Bradyrhizobium STRAIN AND THE ^{15}N NATURAL ABUNDANCE QUANTIFICATION OF BIOLOGICAL N_2 FIXATION IN SOYBEAN

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ABSTRACT: In commercial plantations of soybean in both the Southern and the Cerrado regions, contributions from biological nitrogen fixation (BNF) are generally proportionately high. When using the ^{15}N natural abundance technique to quantify BNF inputs, it is essential to determine, with accuracy, the ^{15}N abundance of the N derived from BNF (the '*B*' value). This study aimed to determine the effect of four recommended strains of *Bradyrhizobium* spp. (two *B. japonicum* and two *B. elkanii*) on the '*B*' value of soybean grown in pots in an open field using an equation based on the determination of $\delta^{15}N$ natural abundance in a non-labelled soil, and estimate of the contribution of BNF derived from the use of ^{15}N-isotope dilution in soils enriched with ^{15}N. To evaluate N_2 fixation by soybean, three non-N_2-fixing reference crops were grown under the same conditions. Regardless of *Bradyrhizobium* strain, no differences were observed in dry matter, nodule weight and total N between labelled and non-labelled soil. The N_2 fixation of the soybeans grown in the two soil conditions were similar. The mean '*B*' values of the soybeans inoculated with the *B. japonicum* strains were -1.84 ‰ and -0.50 ‰, while those inoculated with *B. elkanii* were -3.67 ‰ and -1.0 ‰, for the shoot tissue and the whole plant, respectively. Finally, the '*B*' value for the soybean crop varied considerably in function of the inoculated *Bradyrhizobium* strain, being most important when only the shoot tissue was utilised to estimate the proportion of N in the plant derived from N_2 fixation.
Key words: *Bradyrhizobium elkanii*, *Bradyrhizobium japonicum*, *Glycine max*, '*B*' value

ESTIRPE DO *Bradyrhizobium* E QUANTIFICAÇÃO DA FIXAÇÃO BIOLÓGICA DE NITROGÊNIO EM SOJA UTILIZANDO A TÉCNICA DA ABUNDÂNCIA NATURAL DE ^{15}N

RESUMO: Em plantações comerciais de soja na região Sul e do Cerrado, as contribuições da fixação biológica de Nitrogênio (FBN) são geralmente elevadas. Quando usamos a técnica da abundância natural de ^{15}N para quantificar a FBN, é essencial determinar com exatidão a abundância de ^{15}N do N derivado da FBN (valor *'B'*). Este trabalho buscou determinar o efeito das quatro estirpes de *Bradyrhizobium* spp. (duas *B. japonicum*, duas *B. elkanii)* sobre o valor *'B'* de soja crescida em vasos em ambiente aberto usando uma equação na determinação da abundância natural de ^{15}N em um solo não enriquecido com ^{15}N, e estimativas da contribuição da FBN derivado do uso da técnica de diluição isotópica de ^{15}N em solo enriquecido com ^{15}N. Para avaliar a fixação de N_2 pela soja três plantas referenciam foram crescidas nas mesmas condições. Independente da estirpe de *Bradyrhizobium*, não foi observada diferença para matéria seca, massa de nódulos e N total entre solo marcado e não marcado. A fixação de N_2 em soja crescida nas duas condições de marcação do solo foi semelhante. Os valores médios de '*B*' para plantas de soja inoculadas com estirpes de *B. japonicum* foram, em média, de -1,84 ‰ e -0,50 ‰ enquanto as inoculadas com *B. elkanii* apresentaram médias de -3,67 ‰ e -1,00 ‰, para parte aérea e planta inteira, respectivamente. Finalmente, o valor *'B'* para a cultura da soja variou consideravelmente em função da estirpe de *Bradyrhizobium* inoculada, sendo mais importante quando se utiliza somente a parte aérea da planta para estimar a proporção do N da planta derivado da fixação de N_2.
Palavras-chave: *Bradyrhizobium elkanii*, *Bradyrhizobium japonicum*, *Glycine max*, valor de '*B*'

INTRODUCTION

Using the ^{15}N natural abundance technique to quantify the contribution of biological nitrogen fixation (BNF) to a legume has the almost unique attribute that it can be used without interference to the plant environment (Peoples et al., 1989). The technique is based on the commonly-observed phenomenon that N in most (but not all) soils is slightly naturally enriched with ^{15}N as compared to the natural abundance of atmospheric N_2 (Högberg, 1997; Boddey et al., 2000). In order to determine the proportion of N derived from the air (%Ndfa) it is necessary to determine: the ^{15}N abundance of the legume under study, and the ^{15}N abundance of both the N derived from the soil, and, that derived from the air via BNF (Shearer & Kohl, 1986).

