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REGULAR ARTICLE

Growth patterns of common bean cultivars affect the '*B*' value required to quantify biological N_2 fixation using the ^{15}N natural abundance technique

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Abstract
Background and aims The *'B'* value, the ^{15}N abundance of plants depending completely on biological nitrogen fixation (BNF) for growth, is required for quantifying BNF using the ^{15}N natural abundance technique. This study aimed to investigate how common bean (*Phaseolus vulgaris* L.) cultivars with different growth patterns could affect the *'B'* value calculated for the plant shoot.
Methods Two experiments were conducted in N-free solution. Experiment I had a factorial design with three cultivars and two rhizobium inoculants. Plants were harvested at pre-flowering stage. Experiment II was also factorial with two cultivars and two times of sampling: full flowering and mid-pod filling. Total N and $\delta^{15}N$ analyses of different plant parts were carried out to estimate the *'B'* value.
Results There were differences between cultivars for N accumulation and ^{15}N abundance, but no difference between rhizobium inoculants. Results revealed differences in *'B'* value between cultivars of small and large seeds, the growth stage being also relevant. The '*B*' values of −2.08 and −1.34‰ could be used for BNF estimates in small-seeded cultivars at full flowering and mid-pod filling stages, respectively, while such values should change, respectively, to −1.73 and −1.03‰ for large-seed cultivars. The mean *'B'* value in shoots was −1.90 and −1.20‰ at full flowering and mid-pod filling stages, respectively.
Conclusions There are variations in *'B'* value between cultivars of different cycle length and seed sizes, which are associated with plant architecture, thus a *'B'* for each growth stage of common bean plants is required.

Keywords *'B'* value · N_2 fixation · ^{15}N natural abundance · *Phaseolus vulgaris* · Seed size

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Introduction

Common beans (*Phaseolus vulgaris* L.) are one of the most important edible grain legumes for direct human consumption in the world (Broughton et al. 2003). In Africa and Latin America, this legume is a key component of the basic diet of the population.

Biological nitrogen fixation (BNF) from the rhizobium symbiosis can partially supply the N required by common bean plants, but complementary N fertilizer is usually required in field crops for high yields (Brito et al. 2015). Therefore, reliable estimates of the contribution of BNF to common beans are needed in order to improve the seed inoculation procedures in commercial

crops in an effort to replace mineral N fertilization, bringing positive effects for both economic and environmental sustainability.

The slight increase in the ^{15}N natural abundance ($\delta^{15}N$), compared to that of atmospheric N_2, which occurs in many soils can be measured by a suitable mass spectrometer (Unkovich et al. 2008). This small difference can in many cases be used to distinguish between the N originating from the soil and from the air in N_2-fixing plants and thus to estimate the proportion of N in the plant derived from the air (%Ndfa). This methodology is known as the ^{15}N natural abundance technique (Shearer and Kohl 1986).

Apart from the fact that it is unnecessary to purchase and uniformly apply ^{15}N-labeled fertilizer when the ^{15}N natural abundance technique is employed, it is generally considered that this technique is less subject to errors arising from temporal and spatial variations in the ^{15}N abundance of plant available N (Shearer and Kohl 1986; Boddey et al. 2000; Unkovich et al. 2008) in comparison to what happens when the soil is enriched with ^{15}N by adding labeled fertilizers.

While by definition, atmospheric N_2 has a value of 0.00 delta ^{15}N units (part per thousand ‰—Shearer and Kohl 1986; Chalk et al. 2015); it has been found by many workers that plants relying on BNF can have different values (e.g., Shearer and Kohl 1986; Boddey et al. 2000). It seems to be a consensus that there is no N isotope discrimination in the process of symbiotic N_2 fixation (Unkovich 2013), but different parts of the plant can become enriched or depleted in ^{15}N with respect to atmospheric N_2. Nodules are almost always enriched in ^{15}N and shoot tissue often depleted (Shearer et al. 1980; Boddey et al. 2000). As it is almost impossible to recover 100% of the roots and nodules of legumes in the field, the calculations of the proportion of N derived from N_2 fixation are generally based on shoot tissue only. Thus, it is necessary to determine the ^{15}N abundance of the shoot tissue of legume plants grown solely on N_2 fixation. This value is known as the *'B'* value (Shearer and Kohl 1986) and it is obtained when the ^{15}N abundance in the whole plant stabilizes after several weeks. This is usually explained by the increasing dilution of seed N in the plant by the N derived from BNF (Bergersen et al. 1988). Hence, the amounts of N in seeds that are transferred to the plant and its partitioning between aerial and belowground tissues are required to discount this ^{15}N source to the *'B'* value for the plant shoot. Okito et al. (2004) considered that half of the whole seed N was accumulated in the soybean shoot. López-Bellido et al. (2010) discounted the remaining N in detached cotyledons from the seed N to calculate the chickpea seed N that was transferred to the plant, but also considered a partitioning of seed N between above and belowground material of 50%. Seed N can be negligible when relatively large quantities of N come from BNF or soil (Bergersen et al. 1988), but correction of the estimate of %Ndfa for seed N requires special attention with legumes with low N accumulation and large seeds.

