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Variability of nodulation and dinitrogen fixation capacity among soybean cultivars

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Abstract In order to identify soybean cultivars with higher biological N_2 fixation capacities, North American and Brazilian soybean [G*lycine max* (L.) Merrill] cultivars, belonging to maturity groups VI–VIII, were evaluated for nodulation parameters and N_2 fixation rates. The symbiotic performance of 152 cultivars was evaluated in pots containing 4 kg soil with an established population of the three *Bradyrhizobium elkanii* strains [29w (SEMIA 5019):SEMIA 566:SEMIA 587, 22%:36%:34%] which are established in most Brazilian soils cultivated with soybean. Differences were verified among cultivars, with some accumulating up to twice as much nodule dry weight and N in tissues as others. The variability among cultivars was also confirmed when six of them were used in a field experiment, resulting in differences in nodulation, yield and total N accumulated in grains. The analysis of nodule occupancy in 12 cultivars grown either under sterile conditions and receiving a double inoculum and N-free nutrient solution, or in pots containing soil with an established population of bradyrhizobia, showed the preference of cultivars for specific strains.

Key words *Bradyrhizobium* · *Glycine max* · Nodulation · Symbiosis · Plant breeding

Introduction

Soybean [*Glycine max* (L.) Merrill] is considered one of the oldest crops in the world, with reports of its cultivation in China around 2500 B.C. (Morse 1950). In Brazil, the crop was probably introduced in 1882 as *Soja japonica*, *Soja hispida Soja ochroleuca* and *Glycine soja* (D'utra 1882), but large scale cultivation began in this century, in the early 1960s. Today, the crop is cultivated throughout the entire country, occupies almost 13 million ha and produces more than 30 million t grain. Due to the high protein content of the seeds, the crop requires high levels of N, which can be supplied by symbiosis with the N_2-fixing bacteria *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii*.

Biological N_2 fixation is a complex process, and a successful symbiosis depends on the genetic background of both symbiotic partners and is strongly affected by environmental factors. Brazilian soils were originally free of soybean bradyrhizobia. The first strains were brought from the USA, and large-scale production of inocula started after the mid-1960s at the time of crop expansion. Today, there are few areas cultivated with soybean for the first time, and most soils contain a naturalized population of soybean bradyrhizobia, amongst which predominate the three most competitive strains used as inocula in the past three decades: 29w (SEMIA 5019), SEMIA 566 and SEMIA 587 (Hungria et al. 1994; Vargas and Hungria 1997). Consequently, what holds for several areas of the USA with serocluster USDA 123 (Ham et al. 1971; Weber et al. 1989) holds here: the introduction of new strains more efficient in the process of N_2 fixation can be very difficult. An approach taken by some North American laboratories to solve the competitiveness problem has been to identify soybean genotypes which specifically restrict nodulation to a determined serogroup (Caldwell 1966; Sadowsky and Cregan 1992). However, in an earlier investigation, restricting the nodulation of soybean cultivars to naturalized *Bradyrhizobium* strains from Brazil was not successful (Bohrer and Hungria 1998).

Earlier studies already pointed out the importance of plant genotype to the success of symbiosis (Wilson 1940; Nutman 1946). Several plant factors were considered responsible for the control of the N_2 fixation process, such as photosynthetic ability, hormonal balance, and activity of enzymes related with N assimilation, among others (Wilson 1940; Nutman 1946, 1981). Pioneer studies reported differences among soybean cultivars, including Brazilian soybean cultivars derived from

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North American cultivars, in relation to their symbiotic performance (Döbereiner and Arruda 1967; Brose et al. 1979; Vargas et al. 1982). However, in the last two decades very few studies have investigated the variability among North American and Brazilian soybean cultivars in relation to nodulation and N_2 fixation capacity. Furthermore, although the Brazilian soybean breeding program has been performed in areas previously inoculated with soybean N_2-fixing symbionts, and commonly in soils that are limited in N, the parameters related to symbiotic performance had not been evaluated up until now, with more attention being paid to resistance to diseases and yield.

