen fixation; *Rhizobium*; *Sinorhizobium fredii*; Soyabean

1. Introduction

Soyabean (*Glycine max* (L.) Merrill) is considered one of the oldest crops in the world, with reports of its cultivation in China at around 2500 BC. The genetic primary centre of *G. soja* Sieb. and Zucc., the ancestor of *G. max*, is the Valley of the Yangtze River, in the north and north-east provinces of China, close to Russia, Korea and Japan; with the emigration of tribes to Manchuria, this region became the secondary centre of diversity (Morse, 1950; Hymowitz, 1970). The crop was probably introduced in Brazil in 1882, in the State of Bahia, as '*Soja hispida*' (D'utra, 1882). During the following decades, a few tests were performed in some Brazilian agronomic experimental stations, but it was only in the 1940s that commercial crops of *G. max* were established in the southern region of the country, with a considerable expansion of soyabean areas after the 1960s. Today, the crop is spread throughout the country, occupying almost 13 million hectares and producing about 31 million tons of grain, making Brazil the world's second largest soyabean producer.

At the time of crop expansion, experiments using disinfected seeds reported none or one or two sporadic nodules, with plants showing severe N deficiency symptoms, indicating that Brazilian soils were devoid of rhizobial strains able to establish an effective symbiosis with soyabean (Lopes et al., 1976; Peres, 1979; Vargas and Suhet, 1980; Suhet et al., 1981). That hypothesis has been recently confirmed in the same undisturbed soils used in this study (Ferreira, 1999). Therefore, when large scale production began in the 1960s, inoculants were brought from the USA. Today, more than

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Table 1
Location and agricultural management of the soils used in this study

Soil	District	State	Soil management
1	Ponta Grossa	Paraná (PR)	Conventional tillage and soyabean as summer crop for more than 5 years
2	Ponta Grossa	PR	Previously cultivated with soyabean but now cropped with beans (*Phaseolus vulgaris*) under no-tillage for the last 3 years
3	Ponta Grossa	PR	Undisturbed soil under native subtropical forest
4	Castro	PR	No-tillage system and soyabean as summer crop for more than 5 years
5	Castro	PR	Same as soil 1
6	Londrina	PR	Same as soil 4
7	Londrina	PR	Same as soil 1
8	Passo Fundo	Rio Grande do Sul (RS)	Undisturbed soil covered with native subtropical forest
9	Passo Fundo	RS	Undisturbed soil covered with native grasses
10	Planaltina	Distrito Federal (DF)	Undisturbed soil covered with Cerrado vegetation (an edaphic type of savanna)
11	Planaltina	DF	Undisturbed soil covered with Cerradão vegetation (higher density of trees than the Cerrado)
12	Planaltina	DF	Same as soil 10
13	Manaus	Amazonas (AM)	Undisturbed soil covered with native tropical forest
14	Manaus	AM	Same as soil 13
15	Campinarama	AM	Same as soil 13
16	Campinarama	AM	Same as soil 13
17	Itutinga	Minas Gerais (MG)	Undisturbed soil covered with native vegetation
18	Itutinga	MG	Same as soil 17
19	Lavras	MG	Same as soil 17
20	Goiânia	Goiás (GO)	Same as soil 10
21	Goiânia	GO	Same as soil 10
22	Londrina	PR	Same as soil 4

90% of the soyabean is grown in areas which were previously inoculated and which contain a naturalised population of competitive strains, introduced in the inoculants (Vargas and Hungria, 1997).

The bacteria able to nodulate and establish an effective symbiosis with soyabean were first named as *Rhizobium japonicum* (Fred et al., 1932). Today, they are classified into two species, *Bradyrhizobium japonicum* (Jordan, 1982) and *B. elkanii* (Kuykendall et al., 1992. Soyabean is also nodulated by fast growing rhizobia first isolated from China (Keyser et al., 1982), initially classified as *Rhizobium fredii* (Scholla and Elkan, 1984) and later reclassified as *Sinorhizobium fredii* and *S. xinjiangensis* (Chen et al., 1988). Although it was originally thought that *S. fredii* was specific for Asian soybean lines (Keyser et al., 1982; Stowers and Eaglesham, 1984; Devine, 1985), Balatti and Pueppke (1992) recently showed that several North American genotypes were effectively nodulated by *S. fredii* strain USDA 257.

The presence of *S. fredii* in soyabean nodules in Brazil has not been reported. However, in the last 5 years, while isolating bacteria from soyabean nodules collected in different Brazilian areas, we identified strains with a fast growth rate and acid reaction in yeast-mannitol (YM) medium. Furthermore, an analysis of 80 soyabean cultivars from Brazilian breeding programs showed that 67% were able to form effective nodules with *S. fredii* strains USDA 205 and CCBAU 114 and with *S. xinjiangensis* CCBAU 105 (Chueire and Hungria, 1997). This study was thus carried out to trap and to characterise fast growing soyabean rhizobial strains from several Brazilian soils.

2. Materials and methods

2.1. Isolation of fast growing strains from nodules of soyabean inoculated with Brazilian soils

The field sites used in this study were located in five widely separated states of Brazil. They included undisturbed areas covered with native vegetation and fields traditionally cultivated with soyabean (Table 1). Soil samples were collected at a depth of 0–20 cm and the chemical characteristics, analysed by standard methods (Ferreira et al., 2000), are shown in Table 2. Soyabean seeds of six Asian genotypes, Pickett 71, Peking (= Plant Introduction, PI17852.B), PI 245331, PI 437654, PI 88788 and PI 90763 and of the modern cultivar BR-16, able to form effective nodules with *S. fredii* (Chueire and Hungria, 1997), were used as trap hosts. Seeds supplied by the Embrapa Soja germplasm bank were surface sterilised as described before (Ferreira et al., 2000) and pre-germinated for 2 days. Each seedling was inoculated with 500 μl of diluted soil (10 g of soil mixed with 95 ml of 0.85% w/v NaCl, agitated with glass beads for 30 min), in triplicate, and allowed to grow in glass jars containing filter paper and N free sterile plant nutrient solution (Ferreira et al., 2000). After 4 weeks, all nodules were collected and rhizobia isolated (Somasegaran and Hoben, 1994). The purity of the cultures was confirmed by repeatedly streaking the bacteria on yeast extract-mannitol agar (YMA) medium (Somasegaran and Hoben, 1994) and verifying a single type of colony morphology, absorption of Congo red (25 $\mu g\ ml^{-1}$) and the Gram stain reaction. Bacteria showing a fast growth

Table 2
Chemical properties and number of fast and slow growing rhizobial strains isolated from nodules of six Asian and one modern soyabean genotypes inoculated with 22 Brazilian soils

