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Nitrogen fixation capacity and nodule occupancy by *Bradyrhizobium japonicum* and *B. elkanii* strains

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Abstract In a previous study soybean *Bradyrhizobium* strains, used in Brazilian studies and inoculants over the last 30 years, and strains adapted to the Brazilian Cerrados, a region frequently submitted to environmental and nutritional stresses, were analyzed for 32 morphological and physiological parameters *in vivo* and *in vitro*. A cluster analysis allowed the subdivision of these strains into species *Bradyrhizobium japonicum, Bradyrhizobium elkanii* and a mixed genotype. In this study, the bacteria were analyzed for nodulation, N_2 fixation capacity, nodule occupancy and the ability to increase yield. The goal was to find a relationship between the strain groups and the symbiotic performance. Two strains of Brazilian *B. japonicum* showed higher rates of N_2 fixation and nodule efficiency (mg of N mg^{-1} of nodules) under axenic conditions. These strains also showed greater yield increases in field experiments when compared to *B. elkanii* strains. However, no differences were detected between *B. japonicum* and *B. elkanii* strains when comparing nodule occupancy capacity. The adapted strains belonging to the serogroup *B. elkanii* SEMIA 566, most clustered in a mixed genotype, were more competitive than the parental strain, and some showed a higher capacity of N_2 fixation. Some of the adapted strains, such as S-370 and S-372, have shown similar N_2 fixation rates and nodulation competitiveness to two Brazilian strains of *B. japonicum.* This similarity demonstrates the possibility of enhancing N_2 fixing ability, after local adaptation, even within *B. elkanii* species. Differences in the DNA profiles were also detected between the parental SEMIA 566 and the adapted strains by analyses with the ERIC and REP-PCR techniques. Consequently, genetic, morphological and physiological changes can be a result of adaptation of rhizobia to the soil. This variability can be used to select strains capable of increasing the contribution of N_2 fixation to soybean nutrition.

Key words *Bradyrhizobium japonicum* · *Bradyrhizobium elkanii* · Competitiveness · Nitrogen fixation · Soybean

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Introduction

Biological N_2 fixation with *Bradyrhizobium* strains can supply the N demand of soybean [*Glycine max* (L.) Merr.] plants. In Brazil, large-scale soybean cultivation and inoculation began in the 1960s with strains brought from other countries, espicially the United States. Since then, several promising imported bradyrhizobia have been tested, but emphasis has also been given to programs aimed at selecting strains adapted to Brazilian soils, isolated from areas which have been previously inoculated. The parameters evaluated for such selection are competitiveness and yield in the field. Today, four strains are officially recommended for use in commercial inoculants in Brazil, and all of them were selected from areas previously inoculated with foreign inoculants (Hungria et al. 1994).

From 1932 to 1980 the bacteria able to nodulate soybean were named *Rhizobium japonicum* (Fred et al. 1932; Buchanan 1980). In 1982 they were reclassified to a new genus, *Bradyrhizobium.* During the 1980s, physiological, biochemical and genetic differences among strains within the *Bradyrhizobium japonicum* species were reported. Genetic differences were detected after hybridization with *hup* (Minamisawa 1990), *nod* and *nif* genes (Stanley et al. 1985; Minamisawa 1990; Minamisawa and Fukai 1991), and sequences of 16S rRNA (Young et al. 1991) and Rs_{α} (Minamisawa et al. 1992). Examples of non-symbiotic differences were also reported in colony morphology (Fuhrmann 1990), profile of fatty acids (Kuykendall et al. 1988), intrinsic anti-

biotic resistance (Kuykendall et al. 1988), extracellular polysaccharide composition (Huber et al. 1984) and synthesis of rhizobitoxine and indole acetic acid (IAA) (Minamisawa 1989; Minamisawa and Fukai 1991). Symbiotic differences include infection ability with rj_1rj_1 and Rj_4 soybean genotypes and with peanut (Devine et al. 1983, 1990). The extensive characterization of soybean bradyrhizobia subdivided them into two species: *B. japonicum* and *B. elkanii* (Kuykendall et al. 1992). Strains with intermediate characteristics were reported by Minamisawa (1989). Furthermore, based on the degree of sequence divergence in and around the common *nod* gene region, Yokoyama et al. (1996) showed that some soybean bradyrhizobia originating from Thailand were clustered into two groups which did not correspond to any known homology group. Consequently, taxonomic studies on soybean bradyrhizobia have reported important and new information over the few last years.