Variations in *'B'* value estimates of leguminous plants were reported to occur at the level of cultivar (Nebiyu et al. 2014), the rhizobium strain involved in symbiosis (Guimarães et al. 2008; Pauferro et al. 2010) and the plant phenological stage (Kyei-Boahen et al. 2002; López-Bellido et al. 2010).

There are few studies that give estimates of the *'B'* value of common beans. Mariotti et al. (1980) determined values in shoots ranging from −1.97 to −1.83‰, and Yoneyama et al. (1986) found values ranging from −3.20 to −1.80‰ with differences related to inoculated rhizobium strains. The influence of cultivars was pointed out by Polania et al. (2016) who reported the *'B'* value varying from −3.62 to −2.44‰ in bush bean genotypes of growth habit II and III. In view of the diversity among common bean cultivars, more studies are required, especially as common beans have nodules with a short activity period (Piha and Munns 1987; Almeida et al. 2013) and premature senescence.

The objective of this study was to investigate how common bean cultivars with different growth patterns and rhizobium inoculants could affect the *'B'* value calculated for the plant shoot.

Materials and methods

Experimental conditions

Two experiments were carried out in a temperature-controlled greenhouse under natural lighting at Embrapa Agrobiologia in Seropédica, Rio de Janeiro State, Brazil.

Plants were grown in 1.0-L plastic pots in hydroaeroponic culture with sterilized nutrient solution (Araújo et al. 2008) lacking N, containing 1.65 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.25 mM KH_2PO_4, 0.7 mM K_2SO_4, 8.0 μM Fe-EDTA, 6 μM $MnSO_4.H_2O$, 4 μM

H_3BO_3, 2 μM $ZnSO_4.7H_2O$, 1 μM $CuSO_4.5H_2O$, and 0.2 μM $Na_2MoO_4.2H_2O$. Each pot received 1 g of $CaCO_3$ to maintain the solution pH near 7 (Araújo et al. 2008). The nutrient solution was aerated by intermittent pumping and was changed weekly.

Three common bean cultivars, with different characteristics of growth habit, cycle length, and seed size, were used (Table 1). For each cultivar, seeds were weighed one by one in order to ensure size homogeneity, from which a sample of 100 seeds were pooled and put to dry at 65 °C, grounded and analyzed for N and ^{15}N natural abundance. Seeds were surface sterilized and pre-germinated in a germination chamber. The stems of pre-germinated seedlings were gently wrapped in sterile cotton and attached to polystyrene foam plates covering the plastic pots, leaving the roots in contact with the nutrient solution. Four seedlings were planted and further thinned to two plants per pot.

Experiment I had a 3 × 2 factorial randomized block design with four replicates, comprising three common bean cultivars (Ouro Negro, BRS Radiante and BRS Grafite), and two rhizobium inoculants: CIAT 899 or a commercial strain mixture (the strains CIAT 899, PRF81 and CPAC H12); besides the non-inoculated plants of each cultivar, for checking the absence of N in the growth medium and the absence of cross contamination. The rhizobium strains CIAT 899 (BR 322 syn SEMIA 4077) was isolated in Colombia and PR-F81 (BR 520 syn SEMIA 4080) was isolated from soil of Southern Brazil (Hungria et al. 2000) while CPAC H12 (BR 534 syn SEMIA 4088) was isolated from soil of the Brazilian Cerrado region (Mostasso et al. 2002). All are recommended for common bean crops in Brazil. The strains were obtained from the Embrapa Agrobiologia strain collection. For inoculation, 4 mL of liquid inoculant was transferred to the nutrient solution of each pot. The number of rhizobium cells per milliliter of inoculant (estimated as described by Somasegaran and Hoben 1985) was 5.5×10^9 for strain CIAT 899, and 8.5×10^9 for the commercial strain mixture. Plants were harvested at the pre-flowering stage of each cultivar, i.e., 30 days after transplant (DAT) for Ouro Negro, 26 DAT for Radiante, and 33 DAT for Grafite. At harvest, plants were separated into leaves, stems, roots, and nodules.

Experiment II had a 2 × 2 factorial randomized block design with four replicates, combining two common bean cultivars (BRS Radiante and BRS Grafite) and two sampling times, at full flowering and mid-pod filling stages of each cultivar, i.e., 35 and 42 DAT for Radiante and Grafite at full flowering and 49 and 56 DAT for Radiante and Grafite at mid-pod filling, respectively. The rhizobium strains used for the inoculation were the same commercial strain mixture used in experiment I, with 7.0×10^9 rhizobial cells per milliliter of inoculant (Somasegaran and Hoben 1985). At harvest, plants were separated into reproductive tissues (flowers or pods), leaves, stems, roots, and nodules.

Plant analyses

At harvest, plant tissues were superficially washed with deionized water, and each plant portion was oven dried at 75 °C for 3 days and weighed. Nodules were counted immediately after being detached from roots. A sample of the seeds of each cultivar used to plant the experiments was also processed in the same manner. The cotyledons and seed tegument were also collected when they dropped off the plants. Senesced leaves were collected and pooled with green leaves. Each plant portion (seed, cotyledons + tegument, roots, nodules, leaves, stems, flowers, and pods) was manually ground to a fine powder. All plant samples were analyzed for total N and $\delta^{15}N$ content by an automated continuous-flow isotope ratio mass spectrometer consisting of a Finnigan Delta Plus mass spectrometer coupled to a Costech total C and N analyzer in the "John Day Stable Isotope Laboratory" at Embrapa Agrobiologia. The N accumulation in each plant tissue was obtained by multiplying the N concentration of each tissue and their respective dry mass.

