Symbiosis (2019) 79:213–219
https://doi.org/10.1007/s13199-019-00650-2

The $\delta^{15}N$ value of N_2 fixing actinorhizal plants and legumes grown with N_2 as the only nitrogen source

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Received: 13 December 2018 / Accepted: 24 September 2019 / Published online: 19 November 2019

Abstract
The aim of this study was to investigate the effects of different plant parts and the age of plants at harvest as well as N_2 fixing bacterial strains on the N concentration in symbiotic plant parts, especially on the $\delta^{15}N$ signature of the actinorhizal plants and legumes. The ^{15}N natural abundance method was used. Two actinorhizal plants were studied: *Alnus incana* (L.) infected with the *Frankia* strains ArI3 or "lsF" (local source of *Frankia*) and *Hippophaë rhamnoides* (L.) infected with the *Frankia* strains T1 or E15b. Two legume species were studied: *Hedysarum coronarium* (L.), infected with a soil suspension, and *Robinia pseudoacacia* (L.), infected with a crushed nodule suspension. It was particularly interesting that in *A. incana*, the two *Frankia* strains affected not only N concentration and $\delta^{15}N$ signature of leaves and roots, but also had an impact on plant growth at first harvest. In *Hippophaë rhamnoides* plants inoculated with the *Frankia* strains T1 and E15b, N concentrations and $\delta^{15}N$ values did not differ at any harvest time. However, plants nodulated by the *Frankia* strain T1 showed a higher nitrogen fixation rate and higher plant dry matter at all harvesting times. Based on our results for the quantification of N_2 fixation with the "B" value, that is the $\delta^{15}N$ value of the N_2 fixing plants relying only on N_2 fixation, plant parts, ages and strains should be carefully considered.

Keywords Actinorhizal plants · B value · Leguminous plant · ^{15}N natural abundance · $\delta^{15}N$ · N_2 fixation

1 Introduction

The ^{15}N natural abundance (NA) method is an important technique to measure N_2 fixation, N uptake and N transfer in the soil-plant system. This method is used in plant physiology and ecology (Handley and Raven 1992; Högberg 1997; Robinson 2001), but it can also be applied at the farm level as well as at the landscape level (Unkovich and Pate 2000). The ^{15}N NA method has become more commonly used to quantify N_2 fixation (Okito et al. 2004).

However, to adequately and correctly quantify N_2 fixation, we need to know the $\delta^{15}N$ of soil N; if a legume or actinorhizal plant was totally reliant on soil mineral and organic N, its $\delta^{15}N$ should resemble that of the soil mineral N taken up (Unkovich et al. 2008). Hence, the $\delta^{15}N$ of a legume or actinorhizal plant using a combination of atmospheric N_2, soil mineral and organic N for growth should range between the values of the two possible N sources: soil and atmospheric N_2 (Andrews et al. 2011). Usually, to calculate the %Ndfa (percentage of N derived from the atmospheric N_2), non-N_2-fixing 'reference' plants are used to obtain the $\delta^{15}N$ of soil mineral and organic N and the N_2 fixation by the legume or actinorhizal plant can be calculated using the following formula (Eq. 1):

$$\%\mathrm{Ndfa} = \left(\frac{\delta^{15}\mathrm{N\ of\ reference plant} - \delta^{15}\mathrm{N\ of\ N_2\ fixing plant}}{\delta^{15}\mathrm{N\ of\ reference\ plant} - \delta^{15}\mathrm{N\ of\ N_2}}\right) \times 100$$

In ^{15}N enrichment studies, isotopic discrimination during N_2 fixation is generally considered to be zero; however, isotopic discrimination should be considered when using ^{15}N natural abundance (Unkovich et al. 2008). The natural abundance of atmospheric N_2 will have a $\delta^{15}N$ of 0‰, by definition. The above equation assumes that N_2 fixing plants relying only on N_2 fixation do not have any isotope discrimination, hence they have a $\delta^{15}N$ similar to the $\delta^{15}N$ of atmospheric N_2. However, N_2 fixing plants have a certain discrimination between ^{14}N and ^{15}N, even when relying only on N_2 fixation. Hence, to correctly quantify N_2 fixation, the ^{15}N NA method

