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ORIGINAL PAPER

Importance of correct *B* value determination to quantify biological N_2 fixation and N balances of faba beans (*Vicia faba* L.) via ^{15}N natural abundance

Amsalu Nebiyu · Dries Huygens · Hari Ram Upadhayay · Jan Diels · Pascal Boeckx

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Abstract Quantifying biologically fixed nitrogen (BNF) by legumes through the ^{15}N natural abundance techniques requires correct determination of a so-called *B* value. We hypothesized that significant variations in *B* values exist between faba bean (*Vicia faba* L.) varieties having consequences for BNF and N balance calculations. We experimentally determined *B* values for a range of faba bean varieties and quantified to what extent variety has an effect on *B* values and hence BNF quantification. Seeds of six faba bean varieties released in Ethiopia were inoculated with *Rhizobium fabae* strain LMG 23997-19 and grown in vermiculite with an N-free nutrient solution in a growth room until full flowering. Total N and ^{15}N content of nodules, roots, and shoot components was analyzed separately to determine the weighted whole plant ^{15}N fractionation during N_2 fixation, i.e., the *B* value. Owing to its large seed size and high N content, a correction for seed N was carried out. We then calculated the percentage of N derived from air (%Ndfa), BNF, and N balance for faba beans grown in the field using three *B* value scenarios (variety specific *B* value corrected for seed N, variety specific *B* value without seed N correction, and a literature derived *B* value). Whole plant seed N corrected *B* values were significantly different ($P<0.05$) between varieties and varied between +0.5±0.4 and −1.9± 1.4‰ suggesting a variable isotope fractionation during N_2 fixation. The %Ndfa was significantly ($P<0.05$) different between varieties (59±4.2–84±4.5 %) using seed N corrected B values. BNF (218±26.2–362±34.7 kg N ha^{-1}) was significantly ($P<0.05$) different between varieties for corrected and uncorrected *B* values. Soil N balance did not result in statistically significant ($P>0.05$) difference between varieties for all three *B* value scenarios. Use of inappropriate *B* values masked the difference between varieties and affected their ranking in terms of BNF, resulting from an over- to underestimation of 15 and 19 %, respectively. When applying the ^{15}N natural abundance technique to compare BNF of legume accessions, we recommend determining a *B* value for each accession. For legumes with large seeds such as faba beans, it is moreover essential to account for seed N when determining the *B* value.

Keywords Faba bean · *B* value · ^{15}N natural abundance

A. Nebiyu (✉)
College of Agriculture and Veterinary Medicine, Jimma University, POB 1316, Jimma, Ethiopia
e-mail: anebiy@yahoo.com

D. Huygens · H. R. Upadhayay · P. Boeckx
Isotope Bioscience Laboratory—ISOFYS, Faculty of Biosciences Engineering, Ghent University, Coupure links 653, 9000 Gent, Belgium

J. Diels
Department of Earth and Environmental Sciences, KU Leuven, Celestijnenlaan 200E, box 2411, 3001 Leuven, Belgium

Introduction

Atmospheric nitrogen (N) fixation in crop and pasture legumes plays a key role in providing human and livestock protein and for maintaining soil fertility in agro-ecosystems (Unkovich et al. 2010). Improving N fertilizer efficiency and exploitation of biologically fixed N (BNF) are thus of great importance for long-term sustainability of crop production in agro-ecosystems (Unkovich et al. 2008). The global high price for N fertilizer and the overall environmental impact of excessive fertilizer use (Chianu et al. 2011; Fan et al. 2006) warrant a growing interest in legume BNF, especially for smallholder farmers in the tropics. Many literature reviews document on the magnitude and potential benefits of BNF by legumes within different agro-ecosystems (Herridge et al. 2008; Peoples et al. 2009a; Salvagiotti et al. 2008; Unkovich

and Pate 2000). The use of legume–cereal crop rotation systems, particularly with faba beans, has proven to be an efficient cultivation method to reduce N fertilizer use in tropical highlands (Amanuel et al. 2000; Maidl et al. 1996), and is thus a sustainable option for agricultural intensification. Faba bean is one of the best crop species for atmospheric N_2 fixation, with global annual BNF inputs estimate at around 0.3 Tg year^{-1} (Herridge et al. 2008). Research reports also indicated a substantial grain yield increment for wheat cultivated in rotation with faba beans (Amanuel et al. 1991; Habtemichial et al. 2007; López-Bellido et al. 2010). Nevertheless, in order to improve the N use efficiency of faba-bean-based cropping systems, an accurate quantification of the proportion of N derived from the air (%Ndfa) is required for the range of faba bean varieties.