The ^{15}N natural abundance was calculated by the software of the isotope ratio mass spectrometer (IRMS) according to the formula (Chalk et al. 2015):

$$\delta^{15}N = \frac{R\left(^{15}N/^{14}N\right)_{sample}}{R\left(^{15}N/^{14}N\right)_{standard}} - 1$$

where $R\ ^{15}N/^{14}N$ is the ratio of the number of atoms of ^{15}N to ^{14}N which for the standard (atmospheric N_2) is 0.003676.

The total N in the plants was calculated by summing the N accumulated in roots (N_{roots}), nodules ($N_{nodules}$), stems (N_{stems}), leaves (N_{leaves}), and reproductive structures (N_{rs}). For the weighted mean ^{15}N abundance of the whole plant ($\delta^{15}N_{plant}$) the following calculation was made:

Table 1 Some characteristics of the three common bean cultivars evaluated

Cultivar	Plant characteristics			Seed characteristics				
	Growth habit	Cycle	Cycle length (days)	Color	Unit mass (mg)	N concentration (mg g^{-1})	N content (mg $seed^{-1}$)	$\delta^{15}N$ (‰)
Ouro Negro	PI	Normal	80–90	Black	240 ± 24	3.32	7.71	+2.44
BRS Radiante	ED	Early	<75	Cream stripped	440 ± 44	3.65	15.16	+2.41
BRS Grafite	EI	Late	>95	Black	250 ± 25	3.29	7.90	+3.04

ED erect determinate (type I), *EI* erect indeterminate (type II), *PI* prostrate indeterminate (type III)

$$\delta^{15}N_{plant} = \frac{\left(N_{roots} \times \delta^{15}N_{roots}\right) + \left(N_{nodules} \times \delta^{15}N_{nodules}\right) + \left(N_{stems} \times \delta^{15}N_{stems}\right) + \left(N_{leaves} \times \delta^{15}N_{leaves}\right) + \left(N_{rs} \times \delta^{15}N_{rs}\right)}{N_{roots} + N_{nodules} + N_{stems} + N_{leaves} + N_{rs}}$$

To estimate the *'B'* value, the $\delta^{15}N$ of the N derived from the seed ($\delta^{15}Ndf_{seed}$) was discounted, as follows (Högberg et al. 1994):

$$B = \frac{\left(N_{plant} \times \delta^{15}N_{plant}\right) - \left(Ps \times Ndf_{seed} \times \delta^{15}Ndf_{seed}\right)}{N_{plant} - \left(Ps \times Ndf_{seed}\right)}$$

where Ps is the proportion of the seed N that was incorporated into the plant tissue. When the whole plant tissue was considered, Ps was assumed to be 1.0, whereas when only shoot tissues were considered, Ps was 0.5, assuming that 50% of the seed N was incorporated into the shoot (Okito et al. 2004). The Ndf_{seed} is the seed N effectively transferred to the plant, or the difference between the total seed N (N_{seed}) and the N contained in the remaining cotyledons (+ tegument) naturally detached (N_{dc}) during plant growth.

The $\delta^{15}Ndf_{seed}$ was calculated by the following equation:

$$\delta^{15}Ndf_{seed} = \frac{\left(N_{seed} \times \delta^{15}N_{seed}\right) - \left(N_{dc} \times \delta^{15}N_{dc}\right)}{N_{seed} - N_{dc}}$$

Normal distribution of experimental data was verified, and only the *'B'* value in Experiment I showed slight symmetry deviation (<0.1%), thus no data transformation was performed. Data were submitted to analysis of variance, considering a two-factor scheme, in experiment I between cultivars and inoculants, and in experiment II between cultivars and sampling dates. Means were compared by the Duncan test at the 5% level.

Results

The non-inoculated common bean plants were found to have no root nodules and their shoot and whole plant dry matter were lower (Table 2) than those of inoculated plants at the pre-flowering stage (Table 3). Non-inoculated plants displayed approximately 50% of the seed N allocated in the shoots (Table 2). Total N accumulated by the whole plants (Table 2) was close to that found in the seed (Table 1) differing by only 0.61, 1.36, and 1.00 mg N $plant^{-1}$, respectively, for Ouro Negro, Radiante, and Grafite cultivars (Table 2). However, when the N in detached cotyledons + teguments is also accounted for, very similar N contents to that originally in the seeds were obtained (Fig. 1). This is strong evidence that the nutrient solution was truly N-free and N inputs from any other sources were negligible. The slight difference observed in the large-seed cultivar Radiante (Fig. 1) could be explained by the fact that the seeds analyzed for N content were not identical to those that formed the plants. Although an attempt was made to balance the weight among seeds within the same cultivar, some variability was likely to still exist and this is even less predictable for the N content.

