Plant and Soil **186:** 331–341, 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

M. Hungria[1], C.Y.M. Nishi[1], J. Cohn[2] and G. Stacey[2]
[1]*EMBRAPA-CNPSo, Cx. Postal 231, 86001–970, Londrina, PR, Brazil**, [2]*Center for Legume Research, M409 Walters Life Sciences Building , University of Tennessee, Knoxville, TN 37996–0845, USA*

Received 29 February 1996. Accepted in revised form 23 August 1996

Key words: Bradyrhizobium, competitiveness, *Glycine max*, N_2 fixation, Nod metabolites, symbiosis

Abstract

Soybean is the most important leguminous crop in Brazil and the nitrogen required for plant growth is supplied by *Bradyrhizobium* bacteria through the symbiotic relation established by the inoculation process. Since 1992, two new strains, CPAC 7 and CPAC 15, which have been shown to increase yields in several field experiments, have been recommended in Brazilian commercial inoculants. CPAC 15 is a natural variant of the *B. elkanii* SEMIA 566 strain, and was isolated after several years of adaptation to a Brazilian Cerrado soil, while CPAC 7 is a variant of *B. japonicum* strain CB 1809, selected under laboratory conditions for higher nodulation and yield. The comparison between parental and variant strains, under greenhouse conditions, showed that both CPAC 15 and CPAC 7 increased N_2 fixation rates in relation to the parental strains. The better performance of CPAC 15 was related to an increase in nodule efficiency (mg N_2 fixed mg^{-1} nodule) while with CPAC 7 the higher N_2 fixation rates were due to increased nodulation. Both CPAC 15 and CPAC 7 increased nodule occupancy, when co-inoculated at a ratio of 1:1 with *B. elkanii* 29w, in relation to their parental strains. Variant strains also differed from parental in their ability to increase numbers of root hairs (Hai phenotype) either when inoculated onto plants, or when supernatants of bacteria exposed to seed exudates were used as inoculants. This results lead to the hypothesis that a modification in some of the "common" nodulation genes had occurred. However, the increase in Hai phenotype with CPAC 7 was dependent on the soybean cultivar, indicating a possible alteration in some genotypic specific nodulation gene. Apparently, there were no differences in Nod metabolites produced by strains CPAC 15 and SEMIA 566, but a more detailed chemical analysis would be required to rule out subtle differences. On the contrary, significant differences were found between CPAC 7 and the parental strain CB 1809, in the profile of Nod metabolites. Consequently, it may be possible that diffusable molecules, responsible for Hai phenotype, would be related to nodulation ability, competitiveness, and N_2 fixation, resulting in the higher yields that have been associated with CPAC 7 and CPAC 15. For the CPAC 7 strain, the increase in Hai phenotype could be attributed to the differences found in the Nod molecules. Consequently, a high degree of physiological and genetic variability can result from the adaptation of rhizobial strains to the soil. Also, this variability can be found under laboratory conditions, when searching single colonies with specific properties.

Introduction

The successful symbiosis between leguminous plants and rhizobia starts with the formation of effective nodules, where N_2 fixation takes place. Although nodule formation has been studied for several decades, it was only recently reported that, before any root phenotypic modification can be observed, the symbionts communicate, exchanging molecular signals. These signals can induce or repress the activity of genes that play a role in nodulation or they can modify the products of these genes. First, diffusable substances that induce

* FAX No: + 55433716100. E-mail: hungria@cnpso.embrapa.br

the transcription of nodulation (*nod*) genes in bacteria, the majority of which have been identified as phenolic compounds of the flavonoid family, are released by the host seeds and roots (Hungria et al., 1991a,b; Kosslak et al., 1987; Peters et al., 1986). In a second stage, the bacteria, by the action of *nod* gene products, produce specific molecular signals (Faucher et al., 1989; Sanjuan et al., 1992; Van Brussel et al., 1986; Zaat et al., 1987), called Nod signals, that have been chemically defined as lipo-chitin oligosaccharides, or LCOs (Lerouge et al., 1990). These specific bacterial signals induce modifications on the roots that are typical of the pre-infection process, such as Tsr (thick and short root), Had (hair deformation), Hai (hair induction) and Hac (hair curling) (van Brussel et al., 1986; Zaat et al., 1987).

Soybean [*Glycine max* (L.) Merrill] is the most important leguminous crop in Brazil, and the nitrogen (N) required for plant growth is almost completely supplied by *Bradyrhizobium* bacteria through the symbiotic relation established by the inoculation process (Cattelan and Hungria, 1994; Hungria et al., 1994). Since 1992, two new strains have been used in Brazilian inoculants, CPAC 7 and CPAC 15, which in many field trials, performed over seven years, were able to increase yield by up to 750 kg ha^{-1}, in comparison with the other strains commercially recommended (Vargas et al., 1992). CPAC 15 is a natural variant of SEMIA 566, and this last strain was isolated in southern Brazil in 1966 from a soil that had been inoculated for one year with a North American inoculant. The natural variant CPAC 15 was isolated from a Brazilian savanna soil ("Cerrado") (Vargas et al., 1992), originally free of soybean rhizobia (Vargas et al., 1982), about 15 years after the last inoculation with SEMIA 566. Both strains share serological properties and, since the area has been inoculated exclusively with SEMIA 566, it seems that CPAC 15 is a natural variant obtained by several years of stressful conditions, including high temperatures and low soil moisture, typical of the Cerrado region. CPAC 7 is also a natural variant, but of strain CB 1809 (received from Australia in 1966), and it was obtained during a selection program of screening and testing single colonies for higher nodulation and yield. In contrast to the parental strain, CPAC 7 is also able to nodulate the soybean cultivar IAC-2, which was largely cultivated in the Cerrado region during the last decade (Vargas et al., 1992).