Few studies have correlated *Bradyrhizobium* species with N_2 fixation. Hypothetically, since the Hup^+ phenotype and the absence of rhizobitoxine toxicity are restricted to *B. japonicum,* this species could be more efficient (Fuhrmann 1990; Teaney and Fuhrmann 1992; Vasilas and Fuhrmann 1993). Under axenic conditions, Minamisawa et al. (1993) reported that *B. japonicum* is more competitive than *B. elkanii* with *G. max* but less competitive with *Glycine soja* and *Macroptilium atropurpureum.*

In earlier studies using several non-symbiotic and symbiotic parameters, we classified the main strains, used in Brazilian studies and inoculants over the last 30 years within the species *B. japonicum, B. elkanii* and a mixed genotype group (Boddey and Hungria 1997). Those strains were now investigated in relation to nodulation ability, N_2 fixation capacity and nodule occupancy. The main purpose was to investigate a possible relationship between species and symbiotic performance.

Materials and methods

Rhizobial strains and plant genotypes

Thirty-eight strains were used in this study. Their sources and main characteristics were described previously (Boddey and Hungria 1997). Seven strains represented *B. japonicum* species (USDA 110, USDA 122 and USDA 123), a mixed genotype (USDA 73) and *B. elkanii* (USDA 31, USDA 76 and USDA 94). Another 14 strains represented the main strains used in Brazilian studies and/or inoculants over the last 30 years and were denominated "Brazilian" strains. Finally, 17 strains represented strains adapted to the soils of Cerrado, a Brazilian edaphic type of savanna. These adapted strains are natural variants of strain SEMIA 566 which was used in Brazilian inoculants from 1966 to 1978.

Brazilian strains and adapted strains were previously classified (Boddey and Hungria 1997) as *B. japonicum* (CB 1809 and CPAC 7), a mixed genotype (13 adapted strains: S-127, S-204, S-340, S-370, S-372, S-406, S-452, S-468, S-478, S-481, S-490, S-506, and S-516) or as *B. elkanii* (SEMIA 587, INPA 37, DF 383, DF 395, 29w, SEMIA 566, R 54-a, SM_1b, NC 1005, 532C, 965, CPAC 15, and four adapted strains: S-220, S-273, S-335, S-381).

Two soybean [*G. max* (L.) Merrill] cultivars were utilized (genealogy in parentheses): BR-16 (D69-B10-M58 X Davis) and BR-37 (União (2) X Lo 76-1763).

N_2 fixation capacity

Each strain was grown in a yeast mannitol (YM) medium (Vincent 1970) for 7 days at 28 °C and equalized to a concentration of 10^9 cells ml^{-1}. Soybean seeds of cultivar BR-16 were surface-sterilized (Vincent 1970) and incubated with the inoculum (1 ml $seed^{-1}$) for 30 min. Four seeds were sown per modified Leonard jar (Vincent 1970). Each jar contained sand and vermiculite (1:2, v:v) and was filled with N-free nutrient solution (Somasegaran and Hoben 1985). Plants were grown under greenhouse conditions with a 12 h photoperiod and a temperature of 28/23 °C (day/night, with a standard deviation of ±2.5 °C) and were thinned to two plants per jar 4 days after emergence (DAE). Nutrient solution was completed every other day and plants were harvested at 40 DAE, when maximum nodule mass was achieved with cultivar BR-16. The parameters evaluated were nodule number and dry weight, shoot and root dry weight and total N. The N content was determined by the indophenol blue colorimetric method of Feije and Anger (1972). Total N accumulated by plants was calculated as follows: Total N in shoots + total N in roots − total N in seeds. The experiment was performed in a randomized block design with five replications and statistically analyzed using the Tukey's test ($P \leq 0.05$).

Nodule occupancy

The experiment testing nodule occupancy was performed as described in the previous section except that each strain (10^9 cells ml^{-1}) was inoculated in a 1:1 proportion with strain 29w (10^9 cells ml^{-1}). Strain 29w is highly competitive and belongs to a different serogroup than the other strains. Added to the parameters analyzed in the previous experiment, 60 nodules were randomly collected per treatment and analyzed for serological reactions (Somasegaran and Hoben 1985) against the antisera of each inoculated strain. The experiment was performed in a randomized block design with five replications. Plants were collected at 40 DAE and the results were statistically analyzed using the Tukey's test ($P \leq 0.05$).

Genetic characterization of adapted strains

Analyses were performed with bacterial genomic DNA and amplified by the PCR technique with the primers REP and ERIC, as described by de Bruijn (1992).

Field trials

Field experiments were conducted in Oxisols at Londrina and Ponta Grossa in the State of Paraná, Brazil. The soils contained the chemical properties shown in Table 1.

The experimental plots measured 3.0 m × 2.0 m with 0.5 m between lines. Plots were separated by 2.0 m and small terraces. Five days before sowing, plots received 300 kg ha^{-1} of N-P-K (0-28-20) and 40 kg ha^{-1} 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 counting technique (Vincent 1970) in soybean plants, was estimated in 10^4 cells g^{-1} of soil at Londrina and 10^3 cells g^{-1} of soil at Ponta Grossa.

