



Natural abundance of ^{15}N of N derived from the atmosphere by different strains of *Bradyrhizobium* in symbiosis with soybean plants

Karla Emanuelle Campos Araujo¹ Carlos Vergara Torres Júnior¹ Ana Paula Guimarães²
Mara Alexandre da Silva¹ Bruno José Rodrigues Alves²
Segundo Urquiaga² Robert Michael Boddey^{2*}

¹Universidade Federal Rural do Rio de Janeiro (UFRRJ), Seropédica, RJ, Brasil.

²Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), Centro Nacional de Pesquisa Agrobiologia (CNPAB), 23890000, Seropédica, RJ, Brasil. E-mail: robert.boddey@embrapa.br. *Corresponding author.

ABSTRACT: To quantify the BNF contribution to legumes using the ^{15}N natural abundance technique, it is important to know the abundance of ^{15}N of the plants grown entirely dependent on BNF (value 'B'). The aim of the study was to determine the ^{15}N natural abundance of N_2 fixed by different *Bradyrhizobium* strains in symbiosis with one soybean cultivar. Treatments consisted of soybean plants cultivated with and without inoculation with ten *Bradyrhizobium* strains, in five replicates planted in Leonard jars in a sand/vermiculite mixture. Plants were harvested after 46 days. The 'B' values of the aerial tissue ('B_{pa}') ranged from -2.6 to -3.9 ‰. There was a tendency for the 'B_{pa}' values of plants inoculated with strains of *B. elkanii* to be more negative than plants inoculated with other strains. All 'B' values of the whole plant were less than 1 unit of $\delta^{15}\text{N}$ (‰) different from zero, suggesting that the symbioses have little tendency to show significant isotopic fractionation during N_2 fixation, but there is considerable depletion in ^{15}N of the N translocated to the shoot tissue.

Key words: ^{15}N Isotopic fractionation, 'B' value, Biological nitrogen fixation

Abundância natural de ^{15}N do N derivado da atmosfera por diferentes estirpes de *Bradyrhizobium* em simbiose com plantas de soja

RESUMO: Para quantificar a FBN em leguminosas, através da técnica de abundância natural de ^{15}N , é importante conhecer a abundância de ^{15}N da planta leguminosa crescida inteiramente dependente da FBN (valor 'B'). O objetivo deste estudo foi determinar a abundância natural de ^{15}N do N_2 fixado por diferentes estirpes de *Bradyrhizobium* em simbiose com uma cultivar de soja. Os tratamentos consistiram de plantas cultivadas sem e com inoculação com dez estirpes de *Bradyrhizobium*, em cinco repetições. Foram utilizados vasos de Leonard, substrato de areia e vermiculita. As plantas foram colhidas após 46 dias. Os valores de 'B' parte aérea ('B_{pa}') variaram entre -2.6 to -3.9 ‰. Houve tendência para a abundância de ^{15}N dos valores de 'B_{pa}' das plantas inoculadas com estirpes de *B. elkanii* a serem mais negativos que as plantas inoculadas com as outras estirpes. Todos os valores de 'B' da planta inteira foram menos de 1 unidade de $\delta^{15}\text{N}$ (‰) diferente de zero, sugerindo que as simbioses têm pouca tendência de mostrar fracionamento isotópico significativo, mas houve considerável empobrecimento do isótopo ^{15}N no N translocado a parte aérea.

Palavras chave: fracionamento isotópico ^{15}N , valor 'B', fixação biológica de nitrogênio.

INTRODUCTION

Biological nitrogen fixation (BNF) in soybean is one of the most successful examples of this process in agriculture, since the use of *Bradyrhizobium* inoculants can supply up to 100% of the nitrogen (N) in the plant from BNF. Yields of over four tonnes (Mg) of grain containing 6.5 to 7% N can be achieved, even when reliant almost solely on BNF (ALVES et al., 2003).

The ^{15}N natural abundance technique is currently the most used to quantify BNF in legumes.