Although economically very important in Brazil, since these two new strains have been preferentially used in commercial inoculants, only a few basic studies were performed comparing rhizobial characteristics associated with the higher field performance. The only data available compared some characteristics of *Bradyrhizobium* species, such as Hup phenotype, rhizobitoxine and indole acetic acid synthesis, and restriction to nodulation with Rj_4 soybean cultivar. The results demonstrated that CB 1809 and CPAC 7 show typical properties of the species *B. japonicum*, while SEMIA 566 and CPAC 15 resemble *B. elkanii* (Nishi et al., 1996). Also, genetic differences, between parental and variant strains, were seen in DNA profile, obtained by RAPD analysis with 23 short primers (Nishi et al., 1996).

In this study we have confirmed, under axenic conditions, the higher rates of N_2 fixation obtained with CPAC 15 and CPAC 7, as well as the higher competitiveness, in co-inoculation studies with strain 29w, in comparison with their parental strains. We have also studied the variant strains with regard to parameters related to the exchange of molecular signals between microsymbiont and host plant. The long term goal of this research is to identify characteristics that could be related to the achievement of higher yields observed when soybean is inoculated with the variant strains CPAC 7 and CPAC 15 in the field.

Material and methods

Bacterial strains

- SEMIA 566, isolated in the State of Rio Grande do Sul, Brazil, in 1966, from large, pink nodules on soybean roots grown on a soil that had been inoculated, one year before, with a North American inoculant from Nitragin (today LiphaTech Inc., Milwaukee, WI, USA). SEMIA 566 was recommended in Brazilian commercial inoculants from 1966 to 1978.
- CB 1809 (= SEMIA 586), strain received from CSIRO, Australia, in 1966.
- CPAC 15 (= SEMIA 5079), strain isolated from a savanna soil (Cerrado) in the central region of Brazil, at EMBRAPA-CPAC (Empresa Brasileira de Pesquisa Agropecuária, Centro de Pesquisa Agropecuária do Cerrado), and serologically related to strain SEMIA 566. (Vargas et al., 1992).
- CPAC 7 (= SEMIA 5080), strain selected from a single colony of CB 1809, under laboratory conditions at EMBRAPA-CPAC (Vargas et al., 1992), according to the methodology proposed by Peres

et al. (1984), which tests individual colonies for higher N_2 fixation rates (total N in shoots under axenic conditions), and screened also for higher nodulation. Both CPAC 15 and CPAC 7 increase soybean yield under field conditions and have been used in Brazilian commercial inoculants since 1992 (Vargas et al., 1992).

- *29w* (= SEMIA 5019), *B. elkanii* strain isolated in Brazil, from soybean line IAC-70-559, and used in Brazilian commercial inoculants since 1979.

Plant genotypes

Two soybean [*Glycine max* (L.) Merrill] cultivars were used (genealogy in parenthesis): BR–16 (D69-B10-M58 X Davis) and IAC-2 (La 41-1219 X Yelnanda). The seeds and information about the cultivars were obtained from the EMBRAPA-CNPSo (Centro Nacional de Pesquisa de Soja) germplasm bank.

N_2 fixation rates

Each of the four bacterial strains was grown in YM (Yeast Manitol) medium (Vincent, 1970), for seven days, at 28°C, and equalized to a concentration of 10^9 cells mL^{-1}. Soybean seeds of cultivars BR–16 and IAC–2 were surface-sterilized (Vincent, 1970) and then incubated with the inoculum (1 mL $seed^{-1}$) for 30 min. Five seeds were sown per modified Leonard jar (Vincent, 1970) containing sand and vermiculite (1:2, v/v) and filled with N-free nutrient solution (Somasegaran and Hoben, 1985). Both cultivars, without inoculation, were included as controls. Plants were grown under greenhouse conditions, with a 12 h photoperiod and temperature of 28/23 °C (day/night, with a standard deviation of ± 2.5 °C) and thinned to two per jar, four days after emergence (DAE). Nutrient solution was completed every other day and plants were harvested 40 DAE. The parameters evaluated were nodule number and dry weight of nodules, roots and shoots. Total N of shoots was determined after the indophenol blue colorimetric method of Feije and Anger (1972). The experiment was performed in a randomized block design, with five replicates and statistically analyzed by the Tukey's test ($p \leq 0.05$).

Nodule occupancy

The experiment was performed as described in the study of evaluation of N_2 fixation rates, except that each one of the four strains (10^9 cells mL^{-1}) was inoculated in a proportion of 1:1, 10:1, or 1:10 with strain 29w, which is highly competitive and belongs to a different serogroup. Besides 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 anti-sera of each inoculated strain. Competitiveness was tested with cultivar BR–16, which showed higher nodulation with all strains in the experiment of evaluation of N_2 fixation rates. The experiment was performed in a randomized block design, with five replicates, the plants were collected 40 DAE and the results were statistically analyzed by the Tukey's test ($p \leq 0.05$).