The ^{15}N abundance of the N in the legume derived from BNF is known as the '*B*' value. When contributions of BNF are high and/or the ^{15}N abundance of the soil-derived N is low, it is extremely important to accurately determine this value.

Using plants grown in N-free medium (Steele et al., 1983; Bergersen et al., 1986; Yoneyama et al., 1986) have reported that the '*B*' value of a particular legume may vary with rhizobium strain. Doughton et al. (1992) developed a method to determine '*B*' with plants grown in the field based on the comparison of the estimate of the % Ndfa from the use of the ^{15}N isotope dilution technique on soil enriched with ^{15}N with those derived from the use of the ^{15}N natural abundance technique. With this method Okito et al. (2004) found that there were large differences in '*B*' value of either the shoot tissue or the whole soybean plants between the different strains. The '*B*' values for the *Bradyrhizobium* strains 29 W (SEMIA 5019 - *B. elkanii*) and CPAC 7 (SEMIA 5080 - *B. japonicum*), respectively, were -4.54 and -2.39 ‰ for the shoot tissue. These two strains are both recommended for inoculant manufacture in Brazil and proportional BNF inputs to soybean are generally high (Alves et al., 2003, 2006). Therefore, the objective of this study was to determine the '*B*' values in soybean plants grown in an open field soil inoculated with of all four recommended strains of *B. elkanii* and *B. japonicum*.

MATERIAL AND METHODS

An open field pot experiment was installed on December 13th 2003 in Seropédica, Rio de Janeiro State (22º47' S, 43º40' W, 33 m above sea level). The soybean plants were grown in 140 plastic pots filled with 4 kg of dry topsoil (0-20 cm) taken from an area of the field station which had never been planted before with soybean and was known from previous studies not to possess significant numbers of rhizobium strains capable of nodulating this crop. The soil type was classified as an Orthic Acrisol based on the FAO classification, or as an Argissolo Vermelho - Amarelo distrófico using the Brazilian classification. Before fertiliser addition the main chemical analyses of the soil were: pH in H_2O, 5.1 exchangeable; Al, Ca and Mg were 10, 10 and 8 $mmol_c\ dm^{-3}$; respectively, and P and K, 1 e 61 mg dm^{-3}, respectively (Embrapa, 1997). To each pot, 1.2 g dolomitic lime per kg of dry soil, and subsequently 230 mg P_2O_5 kg and 120 mg K_2O $kg\ soil^{-1}$ were added. Half of the pots were amended with a solution containing 40 mg of ^{15}N-labelled ammonium sulphate enriched at 20 atom $\%^{15}N$ excess.

Soybean seeds (cv Celeste) were surface sterilised in 70% aqueous ethanol for 3 min followed by 2 min in sodium hypochlorite solution (50 g L) and then washed 10 times in sterile distilled water. Pots were seeded with five seeds of each plant and 8 days after emergence these were thinned to two plants per pot.

The experiment was laid out in a randomised complete block design with split plots and five replicates. Main plots consisted of the two ^{15}N treatments (enriched with ^{15}N or not) and subplots of soybean plants inoculated with the four strains of *Bradyrhizobium* recommended for the manufacture of commercial inoculants in Brazil along with non-inoculated plants and three more pots for reference plants. The Brazilian recommended strains are 29W (SEMIA 5019) and SEMIA 587 which belong to the species *Bradyrhizobium elkanii* and CPAC 15 (SEMIA 5079) and CPAC 7 (SEMIA 5080) which are *B. japonicum* (Rumjanek et al, 1993; Sato et al., 1999). The inocula were peat based containing approximately 10^9 colony-forming units g dry $weight^{-1}$. To facilitate the quantification of BNF by the soybean and to evaluate the '*B*' value, three non-N_2-fixing reference plants, non-nodulating soybean (cv. T 201), rice (*Oryza sativa* cv. IAC 4440) and grain sorghum (*Sorghum bicolor*, cv. BR 305) were employed.