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demands a constant, namely the "B" value, which is the $\delta^{15}N$ value of the N_2 fixing plants relying only on N_2 fixation. Therefore, the above Eq. 1, including the B value, can be written as follows (Eq. 2):

$$\%Ndfa = (\delta^{15}N\ of\ reference\ plant - \delta^{15}N\ of\ N_2\ fixing\ plant / \delta^{15}N\ of\ reference\ plant - B) \times 100$$

The use of the equation including the B value will result in a more accurate N_2 fixation contribution and hence a more precise calculation of the percentage of N derived from the atmosphere. Several studies have emphasised the importance of measuring B values (Kurdali 2009; Andrews et al. 2011; Chalk et al. 2016). Previous studies on legumes have shown that the B value can vary with plant species and age (Carlsson et al. 2006; Unkovich et al. 1993), bacterial strains, and growing conditions (Carlsson et al. 2006; Ledgard 1989; Unkovich and Pate 2000). Much less is known about actinorhizal plants; however, Domenach et al. (1989) have investigated $\delta^{15}N$ in *Alnus incana* and *A. glutinosa* inoculated with four different *Frankia* strains. Tjepkema et al. (2000) studied differences in $\delta^{15}N$ values among parts of the actinorhizal plants *Alnus glutinosa* (L.) Gaertn., *Casuarina cunninghamiana* Miq., *Datisca glomerata* (Presl.) Baill. and *Myrica gale* L grown with N-free solution. However, plants were inoculated mainly with crushed nodule suspensions without testing different strains (Tjepkema et al. 2000).

The group of actinorhizal plants includes eight families of mainly woody plants that are infected with N_2-fixing actinomycetes of the genus *Frankia* (Huss-Danell 1997). In the present work, we studied the actinorhizal plants *Alnus incana* L. Moench (grey alder) and *Hippophaë rhamnoides* L. (sea buckthorn). *H. rhamnoides* is a multipurpose plant that has been exploited in East Europe and Asia for several years (Li and Schroeder 1996; Zielinska and Nowak 2017). *Alnus incana* is a fast-growing tree that thrives in alluvial nutrient-poor soils, and it is used to stabilize damaged forest topsoils (Flores Fernández et al. 2019). These two actinorhizal plants have the following different *Frankia* infection pathways: via root hairs in *A. incana* and via intercellular penetration in *H. rhamnoides* (Berry and Torrey 1979; Gentili et al. 2006). Generally, each of actinorhizal plant species has a different infection pathway (Pawlowski et al. 2011), and is infected by a specific *Frankia* strain; however some *Frankia* strains such as UFI 132715 are able to form nodules in actinorhizal plant species having different infections pathways (Lumini and Bosco 1996).

The legumes studied in the present work were *Hedysarum coronarium* (Italian "sainfoin" or "sulla") infected by *Rhizobium sullae* (de Diego-Diaz et al. 2018) and *Robinia pseudoacacia* (black locust) infected by *Mesorhizobium* and *Rhizobium* (Andrews and Andrews 2017). *Hedysarum coronarium* is a good forage plant for Mediterranean regions, and it can grow in arid environments and clay soils (Chisci et al. 2001), while *R. pseudoacacia* is a widespread tree used in agroforestry and land reclamation (Groninger et al. 1997).

The main aim of this work was to determine the B values in the four plant species mentioned above at different plant ages and with different *Frankia* strains for the actinorhizal plants and with crushed nodule or soil suspension for the legumes.