The correct estimation and quantification of BNF by legumes depends on the applied methodology (Hardarson et al. 1993). Stable isotope methods have emerged as one of the more powerful tools to advance the understanding of relationships between plants and their environment (Dawson et al. 2002). The natural abundance of ^{15}N ($\delta^{15}N$) can be used to assess BNF in field conditions without the additional cost and effort of applying ^{15}N-enriched fertilizer (e.g., Houngnandan et al. 2008; Pate et al. 1994; Okito et al. 2004). This technique depends on the fact that the plant available N in most soils is enriched in ^{15}N compared to that in air (Okito et al. 2004); hence a simple two end member isotopic mixing model can be used to quantify the contribution of atmospheric N and soil N to a legume crop. The ^{15}N natural abundance method has a number of advantages over other methodologies. It can be applied in greenhouse or field experiments like other techniques, allows N_2 fixation to be assessed in almost any situation where both N_2 fixing and non-fixing plants are present at the same location and can be easily applied to farmers' fields (Unkovich et al. 2008).

Since the legume growing in the field acquires N from two end members (soil N and air N), it is required to determine the ^{15}N abundance of both the N derived from the soil and that derived from air through fixation. The most reasonable measure of the $\delta^{15}N$ value of soil derived N within the legume plant is to rely on the $\delta^{15}N$ value of a non-N_2 fixing reference plant grown on the same soil (Shearer and Kohl, 1988). However, this measure may be subject to errors and the problems associated to reference plant selection have been reviewed earlier in detail (Boddey et al. 2000; Handley and Scrimgeour 1997; Högberg 1997) and is therefore not the focus of this paper. The other potential limitation of the ^{15}N natural abundance technique is the need to adjust for isotopic fractionation by the legume during N_2 fixation, i.e., the estimation of the so-called *B* value (Doughton et al. 1992; Unkovich and Pate 2000; Unkovich et al. 2008). Hence the *B* value is the isotope fractionation corrected atmospheric N_2 end member. The *B* value is defined as the $\delta^{15}N$ value of a legume when completely dependent on N_2 fixation for growth (Unkovich and Pate 2000). Under these conditions legume shoot N is normally depleted in ^{15}N relative to atmospheric N_2 due to isotope discrimination within plant tissues. Since the *B* value varies with species, plant age at harvest, growing conditions (Unkovich and Pate 2000), and seed size (López-Bellido et al. 2010), a single *B* value could not be satisfactory for all legumes and environments. Reported estimates may thus vary considerably, even for the same plant species (Boddey et al. 2000). The *B* values cited in the literature show a wide range for different legumes (Boddey et al. 2000; Peoples et al. 2009b), and for faba beans specifically (Fan et al. 2006; López-Bellido et al. 2010; Unkovich et al. 2008). The majority of the *B* values for legumes usually lie in between 0 and −2.0‰, with one or two exceptions (Okito et al. 2004).

Large errors in N_2 fixation calculation can be generated by using incorrect *B* values, especially when %Ndfa is higher than 85 % (Unkovich and Pate 2000). Doughton et al. (1992) designed a method to estimate *B* values with plants grown in the field by the combined use of ^{15}N enrichment techniques and natural abundance methodologies on the same crop at the same site. Their method was basically a process of adjusting *B* values until %Ndfa measured by natural abundance best matches %Ndfa as derived from a ^{15}N tracer experiments. However, this type of *B* value estimation technique may not be suitable for BNF measurements when dealing with large number of varieties of a single legume species. An alternative methodology to determine *B* values is based on the cultivation of N-fixing plants that are grown in N-free growth medium (Vincent 1970).

Current estimates of *B* values (e.g., Boddey et al. 2000; Doughton et al. 1992; Houngnandan et al. 2008; Kyei-Boahen et al. 2002; López-Bellido et al. 2010; Nguluu et al. 2001; Okito et al. 2004) are often biased due to two reasons. First, *B* values are typically calculated based on aerial tissues because of ease of sampling. However, such sampling protocols do not take into account the non-uniform distribution of ^{15}N between roots, nodules, and shoot tissue (Boddey et al. 2000). Second, in order to obtain the true *B* value an additional adjustment for seed N at sowing should be considered. The significant impact of seed size has often been overlooked in studies of *B* value and BNF determination. Seed N can constitute a significant proportion of total N accumulated by the crop, especially for large seeded legumes with high seed N contents like faba beans (López-Bellido et al. 2010; Okito et al. 2004). In this study, we test the importance of considering the non-uniform distribution of ^{15}N in legumes, the effect of variety and the seed N contribution for calculating *B* values for a range of faba bean varieties. It is hypothesized that significant variations in *B* values exist between faba bean varieties and that %Ndfa calculations show large variations dependent on the methods applied to calculate *B* values. Most *B* values for

faba beans reported in the literature are determined using above ground biomass only and did not take into account the seed N and seed ^{15}N contribution. We experimentally determined *B* values for a range of faba bean varieties taking into account (1) the non-uniform distribution of ^{15}N both in the above and below ground biomass, (2) the need for an additional adjustment for seed N and seed ^{15}N at sowing; and quantified the effect of variety on *B* values and hence BNF estimates. We also examined the need for seed N correction for correct *B* value, hence BNF estimation.

