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NITROGEN STABLE ISOTOPE COMPOSITION OF LEAVES AND ROOTS OF PLANTS GROWING IN A FOREST AND A MEADOW

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In controlled N-nutrition experiments, differences in $\delta^{15}N$ composition of leaves and roots are regularly found. In this paper we report results from a survey of nitrogen stable isotope signatures of leaves and roots of 16 plant species growing under natural conditions in a meadow and a forest understorey, which differed in nitrate and ammonium availability. Significant differences between leaf and root were observed. The range of $\Delta^{15}N$ [leaf–root] values was −0.97 to +0.86‰, small compared to published values from controlled N-nutrition experiments, but almost as large as the range of leaf $\delta^{15}N$ values (−1.04 to +1.08‰). Forbs showed the largest differences between leaves and roots and showed a significant difference with respect to habitat. Grasses and legumes did not show significant differences in $\Delta^{15}N$ [leaf–root] between the two habitats. Care must be taken when using leaf $\delta^{15}N$ values as representative for whole-plant ^{15}N composition in these two habitats.

Keywords: Ammonium; Forbs; Grasses; Legumes; Natural abundance; Nitrate; Nitrogen

INTRODUCTION

Stable isotope studies can provide powerful clues and insights into ecological and physiological processes in ecosystems and vegetation [1–3]. Ecosystems, soils and plant species often differ markedly in nitrogen isotope composition ($\delta^{15}N$) [1–5]. Precipitation [12] and substrate development [4, 13, 14] are put forward as an explanation for geographically large-scale gradients in nitrogen isotope composition. Hypotheses have only recently been proposed concerning the role of mycorrhizae [6–9], nitrate reductase and nitrogen efflux [10, 11] and other discriminating processes [1, 2] in determining a plant's nitrogen isotope composition. With increasing accuracies of nitrogen isotope ratio measurements, the prospect to better understand $\delta^{15}N$ variation, and its relationship with ecosystem processes, increases.

Enzymes of the nitrogen assimilation pathway, such as nitrate reductase and glutamine synthetase discriminate against the heavier nitrogen isotope [10, 18, 26]. Nitrate reductase

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activity (NRA) in plants is localized in the cytoplasm of root and leaf cells, and its relative distribution over leaves and roots is variable [27, 28], plant-species dependent [27], influenced by the availability of nitrate and ammonium in the root medium [29, 30] and by energy and carbohydrate status of the plant [31]. Mariotti *et al.* [32] were the first to suggest that the distribution of NRA over leaves and roots can have consequences for the nitrogen isotopic composition of these organs when plants are grown with nitrate. This was further confirmed by several authors [10, 18, 20], and modeled by Robinson *et al.* [11]. NRA in the root tissues consumes preferentially the lighter nitrogen isotope, while the heavier isotope accumulates as unused nitrate stored in the vacuoles, is effluxed from the root or transported to the leaves where it is subsequently reduced and used. As a consequence, leaves are ^{15}N-enriched compared to the root (Δ^{15}N [leaf–root] > 0) [11].

An analogous situation exists for ammonium assimilation. Glutamine synthetase fractionates against the heavier isotope [19, 33]. However, since most of the ammonium taken up is assimilated immediately upon entry in the root cell [20], plant tissue δ^{15}N does not vary as much as for nitrate-grown plants [19, 20]. Volatilization of apoplastic ammonium [34], causing enrichment of the remaining ammonium pool, can potentially induce in δ^{15}N variation between tissues. However, it is not likely that this is very important under natural conditions where soil and plant nitrate and ammonium concentrations are usually low [5, but see 35]. Additional processing of nitrogen compounds, such as transamination and deamination, leaching and volatilization of organic nitrogen compounds may also affect the nitrogen isotope composition of plant tissues [11].

In actively nitrogen-fixing plants large enrichments are observed in nodules, [10] associated with the bacteroid's nitrogen metabolism [17, 36]. However, enrichment of the nodules is not always found and may be species dependent [17, 36].