Effects of bacterial strains on root phenotypic traits

The studies were performed under axenic conditions. Soybean seeds of cultivar BR–16 and IAC–2 were surface-sterilized (Vincent, 1970), pre-germinated in a growth chamber at 25 °C for two days and transfered to 500-mL glass flasks, containing 150 mL of N-free nutrient solution and 8 g L^{-1} of agar, paper-covered and topped with cheesecloth. The nutrient solution was modified from Somasegaran and Hoben (1985), adding 0.5 mL. L^{-1} of a stock solution containing the following concentrations: $CaCl_2 \cdot 2H_2O$, 1*M*; KH_2PO_4, 0.5*M*; Fe-EDTA, 10 m*M*; $MgSO_4 \cdot 7H_2O$, 0.25 *M*; K_2SO_4, 0.25 *M*; $MnSO_4 \cdot H_2O$, 1 m*M*; H_3BO_3, 2 m*M*; $ZnSO_4 \cdot 7H_2O$, 0.5 m*M*; $CuSO_4 \cdot 5H_2O$, 0.2 m*M*; $CoSO_4 \cdot 7H_2O$, 0.1 m*M*; $NaMoO_4 \cdot 2H_2O$, 0.1 m*M*, and the final pH was adjusted to 6.8. Bacteria were grown in YM for four days, equalized to 10^5 cells mL^{-1}, and 1 mL was used per seedling. Plants were grown in a growth chamber at 28/23 °C (day/night), 60% relative humidity and a 12h photoperiod. Fifteen days after inoculation the following parameters were evaluated: Tsr (thick and short root, evaluated by the length and thickness of main root), Had (hair deformation, evaluated by the length and thickness of root hairs), Hai (hair induction, determined by the number of root hairs per field of the Neubauer chamber), and Hac (hair curling, evaluated by the presence or absence of root hair curling). Tsr, Had, Hai and Hac were investigated by optical microscopy. Roots were stained with methylene blue (0.01% w/v) in deionized water, incubated for 15 minutes in a moist chamber at 28 °C, carefully washed at least three times with deionized water, and then examined (Vasse and Truchet, 1984). The experiment was performed in a randomized design with four

replicates, and the results were statistically analyzed by the Tukey's test at $p \leq 0.05$.

Effects of seed exudates and supernatants of bacteria on root phenotypic traits

Soybean seed exudates of cultivars BR–16 and IAC–2 were produced from 100-surface-sterilized seeds (approximately 20 g) that were soaked in 35 mL of sterile deionized water, with agitation, at 28 °C, in the dark. After 24 h, about 22 mL of seed exudates were collected and filter sterilized by passing successively through sterile nitrocellulose filters (Millipore) with 0.8, 0.4 and 0.2 μm pore sizes. Before utilization, all seed exudates were tested for contamination in nutrient-agar medium.

Bradyrhizobium strains SEMIA 566, CPAC 15, CB 1809 and CPAC 7 were grown in YM medium for 48h (10^5 cells mL^{-1}). Cultures were then centrifuged at 6,500 *g* for 30 min and resuspended to the initial volume in N-free nutrient solution, described in the study of rates of N_2 fixation. Bacteria were incubated in this solution for 48 h. After incubation, bacteria were centrifuged again at 6,500 *g* for 30 min, and resuspended in N-free nutrient solution, but in half of the initial volume. This suspension, designated inoculum, was mixed 1:1 (v/v) with seed exudate, incubated for 48h, centrifuged again at 6,500 *g* for 30 min, and the supernatant fluid collected. This fluid was filter sterilized as previously described and labelled as bacterial supernatant.

Sterilized seed exudates or bacterial supernatants were mixed, in the proportion of 10% (v/v), with N-free nutrient solution, described in the item of effects of bacterial strains on root phenotypic traits and 8 g L^{-1} of agar. A volume of 150 mL of this solution was added to each 500-mL flask and autoclaved. Soybean seeds were surface-sterilized, pre-germinated, sown in each flask, harvested after 15 days and analyzed as described for the effects of bacterial strains on root phenotypic traits.

Analysis of lipo-chitin Nod signals

Thin layer chromatography analysis of lipo-chitin Nod signals produced by strains SEMIA 566, CPAC 15, CB 1809 and CPAC 7 was performed as described by Sanjuan et al. (1992). Strains were grown in modified YM, changing the carbon source to 5 g of Na-gluconate and the N source to 1 g of L-glutamate, until an OD_{600} of 0.5–0.6. Cells were then pelleted and washed with modified Bergersen's minimal medium (Spaink et al., 1992), lacking a carbon source (glycerol). After that, cells were diluted in the same medium to an OD_{600} of 0.1 and incubated with exudates of soybean cultivar Essex, obtained as previously described, or 2 μM of genistein. Cells incubated in the absence of any *nod* gene inducer served as controls. ^{14}C-acetate was added to the non-induced and induced cultures at the time of incubation, using 50 μCi of [1-^{14}C] acetate (56 mCi $mmol^{-1}$, ICN Pharmaceuticals, Inc. USA; 1 Ci=37 GBq) and the cultures were incubated overnight with shaking (150 rpm) at 30 °C. Nod metabolites were extracted from the cultures by the addition of 50% of the total volume with distilled n-butanol. The butanol was removed by drying under a stream of nitrogen gas and the remaining residue was resuspended in 50% (v/v) acetonitrile:water. Extracts were analyzed on reverse phase TLC pre-coated plates of silica gel with 100% octadesylsilanization (Sigma Chemical Co.) as described by Spaink et al. (1992). Plates were then exposed to X-ray film (Kodak X-Omat AR) for four days at room temperature.

Results

N_2 fixation rates

The comparison of *B. elkanii* strains SEMIA 566 and CPAC 15 did not show statistical differences in relation to the nodulation parameters. However, total N in shoots and nodule efficiency in both soybean cultivars inoculated with the natural variant strain were higher than with the parental strain SEMIA 566 (Table 1). When *B. japonicum* strains CB 1809 and CPAC 7 were compared, nodule number and dry weight were higher with strain CPAC 7 for both cultivars BR–16 and IAC–2, as well as total N content, but these strains did not show statistical differences in the nodule efficiency parameters (Table 1).