In a previous study, the capacity of nodulation and N_2 fixation activity of 152 North American and Brazilian soybean cultivars, belonging to maturity groups VI-VIII, was investigated under axenic conditions (Bohrer and Hungria 1998). Important differences were detected amongst these cultivars and in the study reported here, the performance of these genotypes was evaluated in soils containing three *B. elkanii* strains. The objectives were to both identify cultivars with a higher capacity of N_2 fixation with the naturalized *Bradyrhizobium* strains, and cultivars with contrasting characteristics that could be used as the parental generation in future genetic studies and breeding programs.

Materials and methods

Plant materials

One hundred and fifty two soybean cultivars from the Brazilian germplasm bank, belonging to maturity groups VI–VIII were chosen for this experiment. The genealogies of these cultivars have been described before (Bohrer and Hungria 1998). They included North American cultivars, used during the introduction of soybean into Brazil, as well as cultivars resulting from Brazilian breeding programs. Seeds and information about the cultivars were obtained from the germplasm bank of the Embrapa-Soja (Brazilian Enterprise for Agricultural Research-National Soybean Research Center, Londrina).

Bradyrhizobium strains

Three *B. elkanii* strains were used, representing the majority of the soybean bradyrhizobia population established in Brazilian soils: 29w, SEMIA 566 and SEMIA 587. Strain 29w was isolated by Embrapa-Agrobiologia, (state of Rio de Janeiro), from soybean line IAC-70-559, and has been recommended for use in commercial inocula since 1979. SEMIA 566 was isolated by FEPAGRO (Fundação Estadual de Pesquisa Agropecuária), in the state of Rio Grande do Sul, in 1966, from a North American inoculum, and it was officially recommended from 1966 to 1978. SEMIA 587 was also isolated by FEPAGRO, and recommended from 1968 to 1975, and from 1979 till now. The three strains are serologically distinct and were recently classified as *B. elkanii* by Boddey and Hungria (1997).

Evaluation of N_2 fixing capacity in non-sterile soil

The experiment was performed under greenhouse conditions, in pots containing 4 kg of an oxisol collected from Londrina (state of Paraná, Brazil), with the following chemical properties: pH in $CaCl_2$, 5.1; N, 0.15 g dm^{-3}; Al, 0.0 $cmol_c$ dm^{-3}; K, 0.25 $cmol_c$ dm^{-3}; Ca, 5.2 $cmol_c$ dm^{-3}; Mg, 1.5 $cmol_c$ dm^{-3}; H+Al, 4.16 $cmol_c$ dm^{-3}; C, 2.5 g dm^{-3}; P, 5.1 mg dm^{-3}. The soil had been inoculated for more than 20 years and the naturalized bradyrhizobia population, evaluated by the most probable number technique (Vincent 1970) in soybean plants of cultivar BR-16, was estimated to be 10^5 cells g^{-1} soil. Using 20 soybean plants of cultivar BR-16, the serological reactions of 40 nodules per plant were evaluated, and the bradyrhizobia population estimated as being composed of strains 29w, SEMIA 566 and SEMIA 587, (22%, 36% and 34%, respectively); 5% of the nodules did not react with any known serogroup.

Seeds were surface sterilized, and five seeds of each cultivar were planted per pot. The pots received water daily and N-free plant nutrient solution every 3 days, as described before. After 5 days, plants were thinned to 2 plants pot^{-1}. They were harvested 7 weeks after germination, when the maximum nodule number and N_2 fixation rates occur under the conditions employed, and before nodule senescence started. At harvest, plants were divided into shoots, roots and nodules. Nodulation was evaluated by using the parameters of nodule number and dry weight, as well as nodule distribution on the roots. Shoot, root and nodule dry weight were evaluated after drying them at 65 °C for 4 days. Total N accumulated by shoots, roots and nodules was determined by the indophenol blue colorimetric method of Feije and Anger (1972). N accumulated by plants is presented as N accumulated in shoots plus roots plus nodules. The experiment was performed in a completely randomized block design, with four replicates. Data were analysed with the program SANEST and statistically significant differences determined by Tukey's test (Zonta et al. 1982).