In many studies, leaf δ^{15}N-nitrogen is used as a proxy for whole-plant δ^{15}N [9, 13, 15, 16]. For practical reasons, it is often difficult to access root material and impossible to sample whole-plant δ^{15}N in field experiments. Differences between δ^{15}N of leaves (or whole shoots) and roots are reported in controlled environment studies [9, 17–23]. Similar studies in the field are more rare, but seem to confirm the existence of potentially large within-plant differences [8, 24, 25]. Although it was expected that the tissues (outside the nodules) are homogenous with respect to ^{15}N composition [17], Shearer *et al.* [24] observed that leaf tissue nitrogen was enriched with 3.7‰ compared to root samples for field-grown *Prosopis glandulosa.*

The presence of only a few studies on tissue δ^{15}N variability under natural conditions prompted us to undertake this study. We sampled plants from an open meadow and a nearby forest understorey, classified plant species in three functional groups (legumes, forbs (broad-leaved excluding legumes) and grasses), and related Δ^{15}N [leaf–root] to functional group and habitat nitrogen availability. This research is important because fractionation during N-reduction, assimilation and further processing may cause differences within plants, which potentially makes studies that use δ^{15}N of leaves as a proxy for δ^{15}N of the whole plant ambiguous.

MATERIALS AND METHODS

Two plots were selected not more than 200 m apart, at 2655 m above sea level (mixed conifer zone) on the San Francisco Peaks near Flagstaff, Arizona. One plot of 50 × 20 m was located in a meadow and a similarly sized plot was located in a forest under a canopy of aspen and spruce, with an estimated 20–40% direct sunlight. The main plots were divided into ten 10 × 10 m^2 subplots. Within each subplot, randomly assigned coordinates were used to select 1 m^2, within which all species that could be identified with certainty were harvested (Tab. I,

TABLE I δ^{15}N of Leaves and Roots (±se), and Number of Observations for Different Species Sorted for Habitat and Functional Type.

Habitat	*Functional type*	*Species*	*n*	*δ^{15}N leaves (‰)*	*δ^{15}N roots (‰)*
Meadow	Forbs	*Achillea lanulosa**	8	0.20 (0.49)	0.05 (0.31)
		Antennaria rosulata	10	0.22 (0.25)	0.77 (0.26)
		Cirsium wheelerii	7	−0.04 (0.12)	0.57 (0.19)
		Geum trifolium	10	0.54 (0.08)	0.38 (0.18)
		Potentilla hippeana	10	0.38 (0.26)	0.37 (0.20)
	Grasses	*Elymus elymoides*	9	0.54 (0.14)	1.51 (0.21)
		Festuca arizonica	10	0.35 (0.24)	0.99 (0.21)
		*Poa pratensis**	7	0.95 (0.27)	0.42 (0.16)
	Legumes	*Oxytropis lambertii*	6	−1.04 (0.11)	−0.70 (0.27)
		*Vicia americana**	9	−0.20 (0.07)	−0.63 (0.13)
Forest	Forbs	*Achillea lanulosa**	7	0.39 (0.26)	−0.38 (0.26)
		Geranium richardsonii	9	1.08 (0.19)	0.21 (0.24)
		Pseudocymopteris montanus	8	0.66 (0.37)	0.43 (0.23)
		Taraxacum officinalis	7	0.78 (0.46)	0.24 (0.40)
		Thalictrum fendleri	7	0.22 (0.27)	−0.25 (0.29)
	Grasses	*Bromus anomalus*	7	0.93 (0.30)	1.16 (0.32)
		*Poa pratensis**	8	0.23 (0.41)	−0.08 (0.41)
	Legumes	*Lathyrus sp.*	10	−0.27 (0.08)	−0.28 (0.10)
		*Vicia americana**	10	−0.26 (0.19)	−0.25 (0.14)

*Species present in meadow and forest.