Inoculants were prepared to a density of 10^9 cells ml^{-1} in a semi-solid YM, adding 100 ml of inoculant kg^{-1} of seeds. Treatments consisted of one or two strains representing each of the following groups: *B. japonicum* (USDA 110 and USDA 122),

Table 1 Chemical properties of the soils

Depth cm	pH in $CaCl_2$	N	Al	K	Ca	Mg	H+Al	Al	C	P
		g dm^{-3}	$cmol_c$ dm^{-3}					%		mg dm^{-3}
					Londrina					
0–20	5.20	0.13	0.00	0.35	5.19	1.67	4.16	0.00	2.73	5.70
20–40	4.79	0.08	0.00	0.19	3.56	1.44	4.26	0.00	1.30	1.75
					Ponta Grossa					
0–20	4.99	0.10	0.05	0.25	3.19	2.05	5.71	0.91	2.36	5.20
20–40	4.47	0.06	0.23	0.20	1.81	1.35	6.78	6.40	1.22	1.22

mixed genotype (USDA 73), *B. elkanii* (USDA 31 and USDA 76), Brazilian *B. japonicum* strains (CB 1809 and CPAC 7), Brazilian *B. elkanii* strain SEMIA 566, adapted strains of the mixed genotype (S-370 and S-372), Brazilian *B. elkanii* strains (29w and SEMIA 587) and adapted strains of *B. elkanii* (S-273 and S-381). Two controls were included: one without inoculation and another without inoculation but receiving 200 kg of N ha^{-1} as urea, divided into two applications of 100 kg N ha^{-1} at sowing and at R2 (open flower at one of the two uppermost nodes on the main stem with a fully developed leaf), respectively. The cultivars used were BR-16 and BR-37 at Londrina and Ponta Grossa, respectively.

The experiments were performed in a randomized block design with six replications. At R2 stage, 15 plants of each treatment were harvested, and nodule number and dry weight were evaluated. Sixty nodules were randomly chosen per treatment for serological analysis of agglutination against the antisera of the corresponding strains for that treatment. At the final harvest, yield and N content of grains were evaluated, and the values were corrected for 13% moisture. The results were statistically analyzed using the Tukey's test ($P \leq 0.05$).

Results

Under axenic conditions, at 40 DAE, *B. japonicum* reference strains averaged 61 nodules $plant^{-1}$ weighing 219 mg. *B. elkanii* reference strains formed 69 nodules $plant^{-1}$ weighing 257 mg (Table 2). Mean values of nodule number and dry weight of Brazilian *B. elkanii* strains (55 nodules weighing 233 mg) and adapted strains (59 nodules weighing 260 mg) were slightly higher than the adapted strains classified in the mixed genotype (53 nodules weighing 224 mg). Consequently, the different groups of this study were not characterized by contrasting responses in nodulation parameters.

B. japonicum reference strains allowed an accumulation of 84 mg N $plant^{-1}$. An intermediate position was achieved by USDA 73 with 75 mg N plant. *B. elkanii* reference strains accumulated 47 mg N $plant^{-1}$, a value 44% lower than *B. japonicum* reference strains (Table 2). The highest rate of N_2 fixation, 107 mg N $plant^{-1}$, was verified by the inoculation with the group of Brazilian *B. japonicum* strains. Great variability in the N accumulation capacity was detected within the Brazilian strains classified in the mixed genotype. Accumulation capacity ranged from 30 (S-452) to 108 mg N (S-370) $plant^{-1}$. *B. elkanii* Brazilian strains were a more homogeneous group, fixing a mean value of 78 mg N $plant^{-1}$. A slightly lower content resulted from the inoculation with *B. elkanii* adapted strains (70 mg N $plant^{-1}$).

Remarkable differences were verified between *B. japonicum* and *B. elkanii* reference strains on nodule efficiency, with mean values of 0.387 and 0.182 mg N mg^{-1} of nodules, respectively (Table 2). Brazilian *B. japonicum* and *B. elkanii* strains also differed in relation to this parameter, nodule efficiency being 0.498 and 0.341, respectively. A lower nodule efficiency was observed on plants inoculated with the adapted strains belonging to the mixed genotype (0.289) and to *B. elkanii* (0.271 mg N mg^{-1} of nodules). However, some adapted strains of the mixed genotype achieved a high nodule efficiency, e.g. S-370 and S-468, with 0.514 and 0.485 mg N mg^{-1} of nodules, respectively.

When co-inoculated with the strain 29w, nodule occupancy by reference strains of *B. japonicum* was similar to that of *B. elkanii.* Both species were able to occupy a mean of 45% of the nodules (Table 2). USDA 73, a mixed genotype, was not very competitive. It occupied just 27% of the nodules, while higher values were found with Brazilian *B. elkanii* strains (53%) and adapted strains (69%). Adapted strains of the mixed genotype formed the outstanding group in terms of nodule occupancy. They were able to occupy an average of 85% of the nodules. Some strains of this group were able to occupy up to 90% of the nodules (S-340, S-370, S-406, S-478 and S-506), and S-490 occupied 100% of the nodules.