Nodule occupancy following double inoculation under sterile conditions

Twelve cultivars were chosen to evaluate nodule occupancy, based on previous results obtained under axenic conditions, when each of the 152 cultivars was inoculated with one of the three *B. elkanii* strains used in the present experiment (Bohrer and Hungria 1998). In the previous study, six cultivars were classified as among the 20 best when inoculated with strain SEMIA 566 (EMGOPA-309, EMGOPA-313, IAC-4, IAC-15, OCEPAR 6 and Tropical), and six others were classified among the best when inoculated with SEMIA 587 (EMBRAPA 5, FT-9, Lancer, MSBR-17, OCEPAR 4 and Timbira). In the present experiment for the evaluation of N_2 fixing capacity in non-sterile soil, nodules of these 12 cultivars were sampled (40 nodules per replicate), and analysed for their serological reactions (Somasegaran and Hoben 1985) against the antisera of strains 29w, SEMIA 566 and SEMIA 587.

The preference shown by cultivars for some strains was also verified under sterile conditions. Strains SEMIA 566 and SEMIA 587 were grown in yeast mannitol medium (YM, Vincent 1970), for 7 days, equalized at 10^9 cells ml^{-1}, and a mixed inoculum of the two strains was prepared. Seeds were surface sterilized (Vincent 1970) and each cultivar inoculated by leaving the seeds for 30 min with the mixed inoculum (1 ml $seed^{-1}$). Five seeds were then planted per modified Leonard jar containing sterilized sand and vermiculite (1:2, v:v) and filled with N-free plant nutrient solution, with the pH adjusted to 6.8 (Somasegaran and Hoben 1985). Several non-inoculated controls of randomly selected cultivars were distributed throughout the greenhouse. Jars were filled with sterile N-free nutrient solution every other day. After 5 days plants were thinned to 2 plants jar^{-1} and were harvested 5 weeks after germination. At harvest, nodules were collected and analysed for serological reactions, using the antiserum prepared against each inoculated strain. The experiment was performed in a randomized block design, with five replicates, and the results were analysed with the program SANEST and statistically significant differences determined by Tukey's test.

Field trial

A field experiment was performed in an area with the same oxisol used for the greenhouse experiment. The chemical properties of the soil, number of viable *Bradyrhizobium* cells and distribution of the serogroups were as described above for the evaluation of N_2 fixing capacity in non-sterile soil.

The experimental plots measured 3.0×2.0 m, with 0.5 m between lines. The plots were separated by small terraces and were 2.0 m apart. Five days before sowing, the plots received 300 kg ha^{-1} of N-P-K (0, 28, 20) and 40 kg ha^{-1} of micronutrients (Zn, 9.0%; B, 1.8%; Cu, 0.8%; Fe, 3.0%; Mn, 2.0%; Mo, 0.10%).

Six cultivars were selected, taking into account their N_2 fixing ability evaluated in the previous experiment with non-sterile soil. The cultivars were chosen from among 17 genotypes recommended for the state of Paraná showing a growth cycle of between 109 and 120 days: BR-16, BR-36, Davis, FT-6, Invicta, OCEPAR 6. Inocula of strains 29w, SEMIA 566 and SEMIA 587 were prepared to a density of 10^9 cells ml^{-1} in semi-solid YM. A mixture containing one third of each strain was prepared, to which 100 ml inoculum kg^{-1} seeds was added. The inoculation aimed to guarantee good nodulation, with the strains inoculated in similar proportions to that of the established population. A control receiving 200 kg N ha^{-1} as urea, divided into two applications of 100 kg N ha^{-1}, one at sowing and the other at R2 (open flower at one of the two uppermost nodes on the main stem with a fully developed leaf), was also included.

The experiment was performed in a randomized block design, with six replicates. At R2, 15 plants of each treatment were harvested and nodule number and dry weight were evaluated. At the final harvest, a central area of 1.5 m×1.0 m (three lines) of each of the six replicates was collected; yield and N content of grains were evaluated, and the values were corrected for 13% moisture content. The results were analysed with the program SANEST and statistically significant differences determined by Tukey's test.

Results

Differences in symbiotic performance among the 152 soybean cultivars grown in a soil containing a naturalized population of bradyrhizobia with three *B. elkanii* were verified. In relation to nodulation parameters, nodule number was significantly affected by cultivar ($F=3.12$, $P\leq 0.0001$), with similar effects for nodule dry weight ($F=3.13$, $P\leq 0.0001$) and the percentage of nodules on the tap root ($F=3.23$, $P\leq 0.0001$). Although the 152 cultivars have been used in Brazil as North American parental genotypes in breeding programs (e.g. Davis), or recommended as commercial cultivars, and are largely employed today by farmers (e.g. BR-16, BR-37), differences of up to 100% were detected for nodule number and nodule dry weight (Table 1). Nodules were predominantly localized on the tap root, within the first 3.5×2.5 cm (length× width) of the root region, with a mean of 75% of the nodules in this area. However, some differences were detected between cultivars, e.g. cultivars FT-Abyara and BR-27 showed a higher percentage (40–60%) of nodules on the lateral roots (data not shown).