species names according to Kearney *et al.* [37]). The harvest consisted of removing the plant plus the top 10 cm soil, which were then stored in an icebox until processed in the lab the same day. Roots were selected and cleaned in demineralized water, making sure that they were visibly attached to the plant from which the leaves were sampled. Senescing leaves were excluded. Legume roots did show nodulation, but nodules were excluded from the root samples. Ten grams of soil, associated with the plant sampled, were sieved through a $2 \times 2\,mm^2$ mesh and extracted in 50 ml 1 M KCl solution by shaking for 24 hours at 4 °C. Gravimetric soil moisture content was determined from the same sieved soil. Leaves and roots were dried for at least 24 h at 70 °C, while soil was dried at 105 °C. Collection of plant and soil took place from 31 July 2001 until 14 August 2001.

Soil ammonium and nitrate concentrations were determined using an autoanalyzer (Lachat Quickchem FIA + 8000 autoanalyzer, Lachat Instruments, Milwaukee, WI, USA). Nitrate was determined by diazotizing with sulfanilamide after reduction to nitrite, followed by complexion with N- (1-naphthyl) ethylenediamine dihydrochloride. Ammonium was determined with the alkaline phenol reaction.

Stable isotope ratios (δ^{15}N) and N-content of tissue N were measured via continuous flow using a NC 2100 Elemental Analyzer interfaced with a Finnigan Delta Plus XL isotope ratio mass spectrometer at The Colorado Plateau Stable Isotope Laboratory (http://jan.ucc.nau.edu/∼bah/cpsil.html). The results of the nitrogen isotope composition were expressed in standard notation (δ^{15}N) in parts per thousand (‰) relative to atmospheric N_2, where $\delta^{15}N = 1000*[(R_{sample}/R_{atmosphere}) - 1]$, and R is the molar ratio $^{15}N/^{14}N$. Precisions were better than 0.2‰ for δ^{15}N and 0.1% for nitrogen content. The difference between the δ^{15}N of the leaf and the δ^{15}N of the root is denoted as Δ^{15}N [leaf–root].

Statistical analysis was a 2-factor ANOVA: functional groups (legumes, grasses and forbs) and habitat (forest and meadow). There were three species common to both habitats, a forb: *Achillea lanulosa*, a grass: *Poa pratensis* and a legume: *Vicia americana*. A separate analysis was done on these three species.

TABLE II Moisture Content, Nitrate, Ammonium and Dissolved Inorganic N (DIN) Concentration Per Dry Weight of Soil in a Meadow and Forest (Means ± se, Number of Observations is 78 and 68 for Respectively Meadow and Forest).

Ecosystem	*Moisture* ($g\,g^{-1}$)	*se*	*Nitrate-N* ($mg\,kg^{-1}$)	*se*	*Ammonium-N* ($mg\,kg^{-1}$)	*se*	*DIN* ($mg\,kg^{-1}$)	*se*
Meadow	21.2	0.34	1.02	0.03	3.70	0.21	4.72	0.21
Forest	36.7	0.67	1.65	0.10	12.72	0.77	14.36	0.85

RESULTS

Forest soil contained more nitrate ($P < 0.01$), ammonium ($P < 0.01$), and dissolved inorganic N ($NO_3^- + NH_4^+$) ($P < 0.01$) and had higher gravimetric moisture content ($P < 0.01$) than meadow soil (Tab. II). These differences were also observed when only sites of the three species, common to both habitats, were compared (Fig. 1, moisture content not shown). Leaf ($P < 0.01$) and root ($P < 0.01$) nitrogen content was higher for plants in the forest than in the meadow (Tab. III). The three species, common to both habitats, also exhibited higher nitrogen content in leaf ($P < 0.01$) and root dry weight (NS, $P = 0.26$) in the forest than in the meadow (Fig. 2). Not surprisingly, the leaf and root N-content was higher for legumes than for the other two functional groups (Tab. III).