2 Materials and methods

2.1 Plant cultivation

Alnus incana seeds were obtained from a clone (Huss-Danell 1991), while *H. rhamnoides* from Hungary and *R. pseudoacacia* from Poland were purchased from Sandeman Seeds (UK). *Hedysarum coronarium* seeds were collected in central Italy (Santa Sofia, Forlì, Italy). Seeds of *H. rhamnoides* and *R. pseudoacacia* were surface-sterilised as previously described by Gentili and Huss-Danell (2002). *Alnus incana* seeds were washed in water containing a few drops of detergent, immersed in hydrogen peroxide (30%*v*/v) for 1 h, shaken a few times manually, and then carefully rinsed with sterile water. *Hedysarum coronarium* seeds were not surface-sterilised. *Hippophaë rhamnoides* and *R. pseudoacacia* were sown in Petri dishes containing perlite, *He. coronarium* was sown in 8 × 8 cm plastic pots containing perlite and *A. incana* was sown in Magenta jars (Sigma, St Louis, Mo, USA) containing glass beads. All seeds were watered with modified N-free Evans solution (Huss-Danell 1978) diluted to 1/10 of full strength. The perlite, glass beads and nutrient solution were sterile. The seedlings were transferred to larger pots twice for the legumes *He. coronarium* and *R. pseudoacacia* or three times for *A. incana* and *H. rhamnoides* (Table 1).

During the entire experiment, plants were watered with N-free Evans solution diluted to 1/10 of full strength. During the whole growth period (end of February to beginning of October), the plants were kept in a greenhouse in Umeå, Sweden (63°45'N), with 17 h of supplemental light set to about 25 °C and 7 h of darkness set to about 15 °C. Philips HPI/T 400 W lamps were used. Relative air humidity was set to 40%.

2.2 Inoculation and harvest of plants

Some of the *A. incana* seedlings were inoculated with the *Frankia* strain ArI3 (Berry and Torrey 1979) grown on P + N medium (Van Ghelue et al. 1997). The *Frankia* cells were collected by centrifugation at 7000 g for 10 min at 22 °C, and the supernatant was discarded. The cell pellet was washed twice by centrifugation (as above-mentioned), using N-free Evans solution diluted to 1/10 of full strength. Each seedling was inoculated with approximately 6 mg (wet weight) of *Frankia* cells added into the Magenta jars. Another part of

Table 1 Plants age at different experimental phases. WAS stands for weeks after sowing

Plant	Inoculation WAS	Transfer WAS	Harvest WAS	Harvest number
A. incana	2	4 to 6 × 6 cm pot		
		11 to 10 × 10 cm pot	18.5	1
		19 to 11x11x12 cm pot	32	2
He. coronarium	2	1.5 to 10 × 10 cm pot	14	1
		10.5 to 11x11x12 cm pot	23	2
H. rhamnoides	3.5	3 to 6 × 6 cm pot		
	8	6 to 8 × 8 cm pot	18	1
		19 to11x11x12 cm pot	31	2
R. pseudoacacia	3.5	2 to 8 × 8 cm pot	12.5	1
		13 to 11x11x12 cm pot	26	2

the *A. incana* seedlings was inoculated with the crushed root nodule suspension from *A. incana*, the local source of *Frankia* (lsF) (Huss-Danell 1991).

Hippophaë rhamnoides seedlings were inoculated with the strains E15b (Lumini and Bosco 1996) and T1 (Chaia 1998), both of which were grown in K-medium with casein hydrolysate and yeast extract as N source (Lumini et al. 1996). Both strains were harvested as described above for the *Frankia* strain, ArI3. However, each *H. rhamnoides* seedling was inoculated with 30 mg (wet weight) of *Frankia* cells the first time and 16 mg of cells the second time (Table 1).

Hedysarum coronarium was inoculated with a soil suspension from an Italian area where this legume grows naturally, while *R. pseudoacacia* was inoculated with a crushed root nodule suspension from plants of *R. pseudoacacia* growing in an Italian nursery garden. For inoculation times, see Table 1. All plant species were harvested twice at the different plant ages indicated in Table 1. Once harvested, the plants were immediately dried at 60 °C for 48 h at the first harvest time and for 72 h at the second harvest time. This difference between the drying times was because at the second harvest, plants were much larger and more lignified. The dried shoots and the dried roots, including the nodules, were milled and analysed for N and ^{15}N as previously described (Gentili 2006). All seeds were dried at 60 °C for 24 h, milled and analysed as above-mentioned.