Soil	pH in $CaCl_2$	N (%)	K	Ca	Mg	Al[a]	BS[b]	C	P	Isolates (no.)	
			($cmol_c dm^{-3}$)			(%)			($mg\ dm^{-3}$)	Fast	Slow
1	4.52	0.10	0.30	5.29	2.43	3.60	56.04	2.24	7.0	7	12
2	4.60	0.12	0.28	2.37	1.65	1.83	40.22	1.22	3.6	1	7
3	4.47	0.26	0.20	1.81	1.35	6.40	56.04	2.75	20.3	2	6
4	5.09	0.15	0.38	3.39	1.72	0.00	54.95	3.45	14.4	3	8
5	4.92	0.15	0.25	8.39	3.38	0.00	68.41	1.94	3.4	1	19
6	4.70	0.15	0.32	4.09	1.50	0.17	54.02	2.46	7.5	2	17
7	4.64	0.14	0.85	5.71	1.79	0.00	64.28	1.67	12.8	0	18
8	4.15	0.54	0.50	7.05	2.17	5.35	46.91	4.87	23.9	3	2
9	4.52	0.14	0.13	3.27	2.09	4.85	45.11	2.64	2.7	1	8
10	4.00	0.13	0.08	0.07	0.08	79.09	2.80	1.95	0.6	1	0
11	3.85	0.18	0.16	0.22	0.32	56.25	5.86	2.62	1.1	1	6
12	3.87	0.16	0.08	0.02	0.08	83.01	2.11	2.42	0.6	0	5
13	3.61	0.69	0.05	0.00	0.04	92.91	1.42	1.29	2.0	0	5
14	3.58	0.07	0.03	0.00	0.04	95.03	1.13	1.40	1.1	0	0
15	3.93	0.08	0.02	0.00	0.00	97.77	0.42	0.69	0.6	3	12
16	3.04	0.04	0.01	0.02	0.00	98.07	0.46	0.73	1.3	0	0
17	4.55	0.37	0.33	4.37	1.60	2.32	48.54	4.40	2.1	0	7
18	4.32	0.18	0.16	0.05	0.00	57.14	2.96	2.59	0.8	0	0
19	3.72	0.27	0.17	0.05	0.00	81.19	2.09	3.05	1.7	0	0
20	4.07	0.13	0.11	0.32	0.28	36.60	11.13	1.46	0.6	0	2
21	4.36	0.26	0.28	2.32	1.06	5.67	35.57	2.74	0.9	0	0
22	4.75	0.18	0.60	4.74	2.05	1.08	54.30	1.52	30.3	5	32

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

rate (growth after 2–3 days) and acid reaction in YMA containing bromothymol blue (25 μg ml^{-1}) were reinoculated on cultivar BR-16 and on the Asian host genotype, to confirm their identity and effectiveness. Bacteria grown in YM were mixed with glycerol (1:1, v:v) and stored at −80°C. Working cultures were maintained on YMA slants at 4°C.

2.2. Morphological and physiological characterisation

2.2.1. Colony morphology

An initial inoculum of 10^4 cells ml^{-1} was prepared in YM medium with a pH initially adjusted to 6.8. Colony morphology (colour, mucoidy, transparency, diameter, form, borders, elevation) was evaluated by streaking a loop of the initial inoculum on YMA and allowing the bacteria to grow in the dark at 28°C for 3, 5 and 7 days.

2.2.2. Doubling time

The initial inoculum (100 μl) was transferred to triplicate tubes containing 5 ml of YM or tryptone yeast extract (TY, Somasegaran and Hoben, 1994) medium. Bacteria were allowed to grow at 28°C, with agitation, in the dark, for 7 days. Growth was evaluated by reading optical density at 600 nm and counting the cells after spreading serially-diluted aliquots of 100 μl on plates containing YMA or TYA.

2.2.3. Serological reaction

Somatic agglutination reactions were performed after Somasegaran and Hoben (1994) with antiserum against ten soyabean *Bradyrhizobium* strains carried in Brazilian inoculants during the last decades and of *S. fredii* USDA 205, CCBAU 114 and CCBAU 115.

2.2.4. Acidification of YM medium

Aliquots (100 μl) of the initial inoculum were transferred to tubes containing 5 ml of YM with the initial pH adjusted to 7.0. The final pH was evaluated after 4 days of growth in the dark at 28°C. The acid/alkaline reaction was also verified by spreading the inoculum on YMA plates, pH 7.0, containing 25 μg ml^{-1} bromothymol blue.

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

Aliquots of 100 μl of inoculum were transferred to tubes containing 5 ml of TY and to tubes containing 5 ml of YM**,** with pH values previously adjusted to 4.0, 5.0, 6.5 and 9.5. Growth was verified in triplicate tubes after growth, with agitation, at 28°C, for 7 days. The tolerance was also verified in media containing the nutrients of TY and buffered to pHs 4.0 or 8.9, after Costilow (1981). Tolerance to salinity was verified in tubes containing 5 ml of TY and in tubes with 5 ml of YM medium (pH 6.8), containing NaCl at final concentrations of 0.1, 0.3 and 0.5 M. Controls for acidity,

alkalinity and salinity tolerance included bacteria grown in YM and TY media with pH initially adjusted to 6.8. To verify the tolerance to high temperatures a loop of each bacterium was streaked on triplicate plates containing TYA and YMA and allowed to grow at 28 (control), 37 and 40°C for 7 days.

2.2.6. Growth in LB and synthesis of melanin

The capability of growing in LB medium (Somasegaran and Hoben, 1994) was verified after 7 days of growth at 28°C. Synthesis of melanin was evaluated after Rodriguez-Navarro et al. (1996), in triplicate TYA plates (pH 6.8) supplemented with 1.2 mg ml^{-1} of L-tyrosine and 40 μg ml^{-1} of $CuSO_4$, after 5 days of growth, and reading proceeded before and after bacteria lysis (Cubo et al., 1997).

2.2.7. Growth with different C and N sources

The defined medium of Brown and Dilworth, described by Bergersen (1980), was used for the assays, containing bromothymol blue (25 μg ml^{-1}) and pH adjusted to pH 6.8. C and N sources (Sigma Chemical Co. or Merck) were sterilised by membrane filtration (Millipore, 0.22 μm) and added to the autoclaved medium. For the evaluation of C sources, the medium was supplied with 0.7 g NH_4Cl l^{-1} and the following compounds were added at 1.5 g l^{-1}: casein, D(+)cellobiose, citrate, dulcitol, D(+)fucose, D(−)fructose, D(+)galactose, gluconate, D(+)glucosamine, D(+)glucose, L-glutamic acid, glutaric acid, glycerol, D-lactose, malate, maltose, D-mannitol, D(+)mannose, myo-inositol, pyruvate, D(+)raffinose, αL-rhamnose, D(−)ribose, D-sorbitol, succinate and sucrose. For the N sources the medium was supplied with 2.5 g glucose l^{-1} and the following compounds were added at 0.7 g l^{-1}: adenine, L-alanine, allantoin, L-arginine, L-asparagine, L-cysteine, L-cystine, cytosine, L-glutamine, glycine, guanine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenyalanine, L-proline, L-serine, L-threonine, thymine, L-tryptophan, L-tyrosine, uracil, D-valine. Utilisation of compounds as C and N sources was verified in the defined medium without any addition of C and N and the following compounds were added at 2.5 g l^{-1}: allantoin, L-arginine, L-cysteine, L-glutamine, L-histidine, L-methionine, L-phenyalanine, L-proline, L-serine, L-tryptophan and L-valine. If the addition of a C or N compound acidified or alkalinised the medium, verified by a change of the green colour of the bromothymol blue, sterilised HCl or NaOH was added to reach the initial pH 6.8. 500 μl of the initial inoculum were added to each well of a microtiter plate and the inoculation performed with a replicator. Three media were used as controls: YMA, TYA and defined medium, supplied with 10 g mannitol l^{-1} and 0.7 g NH_4Cl l^{-1}. Growth was verified after 5 and 7 days of incubation at 28°C in the dark and the bromothymol blue also allowed the verification of acidic or alkaline reactions due to the bacterial metabolism.