$\delta^{15}N$ of leaf tissues was higher (NS, $P = 0.13$), while $\delta^{15}N$ of root tissues was lower ($P < 0.05$) in the forest compared to the meadow ($+0.37 \pm 0.10‰$ and $+0.21 \pm 0.08‰$ for leaves in forest and meadow respectively, and $+0.07 \pm 0.09‰$ and $+0.37 \pm 0.09‰$ for roots in forest and meadow respectively). For both leaves and roots, legumes had a significantly lower $\delta^{15}N$ compared to the other two functional groups ($P < 0.01$, Fig. 3A). For roots ($P < 0.05$), but not for leaves (NS, $P = 0.41$) there was a significant interaction between functional group and habitat with respect to tissue $\delta^{15}N$, associated with a lower $\delta^{15}N$ for forbs and grasses but higher $\delta^{15}N$ for legumes in the forest compared to the meadow (Fig. 3B). There was no significant interaction for $\delta^{15}N$ of leaves ($P = 0.32$) or roots ($P = 0.13$) between species and habitat for the three species common to both habitats (Fig. 3C).

Leaf–root differences in N-isotope composition ($\Delta^{15}N$ [leaf–root]) were larger for forest plants than for plants in the meadow ($+0.30 \pm 0.09‰$ for forest and $-0.159 \pm 0.10‰$ for meadow; $P < 0.05$). The forbs showed the largest difference between habitats (interaction

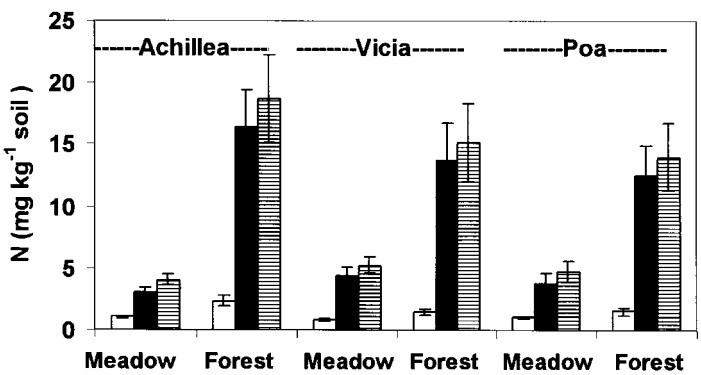

FIGURE 1 Nitrate (open bars), ammonium (closed bars) and dissolved inorganic N (DIN; $NO_3^- + NH_4^+$) content (lined bars) per dry weight of soil from meadow and forest. Means ± se.

TABLE III Leaf and Root Nitrogen Concentration Per Dry Weight of Forbs, Grasses and Legumes Growing in a Meadow and Forest. Means ± se (*n*).

Ecosystem	*Functional type*	*Leaf-N (mg g^{-1})*	*se (n)*	*Root-N (mg g^{-1})*	*se (n)*
Meadow	Forbs	17.0	0.5 (42)	6.6	0.3 (42)
	Legumes	35.0	2.1 (15)	23.0	1.5 (15)
	Grasses	14.0	0.6 (15)	6.8	2.1 (15)
Forest	Forbs	32.2	1.1 (34)	11.3	0.8 (34)
	Legumes	45.4	1.8 (20)	31.1	1.8 (20)
	Grasses	25.3	1.6 (15)	9.0	0.5 (15)

between functional groups and habitat, $P < 0.05$, Fig. 3B). Individual species means likewise exhibited higher Δ^{15}N [leaf–root] for forest plants compared meadow plants (Mann–Whitney *U* test, $P < 0.05$, Fig. 4). When tested separately for each functional group, the habitat difference was significant for forbs ($P < 0.01$), but not for grasses and legumes ($P = 0.56$ and $P > 0.99$ respectively). Although no significant effects were found for the three species common to both habitats, *Achillea lanulosa* had a higher Δ^{15}N [leaf–root] in the forest as compared to the meadow (Fig. 3D).

DISCUSSION

Plants exhibited significant differences in nitrogen isotope composition between leaves and roots in this study (Figs. 3B and 4). In other words, the plants were not homogenous with respect to N-isotope composition. This confirmed experiments in which N-nutrition was carefully controlled [19–23] as well as field observations [8, 24, 25].