Materials and methods

Experimental setup

In order to determine *B* values of faba bean varieties a hydroponic culture without N sources was setup using a modified Leonard jar system (Vincent 1970), consisting of a bottle (330 ml) with the bottom portion cutoff and inverted into a 1-L Mason jar. A cotton lamp wick was inserted through the neck of the inverted bottle, which was placed on the bottom of the Mason jar. A foam plug in the neck of the inverted bottle held the wick in place. The assembled system was autoclaved (120 °C) during 1 h and allowed to cool for 24 h before seed sowing. The inverted bottle was filled with washed and autoclaved (60 min) N-free vermiculite.

Six faba bean varieties (CS-20DK, Degaga, Gebelecho, Moti, Obse, and Walki; Table 1) were used. These varieties were selected because they had good adaptation potential and agronomic performance for the humid tropical highlands of Ethiopia (Nebiyu et al. 2010). The seeds of each variety were surface-sterilized with 10 % H_2O_2 for 30 min and washed and rinsed five times with distilled water. The seeds were then germinated for 3 days on a petri dish with deionized water in the dark at 28 °C and sterile conditions (Rodriguez-Navarro et al. 2000). The germinated seeds were inoculated with *Rhizobium fabae* LMG 23997 (3.5×10^9 CFU mL^{-1}) by dipping them in viscous *Rhizobium* containing specific growth medium (Vincent 1970) for 30 min. Two inoculated seeds were sown in each jar by carefully punching a hole through the rooting medium (vermiculite) and placing the seed into the vermiculite with sterilized forceps. The experimental design used was completely randomized design in six replicates, each jar being a replicate. The seedlings were thinned to one after 4 days of seedling emergence. Each Mason jar was watered with Norris modified N-free nutrient solution (Norris and Date 1976) containing (per liter of de-ionized water) KH_2PO_4 (0.27 g), K_2SO4 (0.35 g), $CaSO_4{\cdot}2H_2O$ (1.0), $MgSO_4{\cdot}7H_2O$ (0.25 g), H_3BO_3 (4.0 mg), $MnCl_2{\cdot}4H_2O$ (0.99 mg), $ZnSO_4{\cdot}7H_2O$ (0.58 mg), $CuSO_4{\cdot}5H_2O$ (0.125 mg), $FeCl_3{\cdot}6H_2O$ (5.4 mg), and $Na_2MoO_4{\cdot}2H_2O$ (0.1 mg).

Table 1 Main characteristics of six selected faba bean varieties

Variety	Variety characteristics						Seed characteristics			
	Pedigree	Source	Days to flowering	Days to maturity	Grain yield potential (Mg ha^{-1})	Adaptation zone ([b]m a.s.l.)	Seed weight (mg)	N-concentration (%)	Total N (mg $seed^{-1}$)	$\delta^{15}N$ (‰)
CS-20DK	CS20DK	Ethiopia	57–67	145–160	1.5–3.0	2,300–3,000	537	4.2±0.5	22.7	−0.5±0.2
Degaga	R878-3	ICARDA[a]	45–62	116–135	2.0–4.5	1,800–3,000	662	4.0±0.6	26.6	−0.9±0.1
Moti	ILB4432×Kuse 2-27-33	ICARDA	43–65	108–165	2.3–3.5	1,800–3,000	587	3.4±1.1	20.3	−0.6±0.1
Gebelcho	Tesfa×ILB4726	ICARDA	51–69	103–167	2.0–3.0	1,800–3,000	757	3.5±0.5	26.7	−0.6±0.1
Obse	CS20DK×ILB4427	ICARDA	43–65	87–166	2.1–3.5	1,800–3,000	897	4.3±0.9	39.2	−0.3±0.1
Walki	Bulga-70×ILB4615	Ethiopia/ICARDA	49–61	133–146	2.0–4.2	1,800–2,800	755	3.9±0.2	29.7	−1.1±0.2

[a] International Center for Agricultural Research in the Dry Areas, Aleppo, Syria

[b] Meters above sea level

Plant growth, harvest, and analyses

The plants were grown in a greenhouse with a 13-h per day length and mean day and night temperatures of about 24 and 16 °C, respectively. The nutrient solution in the Mason jar was replaced every 6 days by 250 to 300 mL of the Norris modified N-free nutrient solution. The plants were harvested at flowering (47 days after sowing). All of the senescent leaves lying on the media of each jar were periodically collected and kept until harvest, and added to the shoot fraction (aerial portion). The aerial portion, root, and nodules were separated during the plant harvest and dried at 65 °C for 72 h to determine the dry matter. The plant parts were ground using a centrifugal mill (Retsch ZM 200, Germany), using a 0.5-mm sieve. The N concentration, total N and ^{15}N of the seed at sowing, and of the shoot, root, and nodules were determined via elemental analyzer isotope ratio mass spectrometry (EA-IRMS; 20-20, SerCon, Crewe, UK). The ^{15}N natural abundance ($\delta^{15}N$) was calculated according to the following formula (Högberg 1997):

$$\delta^{15}N\,(‰) = \left[\left(^{15}N/^{14}N_{sample} - {}^{15}N/^{14}N_{standard}\right)/\left(^{15}N/^{14}N_{standard}\right)\right] \times 1,000$$

where, the standard is air (Eq. 1)