With the use of repetitive primers ERIC (data not shown) and REP (Fig. 1) associated with the PCR technique, it was possible to confirm that the "S" adapted strains were derived from strain SEMIA 566. However, several modifications in the DNA profile were clearly detected and probably resulted from the process of adaptation to the soil.

In field trials at Londrina (Table 3) and Ponta Grossa (Table 4), strains belonging to serogroup SEMIA 566 showed an ability to increase nodule number, mass and occupancy in soils with high populations of naturalized bradyrhizobia. The two strains of serogroup SEMIA 566 classified within the mixed genotype (S-370 and S-372), chosen for the experiment because of the outstanding performance in the greenhouse experiment (Table 2), were able to increase yield and total N in the

Table 2 Nodule number (*NN*, number plant^{-1}), nodule dry weight (*NDW*, mg nodule plant^{-1}), total N accumulated by plants (*TNP*, mg of N plant^{-1}) and nodule efficiency (*NE*, mg of N mg^{-1} of nodules) of soybean cultivar BR-16 inoculated with 38 *Bradyrizobium* strains. Also nodule occupancy by each strain when co-inoculated with 29w (1:1, v:v, 10^9 cells ml^{-1}). Plants harvested at 40 days after emergence

Strains	N_2 fixation capacity				Nodule occupancy (%)
	NN	NDW	TNP	NE	
			Reference strains of B. japonicum		
USDA 110	58.2 d–i[a]	200.5 f–j	96.5 bcd	0.481 ab	37.8 i–l
USDA 122	61.1 c–f	225.4 c–i	77.7 f–j	0.345 d–i	48.4 h–k
USDA 123	62.8 c–f	231.1 b–h	77.1 f–k	0.334 e–i	50.0 g–k
			Reference strain of the mixed genotype		
USDA 73	50.4 h–k	188.2 g–j	75.0 g–l	0.398 b–e	27.4 l
			Reference strains of B. elkanii		
USDA 31	70.4 abc	283.8 a–d	50.4 no	0.178 lmn	37.8 i–l
USDA 76	75.6 ab	263.5 a–f	60.2 lmn	0.228 j–n	49.9 g–k
USDA 94	62.1 c–f	223.6 d–i	31.1 p	0.139 n	48.6 h–k
			Brazilian B. japonicum strains		
CB 1809	66.7 a–d	198.2 f–j	93.5 b–e	0.472 abc	34.8 jkl
CPAC 7	76.7 a	230.0 b–h	120.6 a	0.524 a	50.2 g–k
			Brazilian adapted isolates of a mixed genotype		
S-127	64.9 cde	280.3 a–d	60.1 lmn	0.214 j–n	53.5 g–j
S-204	38.7 m	155.8 j	35.5 op	0.228 j–n	47.6 h–k
S-340	42.5 klm	200.2 f–j	50.3 no	0.251 h–m	97.2 ab
S-370	44.0 j–m	210.6 e–j	108.2 ab	0.514 a	97.3 ab
S-372	60.9 f–j	250.6 a–g	97.6 bc	0.389 b–f	81.6 a–d
S-406	51.1 g–k	230.4 b–h	42.7 nop	0.185 lmn	92.4 abc
S-452	55.3 e–i	180.2 hij	30.4 p	0.169 mn	79.9 b–e
S-468	48.6 i–m	176.2 hij	85.5 c–g	0.485 ab	86.2 a–d
S-478	70.3 abc	290.4 ab	86.3 c–g	0.297 f–k	97.4 ab
S-481	50.7 h–k	250.6 a–g	62.2 k–n	0.248 i–m	89.3 a–d
S-490	59.3 d–h	258.7 a–f	75.5 g–k	0.298 e–k	100.0 a
S-506	60.7 c–g	260.2 a–f	66.9 i–m	0.257 h–m	96.8 ab
S-516	40.2 lm	162.2 i–j	35.5 op	0.219 j–n	86.9 a–d
			Brazilian B. elkanii strains		
NC 1005	66.4 bcd	290.6 ab	88.8 c–g	0.306 e–k	74.9 c–f
532C	68.2 a–d	299.3 a	80.7 e–i	0.270 h–1	61.4 e–h
29w	55.8 e–i	230.8 b–h	90.6 c–f	0.392 b–f	–
R 54-a	53.9 f–i	186.4 g–j	66.3 i–m	0.355 d–h	59.3 fgh
SEMIA 587	69.9 abc	288.8 abc	75.9 f–k	0.263 h–m	56.8 f–i
INPA 37	55.5 e–i	210.8 e–j	80.0 e–i	0.380 c–g	49.4 g–h
DF 395	49.7 h–i	220.0 d–j	75.5 g–k	0.343 d–i	33.3 kl
SM_1b	48.5 i–m	199.5 f–j	78.0 f–j	0.391 b–f	51.3 g–k
965	50.4 h–k	183.6 hij	65.4 j–m	0.356 d–h	48.6 h–k
DF 383	50.3 h–k	177.4 hij	77.7 f–j	0.438 a–d	46.8 h–1
SEMIA 566	48.5 i–m	240.2 a–h	68.5 h–m	0.285 g–k	50.9 g–k
CPAC 15	50.7 h–k	265.7 a–e	82.5 d–h	0.310 e–j	47.8 h–k
			Brazilian adapted isolates of B. elkanii		
S-220	62.2 c–f	265.7 a–e	55.4 mn	0.208 k–n	54.8 ghi
S-273	55.8 e–i	260.0 a–f	59.7 mn	0.230 j–n	56.7 f–i
S-335	65.0 cde	271.4 a–e	90.7 c–f	0.334 e–i	93.5 abc
S-381	53.1 f–g	241.2 a–h	75.6 g–k	0.313 e–j	69.4 d–g
CV (%)	12.1	25.3	13.2	18.5	26.1