We found examples where the leaves were enriched compared to the roots (*Geranium richardsonii*) but also where the leaves were depleted compared to the roots (*Elymus elymoides*, Fig. 4). A relative enrichment of the leaves compared to the roots can be a consequence of nitrate reductase or glutamate synthetase activity divided over both organs [11, 19, 33]. The reverse situation may be related to ammonia volatilization, exudation of organic N from the root [11, 23], or associated with mycorrhizal colonization [21].

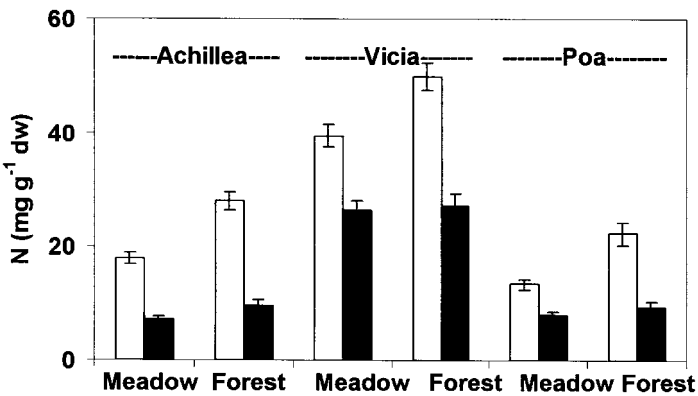

FIGURE 2 Nitrogen content of leaves (open bars) and roots (closed bars) per dry weight of three species common to meadow and forest. Means ± se.

Robinson *et al.* [22] found ^{15}N-depleted shoots compared to roots for wild barley genotypes, while Kolb and Evans [23] found depleted leaves compared to roots for *Quercus* species.

There were significant differences between species in $\Delta^{15}N$ [leaf–root], as also noted by Kolb and Evans [23]. For example *Elymus elymoides*, a grass growing in the meadow, differed from *Geum trifolium* and *Potentilla hippeana*, two forbs growing in the meadow, but also from five species growing in the forest (Fig. 4). This indicates that processes of N-reduction, assimilation and allocation differed between plant species.

Legumes had slightly negative leaf $\delta^{15}N$ values, while the grasses and forbs (with one exception) had positive values (Fig. 3A, Tab. I). From the negative leaf ^{15}N composition of the legumes (Fig. 3A, Tab. I) we conclude that the plants were actively fixing nitrogen. Bergersen *et al.* [38] showed that nitrogen fixation in the nodules supplied slightly negative $\delta^{15}N$ nitrogen to the plant ($\delta^{15}N = -0.59‰$). We hypothesize that the lower value for *Oxytropis lambertii* (leaf $\delta^{15}N = -1.04‰$) indicates a different N-fixation rate and a lesser influence by mineral N compared to the other legume species (leaf $\delta^{15}N = -0.20$ to $-0.27‰$). $\Delta^{15}N$ [leaf–root] of legumes in the forest was close to zero (Figs. 3B and 4). The higher $\Delta^{15}N$ [leaf–root] for legumes in the meadow may indicate the influence of some mineral N uptake specifically nitrate.