Correction for seed N

Owing to its large seed size and high N content (Table 1), faba bean seed N can constitute a significant proportion of total N accumulated in the plant (López-Bellido et al. 2010). Therefore, it was necessary to determine the $\delta^{15}N$ of the seed N, discounting its excess ^{15}N content (using a mass balance) to estimate the *B* value. This correction was made using the following formula given by Högberg et al. (1994):

$$\delta^{15}N\,\text{corrected} \qquad \text{(Eq.2)}$$
$$= \left[\left(\text{whole plant N} \times \delta^{15}N\,\text{whole plant}\right) - \left(\text{seed N} \times Ps \times \delta^{15}N\,\text{seed}\right)\right]$$
$$/\left(\text{whole plant N} - \text{seed N}\right)$$

where $\delta^{15}N$ corrected indicates the correction for seed N and Ps is the proportion of the seed N that was incorporated in the plant tissue. According to Okito et al. (2004), Ps is assumed to be 0.5 when correcting shoot tissue only (i.e., 50 % of the seed N was incorporated into the aerial tissue) and when correcting for the whole plant Ps is assumed to be 1.

The corrected *B* values of each variety were then used to calculate %Ndfa, BNF, and soil N balance using data from unpublished ^{15}N measurements collected from a faba bean experiment with the same varieties carried out on a farmer's field at Dedo (7°28′48″ N and 36°52′19″ E and at an elevation of 2,160 m above sea level), Southwest Ethiopia with wheat as the reference plant. The %Ndfa was calculated as follows (Peoples et al. 2009b):

$$\%\text{Ndfa} = 100 \times \left(\delta^{15}N_{\text{reference plant}} - \delta^{15}N_{\text{legume}}\right) \qquad \text{(Eq.3)}$$
$$/\left(\delta^{15}N_{\text{reference plant}} - B\text{ value}\right)$$

Where $\delta^{15}N_{\text{reference plant}}$ and $\delta^{15}N_{\text{legume}}$ are the $\delta^{15}N$ values of whole plant wheat and faba bean, respectively.

The amount of N_2 fixed by the legume (BNF, kg N ha^{-1})= total N yield×(%Ndfa)/100. The N balance (kg N ha^{-1}) was calculated as whole plant BNF minus N exported via grain N. We compared Ndfa, BNF, and N-balance obtained via variety specific seed N corrected *B* values, variety specific *B* values without seed N correction and a mean of previously published shoot derived B values (−0.85‰) from literature (López-Bellido et al. 2010; Fan et al. 2006; Unkovich et al. 2008).

Statistical analysis

All data were subjected to analysis of variance (one-way ANOVA) using the general linear model procedure of statistical analysis system (SAS) software version 9.2 (SAS Statistical Analysis System 2008). Treatment means for faba bean varieties were compared using the least significant difference (LSD) test at $P<0.05$.

Results

Dry matter yield

The nodule, root, and shoot dry matter yield was significantly different between the faba bean varieties (Table 2). The average whole plant dry matter yield of faba bean varieties was 2.4 g plant^{-1} with significant difference between varieties varying from 1.6±0.1 g plant^{-1} (Gebelcho) to 2.9±0.2 g plant^{-1} (Obse). Varieties CS-20DK, Moti, and Walki produced similar

Table 2 Dry matter yield (mean ± SE) of faba bean plants grown in N-free nutrient medium

Variety	Dry matter (g plant^{-1})			
	Shoot	Root	Nodules	Whole plant
CS-20DK	1.9±0.1ab	0.7±0.1ab	0.06±0.01ab	2.6±0.1ab
Degaga	1.5±0.2a	0.5±0.04bc	0.08±0.01a	2.1±0.3b
Gebelcho	1.1±0.2c	0.5±0.07c	0.02±0.01b	1.6±0.1c
Moti	2.0±0.1a	0.8±0.05a	0.05±0.02ab	2.9±0.2a
Obse	1.9±0.1ab	0.7±0.06a	0.09±0.02a	2.7±0.1a
Walki	1.8±0.1ab	0.7±0.05ab	0.09±0.02a	2.6±0.2ab
Average	1.7	0.6	0.07	2.4
$LSD_{0.05}$	0.4	0.2	0.04	0.5

Means followed by different letters in a column are significantly different at $P<0.05$

($P>0.05$) whole plant total dry matter yield compared with Obse (Table 2).