This technique has the almost unique feature that can be used without addition of ^{15}N -labelled fertilizer or physical disturbance of the plants (PEOPLES et al., 1989). The technique depends on the observation that most soils are slightly enriched with ^{15}N with respect to the atmospheric N_2 . With modern sensitive isotope-ratio mass spectrometers, it is possible to calculate the proportion of plant N derived from BNF from the ^{15}N abundance of plants (SHEARER & KOHL, 1986). The ^{15}N abundance of the N derived from the soil is determined by analyzing plants incapable of obtaining N from BNF growing in the same soil as the legume.

To use this technique, it is necessary to determine the ^{15}N abundance of the legume plant grown entirely dependent on BNF (the 'B' value). For the soybean crop this is especially important, since this crop can obtain a high proportion of its N from BNF in soils with low N contents, which is predominantly the case in Brazil. Under these circumstances, estimation of the percentage of N derived from the atmosphere (%Nd_f) becomes very sensitive to the 'B' value. Several authors have reported that with plants grown in N-free medium, the 'B' value of a particular legume may vary with the inoculated rhizobium strain (BERGERSEN et al., 1986; YONEYAMA et al., 1986; PAUFERRO et al., 2010). Among the four *Bradyrhizobium* strains recommended for the manufacture of commercial inoculants in Brazil, one is *B. diazoefficiens* (CPAC 7), one is *B. japonicum* (CPAC 15) and two are *B. elkanii* (29 W and SEMIA 587). GUIMARÃES et al. (2008) showed that the 'B' value is quite different for soybean plants nodulated with the strains of *B. japonicum* and *B. diazoefficiens* compared to those with *B. elkanii* nodules.

The objective of this experiment was to determine the natural abundance of ^{15}N of the N_2 fixed (the 'B' value), by ten different *Bradyrhizobium* strains in symbiosis with one soybean cultivar.

MATERIALS AND METHODS

The experiment was conducted in a greenhouse at Embrapa Agrobiologia, Seropédica - RJ. The experimental design was completely randomized. Treatments consisted of soybean plants cultivated with the addition of turf inoculants of *B. diazoefficiens* (CPAC 7, USDA 110), *B. japonicum* (CPAC 15, USDA 6), and *B. elkanii* (29 W, SEMIA 587, DF395, SM1b, USDA 31, USDA 46), strains from the Embrapa Agrobiologia culture collection, and without inoculation (control), in five replicates. Seeds of the soybean (*Glycine max* L.) cultivar BRS 133 were surface sterilized by immersion for 30 seconds in ethanol (92.8° INPM), 2 min in hydrogen peroxide (30%) then 10 successive washes with autoclaved distilled water. Five seeds were sown per pot, the thinning was done eight days after emergence, leaving only two plants per pot.

Plants were grown in sterile culture in Leonard jars (SOMASEGARAN & HOBEN, 1985), using a 2:1 (v/v) sand and vermiculite substrate. At planting, 300 mL of autoclaved distilled water was used to fill the jars. Eight days after germination, this was replaced by the nutrient solution free of N (SOMASEGARAN & HOBEN, 1985) at a quarter of the recommended concentration and was gradually increased every seven days during the first four weeks

of the experiment until the full concentration was reached. The harvest was performed 46 days after planting (DAP), at the R4 stage, at the beginning of flowering. The aerial tissue, the roots and the nodules were separated. All plant samples were then dried in an oven at 65 °C for four days to determine the dry matter (DM) content. After this procedure, the samples were processed in a Wiley mill (2 mm sieve), and later in a roller mill similar to that described by ARNOLD & SCHEPERS (2004), until a fine powder was obtained.

Sub-samples of all plant tissues, including seeds, were weighed to determine total N, and ^{15}N natural abundance ($\delta^{15}\text{N}$) in plant tissues and seeds using a continuous-flow isotope-ratio mass spectrometer (Finnigan DeltaPlus or Delta V mass spectrometer Finnigan MAT, Bremen, Germany) coupled to the output of a Costech (model ECS4010) total C and N analyzer in the "John Day Stable Isotope Laboratory" at Embrapa Agrobiologia, as described by RAMOS et al., (2001).