During the first 15 days the plants were irrigated with sterile distilled water to avoid possible contamination of the seedlings with rhizobia. Subsequently, ordinary tap water was used for irrigation. At 87 days after planting (DAP), coinciding with early grain filling, all plants were harvested and separated into shoot tissue, roots and nodules. Great care was taken to recover all roots and nodules by sieving the soil through a 1 mm sieve. All harvested materials were dried at 65ºC for >72 h and ground in a Wiley mill (<0.85 mm). Sub-samples were subsequently ground to a fine powder using a roller mill similar to that described by Smith & Myung (1990).

Samples were analysed for total N content using the semi-micro Kjeldahl procedure as described by Urquiaga et al. (1992). The ^{15}N enrichment/abundance of aliquots of sub-samples containing approximately 35 μg N was determined using an automated continuous-flow isotope-ratio mass spectrometer consisting of a Finnigan DeltaPlus mass spectrometer coupled to the output of a Carlo Erba EA 1108 total C and N analyser (Finnigan MAT, Bremen, Germany). Ground samples of the seeds were also analysed for total N and ^{15}N natural enrichment/ abundance using the same procedures.

The weighted mean of the ^{15}N natural abundance of the whole plants (discounting original seed N) can be calculated using a mass balance as described by Högberg et al. (1994):

Whole plant $\delta^{15}N$ =

$$\frac{\left[\left(Sh\ d^{15}N \times Sh\ N\right) - \left(Sd\ d^{15}N \times Sd\ N\right) + \left(Nod\ d^{15}N \times Nod\ N\right) + \left(Ro\ d^{15}N \times Ro\ N\right)\right]}{\left(Sh\ N - Sd\ N + Nod\ N + R\ N\right)} \quad (1)$$

where Sh, Sd, Nod and Ro represent shoots, seeds, nodules and roots, respectively, and $\delta^{15}N$ is the ^{15}N natural abundance (‰) and N is the total N accumulated by each plant part.

For the soybeans planted in the soil enriched with ^{15}N, the proportion of N derived from the air (%Ndfa) via BNF was calculated according to the equation of Chalk (1985):

$$\%Ndfa = \left[1 - \left(\frac{\%^{15}N_f}{\%^{15}N_r}\right)\right] \times 100 \quad (2)$$

where $\%^{15}N_f$ and $\%^{15}N_r$ are the values of atom $\%^{15}N$ excess of the soybean and the reference plant, respectively. As three non-N_2-fixing crops were used as reference plants, three independent estimates of the %Ndfa for each strains could be calculated.

The addition of a small amount of labelled ammonium fertiliser (40 mg per pot or 2.1 mg N kg soil^{-1}) was insufficient to have any significant effect on N_2 fixation by the soybean. Hence the %Ndfa of non-fertilised plants was assumed to be equal to that of legumes grown in ^{15}N-labelled soil. In this case the '*B*' value can be calculated for the non-labelled soybean plants using the equation (Okito et al., 2004):

$$\text{'}B\text{' value} = [(\delta^{15}N_r \times \%Ndfa) - 100 \times (\delta^{15}N_r - \delta^{15}N_f)] \,/\, \%Ndfa \quad (3)$$

where $\delta^{15}N_r$ and $\delta^{15}N_f$ are the natural ^{15}N abundance of the reference and soybean plants, respectively.

The data were subjected to standard analysis of variance to detect significant differences between treatment means. The Student LSD test was applied to verify similarities and differences of means for the various parameters for the reference plants and the soybean inoculated with the different *Bradyrhizobium* strains.

It was necessary to compare sub-plot treatment means for different main plots in order to evaluate whether there were effects of the *Bradyrhizobium* strain on accumulation of dry matter regime and N and ^{15}N enrichment in the labelled and unlabelled soil individually. This was achieved using the procedure described by Little & Jackson-Hills (1978): The calculation of the least significant difference between means (LSD – Student) becomes:

$$LSD_{0.05} = t_{ab}\sqrt{\{2.[(b-1)Ea + Eb]/rb\}} \quad (4)$$

where b is the number of subplot treatments, r the number of replicates, Ea and Eb are the mean squares of the sub-plot and main plot errors, respectively, and t_{ab} is the weighted t value for main plots and subplots calculated as described by Little & Jackson-Hills (1978).