N concentration

The average total N concentration for the whole plant of faba bean varieties was 4.5 %, with significant differences between the varieties (Table 3). The highest N concentration was recorded in nodules ranging from 5.2±0.3 % (Obse) to 6.7± 0.4 % (Moti) followed by roots ranging from 2.2±0.2 % (Gebelcho) to 3.0±0.2 % (Degaga), with significant differences between varieties. No significant differences were observed between the varieties for the shoot N concentration (Table 3).

The average whole plant total N content of the six faba bean varieties at flowering was 65.3 mg N plant^{-1} without adjusting for the seed N contribution. When adjusting for seed N, the average plant N content was 37.3 mg N plant^{-1} with significant differences between varieties (Table 3). The unadjusted whole plant total N content varied from 35.8±4.6 (Gebelcho) to 78.3±6.3 mg N plant^{-1}(Obse) and from 15.5± 4.6 (Gebelcho) to 52.0±9.8 mg plant^{-1} (CS-20DK) for the adjusted whole plant N content. Total N content of the shoot, root, and nodule components was also significantly different for the varieties (Table 3). The proportion of N in the whole plant that is derived from seed N, from which the plants were grown, represented on average 48.2 % and ranged between 34 % (for CS-20DK) to 61 % (for Gebelcho). The δ^{15}N of the seeds at sowing was between −0.5‰ (for CS-20DK) to −1.1‰ (for Walki).

B value

The δ^{15}N of shoots, roots, and nodules were significantly different between the varieties (Table 4). All the varieties exhibited a positive δ^{15}N value for the nodules (in the range of +3.6±0.9‰ for Degaga to +6.3±0.6‰ for variety Moti) and a negative value for the shoots (ranging from −1.1±0.3‰ in shoots of CS-20DK to −0.2±0.2‰ in shoots of Moti). No significant differences were observed for the uncorrected whole plant δ^{15}N value and the average of the varieties was −0.5‰. The corrected values of δ^{15}N for the whole plant assuming Ps=1 were significantly different between the varieties and varied from +0.5±0.4‰ (Moti) to −1.9±1.4‰ (Degaga).

%Ndfa, BNF, and N Balance

The %Ndfa was significantly ($P<0.05$) different between the varieties when using *B* values corrected for seed N (corrected B; Fig. 1a). Variety Moti had the highest (84±4.5 %) %Ndfa while the lowest %Ndfa values were observed for Degaga (59±4.2 %). Applying the *B* value uncorrected for seed N (uncorrected B) and average *B* value from literature (average B) did not result in significant differences in %Ndfa for faba bean varieties. %Ndfa values ranged from 66.2–78.8 % and 63.3–77.3 % for uncorrected and average *B* values, respectively. Further, BNF was significantly ($P<0.05$) different for the varieties when estimated for two scenarios (corrected and uncorrected *B* values). Moti showed the highest BNF (362±34.7 and 331±33.6 kg N ha^{-1}) and CS-20DK the lowest (227±29.3 and 218±26.2 kg N ha^{-1}), respectively, for both scenarios (Fig. 1b). However, the soil N balance did not differ significantly ($P>0.05$) between varieties when quantified for the three scenarios (Fig. 1c).

Furthermore, it was also shown that calculation of *B* value without correcting for seed N or using an average *B* value from the literature resulted in a biased %Ndfa, BNF, and soil N balance estimation, ranging from an overestimation of +15 % for Degaga to an underestimation of −19 % for Moti (Fig. 1a–c).

Table 3 Nitrogen concentration (%; mean ± SE) and total N content (mg N plant^{-1}; mean ± SE) of shoot, root, nodule, and the whole plant of six faba bean varieties grown in N-free medium

Variety	Nitrogen concentration (%)				Total N content (mg N plant^{-1})				
	Shoot	Root	Nodules	Whole plant	Shoot	Root	Nodules	Whole plant	Whole plant (adjusted)
CS-20DK	2.7±0.3a	2.9±0.3a	6.5±0.2ab	4.5±0.3ab	52.3±9.1a	18.9±1.9a	3.6±0.6ab	74.8±9.9a	52.0±9.9a
Degaga	2.9±0.2a	3.0±0.2a	5.7±0.4bc	4.9±0.2a	47.2±7.7a	15.3±1.8ab	4.5±0.9a	66.9±9.5a	40.4±9.5ab
Gebelcho	2.3±0.4a	2.2±0.2b	5.6±0.1c	3.9±0.4b	24.2±4.7b	10.2±1.4b	1.3±0.2b	35.8±4.6b	15.5±4.6b
Moti	2.3±0.4a	2.5±0.2ab	6.7±0.4a	4.4±0.4ab	48.2±10.2a	19.5±2.1a	5.0±1.5a	72.8±12.4a	46.1±12.4a
Obse	2.8±0.3a	2.9±0.1a	5.2±0.3c	4.9±0.2a	53.5±5.6a	20.3±1.0a	4.5±0.9a	78.3±6.3a	39.1±6.3ab
Walki	2.2±0.2a	2.5±0.2ab	5.5±0.2c	4.1±0.3ab	41.7±6.7ab	16.5±1.6a	4.8±1.1a	63.0±9.1a	33.3±9.1ab
Average	2.5	2.7	5.9	4.5	44.5	16.8	3.9	65.3	37.7
$LSD_{0.05}$	0.8	0.6	0.8	0.9	22.6	5.1	2.9	27.3	27.3