2.2.8. Intrinsic resistance to antibiotics and heavy metals

Intrinsic resistance to antibiotics was evaluated with defined medium supplied with 10 g mannitol l^{-1} and 0.7 g NH_4Cl l^{-1}, pH 6.8 and the gradients of the following antibiotics (in μg ml^{-1}): chloramphenicol, 10, 20, 40; erythromycin, 50, 75, 100; gentamicin sulphate, 10, 20, 40; kanamycin, 10, 20, 30; rifampicin, 5, 10, 20; streptomycin sulphate, 50, 75, 100; tetracycline, 10, 20, 40. Antibiotics were prepared after Sambrook et al. (1989), sterilised by membrane filtration (Millipore, 0.22 μm) and added to the sterilised YM medium. The plates contained 20 ml of the medium and inoculation and growth conditions were as described in the previous item. The control consisted of bacteria inoculated in the medium without antibiotics and strains were considered as tolerant when growth was at least 50% that of the control. The intrinsic resistance to heavy metals was evaluated using the same medium as the antibiotics, but supplied with bromothymol blue (25 μg ml^{-1}). The following heavy metals were tested (in mM): 0.5, 1.0 and 2.0 of cadmium chloride; 0.5, 1.0 and 2.0 of cobalt chloride; 2.5, 5.0, 10.0 of lead acetate; 0.5, 1.0 and 2.0 of nickel sulphate; 0.25, 0.50 and 1.00 of potassium chromate and 5, 10 and 20 of selenium sulphide. Heavy metals sterilization, pH correction, inoculation and growth conditions were as described in item 2.2.7.

2.2.9. Cluster analysis

Twenty parameters were considered for the first phenotypic matrix: soyabean history of the area; host plant; colony colour; mucoidy; type of mucoidy; transparency; diameter of colonies; doubling time; doubling time (very fast growers)); growth in TY and YM media at pH 4.0; growth in TY and YM media at pH 9.5; growth in buffered media at pH 4.0; growth in buffered media at pH 8.9; acidification of YM (low level of acidification); acidification of YM (medium level of acidification); acidification of YMA (strong acidifiers); tolerance to 0.5 M NaCl; growth at 40°C; growth in LB medium; synthesis of melanin. In relation to C and N substrates, only those with different utilisation by the strains were considered. Of the 26 C sources tested, 17 were considered as 1 if the growth was ≥ 0.5 (substrates not considered were citrate, dulcitol, glucosamine, glucose, glutamic acid, mannitol, mannose, succinate and sucrose). All N sources tested (25), except for glycine, were considered as 1 when growth was >1 and 0 when ≤ 1. None of the 11 compounds tested as C and N sources was computed for the cluster analysis. Consequently, the C and N matrix had 41 parameters. For the antibiotic matrix, the three concentrations of seven antibiotics were considered, except for kanamycin at 10 μg ml^{-1}. Finally, for the heavy metals, the data considered were related to three concentrations of three heavy metals (Cd, Co and Se) and two levels of Cr, in a total of 11 parameters. A final dendrogram included all the parameters cited above, except for the data related to intrinsic resistance to heavy metals. The dendrograms were built by the clustering method of

UPGMA (Unweighted Pair-Group Method with Arithmetic Mean) and the coefficient of Jaccard (J) with the statistics program NTSYS (Numerical Taxonomic and Multivariate Analysis System, version 1.80, Applied Biostatistics, New York).

2.2.10. Protein and lipopolysaccharide (LPS) profiles

Protein profiles were determined as described before (Ferreira et al., 2000) and after separation of fragments the presence or absence of bands was used for cluster analysis using the UPGMA method and the Jaccard coefficient, as described in the previous item. LPS profiles were obtained from bacteria grown in 15 ml of YMB for 7 days, at 28°C, with agitation. The bacteria were centrifuged at 10,000 rpm for 15 min and washed twice with 0.85% NaCl. The pellet was resuspended to a concentration of 10^9 cells ml^{-1} and boiled for 45 min at 95°C. The suspensions were centrifuged for 5 min at 12,000 rpm, taking the supernatant and adding the same volume of extraction buffer, consisting of 4% (w/v) SDS in 50 mM Tris-HCl pH 6.8; 20% (v/v) glycerol; 5% (v/v) β-mercaptoethanol and 0.01% bromophenol blue. Samples were boiled for 5 min and kept at −20°C. Samples were run on acrylamide gels as described for protein profiles (Ferreira et al., 2000). Gels were stained overnight by incubation in 40% ethanol: 5% acetic acid; oxidising LPS with 0.7% (w/v) periodic acid, 40% ethanol and 5% acetic acid; four 15-min washes with distilled water; staining for 10 min with a solution of silver nitrate (1 g silver nitrate; 2.8 ml NaOH 1 M; 2 ml NH_4OH; in 150 ml distilled water); three 30-min washes with distilled water, revelation with 0.25% formaldehyde; and finally stopping the revelation with citric acid 1 M.

3. Results

3.1. Isolation of fast growing strains from soyabean nodules

Many of the Brazilian soils used in this study were very acid, frequently saturated with Al and contained small concentrations of available P (Table 2). High saturation with Al (>50%) was observed in nine soils and a slightly higher N content occurred in some undisturbed soils covered with native vegetation (e.g. soils 8, 13). We isolated 196 rhizobia, 30 which were characterised by a fast growth rate and acidic reaction in YMA medium (Table 2). The fast growers were present in 12 of the 22 soils analysed, including areas from the Amazon to Rio Grande do Sul (Table 2). Therefore in ten soils there were no compatible fast-growing rhizobia. Most of the isolates (63%) came from cropped soils (soils 1, 2, 4, 5, 6 and 22), where they represented 17% of all isolates trapped by soybean plants (Tables 1 and 2). When isolated from undisturbed soils covered with native vegetation, the fast growers represented 24% of the population (Table 2). The trap host for each isolate is shown in Table 3, with 83% being trapped by Asian genotypes, but the modern cultivar BR-16 was just as effective for trapping rhizobia. Within the Asian genotypes, Pickett-71 and PI-437654 captured nine and five isolates, respectively, and BR-16 trapped five strains, more than the four other Asian genotypes used in this study, including Peking. Reinoculation tests confirmed that the 30 fast growing strains were able to form effective N_2-fixing nodules with both kinds of genotypes.

3.2. Colony morphology, doubling time and serological reaction

Differences between strains were verified using some morphological parameters, but a high production of mucus was verified in 73% of the strains, 67% formed colonies ≤1 mm of diameter, 73% were white and 43% opaque (data not shown). However, there was no relationship between the morphological data and agricultural management, or the site of isolation, or with any of the soil chemical characteristics analysed (data not shown). The 30 strains can be considered as fast growers, since the doubling times in YM medium were less than 6 h, but they could be divided into three groups (Table 3). A slower growth rate in YM medium was obtained with strains 8, 11 and 16, with doubling times between 205 and 240 min and an intermediate growth rate was obtained with strains 26–30, between 130 and 170 min (Table 3). These five strains also formed small (<1 mm), white, opaque colonies (data not shown) and were isolated from areas traditionally cultivated with soyabean from the state of Paraná (Tables 1 and 2). Most of the strains (73%) showed a very fast growth rate, between 85 and 115 min (Table 3), and mixed morphological characteristics (data not shown). The doubling times were longer in TY (data not shown) than in YM medium. None of the strains reacted serologically with any of the ten antisera tested from the soyabean *Bradyrhizobium* strains carried in Brazilian inoculants in the past decades, or with the antisera of *S. fredii* USDA 205, CCBAU 114 and CCBAU 115 (data not shown).

3.3. Acidification of YM medium

The strains could be grouped into four classes of acidification of YM medium with an initial pH of 7.0: (1) final pH between 6.7 and 6.9 after 4 days of growth, (2) final pH ranging from 6.1 to 6.6, (3) a major group of 13 isolates from cropped and undisturbed areas which acidified the medium to a pH of 5.0–6.1 and (4) six strains, five isolated from undisturbed soils, with a very acid reaction, with final pH values ranging from 3.7 to 4.4 (Table 3). The three strains characterised by a slower mean generation time (8, 11 and 16) were within the less acidifying group.