Table 2 Dry matter and N accumulation and $\delta^{15}N$ abundance in different plant tissues of three non-inoculated common bean cultivars grown in N-free hydroaeroponic culture at pre-flowering stage, in experiment I. Means (± standard error) of 4 replicates for each cultivar

Plant trait	Plant part					Detached cotyledon + tegument
	Root	Stem	Leaf	Shoot	Whole plant	
			Cultivar Ouro Negro			
Dry matter (mg plant^{-1})	234 ± 5.96	67 ± 5.45	217 ± 9.75	285 ± 15.02	563 ± 12.34	45 ± 0.92
N accumulation (mg N plant^{-1})	3.2 ± 0.08	0.6 ± 0.08	3.3 ± 0.30	3.9 ± 0.38	7.1 ± 0.41	0.8 ± 0.13
$\delta^{15}N$ (‰)	+2.59 ± 0.29	+0.78 ± 0.36	+2.23 ± 0.33	+1.99 ± 0.33	+2.22 ± 0.26	+2.06 ± 0.66
			Cultivar Radiante			
Dry matter (mg plant^{-1})	327 ± 36.25	175 ± 36.87	325 ± 31.27	500 ± 67.15	939 ± 79.85	111 ± 21.90
N accumulation (mg N plant^{-1})	4.8 ± 0.49	1.5 ± 0.22	7.5 ± 0.49	9.0 ± 0.69	13.8 ± 0.56	4.2 ± 1.42
$\delta^{15}N$ (‰)	+2.44 ± 0.27	+1.34 ± 0.14	+3.67 ± 0.25	+3.28 ± 0.22	+2.95 ± 0.10	+2.02 ± 0.17
			Cultivar Grafite			
Dry matter (mg plant^{-1})	211 ± 22.66	40 ± 3.95	148 ± 13.85	188 ± 16.09	476 ± 35.31	76 ± 8.43
N accumulation (mg N plant^{-1})	3.4 ± 0.23	0.4 ± 0.05	3.0 ± 0.34	3.5 ± 0.38	6.9 ± 0.59	1.7 ± 0.28
$\delta^{15}N$ (‰)	+3.20 ± 0.37	+1.48 ± 0.29	+5.13 ± 0.29	+4.65 ± 0.25	+3.91 ± 0.27	+2.22 ± 0.15

All tissues of non-inoculated plants showed positive $\delta^{15}N$ values, with some fractionation among organs and a trend of lower $\delta^{15}N$ in stems (Table 2). However, after calculating the weighted $\delta^{15}N$ of the whole plant, discounting that of detached cotyledons, positive values very similar to those of the original seeds were observed (Fig. 1). This reinforces the evidence that the nutrient solution had no significant contamination with external N.

In experiment I, there was no significant difference between inoculation with strain CIAT 899 or the commercial strain mixture, for dry matter and N accumulation of the common bean cultivars. Therefore, data displayed in Table 3 are means of the two inoculation treatments.

There was no difference between the cultivars in the nodule number (data not shown), with an overall mean of 78 nodules per plant. Nevertheless, the cultivar

Table 3 Dry matter and N accumulation in different plant tissues of three common bean cultivars inoculated with rhizobium, grown in N-free hydroaeroponic culture, at pre-flowering stage, in experiment I. Values are means of inoculation with strain CIAT 899 and commercial strain mixture; means of 8 replicates for each cultivar

Plant part	Cultivar					
	Ouro Negro	Radiante	Grafite	Ouro Negro	Radiante	Grafite
	Dry matter (mg plant^{-1})			N accumulation (mg plant^{-1})		
Nodule	113 ab[a]	79 b	147 a	8.4 ab	5.8 b	10.8 a
Root	389 a	289 b	457 a	7.3 b	5.4 b	10.3 a
Stem	219 a	217 a	227 a	4.3 a	4.3 a	4.9 a
Leaf	664 ab	451 b	888 a	21.8 ab	18.6 b	28.9 a
Whole plant	1429 ab	1115 b	1787 a	41.7 ab	34.0 b	54.9 a
Shoot	884 ab	669 b	1116 a	26.1 ab	22.9 b	33.7 a
Detached cotyledon + tegument	43 b	78 a	66 ab	0.5 c	2.5 a	1.4 b

[a] Means in the same line followed by the same letter for each of the measured variables are not significantly different at $P < 0.05$ (Duncan test)