RESULTS AND DISCUSSION

The non-N_2-fixing reference plants and the inoculated soybeans showed no difference in their dry matter accumulation when grown in ^{15}N-labelled or unlabelled soil (Tables 1 and 2). However, the reference sorghum and rice plants accumulated significantly more nitrogen in the soil amended with ^{15}N-labelled ammonium sulphate (Table 1), but the total N accumulated was far less (<6%) than that accumulated by the nodulated soybean plants (Table 3). For the nodulated soybeans, the small labelled-N addition had no significant effect on the dry weight or N content of (or N accumulation by) the nodules or the whole plants. These results indicate that the quantity of labelled N added to the soil in the ^{15}N-enriched treatment had no significant effect on the contribution of BNF to the soybean. This is a necessary prerequisite for the application of the technique used here, originally proposed by Doughton et al. (1992) for the determination of the '*B*' value.

Amongst the nodulated soybean plants, inoculation with the CPAC 15 strain showed the greatest dry matter accumulation with 56.9 g DM per pot in the shoot tissue and 77.5 DM per pot for the whole plant (Table 2). Moreover, the soybean plants inoculated with this *Bradyrhizobium* strain also accumulated significantly more N than all other inoculation treatments (1729 mg N per pot). However, there were no differences in N accumulation between the other three strains (means ranged from 1380 to 1436 mg N per pot - Table 3).

Table 1 - Dry matter accumulation of dry matter and total N by non-nodulating (NN) soybean, sorghum (cv. BRS 305) and rice (cv. IAC 4440), harvested 87 days after planting.

Reference	Shoots			Roots			Whole plant		
Plant	unlabelled	+ ^{15}N	Mean	unlabelled	+ ^{15}N	Mean	unlabelled	+ ^{15}N	Mean
	---------- g MS per pot----------								
NN soybean	2.0 B	2.4 C	2 C	2.3 B	1.3 B	1.8 B	4.3 B	3.8 B	4.0 C
Sorghum	5.2 A	6.5 A	6.8 A	9.4 AB	10.9 A	10.2 A	14.6 A	17.4 A	16.0 A
Rice	3.3 B	5.7 B	4.0 B	9.5 A	8.9 A	8.7 A	11.8 A	13.6 A	12.7 B
CV (%)		28			39			30	
Mean	3^{ns}	5^{ns}		7	7^{ns}		10	12^{ns}	
CV (%)		31			16			16	
	---------- mg N per pot----------								
NN soybean	20.8 A	23.0 B	21.9 A	23.6 A	15.1 B	19.4 B	44.5 A	38.2 B	41.3 B
Sorghum	22.0 A	33.4 A	27.7 A	32.0 bA	46.6 aA	39.3 A	54.0 bA	80.0 aA	67.0 A
Rice	20.2 bA	35.0 aA	27.8 A	34.4 A	38.5 A	36.5 A	55.6 bA	73.9 aA	64.3 A
CV (%)		27			36			25	
Mean	21 a	31 a		30 a	33 a		51 a	64 a	
CV (%)		46			28			24	

Values are means of five replicates. Means within the same column followed by the same upper case letter, or in the same row followed by the same lower case letter are not different at $p < 0.05$ (Student 't' test).

Table 2 - Dry matter accumulation by soybean plants (cv. Celeste) inoculated with four different strains of *Bradyrhizobium* and harvested 87 days after planting.

Reference	Shoots			Roots			Nodules			Whole plant		
Plant	unlabelled	+ ^{15}N	Mean	unlabelled	+ ^{15}N	Mean	unlabelled	+ ^{15}N	Mean	unlabelled	+ ^{15}N	Mean
	---------- g per pot----------											
CPAC 7	52.2 AB	50.7 B	51.4 B	15.8 A	13.9 C	14.9 B	2.52 B	2.70 B	2.61 B	70.5 AB	67.2 B	68.9 B
CPAC 15	56.5 A	57.2 A	56.9 A	16.9 A	18.8 A	17.8 A	2.87 B	2.66 B	2.76 B	76.2 A	78.7 A	77.5 A
29W	48.9 B	51.4 B	50.1 B	15.8 A	17.4 AB	16.6 AB	5.49 aA	4.35 bA	4.92 A	70.2 AB	73.2 AB	71.7 B
SEMIA 587	48.6 B	50.5 B	49.6 B	13.7 A	15.6 AB	14.6 AB	5.25 A	5.28 A	5.26 A	67.6 C	71.3 AB	69.5 B
CV (%)		73			19.2			20.9			15.1	
Mean	51.5 a	52.4 a		15.6 a	16.4 a		4.03 a	3.74 a		71.1 a	72.6 a	
CV (%)		16.3			19.3			25.8			7.9	

Values are means of five replicates. Means within the same column followed by the same upper case letter, or in the same row followed by the same lower case letter are not different at $p < 0.05$ (Student 't' test).