3.4. Tolerance to acidity and alkalinity

All bacteria grew in TY and YM media with pH values of 5.0 and 6.5 (data not shown), but differences were detected

Table 3
Soil of origin and trap host genotype of the 30 rhizobial strains isolated in this study. Also doubling time in YM medium, changes in pH after 4 days of growth in YM and YMA with pH initially adjusted to 7.0, tolerance of the strains to acidity and alkalinity in TY, YM and TY-buffered media, tolerance to high temperature, growth in LB and and synthesis of melanin in vitro

Strain	Soil	Soybean trap host genotype	Doubling time (min)	Final pH in YM	Acid reaction in YMA	TY/YM		Buffered TY		NaCl (M)			Temperature (°C)		LB	Melanin
						4.0	9.5	4.0	8.9	0.1	0.3	0.5	37	40		
1	1[a]	PI 90763	95	6.31	2[b]	− [c]	+	−	+	+	+	+	+	+	+	++++ [d]
2	1	Peking	105	6.08	2	+	+	−	+	+	+	+	+	+	+	+++
3	1	Peking	110	5.55	3	−	−	−	+	+	+	+	+	+	+	++
4	1	Peking	115	5.66	3	−	+	−	+	+	+	+	+	+	+	−
5	1	Pickett-71	90	3.97	4	−	+	−	+	+	−	−	+	+	−	+++
6	1	Pickett-71	100	6.17	3	+	−	−	+	+	+	+	+	+	+	*y*
7	1	Pickett-71	105	5.79	3	+	+	−	+	+	+	+	+	+	+	++
8	2	PI 245331	205	6.91	0	−	−	−	−	+	−	−	+	+	−	−
9	3	Pickett-71	90	5.30	3	+	−	−	+	+	+	+	+	+	+	*y*
10	3	PI 88788	95	5.53	3	+	+	−	+	+	+	+	+	+	+	+
11	4	Peking	240	6.73	0	−	−	−	−	+	−	−	*w*	−	−	−
12	4	Pickett-71	110	6.22	2	+	−	−	−	+	+	+	+	+	+	*y*
13	4	Pickett-71	85	6.00	2	+	−	−	+	+	+	+	+	+	+	−
14	5	PI 437654	85	6.01	2	+	+	−	+	+	+	+	+	+	+	+++
15	6	PI 88788	85	5.99	2	−	+	−	+	+	+	−	*w*	−	+	++
16	6	Pickett-71	225	6.72	0	−	−	−	−	+	−	−	+	+	−	−
17	8	PI 437654	85	3.92	4	+	+	+	+	+	+	+	+	+	+	*y*
18	8	PI 88788	95	5.74	3	−	−	−	+	+	−	−	*w*	−	−	−
19	8	BR-16	105	4.14	4	−	+	−	+	+	−	−	+	+	+	+
20	9	PI 437654	95	6.06	3	−	+	−	+	+	+	+	+	+	+	−
21	10	BR-16	100	3.73	4	+	+	+	+	+	+	+	+	+	+	+
22	11	PI 90763	100	3.76	3	−	+	−	+	+	−	−	+	+	+	*y*
23	15[a]	PI 437654	90	4.38	2[b]	+ [c]	+	−	+	+	+	+	+	+	+	++[d]
24	15	PI 437654	85	6.48	2	−	+	−	+	+	+	+	+	+	*w*	+
25	15	PI 90763	105	5.93	4	−	+	−	+	+	+	+	+	+	+	++
26	22	BR-16	165	6.59	1	−	−	−	−	+	−	−	+	*w*	−	−
27	22	BR-16	155	6.70	0	−	−	−	−	+	−	−	+	*w*	−	+
28	22	BR-16	170	5.86	2	−	+	−	+	+	+	+	+	+	+	−
29	22	Pickett-71	160	6.50	1	−	−	−	−	+	−	−	+	*w*	−	+
30	22	Pickett-71	130	6.44	1	−	+	−	+	+	−	−	+	*w*	−	+

[a] As numbered in Table 1.
[b] Scale ranging from (0) no change in pH to (4) strong acidification.
[c] Absence of growth (−) and growth (+) and *w* indicates weak growth, corresponding to 10–20% of growth in relation to the control.
[d] scale from (+) production of melanin to (++++) very strong production; *y* indicates a yellow dark pigment.

at pH values of 4.0 and 9.5, with similar responses in TY and YM (Table 3). Although bacteria had been isolated from acid soils, some of them with pH 4.0 or below (strains 21, 22, 23, 24 and 25), only 11 strains were able to grow at pH 4.0, while 18 strains tolerated pH 9.5, and seven strains were able to grow at both pH values. The tolerance to acidity was also not related to the soil pH from which the strains were isolated, e.g. only two of the five strains cited above (21 and 23) were acid tolerant. None of the four strains belonging to the group of final pH 6.7–6.9 in YM was able to grow at pH 4.0. In acidification groups 2, 3 and 4, a greater tolerance to pH 4.0 was verified, characterising 28, 46 and 50% of the strains, respectively. Five acid tolerant strains came from undisturbed areas, representing 45% of the isolates from these areas and in cropped soils they represented 32% of the strains. To confirm the high number of strains tolerating alkalinity, a test with buffered medium was performed. An even higher number of strains, 23, were able to grow at pH 8.9, while just two strains, 17 and 21, both from areas never cropped with soyabean, showed growth in buffered medium with pH 4.0 (Table 3).

3.5. Tolerance to salinity, high temperature, growth in LB and synthesis of melanin

Eighteen strains were tolerant to 0.5 M NaCl and all showed at least a weak growth at 37°C (Table 3). Three strains, 11, 15 and 18, were unable to grow at 40°C and did not tolerate higher levels of salinity. These three strains and four others, with weak growth at 40°C (26, 27, 29 and 30), were isolated from cooler subtropical regions, in the states of Paraná and Rio Grande do Sul. However, some strains isolated from these two states (e.g. strains 1 to 10, 17, 19 and 20) were able to grow at 40°C, thus the temperature tolerance was not related to the climate from where the strains came from. Tolerance to high temperature was also not related to mucoidy. The seven strains identified as being able to grow in TY and YM media with pH 4.0 and 9.5 (2, 7, 10, 14, 17, 21 and 23) (Table 3), were also tolerant to salinity and high temperature (Table 3). Twenty strains were able to grow well in LB (Table 3). The synthesis of melanin in tryptophan enriched medium was verified in 16 strains (Table 3) and the treatment to break the cells was not necessary to visualise the dark pigment produced. Five isolates produced an unknown yellow dark pigment and were not considered as positive for this parameter.

3.6. Cluster analysis of morphological and physiological parameters

Twenty parameters shown in Tables 1 and 3 were analysed using the UPGMA method and the Jaccard coefficient. Two great clusters were formed, linked at a 0.218 level of relative similarity (Fig. 1). Cluster II included seven strains isolated from previously inoculated soils from the state of Paraná and had three subclusters considering a 0.383 level of relative similarity. Some common characteristics of strains belonging to cluster II were: white, opaque, <1.0 mm diameter colonies, longer doubling time, inability to grow at pH 4.0 and pH 9.5 (except for strain 30 at pH 9.5), at 0.5 M NaCl and in LB and low acidification of YM (a maximum final pH of 6.44). Within cluster I, considering a level of 0.596, three subclusters were formed and strains 28 and 18 occupied separated positions. Strains from distinct undisturbed areas (AM, DF, RS), shown as circulated numbers, were positioned in subcluster I-2, at the bottom of subcluster I-1 and at the top of subcluster I-3, thus occupying the central part of cluster I, except for strain 18. Strains 17, 21 and 23, tolerant to salinity, acidity, alkalinity and high temperature were also positioned within I-2. Almost all strains from cropped areas under conventional tillage were within subcluster I-1, while in cluster II were most of the strains from soils under the no-tillage management system.