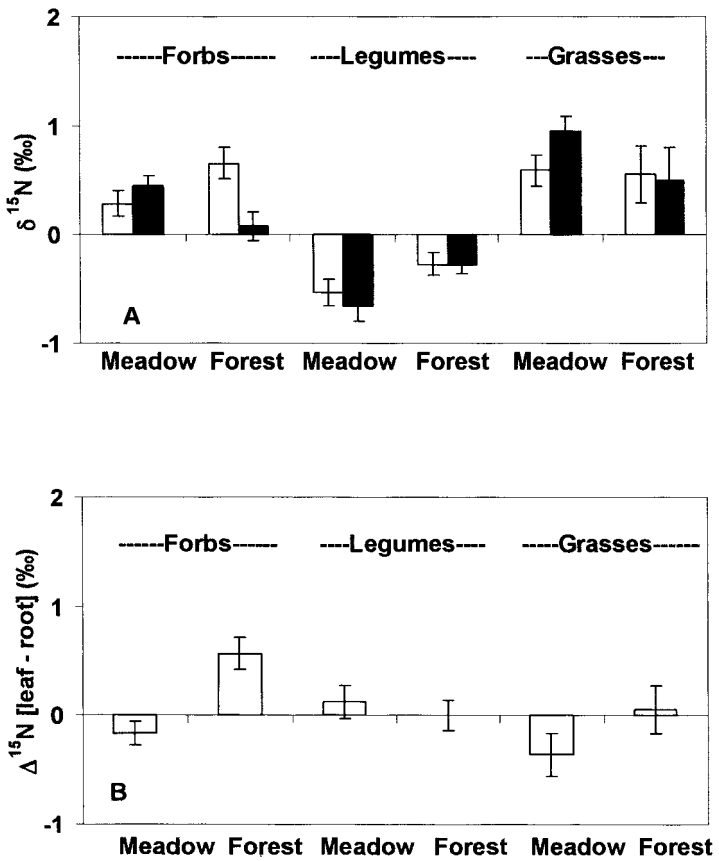

FIGURE 3 $\delta^{15}N$ of leaves and roots (A, C) and $\Delta^{15}N$ [leaf–root] (B, D) for three functional groups (A, B) and three common species (C, D) in meadow and forest. For A and C: Open bars indicate leaves, closed bars indicate roots. Means ± se.

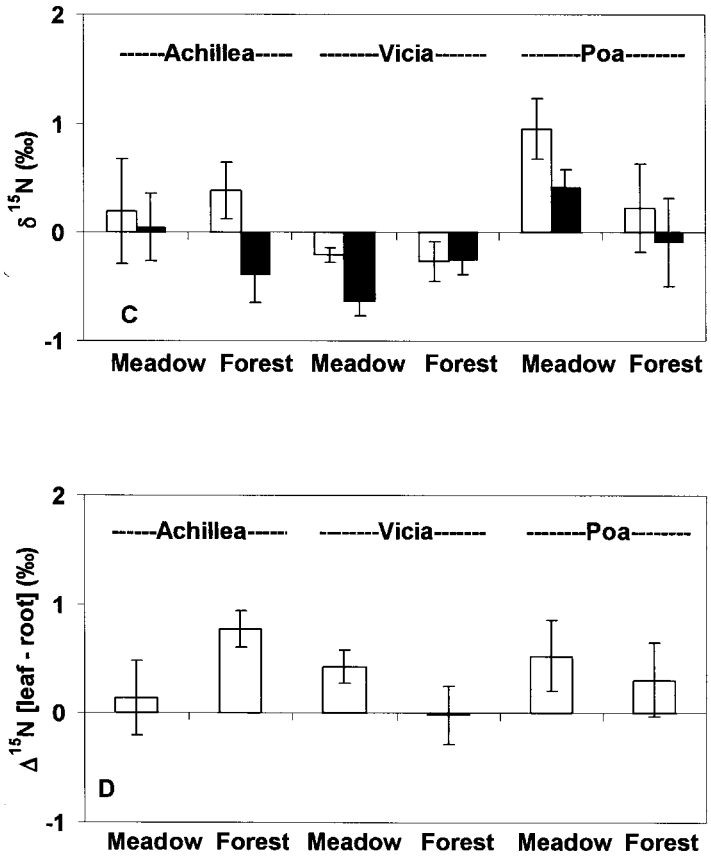

FIGURE 3 (*Continued*)