Nodule efficiency (DÖBEREINER et al., 1970) was defined as the amount of N fixed (mg N accumulated in the whole plant minus the mg N of the seed) per g of DM of nodule. For the 'B' value of the whole plants (B_{wp}), the weighted mean of the ^{15}N abundance of the shoots, roots and nodules was corrected assuming all seed N was present:

$$B_{WP} = \frac{(N_{Tnod} \times \delta^{15}N_{nod}) + (N_{TST} \times \delta^{15}NST) + (N_{TR} \times \delta^{15}NR) - (N_{TS} \times \delta^{15}NS)}{(N_{Tnod} + N_{TST} + N_{TR} - N_{TS})} \quad \text{Eqn. 1}$$

The 'B' value of the shoot tissue (B_s) was calculated assuming that 50% of the seed N was translocated to the shoot, the remainder being found in the roots and nodules (OKITO et al. 2004).

$$B_s = \frac{(N_{TST} \times \delta^{15}NST) - (0.5 \times N_{TS} \times \delta^{15}NS)}{N_{TST} - (0.5 \times N_{TS})} \quad \text{Eqn. 2}$$

where N_{Tnod} , N_{TST} , N_{TR} , N_{TS} are the total N concentration in nodules, shoot tissues, roots and seeds, respectively, and $\delta^{15}N_{nod}$, $\delta^{15}NST$, $\delta^{15}NR$, $\delta^{15}NS$ are the respective $\delta^{15}\text{N}$ values.

The data were submitted to analysis of variance (ANOVA) and to separate the means the Student's LSD test was used. All statistical analyses were performed using the software Sisvar (FERREIRA, 2008).

RESULTS AND DISCUSSION

Accumulation of dry matter and total nitrogen in soybean plants

The DM production of the soybean plants inoculated with the different strains of *B.*

diazoefficiens (CPAC 7, USDA 110), *B. japonicum* (CPAC 15, USDA 6) and *B. elkanii* (29 W, SEMIA 587, DF395, SM1b, USDA 31, USDA 46) were much higher than that of the uninoculated control plants, confirming the contribution of the BNF to the symbioses formed by all strains of *B. diazoefficiens*, *B. japonicum* and *B. elkanii* (Table 1). The fact that no nodules could be reported on the roots of any of the uninoculated plants showed that there was no contamination of this treatment with bacteria derived from the other inoculants and indicated that there was no cross contamination of one treatment with another.

Values of DM accumulation of the whole plant for plants inoculated with *B. diazoefficiens* (CPAC 7, USDA 110) were, on average, 6.07 g DM pot⁻¹. For plants inoculated with *B. japonicum* (CPAC 15, USDA 6) and *B. elkanii* (29 W, SEMIA 587, DF395, SM1b, USDA 31, USDA 46), accumulation of DM was very similar, averaging 5.24 g DM pot⁻¹ for whole plants of *B. japonicum* and 5.35 g DM pot⁻¹ when inoculated with *B. elkanii*. Although, there were no significant differences between the means of the strains when they were grouped as three species of bacteria, the results of analysis of variance showed that there were significant differences ($P < 0.05$) between the symbioses formed by individual strains of *Bradyrhizobium* for DM of shoot, nodules and whole plants.

Plants showing the highest shoot and whole plant DM accumulation were those inoculated with the strains CPAC 7, SEMIA 587, USDA 110 and DF 395. Hence, it was observed that the strains USDA 110 and DF 395 established symbioses that are just as efficient during the first 46 days of growth as the strains currently recommended by the Brazilian Ministry of Agriculture (CPAC 7, CPAC 15, 29 W, SEMIA 587). OKITO et al. (2004), using the variety of soybean cv. Celeste and collecting the plants at 82 days after planting did not find significant difference for accumulation of DM in shoot and whole plant using the strains CPAC 7 and 29 W.