Statistical differences among cultivars were also observed for shoot dry weight ($F=2.12$, $P\leq 0.0001$), root dry weight ($F=2.33$, $P\leq 0.0001$; data not shown) and total N accumulated by plants (shoot plus roots plus nodules) ($F=1.98$, $P\leq 0.0001$). The cultivars accumulated from 96.6 mg N $plant^{-1}$ (J-200) to 42.1 mg N $plant^{-1}$ (EMBRAPA 9; Table 1). The mean total N content was 63.7 mg N $plant^{-1}$, and 33 cultivars accumulated 10% more N than the general mean. Four of these latter cultivars accumulated 30% more N than the general mean: J-200, Bossier, Ivaí and BR-29. In relation to the most ineffective symbiosis, 33 cultivars accumulated 10% less N than the mean reported for the experiment, and two of them, FT-Canarana and EMBRAPA-9, accumulated <44.5 mg N $plant^{-1}$, corresponding to 30% less than the mean:. Differences were also detected for nodule efficiency, defined here as mg N accumulated g^{-1} nodule ($F=8.96$, $P\leq 0.0001$), ranging from 460 mg N g^{-1} nodule with cultivar RS 6, to 246 mg N g^{-1} nodule with EMG.303.

The data were also analysed statistically to obtain the correlation matrices for the parameters (Table 2). Nodule mass was the nodulation parameter which correlated best with N accumulated in shoots and in whole plants (shoots plus roots plus nodules). Highly statistically significant correlations were observed between shoot dry weight and total N accumulated in shoots and in plants (Table 2).

Twelve cultivars were chosen to study nodule occupancy. Six of these cultivars had been previously classified by Bohrer and Hungria (1998) as among the 20 best when inoculated with strain SEMIA 566, and the other six classified among the best when inoculated with SEMIA 587. The preference of cultivars for some strains was shown in pots with soil containing an established population of *Bradyrhizobium* (Table 3). Therefore, when nodule occupancy was analysed, all cultivars belonging to the group which had previously performed well with SEMIA 566 showed a preference for this strain, while five of the six cultivars selected for their good symbiotic performance with SEMIA 587 had more nodules occupied by this strain than any other strain. These preferences were confirmed in the experiment performed with sterile substrate and seeds receiving a mixed inoculum. Under these conditions, five of the six cultivars showed a preference for SEMIA 566, while four of the six cultivars showing a good symbiotic performance with SEMIA 587 had a higher percentage of nodules occupied by this strain than any other strain (Table 4). Consequently, the results obtained under sterile and non-sterile conditions indicated the preference of some cultivars for specific strains.

Variability of nodulation was verified in the field experiment with six cultivars which had showed differences in N_2 fixation capacity, all belonging to the same maturity group and recommended for the state of Paraná due to their high yield, adaptation to the climate and resistance to diseases (Table 5). Differences were also verified for both yield and total N accumulated in grains: these parameters were 27% lower in symbioses with BR-16 than with FT-6 (Table 5).

Table 1 N_2 fixation parameters of 152 soybean cultivars grown in pots containing 4 kg soil with an established population of *Bradyrhizobium elkanii* strains 29w (SEMIA 5019), SEMIA 566 and SEMIA 587. Data are means of four replicates. Plants were harvested 7 weeks after germination. *NN* Nodule no. (no. nodules plant^{-1}), *NDW* nodule dry weight (mg nodule plant^{-1}), *TNP* total N accumulated in plants (shoots plus roots plus nodules; mg N plant^{-1}), *LSD* least significant difference, *CV* coefficient of variation