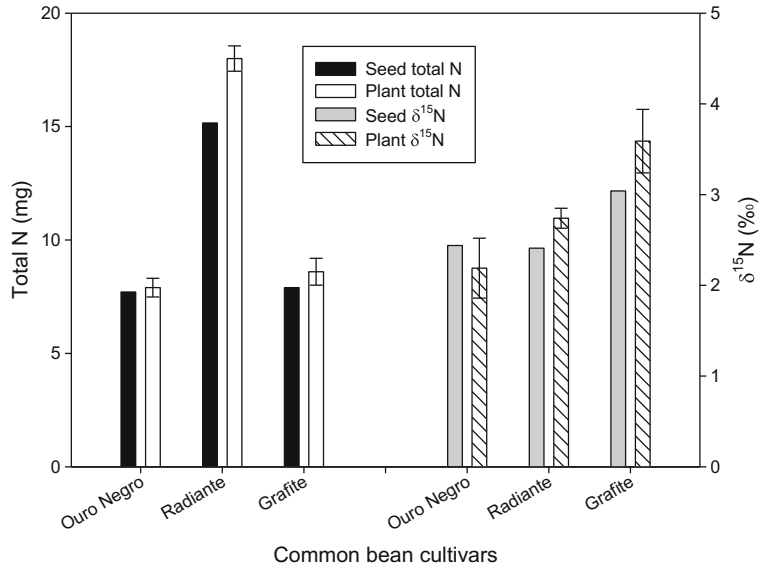

Fig. 1 Total N and $\delta^{15}N$ in seed and whole plants at pre-flowering stage of three non-inoculated common bean cultivars (control plants) grown in N-free hydroaeroponic culture

Table 4 ^{15}N natural abundance and *'B'* value of three common bean cultivars inoculated with different rhizobium strains (CIAT 899 or commercial strain mixture), grown in N-free hydroaeroponic culture, at pre-flowering stage, in experiment I. Means (± standard error) of 4 replicates for each cultivar

Plant part	Rhizobium strain/cultivar							
	CIAT 899				Commercial strain mixture			
	Ouro Negro	Radiante	Grafite	Mean	Ouro Negro	Radiante	Grafite	Mean
				$\delta^{15}N$ (‰)				
Nodule	+8.27 ± 0.10	+6.43 ± 0.16	+7.92 ± 0.22	+7.54 ± 0.25	+7.20 ± 0.76	+6.84 ± 0.32	+8.44 ± 0.20	+7.49 ± 0.34
Root	+0.89 ± 0.25	+2.11 ± 0.15	+0.55 ± 0.15	+1.18 ± 0.22	+1.58 ± 0.35	+1.91 ± 0.18	+0.91 ± 0.20	+1.47 ± 0.19
Stem	−0.92 ± 0.22	+0.05 ± 0.25	−1.18 ± 0.12	−0.68 ± 0.19	−1.06 ± 0.36	−0.27 ± 0.15	−1.38 ± 0.13	−0.90 ± 0.19
Leaf	−0.29 ± 0.26	+2.00 ± 0.29	−0.07 ± 0.18	+0.55 ± 0.33	+0.78 ± 0.41	+1.14 ± 0.48	−0.03 ± 0.13	+0.63 ± 0.26
Detached cotyledon + tegument	+1.01 ± 0.38	+2.56 ± 0.12	+2.66 ± 0.29	+2.08 ± 0.27	+1.01 ± 0.12	+2.68 ± 0.11	+2.57 ± 0.46	+2.09 ± 0.27
Whole plant	+1.30 ± 0.25 b[a]	+2.35 ± 0.43 a	+1.47 ± 0.16 b	+1.71 ± 0.22 A[b]	+1.79 ± 0.18 b	+2.03 ± 0.28 a	+1.54 ± 0.17 b	+1.79 ± 0.14 A
				'B' value (‰)[c]				
Shoot	−0.64 ± 0.25 b	+1.84 ± 0.39 a	−0.39 ± 0.17 b	+0.27 ± 0.36 A	−0.13 ± 0.25 b	+0.87 ± 0.53 a	−0.30 ± 0.12 b	+0.15 ± 0.29 A

[a] Means in the same line for the same rhizobium strain followed by the same lower case letter are not significantly different at $P < 0.05$ (Duncan test)

[b] Different upper-case letters in the same line indicate the existence of significant differences ($P < 0.05$, Duncan test) between overall means of rhizobium strain

[c] Values corrected for discounting the ^{15}N natural abundance of seeds

Radiante showed lower nodule mass than Grafite, both cultivars also differing in nodule, shoot, and whole plant N accumulation (Table 3).

Still in experiment I, leaves, roots, and especially nodules had predominately positive $\delta^{15}N$ values, regardless of the inoculated strain. The opposite was observed for stems (Table 4). The $\delta^{15}N$ for the whole plant discounting the Ndf_{seed} was positive for the three common bean cultivars, varying from +1.30 to +2.35‰, the greatest values registered for Radiante, but the comparison between inoculation treatments was not significant.

The plant reliance on BNF (%Ndfa), estimated by the N difference in N accumulated by the inoculated and uninoculated plants, were 75, 58, and 86%, respectively, to Ouro Negro, Radiante, and Grafite, the value of Radiante being significantly different from the others (Duncan's test, $p < 0.01$). At this stage, the proportion of Ndf_{seed} in the plant total N was 22, 38, and 13%, respectively, for Ouro Negro, Radiante, and Grafite (data not shown).

There was no difference between the inoculated strains for the *'B'* value of the common bean at pre-flowering stage, but there were differences between cultivars (Table 4). The only cultivar with a positive *'B'* value in shoots (+2.19) was Radiante. On the other hand, the cultivars Ouro Negro and Grafite had *'B'* values of −0.39 and −0.35‰, which were not statistically different.