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

grains (Tables 3, 4). A good, but lower, symbiotic performance was also achieved by S-271 and S-281. The adapted strains, classified as *B. elkanii,* gave yields comparable to the mixture of 29w + SEMIA 587, a combination used in Brazilian commercial inoculants.

No differences were detected between the reference strains of *B. japonicum* (USDA 110 + USDA 122) and *B. elkanii* (USDA 31 + USDA 76) on nodulation and nodule occupancy in the field (Tables 3, 4). However, yield and total N accumulated in grains were higher with *B. japonicum* strains. This confirms the higher rates of N_2 fixation and nodule efficiency observed under axenic conditions (Table 2). In field trials at Londrina and Ponta Grossa, the highest yields and N accu-

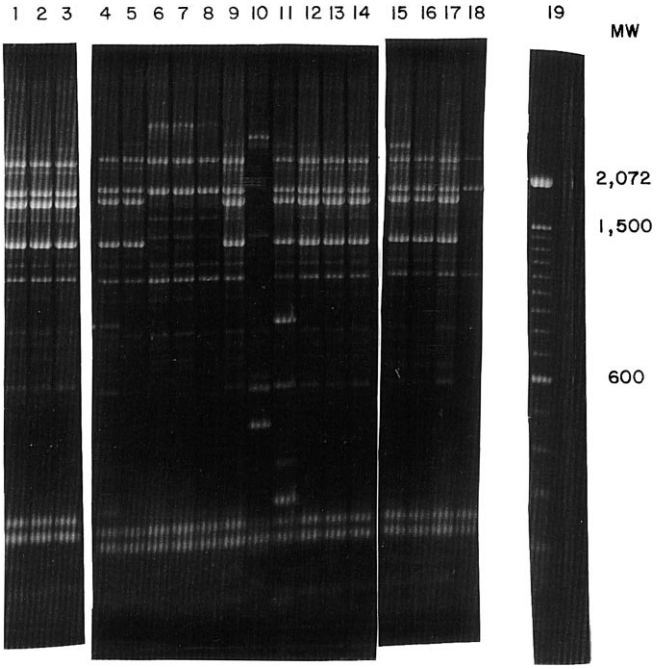

Fig. 1 REP-PCR fingerprint patterns of genomic DNA from the parental *Bradyrhizobium elkanii* strain SEMIA 566 and the adapted strains of this serogroup isolated from Cerrados soils. Lanes: *1* SEMIA 566, *2* S-506, *3* S-516, *4* S-335, *5* S-340, *6* S-370, *7* S-372, *8* S-381, *9* S-406, *10* S-452, *11* S-468, *12* S-478, *13* S-481, *14* S-490, *15* S-204, *16* S-220, *17* S-273, *18* S-204, *19* DNA ladder

mulation were achieved with the combination of CB 1809 and CPAC 7, also classified as *B. japonicum.*

Discussion

In a previous study, these 38 strains were grouped according to 32 morphological, serological and physiological parameters (Boddey and Hungria 1997). The phenotypic grouping positioned most of the strains utilized in Brazilian studies and inoculants within the *B. elkanii* species. Two strains, CB 1809, brought from Australia in 1966, and CPAC 7, which belongs to the same serogroup as CB 1809, fit within the species *B. japonicum.* Several isolates from the Cerrados region, inoculated several years before the study with strain SEMIA 566, have occupied an intermediate position between the two defined species and were called a mixed genotype. The main characteristics of this mixed genotype, in relation to the *B. elkanii* species, were a lower capacity to synthesize IAA *in vitro* and a milder chlorosis in soybean leaves due to the toxicity caused by rhizobitoxine.

