Trees (2015) 29:1069–1078
DOI 10.1007/s00468-015-1186-3

ORIGINAL PAPER

Interspecific variation in leaf–root differences in $\delta^{15}N$ among three tree species grown with either nitrate or ammonium

Lee A. Kalcsits[1,2,3] · Xiangjia Min[1,4] · Robert D. Guy[1]

Received: 8 January 2015 / Revised: 24 February 2015 / Accepted: 4 March 2015 / Published online: 17 March 2015

Key message **Interspecific variation in nitrogen isotope composition of roots and leaves of tree seedlings grown in a steady-state nitrogen environment reflects known variation in sites of assimilation and nitrogen source preference in three tree species.**

Abstract As a time-integrated measure of nitrogen use, discrimination against the heavier stable isotope (^{15}N) during the uptake and assimilation of inorganic nitrogen has the potential to provide information on interspecific differences in inorganic nitrogen source preference. Here, nitrogen isotope composition ($\delta^{15}N$) at natural abundance was measured for the roots and shoots from seedlings of three forest tree species: *Populus tremuloides* (aspen), *Pinus contorta* var. *latifolia* (pine) and *Picea glauca* (spruce). The seedlings were grown hydroponically with low (0.1 mM) or high (1.5 mM) concentrations of NO_3^- or NH_4^+, or in sand with NO_3^-, NH_4^+ or an equal mix of NO_3^- and NH_4^+ (0.1 mM). Whole-plant nitrogen isotope discrimination was observed in hydroponically grown seedlings but not in sand culture. Differences in $\delta^{15}N$ between shoots and roots were greater in aspen when grown with NO_3^- (3.02 ‰) than with NH_4^+ (1.27 ‰). There were no significant differences between the $\delta^{15}N$ of leaves and roots for pine and spruce on either source. Although whole-plant nitrogen isotope discrimination was not observed in sand culture, shoot $\delta^{15}N$ was, again, greater than root $\delta^{15}N$ for NO_3^--grown aspen. Interspecific variation in nitrogen isotope discrimination was observed in both hydroponics and sand culture. The differences in nitrogen isotope composition under steady-state conditions indicate that interspecific differences in nitrogen source preference are consistent with previous experiments using alternative methods to identify differences in nitrogen uptake and assimilation in the same tree species.

Keywords Nitrogen · Source preference · Isotope discrimination · Nitrate · Ammonium · Hydroponics

Communicated by A. Gessler.

✉ Robert D. Guy
rob.guy@ubc.ca

Lee A. Kalcsits
lee.kalcsits@wsu.edu

[1] Department of Forest and Conservation Sciences, University of British Columbia, 2424 Main Mall, Vancouver, BC V6T 1Z4, Canada

[2] Department of Biology, Centre for Forest Biology, University of Victoria, Victoria, BC, Canada

[3] Department of Horticulture, WSU Tree Fruit Research and Extension Center, Washington State University, Wenatchee, WA, USA

[4] Department of Biological Sciences, Center for Applied Chemical Biology, Youngstown State University, Youngstown, USA

Introduction

Nitrogen is one of the most limiting nutrients in most forest ecosystems and tree species have adapted to grow under these limiting conditions. Interspecific variation in uptake and assimilation of nitrate and/or ammonium can vary depending on the ecological niche or adapted environment of a tree species. Variations in nitrogen source preference may, in part, be a result of differences in the tissue-specific location of primary nitrogen assimilation since it can occur in roots, leaves or both (Andrews 1986; Min et al. 1998). The two primary forms of inorganic nitrogen taken up by trees are nitrate and ammonium. Nitrate and ammonium

assimilation are catalyzed by nitrate reductase and glutamine synthetase, respectively. These two enzymes are active in both roots and shoots. In some species, nitrate reductase and glutamine synthetase both have isozymes present in the roots and shoots allowing for multiple assimilatory sites (Glass et al. 2002). Under high NO_3^- concentrations, substantial NO_3^- assimilation can occur in the shoot (Peuke 2010). However, the location of assimilation appears to be dependent on the internal demand of the tree species as well as the external nitrogen environment. Glutamine synthetase is present in high concentrations in roots and leaves. It is critical for photorespiration which results in considerable secondary NH_4^+ turnover in plant tissue (Bauer et al. 1997; Tcherkez and Hodges 2008). As a result of the high activity of glutamine synthetase activity, NH_4^+ assimilation occurs primarily in the roots. However, there is evidence that ammonium is also transported to the shoots and assimilated in leaves (Husted et al. 2000; Schjoerring et al. 2002).