In relation to the mass of the nodules, strains with the highest DM were the strains CPAC 7, USDA 110 (both *B. diazoefficiens*), DF 395, USDA 46, SM1b, 29 W, SEMIA 587 (all *B. elkanii*). A study by OKITO et al. (2004) showed that the 29 W strain of *B. elkanii* showed a greater accumulation of DM of nodules, while the CPAC 7 (*B. diazoefficiens*) strain had a lower accumulation of DM of nodules. GUIMARÃES et al. (2008) and PAUFERRO et al. (2010), in studies performed in pots of soil, reported higher values of DM of nodules formed by 29 W and CPAC 587 (*B. elkanii*) than CPAC 7 (*B. diazoefficiens*) and CPAC 15 (*B. japonicum*). These marked differences seen in the previous research with respect

Table 1 - Accumulation of dry matter per pot (g) of BRS 133 soybean (2 plants per pot) inoculated with different strains of *Bradyrhizobium* and harvested at 46 days after planting.

Treatments	-----Shoot Tissue-----		Root	-----Nodule-----		-----Whole Plant-----	
	-----g DM pot ⁻¹ -----						
----- <i>B. diazoefficiens</i> -----							
CPAC7	4.77	a	1.00	0.59	a	6.36	a
USDA 110 (type strain)	4.16	c	1.05	0.56	a	5.77	b
----- <i>B. japonicum</i> -----							
CPAC 15	3.72	e	0.79	0.44	b	4.95	e
USDA 6 (type strain)	4.04	cd	1.08	0.40	b	5.52	cd
----- <i>B. elkanii</i> -----							
29 W	4.10	c	0.90	0.61	a	5.60	bcd
SEMIA 587	4.55	b	1.32	0.63	a	6.50	a
SM1b	3.41	f	0.79	0.58	a	4.78	e
DF395	4.16	c	0.94	0.57	a	5.67	bc
USDA 46	3.85	de	0.93	0.65	a	5.42	cd
USDA 31	2.79	g	0.93	0.37	b	4.10	f
CV (%)	13		20	11		12	
Not Inoculated	0.65		0.30	0.00		0.95	

Mean values of 5 replicates. Values in each column followed by the same letter do not differ statistically from each other by the (Student) LSD test $P < 0.05$.

to nodule weights of the different *Bradyrhizobium* species may be explained by the increase in nodule mass during plant ontogeny. Plants in these previous studies were harvested from 90 DAP onwards. ARAUJO (2014) also reported only small differences in DM of nodules between the same symbioses at 46 DAP, but the differences were much larger at 76 DAP as was also registered by GUIMARÃES et al. (2008) and PAUFERRO et al. (2010) at 85 to 90 DAP.

Those symbioses with the highest N accumulation in shoot and whole plant were the plants inoculated with the strains CPAC 7 and USDA 110 (both *B. diazoefficiens*) and SEMIA 587, DF 395 (both *B. elkanii*) (Table 2). The total N values for the shoot of soybean plants inoculated with *B. diazoefficiens* were on average 90 mg N pot⁻¹, for plants inoculated with *B. japonicum* and *B. elkanii* the mean total N values were 69 mg N pot⁻¹ to 74 mg N pot⁻¹, respectively.

Values of the nodule efficiency of the soybean plants inoculated with the two strains of *B. diazoefficiens* were 198 and those of *B. japonicum* were 184 mg N g DM nodule⁻¹. With *B. elkanii* strains the mean nodule efficiency was 171 mg N DM nodule⁻¹ and thus somewhat lower than the symbioses formed by *B. diazoefficiens* and *B. japonicum* (Table 2).

However, unlike earlier studies (OKITO et al. 2004; GUIMARÃES et al. 2008; PAUFERRO et al., 2010) the differences between species were not significant ($P < 0.05$ – Student ‘t’ test – analysis not shown).