The uninoculated soybean plants grew more slowly than the inoculated plants. At 30 DAP, the plants were small with pale green leaves suggesting that there was very limited, if any, initial nodulation. Mean final dry matter accumulation was 8.5 g DM per pot (data not shown) approximately 8% of that accumulated by the nodulated plants. Mean N accumulation was 107 mg N per pot approximately 7.3% of that of the inoculated plants. Mean nodule weight of these plants was 0.50 g per pot were again far lower than that of the inoculated plants (mean 3.9 g per pot. These results point out that the native population of *Bradyrhizobium* capable of nodulating soybean in this soil was extremely low and would not have any significant contribution to nodule occupancy.

A marked difference in nodule weight between inoculation treatments was observed.

The two *B. elkanii* strains (29 W and SEMIA 587), presented similar nodule dry weights which were on average 90% greater than the weight of the nodules formed by the two *B. japonicum* strains (CPAC 7 and CPAC 15) (Table 2). A similar division of different "*Rhizobium japonicum*" strains into two groups of low ("group I strains") and high ("group II strains") nodule weights was earlier reported by Döbereiner et al. (1970) and Neves et al. (1985). These authors found

Table 3 - Nitrogen accumulation by soybean plants (cv. Celeste) inoculated with four different strains of *Bradyrhizobium* and harvested 87 days after planting.

Bradyrhizobium	Shoots			Roots			Nodules			Whole plant		
strain	unlabelled	+ ^{15}N	Mean	unlabelled	+ ^{15}N	Mean	unlabelled	+ ^{15}N	Mean	unlabelled	+ ^{15}N	Mean
	mg per pot											
CPAC 7	1140 B	1038 B	1089 B	217 A	212 A	214 A	126 B	122 B	124 B	1483 B	1371 B	1427 B
CPAC 15	1391 A	1279 A	1335 A	264 A	263 A	263 A	129 B	131 B	130 B	1784 A	1673 A	1729 A
29W	990 BC	978 B	984 BC	201 A	248 A	225 A	265 aA	191 bA	228 A	1455 B	1417 B	1436 B
SEMIA 587	905 C	986 B	946 C	193 A	249 A	221 A	216 A	211 A	213 A	1314 B	1446 B	1380 B
CV (%)^{ns}	11.4	11.4		23.9	23.9		25.4			11.2		
Mean	1106	1070^{ns}		219	243^{ns}		184	184^{ns}		1494	1437^{ns}	
CV (%)	19.2			35.0			21.1			18.2		

Values are means of five replicates. Means within the same column followed by the same upper case letter, or in the same row followed by the same lower case letter are not different at $p < 0.05$ (Student 't' test). ^{ns}Differences between means not significant at $p < 0.05$.

only minor differences between strains in the quantities of plant N derived from BNF. Those strains (Group I) inducing low nodule weights were described as highly efficient (higher ratio of N_2 fixed to nodule weight) and included the strain CB 1809 which has subsequently been found to be genetically almost identical to CPAC 7 (SEMIA 5080 - Nishi et al., 1996). Those of lower efficiency (Group II, high nodule weight) included the two strains used in this present study and are now classified as *B. elkanii* (SEMIA 587 and 29 W = SEMIA 5019 - Sato et al., 1999). Both strains that induced low nodule weight (CPAC 7 and CPAC 15) are now classified as *B. japonicum* (Rumjanek et al., 1993; Sato et al., 1999). This difference in nodule weight induced by the strains 29 W and CPAC 7 was also confirmed by Okito et al. (2004).

The three reference plants accumulated very small quantities of N, ranging from 4 to 16 mg per pot. In contrast the inoculated soybean plants accumulated over 1700 mg of N per pot indicating extremely large contributions of biologically-fixed N to them.