Means followed by different letters in a column are significantly different at $P<0.05$. Whole plant (adjusted) refers to the total N content of whole plant minus seed N at sowing

Table 4 *B* values (mean ± SE) of the shoot, root, nodules, and the whole plant of six faba bean varieties grown in N-free medium

Variety	(*B* value–$\delta^{15}N$ (‰))				
	Shoot	Root	Nodules	Whole plant	Whole plant (corrected)
CS-20DK	−1.1±0.3b	+0.4±0.4a	+6.3±0.6a	−0.5±0.3a	−0.3±0.6ab
Degaga	−0.7±0.2ab	−0.5±0.2bc	+3.6±0.9b	−0.5±0.1a	−1.9±1.5b
Gebelcho	−0.9±0.2ab	−0.6±0.1bc	+4.2±0.1ab	−0.9±0.1a	−0.6±0.4ab
Moti	−0.2±0.2a	−0.1±0.2ab	+6.3±0.6a	−0.04±0.2a	+0.5±0.4a
Obse	−0.9±0.3ab	−0.3±0.20bc	+4.6±1.1ab	−0.6±0.3a	−1.1±0.7ab
Walki	−0.7±0.2ab	−0.8±0.2c	+5.9±0.7a	−0.6±0.2a	−0.02±0.3ab
Average	−0.7	−0.3	+5.2	−0.5	−0.6
LSD0.05	0.8	0.7	2.1	0.7	2.3

Means followed by different letters in a column are significantly different at $P<0.05$. Whole plant (corrected) refers to the *B* value obtained for whole plant after correction for the initial seed N contribution using a mass balance approach

Discussion

It is often reported that dry matter production potential of a plant is the driving factor behind N_2 fixation when there is effective plant–rhizobium symbiosis (Unkovich and Pate 2000). We determined dry matter yield (g plant^{-1}) of six faba bean varieties grown until full flowering in nutrient medium lacking N but inoculated with rhizobium. Significant differences were shown for the dry matter yield in the whole plant (1.6±0.1 to 2.9±0.2 g plant^{-1}) for the varieties. These differences in whole plant total dry matter yield could be attributed to the differences in dry matter yield produced by shoots, roots, and nodules. The shoots represented 68–72 % of the total dry matter of the whole plant whereas, the nodules and roots contributed for 1.3–3.8 and 23–30 % to the total whole plant dry matter, respectively. The differences between varieties in terms of dry matter content of each plant part and the whole plant will therefore potentially influence the amount of atmospheric N_2 fixation between faba bean varieties. Dry matter yield is reported to be the driving factor behind N_2 fixation and that plant growth creates the demand for N (Unkovich and Pate 2000).

The total N contributed by the seed at sowing to the whole plant N at flowering was in the range from 34 % (CS-20DK) to 61 % (Gebelcho), suggesting that seed size in faba bean is important for the accurate estimation of *B* values and derived parameters such as BNF. The varieties used in the present study had average seed weight ranging from ca. 530 mg (CS-20DK) to 900 mg (Obse). The adjusted total N content for the whole plant was significantly different for the varieties ranging from 15.5±4.6 mg N plant^{-1} (Gebelcho) to 52.0± 9.8 mg N plant^{-1} (CS-20DK). The adjusted values for total N content and $\delta^{15}N$ for the whole plant according to the mass balance model with Ps=1 indicate that the total N and ^{15}N contributed by the seed at sowing to the total plant N was 48 %, on average for the varieties, which was somewhat lower than the 54 % reported by López-Bellido et al. (2010) for faba beans. This difference (48 vs. 54 %) may be due to the differences in the type of varieties used, growing conditions, and age of plants at harvest.