Cultivar	NN	NDW	TNP	Cultivar	NN	NDW	TNP
Andrews	55	252	78.0	EMBRAPA 1	47	215	66.5
BA BR-31	47	241	71.1	EMBRAPA 2	49	203	65.2
Bossier	64	289	93.1	EMBRAPA 3	52	169	56.8
BR/EMG.312	52	215	66.3	EMBRAPA 4	59	247	68.0
BR-1	53	197	59.2	EMBRAPA 5	49	217	66.9
BR-2	55	153	58.0	EMBRAPA 9	44	131	42.1
BR-3	67	170	53.1	EMG.-301	53	207	59.4
BR-4	54	182	58.2	EMG.-302	57	192	53.4
BR-5	49	170	57.5	EMG.-303	52	214	52.7
BR-6	51	208	64.3	EMG.-304	52	172	60.0
BR-7	53	155	47.7	EMG.-305	49	186	53.2
BR-8	48	161	58.2	EMG.-306	51	192	57.6
BR-12	43	157	52.8	EMG.-307	52	197	63.2
BR-13	56	240	69.3	EMG.-309	59	164	53.2
BR-14	66	248	72.3	EMG.-313	45	153	48.2
BR-15	53	187	55.0	FT-1	53	207	62.8
BR-16	59	190	57.7	FT-2	50	209	65.7
BR-23	54	193	62.1	FT-3	49	204	64.3
BR-24	50	213	70.0	FT-4	53	206	71.9
BR-27	53	230	68.2	FT-5	50	194	57.6
BR-28	54	165	46.4	FT-6	64	246	77.8
BR-29	58	237	88.8	FT-7	47	164	60.0
BR-30	65	238	63.3	FT-8	72	207	57.1
BR-32	44	156	52.4	FT-9	54	188	74.2
BR-35	46	174	55.7	FT-10	47	207	68.8
BR-36	46	206	67.0	FT-11	62	198	71.2
BR-37	68	222	65.4	FT-12	55	196	63.6
BR-38	47	170	61.6	FT-14	60	210	72.0
Bragg	56	240	69.3	FT-16	51	182	63.4
C. Gerais	61	211	67.3	FT-17	62	267	75.0
CAC-1	52	192	53.1	FT-18	65	152	58.3
CEP 10	53	186	71.1	FT-19	61	190	64.7
CEP 12	59	213	66.6	FT-20	52	206	75.5
CEP 20	51	147	47.6	FT-Abyara	65	220	65.1
Cobb	45	145	57.2	FT-Bahia	54	217	63.2
Davis	56	231	75.4	FT-Canarana	46	157	42.8
Doko	48	145	58.2	FT-Cometa	57	177	61.6
Dourados	54	208	64.1	FT-Cristalina	52	231	61.1

Discussion

Poor N_2 fixation rates of soybean in the field have often been attributed to its symbiosis with non-efficient bradyrhizobial strains established in soils by previous inoculation. More efficient strains introduced via inocula can hardly compete with the naturalized population, and occupy just a small percentage of root nodules (Kvien et al. 1981; Dunigan et al. 1984). Many laboratories have thus concentrated their efforts on ways of solving the competitiveness problem. In relation to microsymbionts, there is a search for more competitive strains and recommendations for large-scale inoculation with desirable strains (Duningan et al. 1984; Vargas and Hungria 1997). In relation to the host plant, some genes of North American cultivars have been identified which restrict nodulation by certain inefficient serogroups which are established in traditional soybean areas of the USA (Caldwell 1966; Devine 1976). However, when 152 North American and Brazilian cultivars were analysed, restriction of nodulation to the three *B. elkanii* strains established in Brazilian soils cultivated with this crop was not detected (Bohrer and Hungria 1998).

Cultivars with different levels of preference or which exclude certain serogroups have also been reported (Kvien et al. 1981; Weiser et al. 1990); these results can be used as a starting point when exploring the benefits of a specific strain. Preference for specific microsymbionts was also shown with 20 of the cultivars used in the present study, both when seeds were double inoculated under sterile conditions and when plants were grown in pots containing non-sterile soil with an established population of *Bradyrhizobium*.