3.7. Growth with different C and N sources

All strains were able to grow well with glucosamine, glucose, glutamic acid, mannitol, mannose, succinate and sucrose, but were unable to use citrate and showed poor growth with dulcitol (data not shown). Differences were detected among the strains in relation to the ability to use the other C sources tested, e.g. 67% were able to grow with fructose and galactose and 43% used ribose. The strains used all compounds tested as sole N sources, except for glycine, but differences were detected in relation to a higher or lower growth capacity with each source (data not shown). However, none of the strains grew satisfactorily on allantoin or any of ten amino acids tested as sole C and N sources. In general the use of C sources strongly acidified the medium, except for an alkaline reaction with malate, pyruvate and succinate, but no appreciable changes in pH were verified with glycerol and starch (data not shown). The cluster analysis, considering 17 C and 24 N sources, produced two major clusters linked at a 0.104 level of relative similarity (Fig. 2). Cluster II included seven strains with a lower capacity for using C sources and a weaker growth with the N compounds tested. Six of these strains came from areas previously inoculated and under the no-tillage management system. Within cluster I, three subclusters were joint at a level of 0.320, and one separate strain, 27, at the level of 0,191. The first six strains from cluster I were isolated from Paraná soils previously cultivated with soyabean, five of them from areas under conventional tillage management; these strains were the most effective in using the C and N sources. Eight of the eleven strains isolated from undisturbed soils were placed in subcluster I-1. The comparison of the matrices with phenotypic and C/N utilisation data resulted in a correlation of $r = 0.626$ ($P < 0.01$).

3.8. Intrinsic resistance to antibiotics and heavy metals

Most strains were resistant to the lower levels of erythromycin (50 $\mu g\ ml^{-1}$), kanamycin (10) and rifampicin (5)

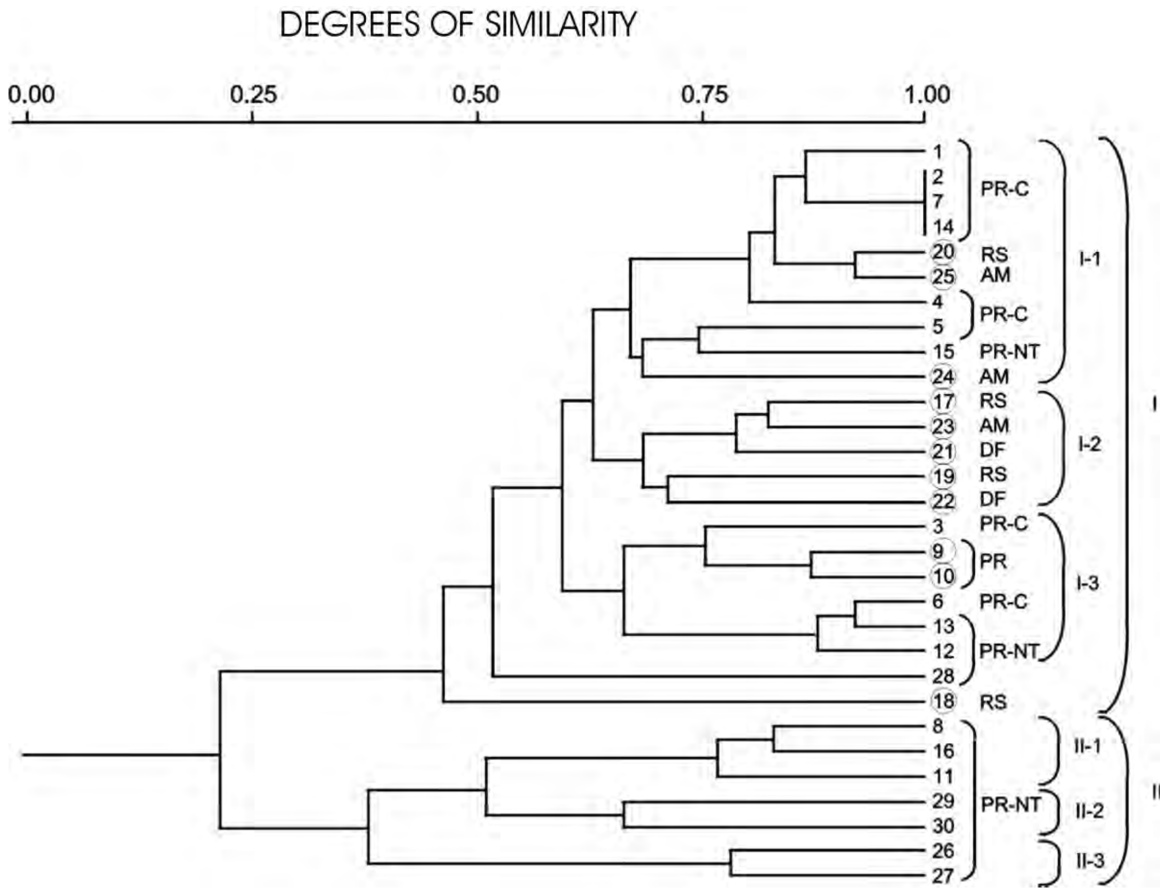

Fig. 1. Dendrogram showing phenotypic similarities of Brazilian rhizobial strains considering 20 morphological and physiological characteristics (UPGMA method and Jaccard coefficient). Strains from undisturbed areas are circulated and the state of origin is shown for each strain; C and NT indicate conventional or no-tillage management; abbreviations for the states are described in Table 1.

(data not shown). The higher levels of each antibiotic tolerated by the majority of the strains were: 10 $\mu g\ ml^{-1}$ of chloramphenicol (57% of the strains), 50 of erythromycin (97%), 20 of gentamicin (60%), 30 of kanamicin (70%), 20 of rifampicin (60%) and 10 of tetracycline (70%). In general, resistance to the levels tested of streptomycin was low. Strains USDA 205 and CCBAU 114 were susceptible to kanamycin at 20 $\mu g\ ml^{-1}$ (data not shown). The cluster analysis has shown that strains fitted into two clusters at a level of 0.408 (data not shown). Cluster I included strains in the following order 1, 5, 7, 15, 2, 10, 21, 24, 14, 20, 19, 23, 28, 26, 27, 30 and 25. Cluster II included the strains 3, 11, 16, 4, 6, 13, 9, 12, 8, 17 and 18. Strains 22 and 29 were separated from the two clusters at relative similarity levels of 0.240 and 0.229, respectively. The correlations obtained between the matrix of intrinsic resistance to antibiotics and the matrices with phenotypic and of C/N utilisation data were $r = 0.333$ and $r = 0.172$, respectively ($P < 0.01$).