The ^{15}N abundance of N_2-fixing plants can vary with variety, growth stage, and the plant part sampled (Boddey et al. 2000; Kyei-Boahen et al. 2002; Unkovich and Pate 2000). In the present study, we analyzed the ^{15}N abundance of faba bean nodules, roots, shoots, and whole plant at flowering stage. The $\delta^{15}N$ values were negative for roots, shoot, and whole plant components but the strongly positive values for nodules showing significant isotopic discrimination against the heavier isotope during atmospheric N_2 fixation and during metabolism and N translocation in the plant system. The ^{15}N enrichment of the legume nodules is in agreement with Boddey et al. (2000), Okito et al. (2004), and Wanek and Arndt (2002). This enrichment has been related to nodule metabolism (Wanek and Arndt 2002). However, the fundamental isotope effects and discrimination processes leading to this non-uniform ^{15}N discrimination within legumes has not been well described, despite a model developed by Robinson et al. (1998) for ^{15}N signatures of nitrate-grown plants. The $\delta^{15}N$ values obtained in our study for nodules (+3.6 to +6.3‰), roots (−0.8 to +0.4‰), and shoots (−0.2 to −1.1‰) suggested wide deviations in $\delta^{15}N$ signatures among plant parts and are similar to those provided by Boddey et al. (2000). $\delta^{15}N$ values in the shoot (with mean *B* value of −0.7‰) was similar to values reported by Unkovich et al. (2008) (−0.3 to −0.6‰) and Fan et al. (2006) (−0.7‰) but higher than values reported by López-Bellido et al. (2010) (−1.7‰).

Regarding the $\delta^{15}N$ of the whole plant, some authors (Nguluu et al. 2001) indicated that the values tend to be close to the $\delta^{15}N$ of atmospheric N (0.0‰). However, it cannot be 0.0‰ due to isotopic fractionation associated with biochemical processes in N_2 fixation (Shearer and Kohl, 1986). López-Bellido et al. (2010) reported a mean value of −1.3‰ (uncorrected for seed N) and −1.5‰ (corrected for seed N) for the whole plant in faba beans. In our study, the uncorrected *B* value for the whole plant of six faba bean

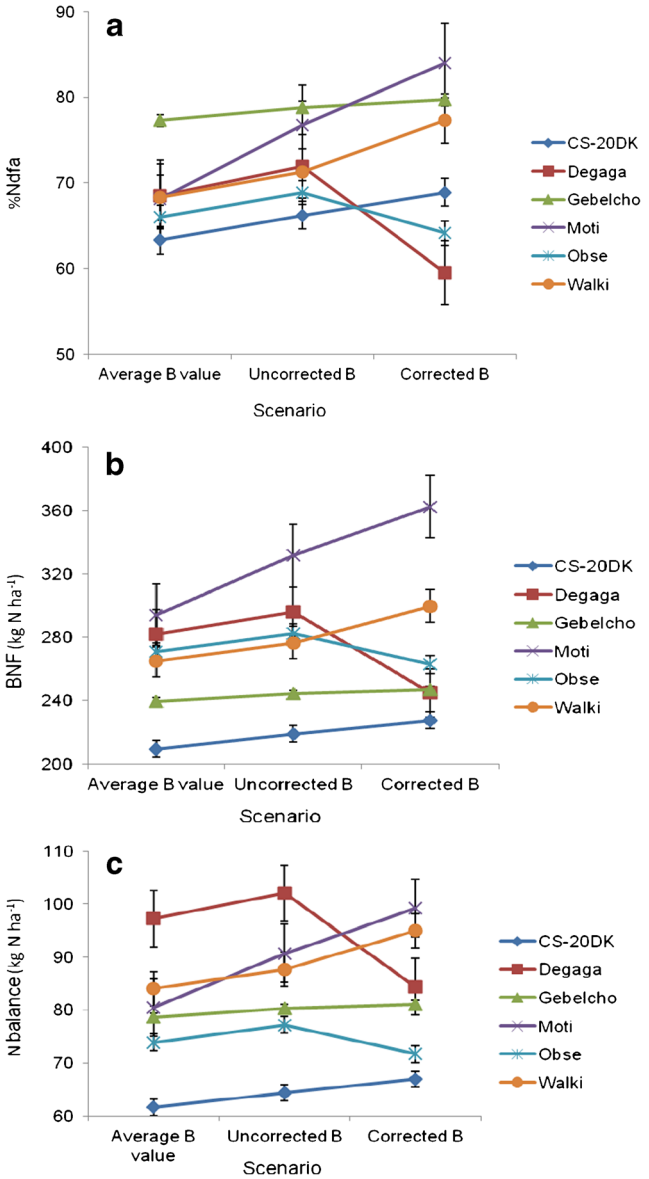

Fig. 1 Effect of *B* value method on **a** %Ndfa, **b** BNF, and **c** soil N balance of six faba bean varieties grown on farmer's field at Dedo, Southwest Ethiopia estimated via ^{15}N natural abundance using wheat as reference. *Average B value* is a *B* value of −0.85‰ obtained from literature, *Corrected B* and *Uncorrected B* are the observed whole plant *B* values corrected or not for the initial seed N, respectively. %Ndfa (**a**) of varieties was significantly different for *Corrected B* only ($LSD_{0.05}$=14.7; P<0.05) and BNF (**b**) for both *Corrected B* ($LSD_{0.05}$=81.2; P<0.05) and *Uncorrected B* ($LSD_{0.05}$=80.8; P<0.05). N balance (**c**) of varieties was not significantly different for three of the scenarios (P>0.05). The *error bars* denote standard errors