Nodule occupancy

In relation to the pair of *B. elkanii* strains, CPAC 15 increased nodule occupancy by 34%, when inoculated in a ratio of 1:1 with 29 w, in relation to the parental strain SEMIA 566. There were no statistical differences between this pair of strains at the ratios of 10:1 and 1:10 (Table 2). For the *B. japonicum* strains, the variant CPAC 7 increased nodule occupancy by 71% in relation to its parental strain CB 1809, when inocu-

Table 1. Effects of inoculation of soybean cultivars BR–16 and IAC–2 on the nodule number (NN, number/plant), nodule dry weight (NDW, mg/plant), total N accumulated in the shoots (TNS, mg N/plant) and nodule efficiency (TNS/NDW). Comparisons were made between the *B. elkanii* strain SEMIA 566 and its variant CPAC 15 and between the *B. japonicum* strain CB 1809 and its variant CPAC 7. Plants were grown under greenhouse conditions in modified Leonard jars containing N-free nutrient solution and harvested 40 days after emergence

Strain	BR–16				IAC–2			
	NN	NDW	TNS	NE	NN	NDW	TNS	NE
B. elkanii SEMIA 566 × CPAC 15								
SEMIA 566	48.7 a[a]	136.2 a	26.2 b	0.192 b	35.3 a	112.1 a	22.1 b	0.197 b
CPAC 15	42.8 a	129.4 a	37.7 a	0.291 a	32.1 a	100.7 a	30.6 a	0.304 a
Control[b]	0.0	0.0	3.9	-	0.0	0.0	3.8	-
CV (%)	23.3	15.5	9.9	20.8	22.2	14.9	11.1	20.7
B. japonicum CB 1809 × CPAC 7								
CB 1809	21.4 b[a]	69.1 b	23.1 b	0.334 a	5.2 b	19.6 b	8.1 b	0.413 a
CPAC 7	41.3 a	118.6 a	35.7 a	0.301 a	11.1 a	29.4 a	13.1 a	0.445 a
Control[b]	0.0	0.0	4.1	-	0.0	0.0	3.7	-
CV (%)	18.6	17.2	11.3	18.8	28.0	16.1	12.3	18.7

[a] Means of five replicates followed by the same letters, in the same column, for each pair of strains, did not show statistical differences (Tukeys test, $p \leq 0.05$).
[b] Non-inoculated plants and these values were not considered for statistical analysis.

lated at a ratio of 1:1 with 29w. At a ratio of 10:1, this increase was in the order of 102% (Table 2).

Effects of bacterial strains on root phenotypic traits

Inoculation of soybean cultivars BR–16 and IAC–2 with strains SEMIA 566 and CPAC 15 showed the same response in root phenotypes Tsr, Had and Hac. However, differences between the two strains were observed in the Hai phenotype, with the natural variant CPAC 15 significantly increasing the number of root hairs in relation to the parental strain (Table 3).

No differences were found in any of the root phenotypes when cultivar BR–16 was inoculated with strains CB 1809 or CPAC 7. However, when inoculated on cultivar IAC-2, differences were found again in Hai phenotype, with higher root hair number caused by the natural variant CPAC 7 (Table 4).

Effects of seed exudates and supernatants of bacteria on root phenotypic traits

Tsr, Had (data not shown) and Hai root phenotypes observed with the addition of sterilized supernatants of bacteria exposed to seed exudates were similar to the phenotypes observed by the inoculation of bacteria cells (Table 5). Consequently, the bacterial supernatant of variant strain CPAC 15, exposed to seed exudates of cultivars BR–16 and IAC–2, increased Hai phenotype in relation to the parental SEMIA 566. Also, the bacterial supernatant of CPAC 7, exposed to exudates of cultivar IAC–2, but not of cultivar BR–16, increased Hai in relation to the parental CB 1809 (Table 5). The parameters of Tsr and Had did not differ between parental and variant strains (data not shown). The Hac phenotype required the presence of bacteria and was not observed with the addition of supernatant of induced bacteria (Table 5).

TLC analysis of the lipo-chitin Nod signals produced by parental and variant strains

An autoradiogram of ^{14}C-acetate-labelled Nod metabolites produced by strains CB 1809, CPAC 7, SEMIA 566, and CPAC 15 and separated by reverse-phase, thin-layer chromatography is displayed in Figure 1. The production of the lipo-chitin Nod signals requires the induction of *nod* gene expression, therefore Nod metabolites can be identified by comparing the profile of compounds made in the presence and absence of *nod* gene inducing compounds. Some of the metabolites were common to all four strains and, as can be seen in Figure 1, the profile of compounds produced by strains SEMIA 566 and CPAC 15 were identical. However,

Table 2. Nodule occupancy (% of nodules occupied by each of the inoculated strains) by *B. elkanii* strain SEMIA 566 and its variant CPAC 15 and by *B. japonicum* strain CB 1809 and its variant CPAC 7, when mixed in ratios of 1:1, 10:1 and 1:10 with *B. elkanii* strain 29w. Occupancy was evaluated by the serological analysis of 60 nodules per treatment and plants were grown under greenhouse conditions, in modified Leonard jars containing N-free nutrient solution, and harvested 40 days after emergence

Ratio	Nodule occupancy (%)					
	B. elkanii			*B. japonicum*		
	566	29w	Double	1809	29w	Double
	SEMIA 566:29w[a]			CB 1809:29w[a]		
1:1	48.1 c[b]	40.2 b	11.7 a	45.7 b	42.7 b	11.6 a
10:1	82.7 a	6.0 d	11.3 a	83.5 a	5.3 c	11.2 a
1:10	18.7 d	71.2 a	10.1 a	19.6 c	65.7 a	14.7 a
	CPAC 15:29 w			CPAC 7:29w		
1:1	64.6 b	24.6 c	10.8 a	78.3 a	9.5 c	12.2 a
10:1	85.3 a	3.5 d	11.2 a	83.3 a	4.0 c	12.7 a
1:10	29.3 d	59.6 a	11.1 a	39.7 b	49.4 b	10.9 a
CV (%)	27.1	23.4	26.5	26.3	25.4	27.2

[a]The parental SEMIA 566 is serologically related to CPAC 15 and the parental strain CB 1809 with the variant CPAC 7.
[b]Means of five replicates and, when followed by the same letter, within each column, did not show statistical differences (Tukeys test, $p \leq 0.05$).