In experiment II, the cultivar Radiante showed lower nodule number than Grafite, with 138 and 236 nodules $plant^{-1}$, respectively, at full flowering (data not shown), and the same was true for nodule mass (Table 5). At mid-pod filling, nodule number increased for both cultivars (265 and 469 nodules $plant^{-1}$) though still lower in Radiante (data not shown). Nodule mass also increased in mid-pod filling, but no differences were observed between cultivars (Table 5).

At full flowering, whole plant dry matter of Grafite plants were more than twice the dry matter in Radiante. However, at mid-pod filling, no differences in whole plant dry matter were observed, because Radiante had a pod dry matter about three times greater than Grafite (Table 5). Irrespective of growth stage, total N accumulated in the plants was always significantly greater in Grafite (Table 5).

Large differences in ^{15}N natural abundance between plant organs were verified in both cultivars, with shoot tissues having negative $\delta^{15}N$ but roots and principally nodules having positive $\delta^{15}N$ values, irrespective of growth stage (Table 6). This internal isotopic fractionation resulted in variation of $\delta^{15}N$ in the following order: leaves < reproductive structures < stems < roots < nodules, with very positive $\delta^{15}N$ values for nodules (Table 6). The $\delta^{15}N$ values for the whole plant discounted from Ndf_{seed} were again generally positive but closer to zero (Table 6) and were different from experiment I when plants were at the pre-flowering stage (Table 4). Only the $\delta^{15}N$ in Grafite plants at full flowering was slightly negative and not different from zero (Table 6).

For full flowering and mid-pod filling stages the cultivar Grafite exhibited a %Ndfa above 90% using the N difference technique as in experiment I, while the reliance on BNF by the cultivar Radiante was 76% at full flowering and only increased to 91% at mid-pod fill (data not shown).

Differences between cultivars for the *'B'* value were observed at both growth stages, with Grafite showing more negative values. The mean *'B'* value in shoots was −1.90 and −1.20‰ at full flowering and mid-pod filling stages, respectively (Table 6).

Discussion

For both experiments, plant dry matter and total N accumulation by common bean plants were not different from other studies under controlled conditions (Piha and Munns 1987; Olivera et al. 2004; Almeida et al. 2013), indicating that the hydroaeroponic system was as suitable for *'B'* value experiments as sand-vermiculite mixtures or other substrates. Moreover, the growing system allowed plants to grow free from any contamination by external N, which was highlighted by the symptoms of N deficiency in non-inoculated plants such as little growth with severe chlorosis. In the same control plants, the similarity between seeds and whole plant in N content and $\delta^{15}N$ signal (Fig. 1) reinforces the experimental conditions were adequate for studies of *'B'* value, free of any external N except BNF.

At pre-flowering, total N accumulated by inoculated plants was 6, 2.5, and 8 times greater than the non-inoculated plants of Ouro Negro, Radiante, and Grafite, respectively, indicating a significant contribution of BNF to the plants. However, the ^{15}N balance for the whole plant resulted in positive $\delta^{15}N$ values after discounting the contribution of N from seeds, indicating a positive discrimination of ^{15}N due to BNF, which is

Table 5 Dry matter and N accumulation in different plant tissues of two common bean cultivars inoculated with commercial strain mixture, grown in N-free hydroaeroponic culture, at full flowering and mid-pod filling growth stages, in experiment II. Means of 4 replicates for each cultivar

Plant part	Growth stage/cultivar							
	Full flowering		Mid-pod filling		Full flowering		Mid-pod filling	
	Radiante	Grafite	Radiante	Grafite	Radiante	Grafite	Radiante	Grafite
	Dry matter (mg plant^{-1})				N accumulation (mg plant^{-1})			
Nodule	147 b[a]	351 a	337 a	502 a	11.3 b	24.1 a	28.9 b	42.6 a
Root	551 b	850 a	943 b	1149 a	10.9 b**	28.1 a	22.9 b	41.0 a
Stem	261 b	644 a	887 b	1245 a	4.3 b	11.0 a	9.7 b	22.5 a
Leaf	846 b	2495 a	1882 b	3121 a	41.5 b	80.2 a	56.7 b	143.5 a
Reproductive structure[b]	86 a	64 b	3750 a	1250 b	3.6 a	3.4 a	92.9 a	45.2 b
Whole plant	1892 b	4404 a	7800 a	7267 a	71.6 b	146.7 a	211.1 b	294.9 a
Shoot	1193 b	3203 a	6519 a	5616 a	49.4 b	94.6 a	159.3 b	211.2 a

[a] Means in the same line for the same growth stage for each of the measured variables followed by the same letter are not significantly different at $P < 0.05$ (Duncan test)

[b] Full flowering: flower; mid-pod filling: pod

not possible according to Unkovich (2013). This author also suggested that the nitrogenase enzyme does not discriminate between the N isotopes, which means the $\delta^{15}N$ abundance for the whole plant should always be zero. Hence, the seeds as source of N and ^{15}N were not adequately dimensioned, which is likely to incur larger errors especially at pre-flowering when seed N represented 17, 37, and 12% of whole plant N, respectively for Ouro Negro, Radiante, and Grafite. The uncertainty related to seed N may be even greater depending on the