The effects of soybean cultivars on N_2 fixation have been frequently reported (Kvien et al. 1981; Senaratne et al. 1987; Mytton and Skøt 1993). In a previous study,

Table 1 Continued

Cultivar	NN	NDW	TNP	Cultivar	NN	NDW	TNP
FT-Estrela	54	209	65.0	MS BR-39	45	192	64.8
FT-Eureka	54	212	71.7	Nova IAC-7	47	180	58.8
FT-Guaíra	55	206	70.3	Numbaíra	57	211	74.3
FT-Jatobá	52	190	65.3	OCEPAR 2	58	199	68.7
FT-Manacá	55	215	71.1	OCEPAR 3	58	198	56.1
FT-Maracaju	51	172	68.6	OCEPAR 4	59	243	76.6
FT-Seriema	46	167	51.6	OCEPAR 5	45	156	58.1
GO BR-25	62	182	69.2	OCEPAR 6	56	217	59.4
IAC-2	53	215	61.1	OCEPAR 7	54	215	59.0
IAC-4	54	212	67.2	OCEPAR 8	50	200	66.2
IAC-5	55	215	64.3	OCEPAR 9	46	171	57.5
IAC-6	46	186	55.5	OCEPAR 10	50	154	55.7
IAC-7	56	229	59.2	OCEPAR 11	43	151	61.1
IAC-8	52	225	73.1	OCEPAR 13	53	230	64.4
IAC-9	47	165	70.6	OCEPAR 14	60	227	56.4
IAC-11	53	182	54.2	Paranagoiana	45	150	46.5
IAC-12	48	170	64.2	Paranaíba	45	160	51.5
IAC-13	53	201	71.1	Paraná	55	231	59.2
IAC-15	53	173	69.9	Pérola	45	176	55.5
IAC-16	35	162	57.7	Planalto	45	162	55.5
IAC-100	39	157	56.0	RS 5	52	162	53.2
IAC-Foscarin	55	195	66.6	RS 6	44	151	69.5
IAC-PL-1	46	182	69.7	RS 7	52	192	65.6
IAS 4	45	180	70.1	Santa Rosa	61	235	75.6
IAS 5	47	171	61.4	Sertaneja	53	215	72.1
Invicta	65	199	65.3	SP BR-41	54	226	63.1
IPAGRO 20	51	182	65.4	Stuart	60	277	83.7
IPAGRO 21	67	229	71.1	Tiaraju	55	204	70.1
Ivaí	54	226	89.9	Timbira	49	169	64.2
J-200	56	272	96.6	Tropical	55	176	60.1
Lancer	55	245	73.3	UFV/ITM-1	55	211	59.2
MG BR-22	60	238	74.2	UFV-1	40	157	61.1
MS BR-17	59	215	65.3	UFV-5	45	156	66.4
MS BR-18	57	238	75.5	UFV-8	60	226	65.2
MS BR-19	47	188	65.2	UFV-9	52	217	59.2
MS BR-20	46	195	68.1	UFV-10	41	154	56.0
MS BR-21	48	217	63.3	União	50	176	60.0
MS BR-34	50	171	52.1	Viçoja	47	155	63.3
Mean	50.7	189.2	60.9				
LSD[a]	25.2	72.5	23.6				
CV (%)	38	29	22				

[a] LSD for Tukey's test ($P=0.05$)

the cultivars used in this study were evaluated under axenic conditions for nodulation parameters and N_2 fixation rates when inoculated with each of the three *B. elkanii* strains examined here (29w, SEMIA 566 and SEMIA 587), which are established in most Brazilian soils cultivated with this legume. The effects of cultivar, strain and interaction among cultivar and strain were reported, with up to fourfold differences in nodule number, nodule mass and total N accumulated in tissues (Bohrer and Hungria 1998). However, it was necessary to confirm these results in an experiment carried out under non-sterile conditions. Therefore, in this study, plants were grown in pots containing soil with an established population of bradyrhizobia, and the 152 soybean cultivars used showed good nodulation, but up to twofold differences in nodule number, nodule dry weight and total N accumulated in tissues were detected. Most nodules were localized on the tap root, which could be associated with a better symbiotic performance (McDermott and Graham 1990). However, the role of nodulation on the secondary roots is not clear and, at least for common bean (*Phaseolus vulgaris*), nodulation of these roots implied higher N accumulation in tissues (Wolyn et al. 1989; Hardarson et al. 1993). Some cultivars showed a higher percentage of nodules on lateral roots, confirming previous results obtained under sterile conditions (Bohrer and Hungria 1998). These cultivars should be used in future experiments to clarify the importance of nodulation on main or lateral roots to the N nutrition of soybean. When six cultivars with the same growth cycle and recommended for the state of Paraná were used in the field experiment, differences in nodulation were confirmed, as well as differences of up to 27% in yield and total N accumulated in grains.