Table 3. Effects of inoculation of soybean, cultivars BR-16 and IAC-2, with the parental *B. elkanii* strain SEMIA 566 and its natural variant CPAC 15, on root phenotypes Tsr, Had, Hai and Hac. Plants were grown in a growth chamber, in glass jars containing N-free nutrient-agar medium, and harvested 15 days after transplant and inoculation

Strain	Tsr		Had		Hai	Hac
	Length (cm)	Thickness (mm)	Length (μm)	Thickness (μm)	(n° field^{-1})	
BR-16						
SEMIA 566	15.6 b[a]	2.6 a	73.4 b	21.7 a	50.8 b	+
CPAC 15	17.1 b	2.5 a	65.6 b	23.6 a	>100 a	+
Control[b]	23.6 a	1.9 b	198.7 a	10.7 b	21.7 c	-
CV (%)	18.6	12.1	15.3	14.2	20.8	
IAC-2						
SEMIA 566	16.7 b[a]	2.6 a	60.6 b	22.7 a	61.7 b	+
CPAC 15	15.9 b	2.4 a	59.7 b	20.3 a	>100 a	+
Control[b]	24.7 a	1.8 b	200.3 a	11.4 b	21.4 c	-
CV (%)	17.1	11.7	14.4	17.6	14.8	

[a] Means of four replicates and, when followed by the same letter, within each column and for each cultivar, did not show statistical differences within each column (Tukeys test,$p \leq 0.05$).
[b] Non-inoculated plants.

Table 4. Effects of inoculation of soybean, cultivars BR–16 and IAC–2, with the parental *B. japonicum* strain CB 1809 and its natural variant CPAC 7, on root phenotypes Tsr, Had, Hai and Hac. Plants were grown in a growth chamber, in glass jars containing N-free nutrient-agar medium, and harvested 15 days after transplant and inoculation

Strain	Tsr		Had		Hai	Hac
	Length (cm)	Thickness (mm)	Length (μm)	Thickness (μm)	(n° field^{-1})	
BR–16						
CB 1809	16.6 b[a]	2.4 a	57.5 b	17.5 a	>100 a	+
CPAC 7	17.1 b	2.5 a	62.5 b	17.5 a	>100 a	+
Control[b]	22.7 a	1.9 a	205.0 a	10.0 b	23.8 b	-
CV (%)	9.6	22.6	17.5	15.7	2.0	
IAC–2						
CB 1809	15.4 b[a]	3.1 a	60.0 b	18.8 a	45.5b	+
CPAC 7	14.8 b	2.9 a	52.5 b	20.0 a	>100 a	+
Control[b]	21.2 a	2.0 b	207.5 a	10.0 b	23.3 c	-
CV (%)	11.1	7.7	12.3	17.0	2.6	

[a]Means of four replicates and, when followed by the same letter, within each column and for each cultivar, did not show statistical differences (Tukeys test, $p \leq 0.05$).
[b] Non-inoculated plants.

Table 5. Effects of soybean seed exudates and of the sterilized supernatants of bacteria previously exposed to seed exudates on root phenotypes Hai and Hac. Plants were grown in a growth chamber, in glass jars containing N-free nutrient-agar medium, and harvested 15 days after transplant

Supernant of strains	Seed exudate used for bacterium induction	Cultivar used in the assay	Hai (n° field^{-1})	Hac
B. elkanii SEMIA 566 × CPAC 15				
SEMIA 566	BR–16	BR–16	72.8 b[a]	-
CPAC 15	BR–16	BR–16	>100 a	-
SEMIA 566	None	BR–16	27.8 d	-
CPAC 15	None	BR–16	23.0 d	-
SEMIA 566	IAC–2	IAC–2	48.7 c	-
CPAC 15	IAC–2	IAC–2	>100 a	-
SEMIA 566	None	IAC–2	24.3 d	-
CPAC 15	None	IAC–2	19.9 d	-
B. japonicum CB 1809 × CPAC 7				
CB 1809	BR–16	BR–16	>100 a	-
CPAC 7	BR–16	BR–16	>100 a	-
CB 1809	None	BR–16	27.8 c	-
CPAC 7	None	BR–16	23.8 c	-
CB 1809	IAC–2	IAC–2	48.5 b	-
CPAC 7	IAC–2	IAC–2	>100 a	-
CB 1809	None	IAC–2	26.8 c	-
CPAC 7	None	IAC–2	24.5 c	-

[a]Means of four replicates and, when followed by the same letter, did not show statistical differences for each pair of strains (Tukeys test, $p \leq 0.05$).

there were notable differences in the profile of Nod metabolites produced by strains CB 1809 and the natural variant CPAC 7. Most notable, was the apparent absence of two major hydrophobic bands (bottom, on the left side) in the profile produced by strain CPAC 7. These bands were present in the extracts obtained from strains CB 1809, SEMIA 566, and CPAC 15. The band marked by the arrow on the right side migrates with the same relative mobility as the major Nod metabolite produced by *B. japonicum* strain USDA 110 (data not shown).

Discussion

There is a constant search for superior *Bradyrhizobium* strains, characterized by higher rates of N_2 fixation, higher nodule efficiency and competitiveness, which would allow an increase in soybean yields and N content. Parameters such as hydrogenase activity (Hanus et al., 1981; Hungria et al., 1989) and ureide transport (Hungria et al., 1989; Neves and Hungria, 1987) have been associated with high efficiency, while motility and chemotaxis (Liu et al., 1989), polysaccharides (Streeter et al., 1992), synthesis of bacteriocin (Fuhrmann, 1990), rate of infection (Smith and Wollum II, 1989), and growth in different soil substrates (Viteri and Schmidt, 1987), among others, have been related to high competitiveness. However, seldom have individual strains been analyzed for a variety of traits nor has this information been correlated to agronomic performance.