The strains were not tolerant to the tested concentrations of nickel and lead, but the majority of the strains showed an intrinsic resistance to 0.5 mM of cobalt chloride (67% of the strains) and 0.25 mM of potassium chromate (65%) (data not shown). Five clusters were formed with these data (not shown). Four clusters were linked at a level of 0.151 and at the top of cluster I were strains 1 and 2, tolerant to six levels of heavy metals. The fifth cluster was far distant and included the strains with no intrinsic resistance to the heavy metals (8, 11, 24 and 27). Low and non-significant coefficients of correlation were obtained between the matrix of tolerance to the heavy metals and the matrices with the phenotypic ($r = -0.066$), C/N utilisation ($r = -0.099$) and intrinsic antibiotic resistance ($r = -0.069$) data. Thus the results for the test of heavy metal tolerance were not considered in the final phenetic dendrogram. However, it is important to state that the cophenetic values obtained for each one of the four matrices cited here were higher than 0.91, indicating an excellent fit of the clusterings to the set of data.

3.9. General phenetic dendrogram

The dendrogram produced with 81 morphological and physiological parameters can be seen in Fig. 3 and the cophenetic value obtained for the matrix was 0.95. Two major phenetic clusters were linked at a relative similarity level of 0.182. Within cluster I, four subclusters were linked at a level of 0.388. The strains isolated from undisturbed soils covered with native vegetation, represented in circulated numbers, were all positioned in the central part of the

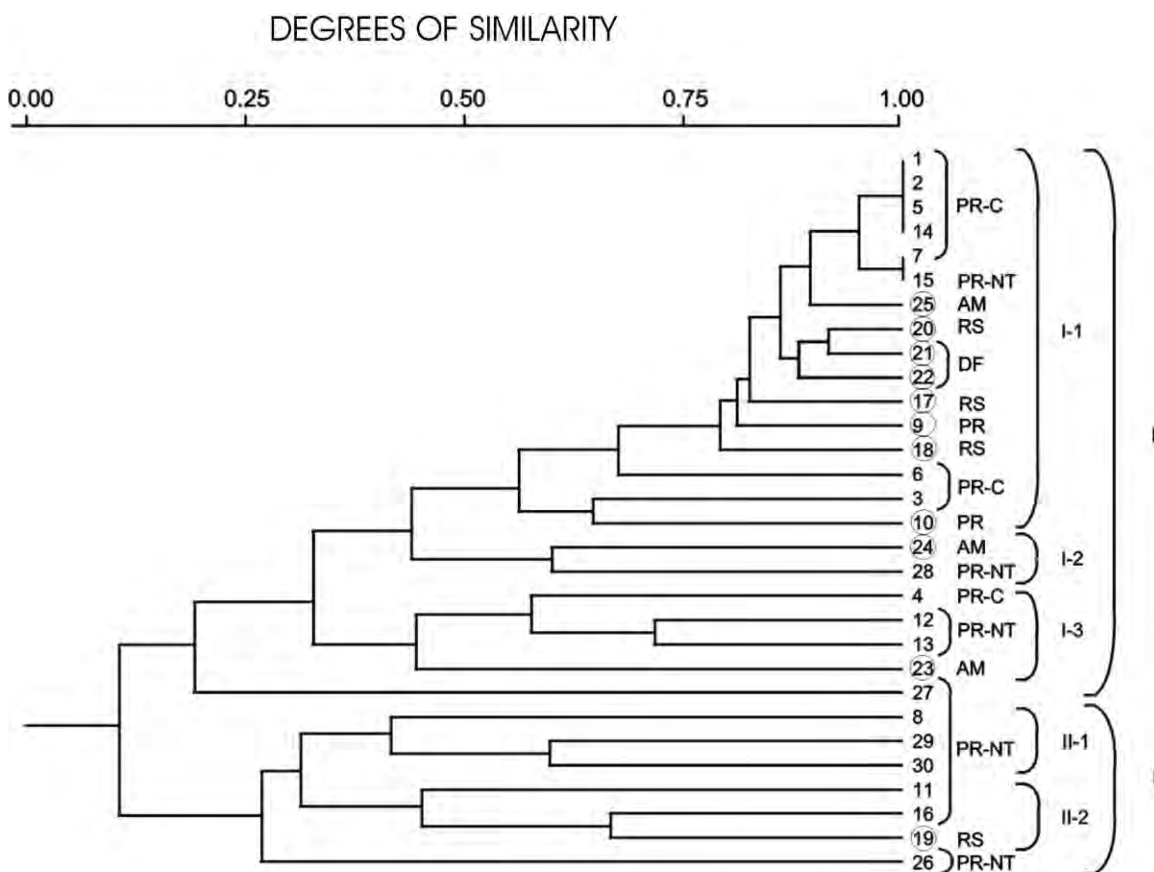

Fig. 2. Dendrogram showing phenotypic similarities of Brazilian rhizobial strains considering the utilisation of 41 compounds as C or N sole sources (UPGMA method and Jaccard coefficient). Strains from undisturbed areas are circulated and the state of origin is shown for each strain; C and NT indicate conventional and no-tillage management; abbreviations for the states are described in Table 1.

dendrogram, at the bottom of subcluster I-1 and within subclusters I-2 and I-4. At the top of subcluster I-1 were positioned six strains from areas cropped with soyabean, five of them from soils under conventional tillage. Cluster II included two subclusters linked at a level of 0.308, with seven strains, all from cropped areas under the no-tillage system.

3.10. Protein and lipopolysaccharide profiles

Strains 7 and 8, both from Paraná, but from soils under different tillage management systems, were the only ones to show similar protein profiles. Every other strain produced a different protein profile and the bands were combined to produce a dendrogram that shows that a low level of relatedness was observed between most of them. The relatedness of the protein profiles of strains from uncropped areas was also very low (Fig. 4). A lower variability was detected in the lipopolysaccharide profiles, but eight different profiles were detected (Fig. 5). Although showing similar protein profiles, strains 7 and 8 were positioned in different LPS groups. The strains with a slower rate of growth, 8, 11, and 16 were classified in groups IV and V of LPS profiles, while most of the fast growers were in groups II, III and IV.

4. Discussion

In Brazil, the combination of a high diversity of native legumes and the low content of soil N result in frequent descriptions of new, effectively nodulating legume species and genera (Moreira, 1994), as well as a great variety of rhizobial strains still to be classified (Moreira et al., 1993). The first experiments performed during crop expansion showed that Brazilian soils were largely devoid of bradyrhizobial strains able to establish an effective symbiosis with soyabean (Lopes et al., 1976). In other field experiments, after some years of soybean cropping, up to one third of the nodules did not react with any known serogroup of bradyrhizobia (Vargas and Hungria, 1997); bacteria inside those nodules could represent indigenous rhizobial strains stimulated by the host plant or that received symbiotic genes from inoculant strains.

There are also no reports of fast growing strains being isolated from soyabean nodules in Brazil. The fast grower *S. fredii* seems to have coevolved with the soyabean plant (Devine, 1985), but the bacteria has a large host range (Stowers and Eaglesham, 1984; Buendía-Clavería et al., 1989) and therefore that species could be found in Brazil, nodulating other native legumines. Furthermore, fast