The nitrogen isotope composition of plants acts as an integrated measure of nitrogen uptake and assimilation (Robinson 2001; Evans 2001; Kalcsits et al. 2014). Deviations in plant tissue $\delta^{15}N$ from substrate $\delta^{15}N$ arise through isotopic discrimination, which is the kinetically or thermodynamically determined process whereby the lighter isotope (^{14}N) is favored, causing a depletion of the heavier isotope (^{15}N) in the product (Handley and Raven 1992; Comstock 2001; Evans 2001; Robinson 2001; Pritchard and Guy 2005; Kalcsits et al. 2014). Discrimination against ^{15}N can only occur when a substrate is partially consumed (Comstock 2001). Partial depletion of soil nitrogen pools can affect organ-specific $\delta^{15}N$. Therefore, discrimination under field conditions is often not observed. Internally, partial consumption may also occur when there is substrate loss to an alternative pool or at a branch point where two reactions utilize the same substrate (Comstock 2001; Robinson 2001; Kolb and Evans 2003). In plants, partial consumption of unassimilated inorganic nitrogen occurs when it is lost from the cytoplasmic pool and returned to the rooting medium by way of efflux or, alternatively, is translocated to the leaves through xylem transport (Comstock 2001; Evans 2001; Luo et al. 2013; Kalcsits et al. 2014).

Whole-plant nitrogen isotope discrimination can only occur when nitrogen returns to the bulk medium through efflux and gross nitrogen influx exceeds nitrogen utilization (Waser et al. 1999; Evans 2001; Pritchard and Guy 2005; Kalcsits et al. 2014). Therefore, nitrogen isotope discrimination is thought to be a function of substrate nitrogen concentration and assimilatory demand of the plant. High assimilatory demand may reduce isotopic fractionation since most of the cytoplasmic nitrogen will be assimilated and little lost back to the substrate. Fractionation will be maximal when cytoplasmic supply significantly exceeds demand, providing ample opportunity for efflux. Flux values can vary diurnally where variation in nitrogen uptake and assimilation can impact efflux and influx of nitrogen and, therefore can impact plant $\delta^{15}N$ (Peuke et al. 2013). Overall, whole-plant N isotope discrimination is inversely proportional to the efflux/influx ratio (Handley and Raven 1992; Evans 2001; Pritchard and Guy 2005; Kalcsits et al. 2014). Ariz et al. (2011) observed increased nitrogen isotope discrimination in plant species that are susceptible to ammonium toxicity. Ammonium toxicity is thought to arise during the loss of the control of cytoplasmic ammonium concentration and subsequent futile cycling of ammonium across the plasma membrane of roots (Britto et al. 2001). This efflux can also occur during the assimilation of nitrate. Recently, Karsh et al. (2014) reported that in algae, nitrogen isotope discrimination during nitrate uptake and assimilation is primarily a result of the kinetic isotope effect during nitrate assimilation and is a function of efflux of unassimilated nitrate to the environment.

The difference in the isotopic composition between the roots and shoot are thought to be caused by translocation of unassimilated NO_3^- or NH_4^+ from the root to the shoot, since unassimilated inorganic nitrogen in the root cytoplasm will be isotopically enriched relative to root-assimilated nitrogen (Evans 2001; Comstock 2001; Kalcsits et al. 2014). Once leftover inorganic nitrogen is translocated to the shoot, it must be fully assimilated, and therefore leaf-assimilated and root-assimilated organic nitrogen will have different nitrogen isotope compositions (Bergersen et al. 1988; Evans et al. 1996). As an example, in *Lycopersicon esculentum* grown with NO_3^-, leaf $\delta^{15}N$ was 8 ‰ greater than root $\delta^{15}N$ (Evans et al. 1996) indicating that some NO_3^- must have been assimilated in the leaves. In hydroponic, steady-state nitrogen environments, enrichment of the shoot relative to the root is to be expected. However, under heterogeneous field or pot-grown nitrogen environments, these differences are difficult to interpret because of heterogeneities that exist within the growing substrate (Högberg et al. 2014). Even still, differences between leaves and roots have been observed in field conditions (Dijkstra et al. 2003; Peuke et al. 2006) and greenhouse pot-grown plants (Luo et al. 2013).

Under steady-state nitrogen conditions, differences between root and leaf $\delta^{15}N$ may reflect differences in the location of assimilation (Kalcsits et al. 2014) and differences in nitrogen source preference. However, the heterogeneity in nitrogen availability, nitrogen cycling within the plant and tissue $\delta^{15}N$ under field conditions makes interpretations of field-collected $\delta^{15}N$ samples problematic. Our primary objectives were: (1) to determine interspecific variability in whole-plant $\delta^{15}N$ and root-to-leaf differences

in $\delta^{15}N$ for plants grown under a controlled nitrogen environment in trembling aspen (*Populus tremuloides* Michx.), lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm.), and spruce (*Picea glauca* [Moench] Voss) and (2) to determine whether leaf to root differences in nitrogen isotope composition are observed in both hydroponic and sand culture. From previous research using compartmental analysis and enzyme assays (Min et al. 1998, 2002), we hypothesize that aspen will demonstrate a higher leaf–root difference in $\delta^{15}N$ than the two conifer species indicating a greater proportion of assimilation occurring in the leaves of aspen relative to the two conifer species.