varieties ranged from −0.04 to −0.9‰, while the corrected *B* value ranged from +0.5 to −1.9‰. This shows a clear variety effect for the seed N corrected *B* value. The findings of this study are in contrast to López-Bellido et al. (2010) who showed a nonsignificant ^{15}N discrimination pattern between the different faba bean plant parts with negative $\delta^{15}N$ values for the nodules, root, and shoot. Nevertheless, our results are in agreement with the reports of Boddey et al. (2000) and Okito et al. (2004) that showed significant isotopic discrimination between the nodules and the rest of plant parts. Our results further suggest that whole plant corrected *B* values are the most correctly estimated *B* values and therefore are more appropriate for the application of the ^{15}N natural abundance technique to quantify BNF under field conditions.

Literature shows a wide range for %Ndfa and BNF by faba beans. Köpke and Nemecek (2010) reported %Ndfa up to 96 % and BNF values ranging from 15 to 648 kg N ha^{-1}. Unkovich and Pate (2000) have shown %Ndfa of 20 to 97 % and BNF of 12–330 kg N ha^{-1} for faba beans. Peoples et al. (1997) in a survey of the on-farm contribution of BNF to soybean and mung bean in Pakistan and Nepal found physiologically incongruous values of over 100 % for %Ndfa using a *B* value determined for Australian soybean and mung bean varieties. This indicates that utilization of *B* value determined for one variety at one site or geographic region may not be valid for all situations even if dealing with the same legume species. The wide range of variation in %Ndfa and BNF may not only be a result of variations in growing conditions and varieties used, but also due to the methodology used for BNF determination including *B* values. Peoples et al. (1997) and Unkovich et al. (1994) have indicated the likely magnitude of errors associated with the determination and application of *B* values for estimates of N_2 fixation using the natural abundance technique. As a general rule, Unkovich et al. (1994) pointed out that the errors associated with an inaccurate *B* value are likely to be high when %Ndfa estimates are greater than 85 % and hence survey reports of Peoples et al. (1997) suggested that this is the case. The use of an average *B* value from literature or a *B* value that does not account for seed N contribution could also be a factor for the wide variation and incongruous estimates of %Ndfa and BNF in the literature.

In order to validate our estimated *B* values, %Ndfa, BNF, and soil N balance were calculated using field experiment data on $\delta^{15}N_{reference\ plant}$ (wheat), $\delta^{15}N_{legume}$ of the same six faba beans (Eq. 3), and three scenarios for *B* values (corrected for seed N, uncorrected for seed N, and *B* value obtained from literature). The three *B* values gave different results as depicted in Fig. 1a–c. We observed that overlooking seed N contribution during calculation of *B* value or using an average *B* value from the literature resulted in overestimation of %Ndfa, BNF, and soil N balance by 15 % for Degaga or underestimation by 19 % for Moti. Moreover, it masked the real difference and rank that exists between faba bean varieties. It is possible to note that %Ndfa of Degaga, Moti, and Walki (68 % each) overlapped with each other with same rank at the average *B* value and that of Degaga and Walki (71 % each) overlapped at the uncorrected *B* value in the same manner. This overlap was unveiled however at the corrected *B* value with clear difference and ranking of varieties, Moti being the top (Ndfa=84 %) and Degaga lower (Ndfa=

59 %). A similar trend was noted also for BNF. This shows that *B* values have differential influences on the estimated quantities of %Ndfa, BNF, and soil N balance. This confirms our hypothesis that a single *B* value taken from the literature or *B* value determined without correcting for the initial seed N at sowing may result in inaccurate field estimates of BNF. To the best of our knowledge, we found only one published work in literature (López-Bellido et al. 2010) that estimated *B* values of faba beans taking into account the seed N contribution.

Conclusion

Quantifying biologically fixed N by legumes through the ^{15}N natural abundance techniques requires the determination of a so-called *B* value. The *B* values in our study differed significantly between varieties when the whole plant and seed N corrected value is used; which is the most correct and unbiased *B* value approach. If the seed N corrected whole plant *B* values are assessed against other approaches then it is noticed that BNF estimates could be biased by 34 % (15 % overestimation to 19 % underestimation) when not accounting for varietal differences and seed-N. Moreover, real differences in varieties are masked, leading to erroneous rankings among varieties. This has especial consequences for correctly assessing N balances which is crucial for agronomic efficiencies. For this reason, the total N and ^{15}N content of large seeded legumes (e.g., faba beans) at sowing should have to be considered in the *B* value determination to eliminate this error and minimize the distortion caused by the possible differences in N content of the seeds. Taking into account variety and seed N will result in more accurate %Ndfa and BNF under field conditions.

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