In an effort to increase soybean nodulation in Brazilian Cerrado soils (an edaphic type of savanna that occupies the central region of Brazil and represents the most important area for soybean cultivation), two new strains were obtained, which significantly increased yields in several field trials (Vargas et al., 1992). Strain CPAC 15 is probably a variant of SEMIA 566, with morphological, physiological and genetic changes caused by several years of adaptation to the Cerrado conditions (Nishi et al., 1996). CPAC 7 was obtained under laboratory conditions when individual colonies were screened from a single culture of CB 1809 for higher nodulation and yield. Since CPAC 15 and CPAC 7 have been tested mainly in the field and have been used in large scale as Brazilian inoculants after 1992, the experiments performed here were designed to characterize the physiological and genetic parameters of these strains that could be related to their better agronomic performance.

The greenhouse experiment confirmed the higher N_2 fixation rates of both *B. elkanii* CPAC 15 and *B. japonicum* CPAC 7 in relation to the parental strains. However, the mechanisms that allowed higher N accumulation in shoots were different. Both SEMIA 566 and CPAC 15 nodulated very well, and the better symbiotic performance of the variant strain was related to an increase in nodule efficiency (mg of N accumulated mg^{-1} of nodule), by a process that we have not identified. In contrast, the increase in N_2 fixation by strain CPAC 7 resulted from an increase in nodule mass (Table 1). Both CPAC 15 and CPAC 7 also increased nodule occupancy, in relation to the parental strains, when co-inoculated, at a ratio of 1:1, with another competitive strain. For CPAC 7, this trait was also observed at a ratio of 1:10 (Table 2).

Since nodulation parameters differed, root phenotypes Tsr, Had, Hai and Hac, which have been frequently reported in studies related to the steps that precede infection and nodulation were analysed (Canter Cremers et al., 1986; Faucher et al., 1988; Vincent, 1980; Zaat et al., 1987). These phenotypes are related to the activity of the protein products of the common nodulation (*nod*) genes, *nodA*, *nodB*, *nodC* and *nodD* (Van Brussel et al., 1986; Zaat et al., 1987) which, in conjunction with products of host-specific nodulation genes (i.e., *hsn*; e.g., Faucher et al., 1988), synthesize lipo-chitin oligosaccharides. These molecules have been termed Nod signals and are responsible for many of the root phenotypes induced during the early stages of rhizobial infection (e.g., Lerouge et al., 1990). The results obtained in this study demonstrate that the natural variant strain CPAC 15 differed from the parental SEMIA 566 in Hai phenotype (Table 3), leading to the supposition that the former had a modification in some of the common *nod* genes. In relation to the pair of strains CB 1809 and CPAC 7, an increase in Hai phenotype was observed only when tested on soybean cultivar IAC-2 (Table 4), indicating that the selection program could have altered some genotypic specific nodulation (GSN) gene. An example of such a gene is *nolA*, identified in North American *B. japonicum* strains and essential for nodulation of specific soybean genotypes (Sadowsky et al., 1991). The fact that this phenotype is only manifested with soybean cultivar IAC-2 is not surprising, since one goal of the selection program in which strain CPAC 7 was obtained was the ability to show higher nodulation on this cultivar.

Root phenotypes Tsr, Had and Hai, obtained with sterilized-supernatant of bacteria previously exposed

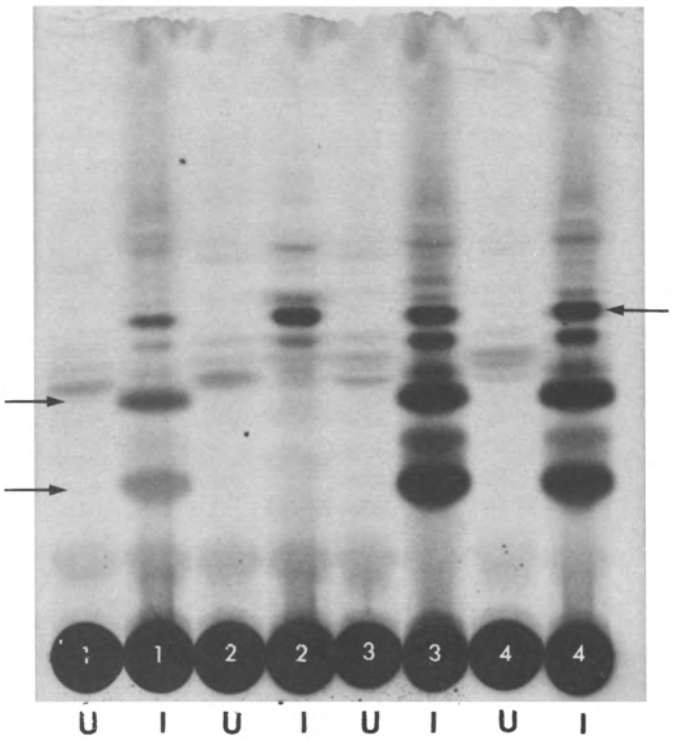

Figure 1. Thin layer chromatography (TLC) of radiolabelled Nod metabolites. Cells were grown in the presence (I, induced) or absence (U, uninduced) of soybean seed exudates and cultures were extracted with n-butanol. The extract was applied to reverse phase silica TLC plates, using 50% (v/v) acetonitrile:water as a solvent. After chromatography, plates were dried and then exposed to X-ray film for four days. The strains tested were as follows: lane 1, strain CB 1809; lane 2, strain CPAC 7; lane 3, SEMIA 566; lane 4, CPAC 15. The band marked by the arrow, on the right side, migrates in a position identical to the major Nod metabolite produced by *B. japonicum* strain USDA 110. Bands marked by the arrow, on the left side, indicate the hydrophobic compounds lacking in CPAC 7.

to seed exudates (Table 5), were similar to those obtained with the cells, confirming that they were caused by soluble molecular signals synthesized by the bacteria in response to plant signals. The only root hair modification which was not observed with the addition of supernatants was Hac, confirming observations of Sprent and Sprent (1990), that this phenotype requires the physical presence of bacteria.