Table 6 ^{15}N natural abundance and *'B'* value of two common bean cultivars inoculated with commercial strain mixture, grown in N-free hydroaeroponic culture, at full flowering and mid-pod filling growth stages, in experiment II. Means (± standard error) of 4 replicates for each cultivar

Plant part	Growth stage/cultivar					
	Full flowering			Mid-pod filling		
	Radiante	Grafite	Mean	Radiante	Grafite	Mean
	$\delta^{15}N$ (‰)					
Nodule	+7.54 ± 0.50	+7.79 ± 0.54	+7.67 ± 0.37	+9.11 ± 0.35	+8.36 ± 0.54	+8.73 ± 0.35
Root	+1.37 ± 0.27	+0.07 ± 0.07	+0.72 ± 0.32	+1.47 ± 0.26	+1.81 ± 0.09	+1.64 ± 0.15
Stem	−0.32 ± 0.05	−1.77 ± 0.09	−1.04 ± 0.26	−0.42 ± 0.12	−0.66 ± 0.06	−0.54 ± 0.08
Leaf	−1.00 ± 0.22	−1.98 ± 0.18	−1.49 ± 0.22	−1.22 ± 0.11	−1.61 ± 0.13	−1.42 ± 0.11
Reproductive structure[c]	−0.53 ± 0.02	−1.58 ± 0.05	−1.06 ± 0.19	−0.81 ± 0.14	−0.76 ± 0.21	−0.79 ± 0.13
Whole plant	+0.30 ± 0.10 a[a]	−0.11 ± 0.12 b	+0.10 ± 0.11 A[b]	+0.60 ± 0.08 a	+0.47 ± 0.13 b	+0.54 ± 0.08 B
	'B' value (‰)[d]					
Shoot	−1.73 ± 0.26 a	−2.08 ± 0.17 b	−1.90 ± 0.16 A	−1.03 ± 0.07 a	−1.34 ± 0.05 b	−1.20 ± 0.07 B

[a] Means in the same line for the same growth stage followed by the same letter are not significantly different at $P < 0.05$ (Duncan test)

[b] Different upper-case letters in the same line indicate the existence of significant dfferences ($P < 0.05$, Duncan test) between overall means of growth stages

[c] Full flowering: flower; mid-pod filling: pod

[d] Values corrected for discounting the ^{15}N natural abundance of seeds

variability of ^{15}N natural abundance among seeds within the same cultivar being used in the experiment (Nebiyu et al. 2014). Although the seeds were not analyzed for N and $\delta^{15}N$ individually, the non-inoculated plants were harvested at the pre-flowering stage of each cultivar. As the results show that all N in the control plants was exclusively derived from seeds (Fig. 1), the variation in values of total N and ^{15}N abundance should reflect that of the seeds. Using such data, the estimates of whole plant ^{15}N natural abundance of nodulated (inoculated) plants varied from +0.71 to +3.99‰ in Ouro Negro, from +0.85 to +4.59‰ for Radiante, and from +0.82 to +2.19‰ in Grafite at pre-flowering.

As was observed in this study and many others, the nodules were highly enriched with ^{15}N (between +6 and +9‰). This implies that the N exported from the nodules would be ^{15}N depleted. Hence, another potential interference would be root N exudation if such N were lost to the growth medium (Ta et al. 1986), which was replaced weekly in our study.

During plant growth under BNF, there is a fractionation of the ^{15}N between plant parts, the nodules being much more enriched and the aerial tissues depleted (Unkovich et al. 2008) as shown in Table 4, for instance. Hence, owing to this fractionation, the *'B'* value required to correct the estimation of BNF when only plant shoots are sampled is expected to be negative.

In this study, the $\delta^{15}N$ of shoots, or the *'B'* value, was negative for the varieties with smaller seeds, but was still positive for the larger seed variety Radiante at pre-flowering. This indicates that the partitioning of seed N between aerial and belowground tissues can significantly affect the *'B'* value depending on the importance of seed N for shoot N accumulation.

In most studies, use of seed N by the plant is assumed to be 100% in studies with chickpea (Kyei-Boahen et al. 2002), soybean (Okito et al. 2004), and faba bean (Nebiyu et al. 2014). Fewer studies tried to estimate more precise values by determining the difference between seed N and the N in remaining fallen cotyledons (López-Bellido et al. 2010). We demonstrated that the N in fallen cotyledon + tegument represented from 10 to 28% of the N in seeds of common beans, being greater for the larger seed cultivar, which is similar to the 20% determined by López-Bellido et al. (2010) for chickpea, an intermediate seed-size legume species. However, how much seed N is partitioned between aerial and belowground tissues is still a matter for study. Experiments with ^{15}N-enriched seed are a suggestion for future research on this question.

In our case, assuming that all plant N came from seeds, shoot N represented 59, 65, and 51% of whole N accumulated by control plants for Ouro Negro, Radiante, and Grafite, respectively, but this proportion could have been influenced by the decrease in shoot:root ratio that is observed when plants are under N stress (Naegle et al. 2005). Usually, half of the seed N is considered to be part of the aerial tissue (Okito et al. 2004), which was assumed in our calculations. This assumption corroborates the results of Jensen et al. (1985) in studies with ^{15}N-labeled seeds in pea and field bean showing that approximately 50% of this N was recovered in the shoots harvested 3 weeks after seedling emergence. This indicated that the determination of the distribution of seed-borne N in plants grown a few weeks with the seed as the only N source is appropriate for obtaining a reliable estimate of the seed N distribution within the plants.