Natural abundance of ¹⁵N and ‘B’ value

There was no clear pattern in the differences in the ¹⁵N-abundance of shoot tissue of the symbioses formed by the three different *Bradyrhizobium* species (Table 3). This is in contrast to the previous studies by the team at Embrapa Agrobiologia (OKITO et al. 2004; GUIMARÃES et al. 2008; PAUFERRO et al., 2010) and, once again, may be explained by the stage of development of the plants which were harvested much later at 80 to 90 DAP in these previous studies.

The δ¹⁵N values of the roots, regardless of the inoculation treatment, were all negative. The same result was observed by OKITO et al. (2004) with the *Bradyrhizobium* strains 29 W and CPAC 7 when the plants were grown in sterile (gnotobiotic) Leonard jars with no mineral N supply. However, in studies by this same team at Embrapa Agrobiologia, when the plants were grown in soil with a natural abundance of plant available N of between +6.0 and +9.0 ‰, the roots showed slightly positive values (OKITO et al. 2004; GUIMARÃES et al. 2008; PAUFERRO et

Table 2 - Total nitrogen accumulation per pot (mg) and nodule efficiency (mg g⁻¹) of BRS 133 soybean (2 plants per pot) inoculated with different strains of *Bradyrhizobium* and harvested at 46 days after planting.

Treatments	--Shoot Tissue--		-----Root-----		-----Nodule-----		---Whole Plant--		--Nodule Efficiency*#--	
	-----mg N pot ⁻¹ -----									
----- <i>B. diazoefficiens</i> -----										
CPAC7	91	a	21	a	33	bc	145	a	203	a
USDA 110 (type strain)	88	ab	21	a	30	cd	138	bc	192	abc
----- <i>B. japonicum</i> -----										
CPAC 15	64	d	15	c	21	f	100	f	171	bc
USDA 6 (type strain)	73	c	17	b	22	ef	113	e	196	ab
----- <i>B. elkanii</i> -----										
29 W	84	b	18	b	33	bc	135	c	185	bc
SEMIA 587	88	ab	21	a	33	bc	143	ab	182	bc
SM1b	72	c	15	c	34	ab	121	d	159	cd
DF395	90	a	19	b	30	bcd	139	bc	188	bc
USDA 46	63	d	18	b	38	ab	118	de	154	d
USDA 31	47	e	18	b	26	de	91	g	155	d
CV (%)	4		7		11		4.00		13	
Not inoculated	7		4				11.00			

Mean values of 5 replicates. Values in each column followed by the same letter do not differ statistically from each other by the (Student) LSD test $P < 0.05$.

*Nodule efficiency = (mg total N accumulated by plants – seed N)/(g DM nodule).

#Values for mean seed DM was 226.5 mg and seed N content 14.7 mg N seed⁻¹.

Table 3 - Natural abundance of ^{15}N (‰) and 'B' values of BRS 133 soybean (2 plants per pot) inoculated with different strains of *Bradyrhizobium* and harvested at 46 days after planting.

Treatments	---Shoot Tissue---		-----Root-----		-----Nodule-----		----Whole Plant----		---Shoot Tissue---	
	----- $\delta^{15}\text{N}$ (‰)-----									
	-----'B' Value (‰) [#] -----									
----- <i>B. diazoefficiens</i> -----										
CPAC7	-2.63	c	-0.51	bc	+8.17	a	+0.07	ab	-2.77	a
USDA 110 (type strain)	-2.42	b	-1.53	e	+6.13	ef	-0.53	e	-2.56	a
----- <i>B. japonicum</i> -----										
CPAC 15	-2.63	c	-0.28	ab	+5.93	fg	-0.65	e	-2.84	b
USDA 6 (type strain)	-2.74	c	-0.76	cd	+6.41	de	-0.74	e	-2.92	b
----- <i>B. elkanii</i> -----										
29 W	-2.36	ab	-0.50	bc	+5.66	g	-0.24	cd	-2.72	a
SEMIA 587	-2.65	c	-0.36	ab	+5.80	fg	-0.47	de	-3.02	b
SM1b	-2.65	c	-0.52	bc	+6.89	c	+0.24	b	-3.12	bc
DF395	-2.73	c	-0.15	a	+7.52	b	-0.23	cd	-3.10	bc
USDA 46	-2.22	a	-0.81	d	+3.84	h	-0.17	bc	-2.71	a
USDA 31	-3.07	d	-0.51	bc	+6.57	cd	+0.14	a	-3.94	c
SEM *	0.20		0.29		0.35		0.95		0.66	