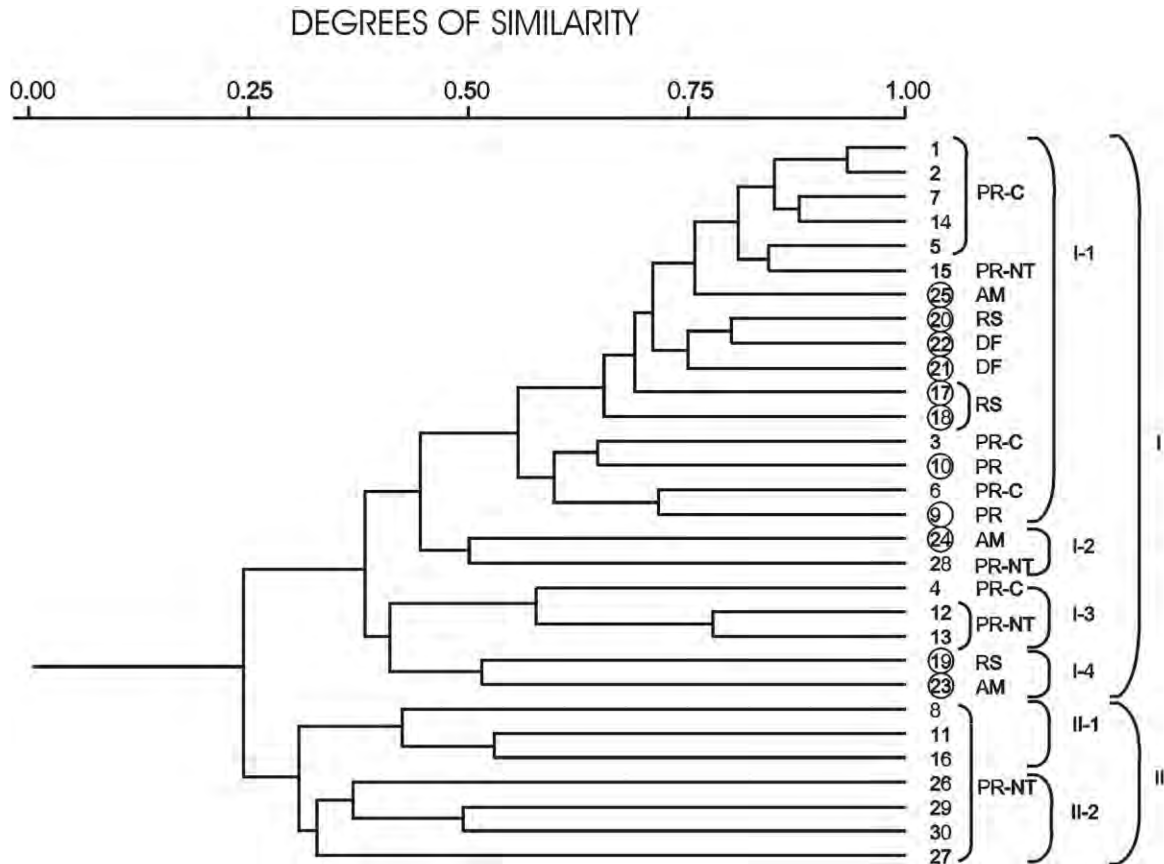

Fig. 3. Phenetic dendrogram (UPGMA method and Jaccard coefficient) showing the similarities among Brazilian rhizobial strains when 81 parameters were considered (20 morphological and physiological characteristics + 41 uses of C and N as sole sources + 20 results of intrinsic antibiotic resistance). Strains from undisturbed areas are circulated and the state of origin is shown for each strain; C and NT indicate conventional and no-tillage management system; abbreviations for the states are described in Table 1.

growing indigenous rhizobia belonging to other species and genera could also be able to effectively nodulate soybean.

In this study, fast growing rhizobial strains were detected in 12 out of the 22 Brazilian soils investigated, being trapped by both Asian and modern soyabean genotypes, and represented up to 24% of the bacteria isolated from nodules. Bacteria had a doubling time of 85–225 min. They showed circular form and convex elevation in YMA medium, as described by Scholla and Elkan (1984) for *S. fredii*, but in contrast to previous reports, abundant mucoidy was verified for most (73%) of the strains. However, the later description of the genus *Sinorhizobium* by Lajudie et al. (1994) also states a copious extracellular polysaccharide slime production.

As for USDA 205 and CCBAU 114, five strains were susceptible to kanamycin at 20 μg ml^{-1} and with three of them the pH in YM after 4 days decreased to values below 6.1, so they would fit into the definition of chemovar *fredii* (Scholla and Elkan, 1984). Within the 25 tolerant strains, only nine produced a pH higher than 6.1 (14 strains considering a pH of 5.99), and could fit into the definition of chemovar *siensis*. Six strains were strong acidifiers, as not previously reported, decreasing the pH to levels below 4.4; the other strains resulted in a final pH of 5.0–6.1.

The majority of the Brazilian strains (60%) showed an intriguing characteristic of growing at pH 9.5, contrary to previous reports by Scholla and Elkan (1984) and of more than 95% of the strains studied by Chen et al. (1988). Although the strains were isolated from acid soils (pH 3.04–5.09), the capacity to grow at pH 4.0 was low, as observed for other *S. fredii* strains (Chen et al., 1988); however, the activity of H^+ ions in culture medium is different from that in the soil, where the charges of the colloids can partially neutralise the activity of the ions. The tolerance to acidity was higher within the strong acidifier group. Although salinity is usually not a problem in Brazilian soils, 60% of the strains were tolerant to 0.5 M NaCl. Other *S. fredii* strains were inhibited by a concentration of 2–3% (0.5 M) NaCl (Scholla and Elkan, 1984; Stowers and Eaglesham, 1984; Buendía-Clavería et al., 1989), but the Vietnamese strain HH 103 also showed some tolerance to salinity (Rodriguez-Navarro et al., 1996). The majority of the strains (77%) tolerated high temperatures, growing at 40°C. Growth in LB was verified in 67% of the strains and this parameter could be used to distinguish groups of strains, e.g. *R. tropici* IIA from *R. tropici* IIB (Martínez-Romero et al., 1991),

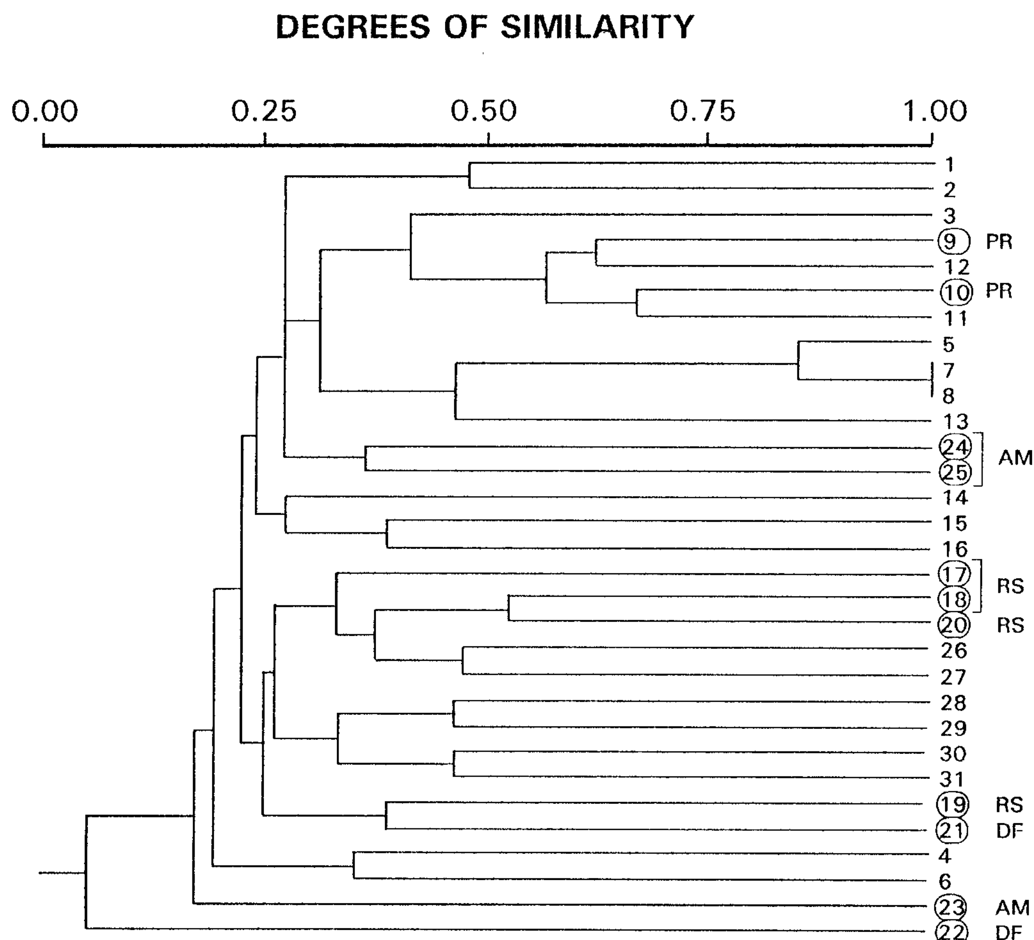

Fig. 4. Dendrogram showing the similarities in protein profiles of the Brazilian rhizobial strains after cluster analysis using the UPGMA method and the Jaccard coefficient. Strains from undisturbed areas are circulated.