2.3 Nitrogenase activity

Acetylene-reducing activity (ARA) was measured in five randomly chosen *H. rhamnoides* plants for the two *Frankia* strains at 21.5 weeks after sowing. The measurements were done three times a week, with one day in between measurements. Each intact individual plant was placed in a gastight chamber (Huss-Danell 1978) during exposure to 10% (*v*/v) C_2H_2 in air. During these incubations, the plants were kept in the greenhouse. The formation of C_2H_4 was measured by gas chromatography as described previously (Valverde et al. 2000).

2.4 Statistical method

One-way ANOVA was used to identify statistically significant differences. Statistical calculations were performed using the software package Minitab (Minitab Inc., State College Pennsylvania, PA, USA, 2000), with $P < 0.05$ as the significance level.

3 Results

The legume seeds had a higher nitrogen concentration than the actinorhizal seeds (Table 2). Only *Robinia pseudoacacia* seeds had a positive $\delta^{15}N$ value when compared to the other seeds analysed (Table 2). However, regarding the weight of a single seed in comparison with the plant dry matter and leaf plus root dry matter (Tables 2 and 3), the influence of the N and $\delta^{15}N$ of the seeds had a negligible impact on the N and $\delta^{15}N$ of the plants. There was a great difference in N concentration among the plants analysed, ranging from 0.94% in roots of *Alnus incana* to 3.39% in leaves of *Hippophae rhamnoides*. However, these differences were not only due to differences in plant species, but also there were significant differences among plant parts, time of harvest

Table 2 Dry seeds mass, nitrogen concentration (% of seed dry mass) and $\delta^{15}N$ values in dry seeds of the studied plant species. The N and $\delta^{15}N$ values are the mean ± SE of 5 replicates

Plant	Dry weight of 100 seeds (g)	N %	$\delta^{15}N$
A. incana	0.05	3.26 ± 0.04	−0.35 ± 0.09
H. rhamnoides	0.89	4.1 ± 0.1	−0.24 ± 0.04
He. coronarium	0.46	7.72 ± 0.11	−0.33 ± 0.13
R. pseudoacacia	2	6.6 ± 0.08	4.22 ± 0.02

Table 3 Plant dry matter (g) and leaf+root (g) of the two actinorhizal plant species inoculated with two different *Frankia* strains and of the two leguminous plants inoculated. All the plants were harvested at two different time. Values are the mean ± SE of 5 replicates. na stands for not available

Plant	Plant DM (g) 1st harvest	Plant DM (g) 2nd harvest	Leaf+root DM (g) 1st harvest	Leaf+root DM (g) 2nd harvest
A. incana x ArI3			2.46 ± 0.4	13.34 ± 1.47
A. incana x lsF			1.73 ± 0.18	13.02 ± 1.81
H. rhamnoides x E15b	2.2 ± 0.27	10.49 ± 0.24		
H. rhamnoides x T1	3.21 ± 0.14	17.59 ± 1.41		
He. coronarium	na	10.08 ± 0.46		
R. pseudoacacia			1.97 ± 0.29	24.31 ± 2.98

and *Frankia* strains (Table 4). In particular, *A. incana* inoculated with *Frankia* strain ArI3 had a significantly higher N concentration in both leaves and roots at first harvest than at second harvest (Table 4). However, *A. incana* inoculated with *Frankia* strain lsF had a significantly higher N concentration in leaves at second harvest than at first harvest (Table 4).

In *H. rhamnoides*, there were no effects on N concentration due to harvest time or strains used (Table 4). Even in *Hedysarum coronarium*, harvest time had no effect on leaf N concentrations (Table 4). However, in *R. pseudoacacia*, roots had higher N concentrations than leaves at both harvest times, while harvest time did not have any clear effect on N concentrations (Table 4).