Mean values of 5 replicates. Values in each column followed by the same letter do not differ statistically from each other by the (Student) LSD test $P < 0.05$.

*SEM = Standard error of the mean (sub-plot). It is not possible to use coefficients of variation as the means may be negative or zero.

[#]'B' values for the whole plants and shoot tissue were calculated using Equations 1 and 2 (Materials and Methods section) and the values for mean seed DM was 226.5 mg, seed N content 14.7 mg N seed⁻¹ and ^{15}N natural abundance of +0.089 ‰.

al., 2010). This suggested that roots may have had a much higher contribution from soil N than the shoots.

All strains presented high $\delta^{15}\text{N}$ values in the nodules (Table 3). Other authors reported the same result for soybean and for other legumes (e.g TURNER & BERGERSEN, 1983; SHEARER & KOHL, 1986; YONEYAMA et al., 1986; BODDEY et al., 2000; PAUFERRO et al., 2010).

The values of 'B_s' in this experiment were approximately of the same magnitude, between -2.6 and -3.9 ‰, to those values previously reported by the team of Embrapa Agrobiologia (OKITO et al. 2004; GUIMARÃES et al. 2008; PAUFERRO et al., 2010). However, in these previous studies, values for 29 W and/or SEMIA 587 strains (both *B. elkanii*) were reported to be significantly more negative for plants nodulated with CPAC 7 (*B. diazoefficiens*) and CPAC 15 (*B. japonicum*) strains.

With respect to the estimates of the 'B' values for the whole plant, values that were more negative were reported in the plants inoculated with the strains, USDA 110 (*B. diazoefficiens*) CPAC 15, USDA 6 (both *B. japonicum*) and SEMIA 587 (*B. elkanii*). All 'B' values of the whole plant (termed 'B_{wp}') in all treatments were less than 1 unit of $\delta^{15}\text{N}$ (‰) from zero, suggesting that symbioses have little tendency

to show significant isotopic fractionation, which is in agreement with the suggestion of UNKOVICH (2013). This result was in contrast to previous studies by the team of Embrapa Agrobiologia (GUIMARÃES et al. 2008; PAUFERRO et al., 2010) who reported values more negative when using the 29 W strain and less negative when using the strain CPAC 7.

CONCLUSION

As it is impossible to remove completely soybean roots and nodules from the soil in field studies, for quantification of contributions of BNF to soybean in the field the 'B' value of the shoot tissue ('B_s') must be used. All estimates of this value were strongly negative (from -2.56 to -3.94 ‰) but unlike previous studies on soybean micro-symbionts, there were no overall significant difference between *Bradyrhizobium* species, only between individual strains.

Despite the statistically significant differences in the values of 'B_{wp}' recorded among the symbioses formed by the different *Bradyrhizobium* strains in the experiment, the magnitudes were small (less than + or -1.0 ‰). This indicated that there was little tendency for isotopic fractionation during the BNF process in the symbiosis of soybean with *Bradyrhizobium* strains,

but there was considerable depletion in ^{15}N of the N translocated to the shoot tissue.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

AUTHORS' CONTRIBUTIONS

KECA, CVTJ, APG, MAdaS, BJRA, SU, and RMB: designed, performed experiments, and analyzed data. KECA, CVTJ, and RMB: conceived the experiments and wrote the paper. All authors read, edited, and approved the final manuscript.

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