Since some parameters evaluated in the previous study were easily detected and quantified, such as synthesis of IAA, a possible relationship between any parameter evaluating, *in vitro* or at an early stage of plant growth, N_2 fixation capacity or competitiveness would mitigate the selection program for better soybean bradyrhizobia.

Under axenic and field conditions, *B. japonicum* showed a higher capacity for N_2 fixation and higher nodule efficiency than *B. elkanii* strains. Although only two strains of Brazilian *B. japonicum* were used, Brazilian *B. japonicum* and *B. elkanii* strains showed the same patterns. Previous studies indicated that *B. japonicum* strains would show a higher capacity of N_2 fixation (Minamisawa 1989; Fuhrmann 1990; Teaney and Fuhrmann 1992; Vasilas and Fuhrmann 1993). The higher efficiency of *B. japonicum* could be the result of hydrogenase activity or the absence of rhizobitoxine (Minamisawa 1989). The importance of hydrogenase for a better symbiotic performance with soybean has been shown by comparing plants inoculated with isogenic strains for the Hup phenotype (Albrecht et al.

Table 3 Effects of inoculation of soybean cultivar BR-16 with eight groups of *Bradyrhizobium japonicum/B. elkanii* strains on nodule number (*NN*), dry weight (*NDW*) and nodule occupancy by the inoculated strains at the R2 stage and yield and total N in grains (*TNG*). Experiment performed on an Oxisol of Londrina, State of Paraná, containing 10^4 cells g^{-1} of soil

Treatment	NN	NDW	Occupancy (%)		Yield	TNG
	(number pl^{-1})	(mg pl^{-1})	Before[a]	R2[a]	(kg ha^{-1})	(kgN ha^{-1})
USDA 110 + USDA 122	38 ab[b]	125 ab	3	32	3248 bc	211 bcd
USDA 73	25 b	95 b	0	15	2880 c	170 fg
USDA 31 + USDA 76	40 ab	121 ab	2	35	3005 c	186 def
CB 1809 + CPAC 7	39 ab	118 ab	11	45	3789 a	248 a
SEMIA 566	53 a	140 a	33	54	2998 c	175 efg
S-370 + S-372	54 a	144 a	33	78	3699 a	230 ab
29w + SEMIA 587	47 a	128 ab	35	65	3110 bc	190 def
S-271 + S-381	52 a	155 a	33	60	3186 bc	197 cde
Non-inoculated	38 ab	99 b	–	–	2549 d	153 g
Non-inoculated + N[c]	11 b	28 c	–	–	3455 ab	221 bc
CV (%)	29	31	18	38	11.2	9.5

[a] Nodule occupancy by the serogroup of inoculated strains when the experiment was setup and after inoculation at the R2 stage. All treatments significantly increased nodule occupancy (Tukey, $P \leq 0.05$)

[b] Means of six replicates and values followed by the same letter were not statistically different (Tukey, $P \leq 0.05$)

[c] 200 kg of N ha^{-1} as urea, split twice, half at sowing and half at flowering

Table 4 Effects of inoculation of soybean cultivar BR-37 with eight groups of *B. japonicum/B. elkanii* strains on nodule number (*NN*), dry weight (*NDW*) and nodule occupancy by the inoculated strains at the R2 stage and yield and total N in grains (*TNG*). Experiment performed on an Oxisol of Ponta Grossa, State of Paraná, containing 10^3 cells g^{-1} of soil

Treatment	NN	NDW	Occupancy (%)		Yield	TNG
	(number pl^{-1})	(mg pl^{-1})	Before[a]	R2[a]	(kg ha^{-1})	(kgN ha^{-1})
USDA 110 + USDA 122	44 abc[b]	145 a	5	41	2880 ab	190 ab
USDA 73	38 bc	115 ab	0	18	2012 d	119 d
USDA 31 + USDA 76	52 ab	151 a	0	37	2420 c	162 c
CB 1809 + CPAC 7	45 abc	140 a	8	44	3215 a	209 a
SEMIA 566	60 ab	150 a	35	52	2010 d	120 d
S-370 + S-372	65 ab	162 a	35	81	3200 a	198 a
29w + SEMIA 587	58 ab	149 a	35	54	2850 b	174 bc
S-271 + S-381	69 a	168 a	35	63	2845 b	176 bc
Non-inoculated	24 cd	78 bc	–	–	1834 d	110 d
Non-inoculated + N[c]	8 d	25 c	–	–	2925 ab	189 ab
CV (%)	38	35	24	39	10.8	9.8

[a] Nodule occupancy by the serogroup of inoculated strains when the experiment was setup and after inoculation at the R2 stage. All treatments significantly increased nodule occupancy (Tukey, $P \leq 0.05$)

[b] Means of six replicates and values followed by the same letter were not statistically different (Tukey, $P \leq 0.05$)

[c] 200 kg of N ha^{-1} as urea, split twice, half at sowing and half at R2

1979; Hanus et al, 1981; Hungria et al. 1989). As to the rhizobitoxine, any stress at the initial stage of nodulation and plant growth may prejudice plants.