The range of leaf δ^{15}N values observed in this study was -1.04 to $+1.08$‰ (Tab. I). This is not a large range compared to some studies in the literature where differences of up to 20‰ were noted [4, 12]. The range of Δ^{15}N [leaf–root] values, -0.97 to $+0.86$‰, was almost as large as the range of the leaf δ^{15}N values. When we want to study the nitrogen cycling in these two habitats, we ideally want to use whole-plant nitrogen isotope composition. However, in these dense and species-rich grasslands this is an impossible task: root identity is often questionable, while sampling the total root biomass would be an enormous task. It is preferred that δ^{15}N of leaf samples can be used to estimate whole-plant δ^{15}N. Based on the δ^{15}N of the leaf samples we would conclude that there was no significant and only small difference between the two habitats ($\delta^{15}\text{N} = +0.37$‰ for the forest and $+0.21$‰ for the meadow). However, if we based our research on root tissue, we would conclude that the plants growing in the forest were slightly depleted compared to the plants growing in the meadow. We conclude that when comparing these two habitats, we need to be very careful when interpreting plant N-isotope data using leaf material alone.

The range of Δ^{15}N [leaf–root] values found in this study (-0.97 to $+0.86$‰) was small compared to results obtained under controlled nutrition experiments. Under two nitrate regimes (10 and 100 μM), shoot and root δ^{15}N differed about $+4$‰ in *Raphanus sativum* × *raphanistrum*, with the shoot being enriched compared to the root (Dijkstra, Hungate and Koch, unpublished). Evans *et al.* [20] reported $+5.8$‰ enrichment in the leaves compared to the roots of tomato plants grown with 50-μM nitrate, while leaf–root differences

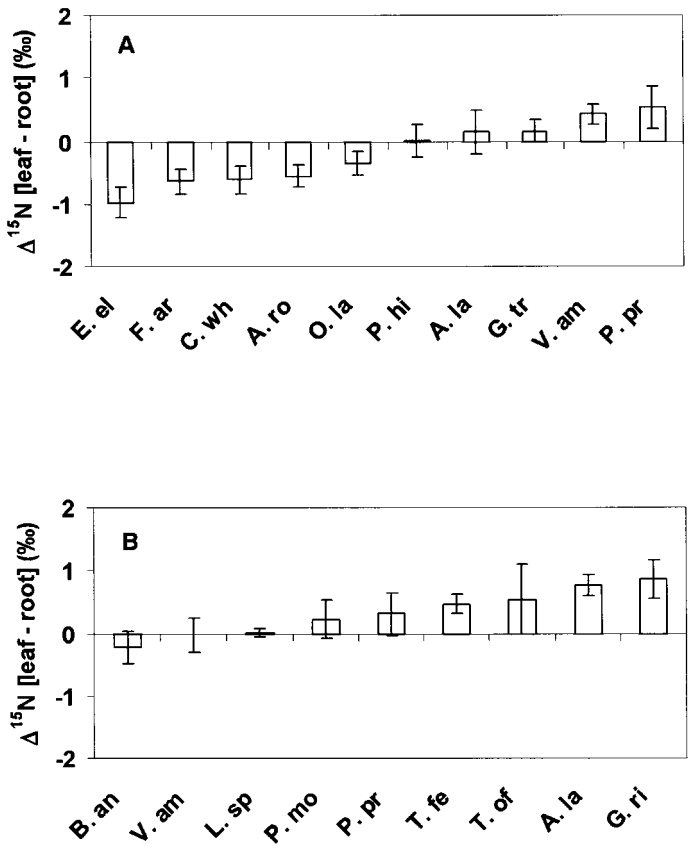

FIGURE 4 $\Delta^{15}N$ [leaf–root] for individual species in meadow (A) and forest (B). Mean ± se. First letter indicates genus name, last two letters are the first two letters of the species epithet (see Tab. I). Means ± se.

were absent when plants were grown with a similar concentration of ammonium. In an experiment by Yoneyama and Kaneko [18], *Brassica campestris* leaves were 2–5‰ enriched compared to roots under different nitrate concentrations (200–1200 μM), while exposure to ammonium resulted in a leaf–root difference of less than 2‰ for rice [19]. The smaller differences found in the field may be related to lower available nitrogen concentrations that are normally found in the field [5], or be caused by the uptake of mixed portions of ammonium and nitrate. Robinson *et al.* [22], growing wild barley genotypes with nitrate, found that $\Delta^{15}N$ [leaf–root] ranged from slightly below zero to +2‰ depending on genotype. Interestingly, all genotypes exhibited enriched roots compared to leaves when plants were transferred to a nutrient solution without N. N-starvation and the resulting remobilization of nitrogen [11, 23] could perhaps explain some of the negative $\Delta^{15}N$ [leaf–root] values found in our study (Fig. 4).