Many of the early root phenotypes caused by *Rhizobium/Bradyrhizobium* infection can be induced by the addition of lipo-chitin Nod signals, so the profile of such signals made by parental and variant strains were examined, since variations in such signals could be a possible explanation for the differences observed in Hai phenotype. Using ^{14}C acetate-labelling and TLC analysis, there were no apparent differences in the Nod metabolites produced by *B. elkanii* strains SEMIA 566 and CPAC 15 (Figure 1). Consequently, differences found between these two strains in Hai phenotype and competitiveness could be related to other stages of the infection, although a more detailed chemical analysis would be required to rule out subtle differences in Nod signals produced by the individual strains.

Significant differences were found in the TLC profile of Nod metabolites produced by strains CB 1809 and CPAC 7 (Figure 1). This is surprising, due to the fact that CPAC 7 was isolated as a single colony from a culture of CB 1809. At this stage, it is difficult to assign differences in Nod metabolites as the cause of the observed variation in nodulation phenotypes and agronomic performance. This is especially true since it is likely that these characteristics are quantitative genetic traits. However, the specific Nod metabolites produced by a given strain may be a contributing factor to nodulation ability and yield performance.

One band common to all strains migrated with the same relative mobility as the LCO produced by *B. japonicum* strain USDA 110, identified as a chitin pentamer acylated with vaccenic acid on the non-reducing terminal sugar and substituted with a 2-*O*-

methylfucose on the terminal, reducing sugar [i.e., NodBj-V(C18:1); Sanjuan et al., 1992]. The metabolites of soybean bradyrhizobia identified so far include four other molecules produced by *B. japonicum* strain USDA 135 and eight different molecules synthesized by *B. elkanii* strain USDA 31 (Carlson et al., 1993). The results obtained here have also shown a high number of molecules produced by the strains in the presence of seed exudates, but the importance of a low or high abundance of LCOs, as well as the cooperativity between multiple LCOs to the nodulation and/or competitiveness is still poorly understood.

Recent reports have also found morphological and physiological differences between CPAC 7 and CPAC 15 and their respective parental strains. Specifically, the variant strains produce more mucus in culture, have reduced synthesis of indole acetic acid and rhizobitoxine, and subtle changes in DNA pattern, as evidenced by the RAPD method (Nishi et al., 1996). Consequently, a great variability can be detected after the adaptation of *Bradyrhizobium* strains to the soil. This variability can also be found just by searching single colonies with specific properties. The results also showed that the diffusable molecules, responsible for Hai, could be highly related to nodulation ability, nodule occupancy and N_2 fixation. For the strain CPAC 7, those differences could be related to the differences shown on Nod profiles.

Acknowledgements

The authors acknowledge Dr Milton A T Vargas and Dr Ieda D C Mendes, from EMBRAPA-CPAC, for their helpful cooperation during this work. M Hungria acknowledges CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil) for the research fellowship and for the financial support (project 500012/92-0). Work conducted in the laboratory of GS was supported by a grant from the US Dept. of Energy (#92ER20072). The authors acknowledge also to Ligia M de O Chueire, Rinaldo B Conceição, José Z Moraes and Rubson N O Sibaldelle for technical help.

References

Canter Cremers H C J, van Brussel A A N, Plazinski J and Rolfe B G 1986 Sym plasmid and chromosomal gene products of *Rhizobium trifolii* elicit developmental responses on various legume roots. J. Plant Physiol. 122, 25–40.

Carlson R W, Sanjuan J, Bhat U R, Glushka J, Spaink H P, Wijfjes A H M, van Brussel A A N, Stokkermans T J W, Peters N K and Stacey G 1993 The structures and biological activities of the lipo-oligosaccharide nodulation signals produced by type I and type II strains of *Bradyrhizobium japonicum*. Proc. Natl. Acad. Sci. USA 268, 18372–18381.

Cattelan A J and Hungria M 1994 Nitrogen nutrition and inoculation. *In* Tropical Soybean - Improvement and Prodution. Ed. FAO. pp 201–215. FAO, Rome.

Faucher C, Camut S, Dénarié J and Truchet G 1989 The *nodH* and *nodQ* host range genes of *Rhizobium meliloti* behave as avirulence genes in *R. leguminosarum* bv.*viciae* and determine changes in the production of plant-specific extracellular signals. Mol. Plant-Microb. Interact. 2, 291–300.

Faucher C, Maillet F, Vasse J, Rosenberg C, van Brussel A A N, Truchet G and Dénarié J 1988 *Rhizobium meliloti* host range *nodH* determines production of an alfalfa-specific extracellular signal. J. Bacteriol. 170, 5489–5499.

Feije F and Anger V 1972 Spot tests in inorganic analyses. Anal. Chem. Acta 149, 363–367.

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

Hanus F J, Albrecht S L, Zablotowicz R M, Emerich D W, Russell S A and Evans H J 1981 Yield and N content of soybean seed as influenced *by Rhizobium japonicum* inoculants possessing the hydrogenase characteristic. Crop Sci. 73, 368–372.