All the inoculated soybean plants grown in pots of soil enriched with ^{15}N showed ^{15}N enrichments of approximately 0.01 atom %^{15}N excess (varying from 0.0091 to 0.0149 atom %^{15}N excess), but the ^{15}N enrichments of the different reference plants, non-nod soybean, sorghum and rice were 0.618, 0.616 and 0.395 atom %^{15}N excess, respectively (Table 4). The fact that the inoculated soybean plants had much lower ^{15}N enrichment than the three reference species shows conclusively that the soybean plants obtained very high proportions of their N from BNF. The rice plants showed considerably lower ^{15}N enrichment than the other two reference plants probably due to different spatial and temporal patterns of soil N uptake in soil which was not uniformly labelled with ^{15}N (Boddey et al., 1995), although some input of unlabelled atmospheric N from N_2-fixing bacteria associated with rice plants cannot be ruled out. The lower ^{15}N enrichment of the rice reference crop resulted in lower estimates of %Ndfa to the inoculated soybean than those resulting from the use of the other two reference crops. Although, this difference was very small (<1.4%) as the proportion of N derived from BNF was extremely high.

The %Ndfa was very high not only because soybean is an efficient N_2 fixer, but also because the amount of soil explored by the plant roots (2 kg per plant) was intentionally much less than would normally be exploited in the field. When the %Ndfa is high the ^{15}N enrichment of the reference plants can vary considerably with little impact on the resulting estimate of %Ndfa (Hardarson et al., 1988; Boddey & Urquiaga, 1992). Furthermore, the sensitivity of the %Ndfa estimate to small difference in '*B*' value is maximised, so that this technique using labelled and unlabelled soil (Doughton et al, 1992; Okito et al., 2004) will give the most accurate values under these conditions.

While the differences in the estimates %Ndfa of the shoot tissue between inocula were very slight (Table 4), because of the much higher total N accumulation by the soybean plants nodulated with the *B. japonicum* strains, they obtained on average 26% more N from BNF than those nodulated by *B. elkanii* (Table 5). The *B. japonicum* strain CPAC 15 exceeded by 39% the mean of the N fixed by the two *B. elkanii* strains. This indicates that if these *B. japonicum* strains can compete with strains already established in the soil to dominate the nodule occupancy soybean growers could find that the use of single-strain inoculants of CPAC 7 or CPAC 15 will promote agronomically significant yield increases.

Table 4 - ^{15}N enrichment* of the shoot tissue of the three reference plants (non-nod soybean, sorghum and rice) and of the soybean (cv. Celeste) inoculated with four different *Bradyrhizobium* strains grown in soil enriched with ^{15}N, and the three estimates of the proportion of N derived from BNF (%Ndfa) by the soybean calculated using the ^{15}N enrichment of the soybean and the three reference plants.

Bradyrhizobium strain	Atom %^{15}N excess	%Ndfans			
		Non nod soybean	Sorghum	Rice	Mean
CPAC 7	0.0128 AB	97.8 AB	97.9 AB	96.7 AB	97.5 AB
CPAC 15	0.0108 AB	98.1 AB	98.2 AB	97.2 AB	97.9 A
29W	0.0149 A	97.4 B	97.5 B	96.1 B	97.0 B
SEMIA 587	0.0101 B	98.2 A	98.3 A	97.3 A	97.9 A
CV (%)	0.3	0.5	0.9	0.5	0.7
Reference plant					
NN soybean	0.6175 A				
Sorghum	0.6156 A				
Rice	0.3953 B				
CV (%)	30.3				

Values are means of five replicates. Means within the same column followed by the same upper case letter are not different at $p < 0.05$ (Student 't' test). ^{ns} Values of %Ndfa calculated from the three different reference plants are not different at $p < 0.05$. *All values corrected for ^{15}N content of the seeds.

Table 5 - Total nitrogen derived from BNF in shoot tissue by soybean plants (cv Celeste) inoculated with four different strains of *Bradyrhizobium* and harvested 87 days after planting. Values are means of five replicates.

	Ndfa (mg N per pot)		
Estirpes	unlabelled	+ ^{15}N	Mean
CPAC 7	1113 B	1013 B	1063 B
CPAC 15	1364 A	1253 A	1308 A
29 W	962 C	950 B	956 C
SEMIA 587	893 C	967 B	930 C
CV (%)	11.35		
Mean	1083		
CV (%)^{ns}	19.6		

Means in the same column followed by the same upper case letter are not different at $p < 0.05$ (Student 't' test). ^{ns}Values of Ndfa calculated from the two different soils are not different at $p < 0.05$.