The soil moisture content was higher in the forest than in the meadow. Also the nitrogen content was higher under the trees (Tab. II) [39]. Since soil moisture is a major determinant of decomposition and mineralization [40], it may have stimulated microbial activity, increasing the rate of litter turnover and mineralization in the forest floor as compared to the meadow, and therefore explaining the high nitrate and ammonium concentrations (Tab. II, Fig. 1). We also observed that the nitrogen content of root and leaf tissues was higher in the forest than in the meadow (Fig. 2, Tab. III). This was either the direct consequence of

higher soil nitrogen availability or indirectly caused by other environmental factors (shade, soil moisture availability). $\Delta^{15}N$ [leaf–root] of forbs increased in parallel with this increased N-availability (Fig. 3B). Legumes showed a lower (not significant), while grasses showed a higher (not significant) $\Delta^{15}N$ [leaf–root] value in the forest compared to the meadow (Fig. 3B). A higher $\Delta^{15}N$ [leaf–root] was in accordance with expectations for a shift of the NRA activity towards the leaves in response to increased nitrate availability [11]. However, ammonium increased more than nitrate did from meadow to forest (Tab. II). Since ammonium nutrition did not result in substantial leaf–root isotope differences [19–21], $\Delta^{15}N$ [leaf–root] of forbs may have responded mainly to nitrate.

Of course it is unlikely that all differences between the meadow and the forest were solely due to nitrogen availability. In addition to nitrogen, moisture content of the soil was different (Tab. II), and light levels were lower in the forest than in the meadow. Robinson *et al.* [22] showed that drought increased the isotope difference between leaves and roots for wild barley genotypes. Applied to our situation, this should result in a higher $\Delta^{15}N$ [leaf–root] in the meadow compared to the forest, the reverse of what we found (Figs. 3B and 4). Light climate in the forest was also very different from that in an open meadow. In a nutrition × day length × intensity factorial experiment with *Raphanus sativus* × *raphanistrum*, only nitrate concentration affected the $\Delta^{15}N$ [shoot–root] (Dijkstra, Hungate and Koch unpublished). It is clear from this discussion that of the considered environmental factors, nitrogen, and especially nitrate, is the most probable candidate for the higher $\Delta^{15}N$ [leaf–root] observed in the forbs and (not significantly) grasses in the forest compared to the meadow.

Once the conclusion is accepted that the plant is not homogenous with respect to ^{15}N, it is a small step to accept differences within the root system itself. It is often found that soil and root isotope ^{15}N composition increases with soil depth [8, 41]. We are open to criticism that the root samples analyzed may not be representative for the whole root system. However, the root zone that we sampled likely contained the majority of roots, as roots normally decrease exponentially or linearly with depth [42].

CONCLUSIONS

Leaf–root differences in $\delta^{15}N$ were found in plants growing in a natural unmanaged meadow and forest understorey. Some species exhibited ^{15}N enrichment of the leaves, while others showed ^{15}N depleted leaves compared to the roots. The differences between leaves and root were smaller than reported for controlled N-nutrition studies and some field experiments.

Forb species growing in the understorey of the forest exhibited greater $\Delta^{15}N$ [leaf–root] than for comparable plant species growing in the meadow, while there were no significant habitat differences for legumes and grasses. It is proposed that this higher $\Delta^{15}N$ [leaf–root] was in response to higher soil nitrate availability in the forest.

Care must be taken when using leaf $\delta^{15}N$ values as a proxy for whole-plant ^{15}N composition in these two habitats, since the range of leaf–root differences in isotopic composition was as large as the range of leaf $\delta^{15}N$ values.

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