More time for plant growth would be necessary to allow a greater N accumulation from BNF by the bean plants to make the errors associated with seed N to be negligible in the determination of the *'B'* value. The fact that there was a similar response of plant dry matter and N accumulation and ^{15}N abundance in both shoots and roots of all cultivars suggested that the inoculant was irrelevant for the *'B'* value for common beans. However, caution is required since the number of strains tested was small (three strains), although strain CIAT 899 was also part of the commercial inoculant. Moreover, there is strong evidence from studies on other legume species that shows a large influence of different rhizobium strains on *'B'* values (Steele et al. 1983; Okito et al. 2004; Guimarães et al. 2008).

At full flowering, Radiante plants accumulated twice as much N as at pre-flowering, equivalent to 6.5 times the N coming from seeds. At mid-pod filling, such differences were much greater, with N in the whole plant being 19 times greater than that from seeds. For the small-seeded cultivar Grafite, N in whole plants was 24 and 48 times that in seeds at full flowering and mid-pod filling, respectively. For Grafite, there was a much lower influence of seed N in the final ^{15}N balance and the whole plant at full flowering had a negative value, but not different from zero (Student's t test, $p < 0.05$), which supports that BNF does not discriminate against ^{15}N (Unkovich 2013). For the large-seeded Radiante, the ^{15}N in whole plant was close to zero, but still positive,

which could be again explained by some influence of seed N. At mid-pod filling, the even greater N accumulation by the two cultivars did not improve the results. Even though close to zero, the whole ^{15}N balance was significantly more positive irrespective of cultivar.

On average, the ^{15}N abundance of the whole plants (corrected for seed N) was significantly greater at mid-pod filling (Table 6). Generally, common bean plants have a short growth cycle and therefore, at mid-pod filling, bean plants are in the process of senescence with N being translocated from leaves to pods (Araújo et al. 2012) while nodule mass starts to decrease (Almeida et al. 2013). While a careful sampling was performed to account for all plant material, potential losses of NH_3 due to protein breakdown during senescence could have occurred, which could bring about ^{15}N enrichment in aerial tissues due to losses of ^{15}N-depleted NH_3 (Farquhar et al. 1983). A tendency for ^{15}N abundance to increase from full flowering to mid-pod filling was observed in Grafite leaves and stems (Table 6).

The '*B*' value estimated from the plant shoot was always negative at full flowering and mid-pod filling, as this value does not include root and nodule tissues enriched in ^{15}N. At full flowering, the *'B'* values were −1.73 and −2.08‰ for Radiante and Grafite, respectively, illustrating a significant difference between the two cultivars which had contrasting growth habits and cycle duration. For mid-pod filling, there was a significant increase in the values to −1.03 and −1.34‰, respectively, for each cultivar, probably due to the aforementioned potential N losses related to internal translocation and senescence processes.

Differences between common bean cultivars of different growth habit with regard to the *'B'* value were observed by Polania et al. (2016) although these differences were not discussed. In addition, for mid-pod filling they found more negative *'B'* values (−3.09 and −3.62‰). Unkovich et al. (2008) reported *'B'* values for common beans varying from −1.97 to −2.50‰, closer to those reported here. Our results support the idea that apart from cultivar, the determination of a *'B'* value to be used in calculations of BNF for common beans should also consider the stage of the plant growth at sampling.

Concluding remarks

The scientific literature lacks information on the *'B'* value for common beans required to employ the ^{15}N natural abundance technique for the quantification of BNF. In this study, there was evidence that the inoculation of a same bean cultivar with the strain CIAT 899 or with a commercial strain mixture would not result in contrasting $\delta^{15}N$ signal of shoots or whole plant. However, our data indicated that a general *'B'* value for common bean is not the best recommendation. Although more studies are always welcome to reduce uncertainties, our results pointed out differences between cultivars of different cycle length and small or large seeds, which were associated with plants of growth habit types II and III and plants of type I, the latter having larger seeds and short cycle duration.

The growth stage was also relevant when determining the *'B'* value. At pre-flowering, plants did not accumulate enough N to make the influence of seed N negligible on the shoot and whole plant $\delta^{15}N$ and this uncertainty impairs the establishment of a reliable *'B'* value. On the other hand, at full flowering and mid-pod filling stages, consistent *'B'* values were obtained. However, our results indicated the need for a value for each growth stage, which is likely to be related to the narrow time frame of BNF activity of this crop.

In view of the scarcity of data for common beans, *'B'* values estimated for the cultivar Grafite could be used for any small seed cultivar while results for Radiante would be feasible for large-seed cultivars. In this case, the *'B'* values are −2.08‰ for small seed and −1.73‰ for the larger seed cultivars when at full flowering stage. These values should be −1.34 and −1.03‰, respectively, for the small and large-seed cultivars when sampling is to be made at mid-pod filling.

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