The shoot tissue of the reference plants grown in the non-^{15}N-labelled soil showed positive values of ^{15}N abundance with values ranging between +6.1 and +8.8 ‰ (Table 6). However, there were smaller differences in the ^{15}N abundance for the whole plants between the three different reference plants indicating that these species tapped similar N pools and/or there was only a small temporal or spatial variation in soil ^{15}N abundance. In contrast, the ^{15}N abundance of shoot tissue of the inoculated soybean plants was always negative and plants nodulated by the *B. elkanii* strains (29 W and SEMIA 587) had more negative ^{15}N abundance values than those nodulated by the *B. japonicum* strains (CPAC 7 and CPAC 15) (Table 6). The roots of the soybean plants nodulated by the *B. elkanii* strains were slightly depleted in ^{15}N compared to that of the air, whereas those formed by the *B. japonicum* were slightly enriched. All nodules were strongly enriched with ^{15}N as has been observed by many authors for soybean as well as for several other legumes (Turner & Bergersen, 1983; Shearer & Kohl, 1986; Boddey et al., 2000). However, the weighted mean value (calculate as described in Equation 1 - Material and Methods) of the whole plants of all inoculated soybeans remained negative.

The ^{15}N natural abundance in the shoot tissue of the soybeans inoculated with the *B. elkanii* strains (29W and SEMIA 587) was more negative than those inoculated with the *B. japonicum* strains (CPAC 7 and CPAC 15) but there were no differences in $\delta^{15}N$ of the whole plants (Table 6). The ^{15}N abundance of the shoot tissue of the reference plants was +7.4 ‰, while the inoculated soybeans ranged from -3.54 and e -1.27 ‰ which confirms that the %Ndfa of all soybeans was very high and that the '*B*' values of the shoot tissue (termed 'B_s' value by Okito et al., 2004) must be considerably negative.

The '*B*' value of the shoot tissue ('B_s') and the whole plants ('B_{wp}') were calculated utilising the %Ndfa of the soybeans determined in the ^{15}N-enriched soil, along with the ^{15}N abundance of the soybean and the mean ^{15}N abundance of all three reference crops grown in the non-labelled soil. The 'B_s' values of the soybeans inoculated with the *B. elkanii* strains 29W

Table 6 - ^{15}N natural abundance (‰) of the reference plants and of the soybean (cv. Celeste) inoculated with 4 different strains of *Bradyrhizobium*, and the '*B*' values calculated from these data and the %Ndfa derived from the ^{15}N-labelled soil treatment. Plants harvested at 87 days after planting. Values are means of five replicates.

Reference plants	$\delta^{15}N$(‰)				'B' Value*	
	Shoot	Root	Nodules	Whole plant*	Shoot	Whole plant
NN soybean	+7.21 AB	+6.82 B		+8.59 A		
Sorghum	+6.16 B	+8.02 AB		+7.45 A		
Rice	+8.75 A	+9.26 A		+8.59 A		
CV (%)	19.4	14.1		2.35		
Bradyrhizobium strain						
CPAC7	-1.41 A	+1.17 A	+9.59 A	-0.12 A	-1.63 A	-0.32 A
CPAC15	-1.88 A	+0.99 A	+11.27 A	-0.52 A	-2.07 A	-0.69 A
29W	-3.54 B	-0.68 B	+9.41 A	-0.79 A	-3.86 B	-1.05 A
SEMIA587	-3.34 B	-0.21 B	+9.30 A	-0.83 A	-3.49 B	-0.96 A
CV (%)	6.5	5.3	19.3	6.1	6.2	5.8

Means for the same parameter in the same column followed by the same upper case letter are not different at $p < 0.05$ (Student 't' test). *Values corrected to discount the ^{15}N abundance of the original seeds.

and SEMIA 587 were -3.86 and -3.49 ‰, respectively, and were significantly lower than those plants inoculated with the *B. japonicum* strains CPAC 7 and CPAC 15 (-1.63 and -2.07 ‰, respectively). The values for the strains 29W and CPAC 7 were similar to, but slightly less negative than, those determined using the same technique by Okito et al. (2004) for same strains (-4.54 and -2.39 ‰, respectively, – difference between strains significant at $p < 0.05$). These authors also determined the 'B_s' values for the shoot tissue of soybean inoculated with these two strains grown in N-free media in the greenhouse and again found that the value for *B. elkanii* strain 29W (-2.58 ‰) was lower than that of the *B. japonicum* strain CPAC 7 (-1.31 ‰). The less negative value of the 'B_s' values determined for plants grown in N-free medium may have been due to the lower total N accumulation of the plants under greenhouse conditions. Therefore Okito et al. (2004) suggested that determination of 'B_s' for plants gown in the open field was more appropriate for the application of the ^{15}N natural abundance technique.