Minamisawa et al. (1993) observed that *B. japonicum* was more competitive with several soybean cultivars, white *B. elkanii* was more competitive with the non-bred cultivar Peking, *G. soja* (the ancestral soybean), and *M. atropurpureum.* In surveys of soybean bradyrhizobia in several soils, Carter et al. (1978) and Brewin (1984) found that Hup^- strains represented the majority of the strains. This suggested a higher ability to survive or compete against soil microorganisms. A similar ecological role could also be associated with the synthesis of rhizobitoxine, as suggested by Minamisawa (1990). However, in this study, no differences were detected, under axenic or field conditions, between the two species when comparing nodule occupancy. Consequently, competitiveness seems to be a more complex trait, and an apparent relationship with the species was not shown by the results obtained here.

Strain SEMIA 566 was isolated in 1966 in an area which had been previously inoculated with a North American inoculant. This strain was used in commercial inoculants until 1978. The "S" strains were isolated from a Brazilian Cerrados soil from an area which had been inoculated a decade before with SEMIA 566. Since the area was originally free of soybean bradyrhizobia and the "S" strains are serologically related to SEMIA 566, they should all be genetically related. This was confirmed by the ERIC and REP-PCR profiles. However, the DNA amplification also detected several modifications in the adapted strains. This data is similar to reports for strains of serogroup *B. japonicum* USDA 123 in the United States (Judd et al. 1993). The Cerrados often experience stressful conditions, such as high temperatures (>40 °C), low soil moisture, poor soil fertility and aluminum toxicity. Consequently, during the process of adaptation to inhospitable environmental conditions, several morphological, physiological and genetic characteristics can be changed, as observed in this and other studies (Hungria and Vargas 1996; Hungria et al. 1996; Nishi et al. 1996; Boddey and Hungria 1997). Physiological changes of "S" strains, in relation to the parental SEMIA 566, include a lower synthesis of IAA and rhizobitoxine (Boddey and Hungria 1997), but the ecological importance of these characteristics is still unknown.

This study also showed differences between the parental SEMIA 566 and the adapted "S" strains in symbiotic properties such as N_2 fixation capacity, competitiveness and agronomic traits. Some of the adapted strains were characterized by higher rates of N_2 fixation, and all of them were equally or far more competitive than the parental SEMIA 566. In soils with naturalized populations, a combination of S-370 and S-372, identified as good N_2 fixers under axenic conditions, was able to increase nodule occupancy, resulting in higher yield and total N in grains. Consequently, a great variability was detected after the adaptation of *Bradyrhizobium* strains to the soil. This variability can be used to select strains that are able to increase the contribution of N_2 fixation to soybean nutrition.

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References

Albrecht SL, Maier RJ, Hanus FJ, Russell SA, Elmerich DW, Evans HJ (1979) Hydrogenase of *Rhizobium japonicum* increases nitrogen fixation by nodulated soybeans. Science 203:1255–1257

Boddey LH, Hungria M (1997) Determination of characteristics of *Bradyrhizobium japonicum* and *B. elkanii* species in Brazilian strains which nodulate soybean. Biol Fertil Soils 25:407–415

Brewin NJ (1984) Hydrogenase and energy efficiency in nitrogen fixing symbionts. In: Verma D, Hohn T (eds) Genes involved in plant-microbe interactions. Springer, New York Berlin Heidelberg, pp 179–203

Bruijn FJ de (1992) Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergenic consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl Environ Microbiol 58:2180–2187

Buchanan RE (1980) Approved lists of bacterial names. Int J Syst Bacteriol 30:225–240

Carter KR, Jennings NT, Hanus J, Evans HJ (1978) Hydrogen evolution and uptake by nodules of soybeans inoculated with different strains of *Rhizobium japonicum.* Can J Microbiol 24:307–311

Devine TE, Kuykendall LD, Breithaup BH (1983) Nodule-like structures induced on peanut by chorosis producing strains of *Rhizobium* classified as *R. japonicum.* Crop Sci 23:394–397

Devine TE, Kuykendall LD, O'Neill JJ (1990) The *Rj4* allele in soybean represses nodulation by chlorosis-inducing bradyrhizobia classified as DNA homology group II by antibiotic resistance profiles. Theor Appl Genet 80:33–37

Feije F, Anger V (1972) Spot tests in inorganic analyses. Anal Chim Acta 149:363–367

Fred EB, Baldwin IL, McCoy E (1932) Root nodule bacteria of leguminous plants. University of Wisconsin, Madison

Fuhrmann J (1990) Symbiotic effectiveness of indigenous soybean bradyrhizobia as related to serological, morphological, rhizobitoxine, and hydrogenase phenotypes. Appl Environ Microbiol 56:224–229