The $\delta^{15}N$ values of the N_2 fixing plants relying only on N_2 fixation or B values are shown in Table 5. The $\delta^{15}N$ ranged from −2.62 in leaves of *A. incana* inoculated with the "lsF" to 0.77 in roots of *R. pseudoacacia* (Table 5). The $\delta^{15}N$ was negative in the two actinorhizal plants studied with all the *Frankia* strains, while the two legume species studied showed negative (*He. coronarium*) and both negative and positive $\delta^{15}N$ (*R. pseudoacacia*). On the one hand, in *A. incana* inoculated with the ArI3 strain, the only relevant difference among plant parts and time of harvest was a slight, but significantly more negative, $\delta^{15}N$ value in the roots at the second harvest (Table 5). On the other hand, *A. incana* inoculated with the "lsF" did not show any relevant differences among plant parts and times of harvest (Table 5). *Hippophaë rhamnoides* was even more interesting because, although the two strains did not affect N concentrations and $\delta^{15}N$ signatures among plant parts and time of harvest, they greatly affected plant biomass (compare Tables 3, 4 and 5) and acetylene-reducing activity (Fig. 1). Under these experimental conditions, the strain T1 fixed N_2 much more efficiently than the strain E15b.

Interestingly, in *H. rhamnoides*, there were clear differences in $\delta^{15}N$ values due to the time of harvest and the plant parts; roots had a more negative $\delta^{15}N$ than leaves. However, *Frankia* strains did not significantly affect the $\delta^{15}N$ signature of *H. rhamnoides* (Table 5).

He. coronarium showed a significant difference between $\delta^{15}N$ values of leaves at different harvesting times and between leaves and roots (Table 5). In *He. coronarium*, N concentrations and $\delta^{15}N$ values had a different trend (compare Tables 4 and 5).

R. pseudoacacia $\delta^{15}N$ signatures followed a trend similar to that of N concentrations (Tables 4 and 5). In this leguminous species, roots had $\delta^{15}N$ signatures higher than leaves at both harvesting times, while harvesting time did not have any clear effect on N concentrations (Table 4).

4 Discussion

It is particularly interesting that in *A. incana*, the two *Frankia* strains affected not only N concentration and $\delta^{15}N$ signatures

Table 4 Nitrogen concentration in leaves and in roots with nodules at different harvest time and with different *Frankia* strains

Plant	*Inoculum*	Leaf N% First harvest	Leaf N% Second harvest	Root N% First harvest	Root N% Second harvest
A. incana	ArI3	2.1 ± 0.3 **aA**	1.15 ± 0.05 **bA**	1.42 ± 0.05 **aA**	0.94 ± 0.03 **cA**
	ls*F*	1.24 ± 0.07 **aB**	1.78 ± 0.05 **bB**	1.34 ± 0.04 **aA**	1.27 ± 0.03 **aB**
H. rhamnoides	E15b	3.24 ± 0.09 **aA**	3.21 ± 0.14 **aA**	3.02 ± 0.24 **aA**	2.75 ± 0.14 **aA**
	T1	3.39 ± 0.16 **aA**	3.12 ± 0.15 **aA**	3.27 ± 0.19 **aA**	2.96 ± 0.14 **aA**
He. coronarium	Soil suspension	2.18 ± 0.11**a**	1.96 ± 0.06 **a**	na	2.06 ± 0.17 **a**
R. pseudoacacia	Crushed nodules	2.17 ± 0.34 **a**	2.27 ± 0.1 **a**	2.88 ± 0.2 **b**	2.71 ± 0.09 **b**

Different letters mean differences at $p \leq 0.05$ as significance level. Lower case letters represent differences between values of different columns but of the same line. Upper case letters represent differences between *Frankia* strains infecting the same actinorhizal species and compared values of different lines but of the same column. Values are the mean ± SE of 5 replicates. na stands for not available