Hungria M, Joseph C M and Phillips D A 1991a Anthocyanidins and flavonols, major *nod* gene inducers from seeds of a black-seeded common bean (*Phaseolus vulgaris* L.). Plant Physiol. 97, 751–758.

Hungria M, Joseph C M and Phillips D A 1991b *Rhizobium nod* gene inducers exuded naturally from roots of common bean (*Phaseolus vulgaris* L.). Plant Physiol. 97, 759–764.

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

Hungria M, Vargas M A T, Suhet A R and Peres J R R 1994 Fixação biológica do nitrogênio em soja. *In* Microrganismos de Importância Agrícola. Eds. R S Araujo and M Hungria. pp 9–89. EMBRAPA-SPI, Brasília, Brazil.

Kosslak R M, Bookland R, Barkei J, Paaren H E and Appelbaum E R 1987 Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavone isolated from *Glycine max*. Proc. Natl. Acad. Sci. USA 84, 7428–7432.

Lerouge P, Roche P H, Faucher C, Maillet F, Truchet G, Promé J C and Dénarié J 1990 Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature 344, 781–784.

Liu R, Tran V M and Schimidt E L 1989 Nodulating competitiveness of a nonmotile *Tn7* mutant of *Bradyrhizobium japonicum* in non-sterile soil. Appl. Environ. Microbiol. 55, 1895–1900.

Neves M C P and Hungria M 1987 The physiology of nitrogen fixation in tropical grain legumes. CRC Crit. Rev. Plant Sci. 6, 267–321.

Nishi C Y M, Boddey L H, Vargas M A T and Hungria M 1996 Morphological, physiological and genetic characterization of two new *Bradyrhizobium* strains recently-recommended in Brazilian commercial inoculants for soybean. Symbiosis 20, 147–162.

Peres J R R, Vargas M A T and Suhet A R 1984 Variabilidade de eficiência em fixar nitrogênio entre isolados de uma mesma estirpe de *Rhizobium japonicum*. Rev. Bras. Cienc. Solo 8, 193–196.

Peters N K, Frost J W and Long S R 1986 A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science 233, 977–980.

Sadowsky M J, Cregan P B, Gottfert M, Sharma A, Gerhold D, Rodrigues-Quinones F, Keyser H H, Hennecke H and Stacey G 1991 The *Bradyrhizobium japonicum nolA* gene and its involvement in the genotype-specific nodulation of soybeans. Proc. Natl. Acad. Sci. USA 88, 637–641.

Sanjuan J, Carlson R W, Spaink H P, Bhat U R, Barbour W M, Glushka J and Stacey G A 1992 A 2-*O*-methylfucose moiety is present in the lipo-oligosaccharide nodulation signal of *Bradyrhizobium japonicum*. Proc. Natl. Acad. Sci. USA 89, 8789–8793.

Smith G B and Wollum II A G 1989 Nodulation of *Glycine max* by six *Bradyrhizobium japonicum* strains with different competitive abilities. Appl. Environ. Microbiol.55, 1957–1962.

Somasegaran P and Hoben H J 1985 Methods in Legume-*Rhizobium* Technology. Niftal, Hawaii, USA. 367 p.

Spaink H, Aaarts A, Stacey G, Bloemberg G, Lugtenberg B J and Kennedy E P 1992 Detection and separation of *Rhizobium* and *Bradyrhizobium* Nod metabolites using thin layer chromatography. Mol. Plant-Microbe Interact. 6, 72–80.

Sprent J I and Sprent P 1990 Nitrogen Fixing Organisms: Pure and Applied Aspects. Chapman and Hall, New York, USA. 256 p.

Streeter J G, Salminen S O, Whitmoyer R E and Carlson R W 1992 Formation of novel polysaccharides by *Bradyrhizobium japonicum* bacteroids in soybean nodules. Appl. Environ. Microbiol. 58, 607–613.

Van Brussel A A N, Zaat S A J, Canter Cremers H C J, Wijffelman C A, Pees E, Tak T and Lugtenberg B J J 1986 Role of plant root exudate and Sym plasmid-localized nodulation genes in the synthesis by *Rhizobium leguminosarum* of *Tsr* factor which causes thick and short roots on common vetch. J. Bacteriol. 165, 517–522.

Vargas M A T, Mendes I D C, Suhet A R and Peres J R R 1992 Duas novas estirpes de rizóbio para a inoculação da soja. (Comunicado Técnico 62). EMBRAPA-CPAC, Planaltina, Brazil. 3 p.

Vargas M A T, Peres J R R and Suhet A R 1982 Adubação nitrogenada, inoculação e épocas de calagem para a soja em um solo sob Cerrado. Pesq. Agropec. Bras. 17, 1127–1132.

Vasse J M and Truchet G L 1984 The *Rhizobium*-legume symbiosis: Observation of root infection by bright-field microscopy after staining with methylene blue. Planta 161, 487–489.

Vincent J M 1970 Manual for the Practical Study of Root Nodule Bacteria. IBP Handbook 15. Blackwell Scientific Publications, Oxford, UK. 164 p.

Vincet J M 1980 Factors controlling the legume-*Rhizobium* symbiosis. *In* Nitrogen Fixation. Eds. W E Newton and W H Orme-Johnson. pp 103-129. Univ. Park, Baltimore, UK.

Viteri S E and Schmidt E L 1987 Ecology of indigenous soil rhizobia: Response of *Bradyrhizobium japonicum* to readily available substrate. Appl. Environ. Microbiol. 53 1872–1875.

Zaat S A J, van Brussel A A N, Tak T, Pees E and Lugtenberg B J J 1987 Flavonoids induce *Rhizobium leguminosarum* to produce *nodDABC* gene- related factors that cause thick short roors and root hair responses on common vetch. J. Bacteriol. 169, 3388–3391.

Section editor: R O D Dixon