The identification of cultivars with a high capacity for biological N_2 fixation is important when recommending cultivars to farmers, as well as determining

Table 2 Matrix of correlation coefficients for nodulation, plant growth and N accumulation parameters in 152 soybean cultivars grown in soil containing an established population of *B. elkanii* strains 29w, SEMIA 566 and SEMIA 587. Nodulation parameters included the percentage of nodules on the tap root (*NTP*), NN (no. nodules $plant^{-1}$) and NDW (mg nodule $plant^{-1}$). Growth and N parameters were root dry weight (*RDW*) and shoot dry weight (*SDW*; g $plant^{-1}$), percentage of N in shoots (%*NS*), total N in shoots (*TNS*; mg N $shoot^{-1}$) and TNP (mg N $plant^{-1}$). Plants were harvested 7 weeks after emergence. For other abbreviations, see Table 1

Variable	NTP	NN	NDW	RDW	SDW	%NS	TNS	TNP
NTP	1.00[a]	0.005	0.092	0.103	0.108	0.002	0.051	0.042
NN		1.000	0.579	0.286	0.315	0.185	0.358	0.322
NDW			1.000	0.622	0.665	0.161	0.697	0.654
RDW				1.000	0.677	0.277	0.411	0.422
SDW					1.000	0.326	0.911	0.915
%NS						1.000	0.555	0.521
TNS							1.000	0.922
TNP								1.000

[a] Correlation coefficients ≥0.16 are statistically significant at $P \leq 0.05$, and correlation coefficients ≥0.21 are statistically significant at $P \leq 0.01$

Table 3 Percentage nodule occupancy of 12 soybean cultivars[a] grown in pots containing 4 kg soil with an established population of *B. elkanii* strains 29w, SEMIA 566 and SEMIA 587. Plants were harvested 7 weeks after germination and nodule occupancy was evaluated by serology. The data are means of four replicates. Values followed by the *same letter*, within a column, were not statistically different (Tukey test, $P \leq 0.05$). *ns,* no significant differences detected

Cultivar	Strain			Multiple occupancy	No reaction
	SEMIA 566	SEMIA 587	29w		
EMGOPA-309	35 b	28 c	25 a	6 ns[2]	6 ns
EMGOPA-313	55 a	32 c	8 c	5	0
IAC-4	53 a	29 c	14 abc	4	0
IAC-15	51 a	33 bc	15 abc	1	0
OCEPAR 6	49 a	35 bc	12 bc	2	2
Tropical	51 a	34 bc	14 abc	1	0
EMBRAPA 5	48 a	35 bc	10 c	4	3
FT-9	34 b	42 ab	17 abc	7	0
Lancer	30 b	45 a	22 ab	1	2
MS BR-17	31 b	47 a	19 abc	3	0
OCEPAR 4	35 b	51 a	9 c	3	2
Timbira	30 b	42 ab	23 a	2	3
CV (%)	22	25	35	35	42

[a] In a previous study in which 152 cultivars were inoculated with one of these two strains, the first six cultivars were identified among the best N_2 fixers with strain SEMIA 566, and the last six ones among the best N_2 fixers with SEMIA 587 (Bohrer and Hungria 1998)

Table 4 Percentage of nodule occupancy of 12 soybean cultivars[a] inoculated with a mixed inoculum of strains SEMIA 587 and SEMIA 566 (1:1, v:v; 10^9 cells ml^{-1}). Experiment performed in Leonard jars (sterilized sand and vermiculite). Plants were harvested 5 weeks after emergence and nodule occupancy was evaluated by serology. The data are means of five replicates. Values followed by the *same letter*, within a column, were not statistically different (Tukey test, $P \leq 0.05$)

Cultivar	Strain		Double occupancy
	SEMIA 566	SEMIA 587	
EMGOPA-309	42 b	35 de	23 a
EMGOPA-313	83 a	6 g	11 ab
IAC-4	68 a	21 ef	11 ab
IAC-15	68 a	18 fg	14 ab
OCEPAR 6	75 a	4 g	11 ab
Tropical	71 a	22 ef	7 b
EMBRAPA 5	40 b	38 cd	14 ab
FT-9	24 c	66 a	10 ab
Lancer	39 b	58 ab	3 b
MS BR-17	35 bc	55 ab	11 ab
OCEPAR 4	34 bc	51 abc	15 ab
Timbira	33 bc	45 bcd	22 a
CV (%)	28	29	43