Table 5 $\delta^{15}N$ values (B values) of leaves and roots at different harvest times and in plants with different *Frankia* strains

Plant	*Frankia* strain	Leaf $\delta^{15}N$ First harvest	Leaf $\delta^{15}N$ Second harvest	Root $\delta^{15}N$ First harvest	Root $\delta^{15}N$ Second harvest
A. incana	ArI3	−2.18 ± 0.11 **aA**	−2.14 ± 0.05 **aA**	−2.08 ± 0.1 **aA**	−2.43 ± 0.06 **bA**
	ls*F*	−2.54 ± 0.05 **aB**	−2.62 ± 0.04 **aB**	−2.41 ± 0.08**aB**	−2.43 ± 0.12 **aA**
H. rhamnoides	E15b	−1.75 ± 0.04 **aA**	−1.28 ± 0.06 **bA**	−2.22 ± 0.12**cA**	−2.57 ± 0.2 **cA**
	T1	−1.70 ± 0.07 **aA**	−1.04 ± 0.11 **bA**	−2.49 ± 0.06**cA**	−2.40 ± 0.06 **cA**
He. coronarium	Soil suspension	−0.49 ± 0,09 **a**	−1.31 ± 0.26 **b**	na	−0.54 ± 0.1 **ac**
R. pseudoacacia	Crushed nodule	−1.31 ± 0.16 **a**	−1 ± 0.12 **a**	0.77 ± 0.39 **b**	0.03 ± 0.21 **b**

Different letters mean differences at $p \leq 0.05$ as significance level. Lower case letters represent differences between values of different columns but belonging to the same line. Upper case letters represent differences between *Frankia* strains infecting the same actinorhizal species and compared values of different lines but of the same column. Values are the mean ± SE of 5 replicates. na stands for not available

of leaves and roots, but also had an impact on plant growth at first harvest (compare Tables 3, 4 and 5). This could be due to a faster root colonisation and nodule formation by the strain ArI3 compared to the lsF. At the second harvest, the plants inoculated with the lsF had similar dry matter values compared to the plants inoculated with the ArI3 strain (Table 3) and a higher nitrogen concentration in both leaves and roots (Table 4). This could be due to a higher N_2 fixation of the plants inoculated with lsF than plants inoculated with ArI3. Remarkably, plants infected with the lsF strain had the $\delta^{15}N$ value of the leaves at second harvest about 0.5 δ units more negative than plants inoculated with the ArI3 *Frankia* strain. A previous study had shown that the choice of the *Rhizobium* strain significantly influenced the B values in shoots of *Trifolium hybridum* and *Trifolium repens* and the N contents in all *Trifolium* species tested (Carlsson et al. 2006). Furthermore, in legumes, considerable variation was found in $\delta^{15}N$ values of plant herbage (−4.5 to +0.8); the extent of isotopic discrimination was dependent on both the host plant and the infecting rhizobial strain (Steele et al. 1983). In a previous study, *A. incana* had a leaf $\delta^{15}N$ of approximately −1.4, hence less negative than in the present study. However, the inoculating strain and the age of the plants were not specified (Domenach et al. 1989). Working on actinorhizal plants, Tjepkema et al. (2000) found that whole plant $\delta^{15}N$ for the four actinorhizal plants examined ranged from −1.41 to −1.90‰. However, they harvested the plants when they reached a dry mass of between 0.5 and 4 g, which was similar to our results from the first harvest, but significantly smaller than our results from the second harvest, where actinorhizal plant dry matter values ranged from 10.5 to 17.6 g.