When the 'B_{wp}' values of the whole plants was determined these values were less negative than the 'B_s' values, principally because of the highly positive values of the $\delta^{15}N$ of the nodules. However, a single-tailed 't' test on each value showed that for the *B. elkanii* strain 29W and the *B. japonicum* strain CPAC 15 the 'B_{wp}' values were lower than zero. These results along with those of Okito et al. (2004) suggest that the isotope fractionation associated with N_2 fixation in the intact *Bradyrhizobium*/soybean symbiosis is significant and may contradict the conclusion of Unkovich & Pate (2000) from their study on chickpea (*Cicer arietinum*), lupin (*Lupinus luteus*), lentil (*Lens culinaris*) and Burr medic (*Medicago polymorpha*).

The advantage of the technique developed by Doughton et al. (1992), tested by Okito et al. (2004) and used in this study to determine '*B*' values, is that plants can be grown in soil in full sunlight either in pots or even in the field. This means that the plants are essentially in the same environment as they would be in a farmer's field.

In most soybean production areas in Brazil farmers establish approximately 320,000 plants per ha and the mean grain yield of this crop in Brazil is close to 2,500 kg ha^{-1}. Assuming an N content of the grain of 6.5% N, this means that this crop would accumulate 163 kg N ha^{-1} in the grain, or approximately 500 mg N per plant. As both the proportion of N in the grain compared to the whole shoot (the N harvest index) and the %Ndfa are both usually close to 80% (Alves et al., 2002, 2003, 2006), ideally soybeans used to determine '*B*' value should accumulate approximately this amount of N from BNF. In the present study N derived from BNF by the soybeans inoculated with the 4 different *Bradyrhizobium* strains accumulated between 460 and 620 mg N per plant (910 to 1240 mg N per pot) from BNF (mean %Ndfa = 97%) very close to this value. In the study of Okito et al. (2004) the inoculated soybean plants accumulated between 340 and 675 mg N per plant in the shoot tissue when grown in pots in the field, but in monoaxenic N-free medium in the greenhouse these values were lower, ranging from 290 to 305 mg N per plant.

Estimates to date of '*B*' values for soybean reported in the literature have almost all been for shoot

tissue only ('B_s' values) and all were determined in the greenhouse in monoaxenic N free culture (see review of Boddey et al., 2000). With the exception of the early study of Steele et al. (1983), all reported values were less negative than -1.7 ‰.

The results here presented show that the '*B*' values of the shoot tissue ('B_s' values), when determined using plants of realistic size grown in conditions closely approximating those experienced in the field, are considerably more negative than results obtained from determinations in N-free greenhouse culture. Most significantly it was shown that the two *B. elkanii* strains, recommended for inoculant manufacture in Brazil, induced significantly lower 'B_s' values than the two recommended *B. japonicum* strains. In many important soybean-growing regions of Brazil, the ^{15}N natural abundance of plant-available soil N is between +2 and +4 ‰ (Macedo, 2003).

If we assume that the ^{15}N natural abundance in field-grown soybean and reference crop were -1.00 and -3.00 ‰, respectively, %Ndfa will be 60% using a 'B_s' value of -3.68 ‰ (mean for the two *B. elkanii* strains) and 82 % if a 'B_s' value of -1.85 ‰ (mean for the two *B. japonicum* strains). This difference for a total N shoot accumulation of 200 kg N ha^{-1}, would be 44 kg N ha^{-1}. This is a very considerable difference and equivalent to the quantity of N fertiliser usually added to wheat following soybean in Brazil.

This study, illustrates the importance of determining which recommended *Bradyrhizobium* strain is principally responsible for nodule formation if an accurate estimate of the BNF contribution is to be determined. In the case where there is a significant proportion of strains of both *Bradyrhizobium* species, it may become necessary to determine the proportion of nodule occupancy of each using either immunological or molecular techniques to establish the appropriate intermediate value for 'B_s'. Further studies will be required to determine how 'B_s' value varies when nodules are occupied by strains of both *B. elkanii* and *B. japonicum*.

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Received March 22, 2007
Accepted April 11, 2008