[a] See Table 3

Table 5 NN and NDW at the R2 stage, and yield and total N in grains (*TNG*) of six soybean cultivars. Experiment performed on an oxisol of Londrina (state of Paraná) containing 10^5 cells of soybean *Bradyrhizobium* g^{-1} soil. Data are means of six replicates. Values followed by the *same letter,* within a column, were not statistically different (Tukey, $P \leq 0.05$). For other abbreviations, see Table 1

Treatment	NN (no. $plant^{-1}$)	NDW (mg $plant^{-1}$)	Yield (kg ha^{-1})	TNG (kg N ha^{-1})
FT-6	61 a	185 a	2925 a	190 a
Davis	58 a	183 a	2782 a	181 ab
BR-36	52 ab	160 ab	2512 ab	163 bc
Invicta	48 ab	161 ab	2471 ab	158 bc
OCEPAR 6	36 b	113 b	2215 b	145 c
BR-16	35 b	121 b	2219 b	138 c
Mineral N[a]	7 c	25 c	2913 a	180 ab
CV (%)	38	32	13	9

[a] N supplied as urea (200 kg N ha^{-1}) half applied at sowing and half at R2 stage

cultivars for use as parental genotypes in breeding programs. The breeding of soybean for increased N_2 fixation can be successful (Ronis et al. 1985; Burias and Planchon 1990), and breeding programmes can also be used to develop cultivars capable of producing an increased nodule mass with naturalized populations of *Bradyrhizobium* (Greder et al. 1986).

Soybean is the most import legume crop in Brazil. Most of the Brazilian cultivars are derived from North American ones, and some of the latter are still used by farmers today. The first breeding trials performed in Brazil considered N_2 fixation capacity as an important characteristic of cultivars. As a result, although Brazilian soils are poor in N, soybean plants fulfil their N requirements exclusively by biological fixation. However, in recent years, breeders have paid less attention to N_2 fixation capacity than to yield and control of diseases. It is not surprising, therefore, that three out of the four best cultivars (J-200, Bossier, Ivaí) with respect to N_2 fixation represent old and traditional cultivars, and some of the recently released cultivars have considerably decreased nodulation and N_2 fixation rates. For example, in the present study, cultivar Davis, a North American cultivar introduced in Brazil in the early 1960s, was found to accumulated 231 mg nodules plant^{-1}and 75 mg N plant^{-1}, while decreased values were obtained in cultivars which had Davis as a parental genotype, e.g. BR-16 (190 mg nodules plant^{-1}, 57.7 mg N plant^{-1}, respectively) and FT-7 (164 mg nodules plant^{-1}, 60 mg N plant^{-1}, respectively). A decline in nodulation and N_2 fixation was also found when other cultivars frequently used as parental genotypes were considered, e.g. Bossier and Paraná. Consequently, more attention should be given to understanding and evaluating the plant mechanisms controlling and regulating nodule formation and activity.

The correlations between values obtained for symbiotic parameters when plants were grown in non-sterile soil resembled those described for plants grown under axenic conditions by Bohrer and Hungria (1998). Nodule number did not show a good correlation with total N accumulated in shoots or in plants (shoot plus roots plus nodules), confirming previous observations, e.g. by Nutman (1981) and Neves et al. (1985). However, significant correlations were found between nodule mass and total N in shoots or in plants, confirming results of earlier experiments performed in Brazil (Döbereiner 1966). Significant linear correlations were also obtained between shoot dry weight and total N in shoots and total N accumulated in plants. This supported a conclusion of Haydock et al. (1980), who found after performing trials under axenic conditions with several nodulated species to select promising cultivars for biological N_2 fixation, that it was not necessary to carry out Kjeldahl analysis for the determination of N. The results obtained in this study suggest that this conclusion can be extended to trials performed in soils with low levels of mineral N. Consequently, a simple evaluation of shoot dry weight could speed up breeding programs and also decrease research costs.

Based on the results obtained by Bohrer and Hungria (1998) and those of this study, four cultivars which ranged from the best to the worst N_2 fixers are currently being employed in a research program examining parental genotypes. This program aims to find genetic markers for biological N_2 fixation, as well as to detect genotypes with a higher capacity for N_2 fixation.

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