although the basis for this ability is still unclear. Most strains were melanin producers (53%), but this is a widespread characteristic among rhizobial strains, with an unknown ecological role (Cubo et al., 1997) and thus is not frequently used to characterise rhizobia groups (Martínez-Romero et al., 1991).

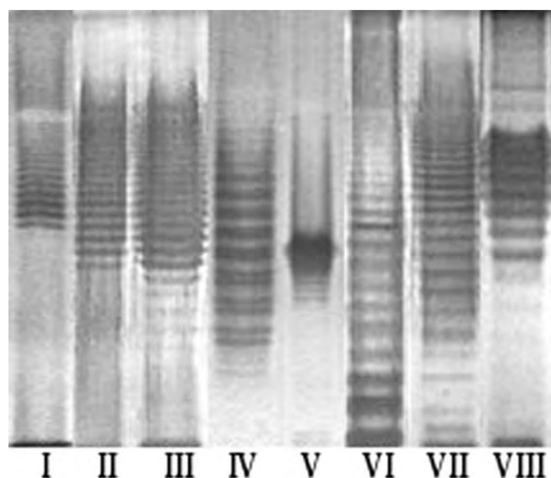

Fig. 5. Groups of lipopolysaccharide profiles of Brazilian rhizobial strains: I (strains 1, 6, 22), II (2, 3, 4, 7, 9, 10, 11, 12, 13, 14), III (5, 15, 23, 24, 25), IV (8, 16), V (11), VI (17, 18, 19, 20, 21), VII (26, 27), VIII (28, 29, 30).

In general, the Brazilian strains were able to use several compounds as sole C sources, as reported for other *S. fredii* strains (Stowers and Eaglesham, 1984; Chen et al., 1988; Buendía-Clavería et al., 1989; Lajudie et al., 1994; Novikova et al., 1994; Rodriguez-Navarro et al., 1996). All strains were able to grow well in the presence of glucosamine, glucose, glutamic acid, mannitol, mannose, sucrose and succinate, but differed in relation to the use of 17 other C sources, e.g. 60% and 43 of the strains were able to use cellobiose and raffinose, respectively. Bacteria were not able to use citrate and had a weak growth with dulcitol, as observed before, for one or both C sources, with other *S. fredii* strains (Stowers and Eaglesham, 1984; Buendía-Clavería et al., 1989; Lajudie et al., 1994; Rodriguez-Navarro et al., 1996; van Berkum et al., 1998). Maltose was used by 87% of the strains, but not by USDA 205 (van Berkum et al., 1998), and they were highly efficient in the use of sucrose, as those described by Buendía-Clavería et al. (1989) and contrary to the sinorhizobia studied by Chen et al. (1988). An acid reaction was produced by the utilisation of most C sources, but an alkaline reaction resulted from the use of malate, pyruvate and succinate, intermediates

of the Krebs cycle, as observed by Stowers and Eaglesham (1984). In a defined medium, supplied with glucose, the strains showed a broader capacity of using several compounds as sole N sources, contrary to USDA 205 (van Berkum et al., 1998). However, although Novikova et al. (1994) reported that USDA 191 was able to use several amino acids as sole C and N sources, none of the Brazilian strains showed this ability.

Both dendrograms obtained with morphological and physiological parameters or with C and N utilisation data, placed the majority of the strains from undisturbed areas into the central subclusters. At the top of the dendrogram were positioned most of the strains from previously inoculated soils maintained under conventional tillage management, while at the bottom were the majority of the strains isolated from soils managed under the no-tillage system. Conventional tillage, involving the traditional practices of plowing and disking to prepare land, leads to reduced soil organic matter and increased erosion, especially in the tropics. As an alternative, no-tillage management protects the soil against erosion by water, improves soil structure stability and moisture content and results in an increase in soil organic matter content (Hungria and Vargas, 2000). It could be that under the lower C content of the no-tillage system the bacteria would have to use a broader range of compounds. Furthermore, the stressing conditions associated with conventional tillage, such as higher temperatures, low soil moisture and low C content (Hungria and Vargas, 2000), could tend to select for strains tolerant to stressful conditions.

Phenotypic characterisation, based on intrinsic antibiotic resistance, has been used both with a view to strain identification and taxonomic classification (Scholla and Elkan, 1984; Chen et al., 1988), as well as for ecological purposes (Josey et al., 1979). The levels of antibiotics used in this study were based on previous studies with *S. fredii* (Stowers and Eaglesham, 1984; Rodriguez-Navarro et al., 1996) and on several studies of natural resistance of Brazilian native rhizobia strains. In general, Brazilian rhizobia showed a higher intrinsic antibiotic resistance than other *S. fredii* strains (Scholla and Elkan, 1984; Stowers and Eaglesham, 1984; Chen et al., 1988; Rodriguez-Navarro et al., 1996), however, in ecological terms, it was not possible to draw a conclusive picture about the results obtained with the antibiotics. Strains were also tolerant to the heavy metals (in mM) cobalt chloride (0.5) and potassium chromate (0.25).

This paper describes, for the first time, the isolation of fast growing strains from soyabean nodules in Brazil. The preliminary phenetic analyses performed with the strains indicate that they have unique properties, differing from *S. fredii* in several characteristics. Protein and LPS profiles indicated that each one was a unique strain. Those strains isolated from undisturbed areas were phenetically related to each other even when isolated from far distant regions, indicating a common background. With soyabean cropping, the relatedness among the strains decreased. The basic criteria of forming effective nodules on Asian and modern soyabean genotypes was confirmed for all 30 strains. Lajudie et al. (1994), using electrophoretic protein patterns, classified other Brazilian strains isolated from *Leucaena leucocephala* nodules (BR 811, BR 817) as *S. fredii*. However, those strains do not establish an effective symbiosis with soyabean (data not shown), so their classification as *S. fredii* should be discussed. There are promiscuous strains, such as NGR 234, able to nodulate a wide range of legumes, some of them evolutionarily divergent (Stanley and Cervantes, 1991); that strain was classified as *S. fredii* by the analysis of cellular fatty acids (Jarvis and Tighe, 1994). Soyabean is an exotic plant in Brazil, and the strains isolated in this study might be broad host range symbionts of native legumes or might belong to other rhizobial species. Indeed, recently Chen et al. (2000) reported the isolation of fast growing strains from soybean nodules in Paraguay, which shares borders with Brazil; the 16S rDNA sequencing of those strains showed genetic similarity with *Rhizobium tropici*, a symbiont of common bean (*Phaseolus vulgaris* L.) thought to be native of South America.

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