Hanus FJ, Albrecht SL, Zablotowicz RM, Elmerich DW, Russell SA, Evans HJ (1981) Yield and N content of soybean seed as influenced by *Rhizobium japonicum* inoculants possessing the hydrogenase characteristic. Agron J 73:368–371

Huber TA, Agarwal AK, Keister DL (1984) Extracellular polysaccharide composition, ex planta nitrogenase activity, and DNA homology in *Rhizobium japonicum.* J Bacteriol 158:1168–1171

Hungria M, Vargas MAT (1996) Exploring the microbial diversity and soil management practices to optimize the contribution of soil microorganisms to plant nutrition. In: Stacey G, Mullin B, Gresshoff PM (eds) Biology of plant-microbe interactions. International Society of Molecular Plant-Microbe Interactions, St. Paul, pp 493–496

Hungria M, Neves MCP, Döbereiner J (1989) Relative efficiency, ureide transport and harvest index in soybeans inoculated with isogenic HUP mutants of *Bradyrhizobium japonicum.* Biol Fertil Soils 7:325–329

Hungria M, Vargas MAT, Suhet AR, Peres JRR (1994) Fixação biológica do nitrogênio em soja. In: Araujo RS, Hungria M (eds) Microrganismos de importância agrícola. EMBRAPA-SPI, Brasília, pp 9–89

Hungria M, Nishi CYM, Cohn J, Stacey G (1996) Comparison between parental and variant soybean *Bradyrhizobium* strains with regard to the production of lipo-chitin nodulation signals, early stages of root infection, nodule occupancy, and N_2 fixation rates. Plant Soil 186:331–341

Judd AK, Schneider M, Sadowsky MJ, Bruijn FJ de (1993) Use of repetitive sequences and the polymerase technique to classify genetically related *Bradyrhizobium japonicum* serocluster 123 strains. Appl Environ Microbiol 59:1702–1708

Kuykendall LD, Roy MA, O'Neill JJ, Devine TE (1988) Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum.* Int J Syst Bacteriol 38:358–361

Kuykendall LD, Saxena B, Devine TE, Udell SE (1992) Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. Can J Microbiol 38:501–505

Minamisawa K (1989) Comparison of extracellular polysaccharide composition, rhizobitoxine production and hydrogenase phenotype among various strains of *Bradyrhizobium japonicum.* Plant Cell Physiol 30:877–884

Minamisawa K (1990) Division of rhizobitoxine-producing and hydrogen-uptake positive strains of *Bradyrhizobium japonicum* by *nifDKE* sequence divergence. Plant Cell Physiol 31:81–89

Minamisawa K, Fukai K (1991) Production of indole-3-acetic acid by *Bradyrhizobium japonicum:* a correlation with genotype grouping and rhizobitoxine production. Plant Cell Physiol 32:1–9

Minamisawa K, Seki T, Onodera S, Kubota M, Asami T (1992) Genetic relatedness of *Bradyrhizobium japonicum* field isolates as revealed by repeated sequences and various other characteristics. Appl Environ Microbiol 58:2832–2839

Minamisawa K, Onodera S, Kobayashi N, Tanimura Y, Yuhashi K, Akao S (1993) Competitiveness of different genotype groups of indigenous *Bradyrhizobium japonicum* for nodule occupancy. In: Palacios R, Mora J, Newton WE (eds) New horizons in nitrogen fixation. Kluwer, Dordrecht, p 632

Nishi CYM, Boddey LH, Vargas MAT, Hungria M (1996) Morphological, physiological and genetic characterization of two new *Bradyrhizobium* strains recently recommended as Brazilian commercial inoculants for soybean. Symbiosis 20:147–162

Somasegaran P, Hoben HJ (1985) Methods in legume-*Rhizobium* technology. Niftal, Hawaii

Stanley JS, Brown GG, Verma DPS (1985) Slow-growing *Rhizobium japonicum* comprises two highly divergent symbiotic types. J Bacteriol 163:148–154

Teaney GB, Fuhrmann JJ (1992) Soybean response to nodulation by bradyrhizobia differing in rhizobitoxine phenotype. Plant Soil 145:275–285

Vasilas BL, Fuhrmann JJ (1993) Field response of soybean to nodulation by a rhizobiotoxine-producing strain of *Bradyrhizobium.* Agron J 85:302–305

Vincent JM (1970) Manual for the practical study of root nodule bacteria. Blackwell, Oxford

Yokoyama T, Ando S, Murakami T, Imai H (1996) Genetic variability of the common *nod* gene in soybean bradyrhizobia isolated in Thailand and Japan. Can J Microbiol 42:1209–1218

Young JPW, Downer HL, Eardly BD (1991) Phylogeny of the phototropic *Rhizobium* strain BTAil by polymerase chain reaction-based sequencing of a 16S rRNA gene segment. J Bacteriol 173:2271–2277