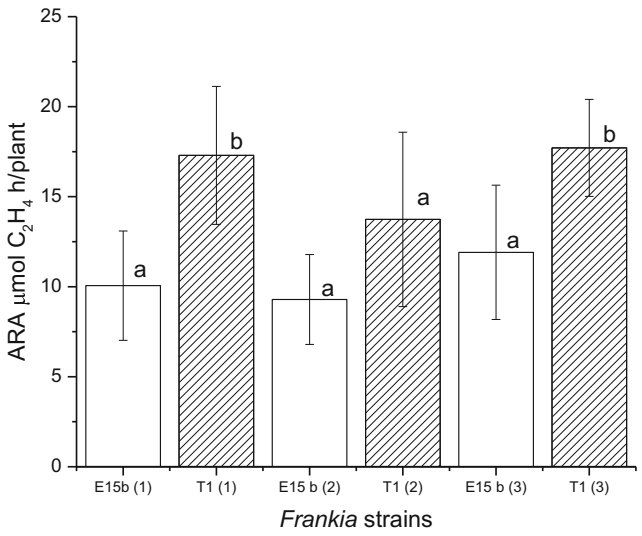

Fig. 1 Acetylene reducing activity in five randomly chosen *H. rhamnoides* plants measured at three different date during one week. Empty bars show ARA for plants infected by the *Frankia* strain E15 b while bars with grid show ARA for plants infected by the T1 *Frankia* strain. Bars stand for mean ± SD of five replicates. Different letters on the top of the bars represent statistically differences among plants infected by different strains at the same measurement date at $p \leq 0.05$ as significance level

Hippophaë rhamnoides plants inoculated with the T1 *Frankia* strain showed a greater biomass and higher ARA than plants inoculated with the E15b strain (Table 3, Fig. 1). Consequently, under these experimental conditions, the T1 strain had a significantly higher efficiency to fix N_2 than the E15b strain. However, for both N concentrations and $\delta^{15}N$ values, plants infected with the E15b and T1 strain, respectively, did not differ at any harvest time (Tables 4 and 5). This means that although T1 fixed N_2 more efficiently than E15b, as also confirmed by the ARA measurements (Fig. 1), which resulted in larger plants at both harvesting times, it had the same ^{15}N discrimination as the less productive E15b. On the one hand, concerning N concentrations in *H. rhamnoides*, there was no significant difference among *Frankia* strains, time of harvest and plant parts. On the other hand, in terms of $\delta^{15}N$, there were differences at time of harvest in leaves and among plant parts, with more negative values for nodulated roots than for leaves. The fact that leaves showed differences over time is extremely relevant, because leaves are generally used to measure N_2 fixation for both actinorhizal plants and legumes. Hence, it would be important in future studies to follow $\delta^{15}N$ values of actinorhizal and legume woody plants grown with N_2 as the only nitrogen source during several growing seasons.

In the present study, leguminous plants showed a higher $\delta^{15}N$ of roots compared to leaves, as had been previously shown (Unkovich et al. 2008). In contrast, the two actinorhizal plants inoculated with four different *Frankia strains* generally had more negative $\delta^{15}N$ values of nodulated roots than leaves (Table 5). It seems that actinorhizal plants have a great variability of ^{15}N fractionation among plant parts, as had also been shown by Tjepkema et al. (2000), where some plants grown on N_2-free medium had more positive $\delta^{15}N$ values for roots than for leaves, while the opposite was true for other actinorhizal plant species.

In conclusion, for the calculation of the %Ndfa, the $\delta^{15}N$ value of N_2 fixing plants relying only on N_2 as nitrogen source (B value, see above Eq. 2) should be used instead of the $\delta^{15}N$ value of atmospheric N_2. This will produce more accurate estimations of %Ndfa. In fact, the use of $\delta^{15}N$ of atmospheric N_2 (0‰) as the B value could result in an underestimation or overestimation of the %Ndfa if the real B value is positive or negative, respectively. Based on our results, the use of the B value of different plant parts, plant age and infecting strains should be carefully considered.

Acknowledgements Open access funding provided by Swedish University of Agricultural Sciences. We thank Anna Maria Ericsson and Håkan Wallmark for valuable technical help. We greatly appreciate the financial support by the Swedish Council for Forestry and Agricultural Research and EC FAIRBM- 972009.

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