Methods

Two experiments were conducted to examine interspecific variation in whole-plant nitrogen isotope discrimination and root vs. leaf differences in $\delta^{15}N$. The plants used for the first experiment were hydroponically cultured; while for the second experiment, they were grown in sand culture. Experiments were carried out in E-15 (Conviron, Winnipeg, MB, Canada)-controlled environment growth chambers. Environmental conditions were maintained at 20 ± 2 °C, 70 % RH, 16 h photoperiod, and 250 μmol m^{-2} s^{-1} PAR.

Hydroponics culture

Three tree species, aspen (Seedlot 42307), spruce (Seedlot 29170), and pine (Seedlot 3847), were obtained from the Tree Seed Resource Centre in Surrey, BC, Canada. Seedlings ($N = 18$) were grown together in a split-plot randomized complete block design from seed in a well-mixed, aerated hydroponic system (40 L) containing a 1/10th Johnson's solution (Johnson et al. 1957) with a final nutrient composition, excluding nitrogen, of 200 μM KH_2PO_4, 200 μM K_2S0_4, 100 μM $MgSO_4$, 100 μM $CaSO_4$, and micronutrients: 5 μM Cl, 2.5 μM B, 0.2 μM Mn, 0.2 μM Zn, 0.05 μM Cu, and 50 μM Fe^{2+}-EDTA complex. Nitrogen was supplied in four separate treatments as 0.05 mM $Ca(NO_3)_2$, 0.75 mM $Ca(NO_3)_2$, 0.05 mM $(NH_4)_2SO_4$, and 0.75 mM $(NH_4)_2SO_4$. Solutions were frequently replaced to avoid significant substrate depletion (no more than 10 %) and the possibility of associated ^{15}N isotope enrichment. To determine the replacement times, inorganic nitrogen concentrations were assayed using perchloric acid (Cawse 1967) and phenol–hypochlorite (Solorzano 1969) for nitrate and ammonium, respectively. Aspen seedlings grew quickly relative to the conifers and were harvested after 4 weeks to limit the effect of nitrogen depletion on substrate $\delta^{15}N$, while the other two species were grown for 8 weeks to allow for sufficient accumulation of biomass for biomass and nitrogen isotope measurements.

Sand culture

Aspen, spruce, and pine were germinated and grown together in acid-washed silica sand media ($N = 18$). The seedlings were watered daily with modified 1/10th strength Johnson's solution identical to plants that were hydroponically grown (Johnson et al. 1957). Nitrogen was supplied at a concentration of 0.1 mM nitrogen as $Ca(NO_3)_2$, $(NH_4^-)_2SO_4$, or a 50:50 molar concentration mix of the two. All three species were harvested simultaneously after 3 months of growth.

Isotope analysis

Following the prescribed treatments, seedlings were separated into roots and shoots and then frozen in liquid nitrogen. Samples were then lyophilized at −50 °C and weighed for biomass. To ensure adequate material for sample preparation and to reduce analytical costs, for the sand-grown seedlings, six seedlings were pooled from each treatment and for hydroponically grown plants, two aspen seedlings and six spruce and pine seedlings were pooled to make three replicates for each treatment. Samples were then ground to sub-micron particle size in a ball mill (Pulverisette, Fritch GMBH, Germany). 3 ± 0.1 mg from prepared leaf and root samples and 1 ± 0.1 mg for calcium nitrate and ammonium sulfate crystals were weighed into tin capsules (Elemental Microanalysis Ltd., 8 × 5 mm, D1008) and analyzed for nitrogen isotopes and nitrogen concentration on an ElementarVario EL Cube elemental analyzer interfaced to a VG Spectrum ratio mass spectrometer (Earth and Ocean Sciences, UBC) and compared to international IAEA standards (internal standard deviation of 0.3 ‰). $\delta^{15}N$ values were calculated according to the following:

$$\delta^{15}N = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000 \quad (1)$$

where R_{sample} is the $^{15}N/^{14}N$ isotope ratio of the sample and $R_{standard}$ is the isotope ratio of a known standard (air). The $\delta^{15}N$ of $Ca(NO_3)_2$ and $(NH_4^-)_2SO_4$ salts were −1.23 and 0.48 ‰, respectively. Isotope discrimination is denoted by the equation:

$$\Delta^{15}N_{sample} = \left[\frac{\left(\delta^{15}N_{source} - \delta^{15}N_{sample}\right)}{\left(1 + \frac{\delta^{15}N_{sample}}{1000}\right)}\right] \quad (2)$$

However, since $1 + \delta^{15}N_{sample}/1000$ will not deviate significantly from 1 when isotope ratios are close to 0 ‰, discrimination ($\Delta^{15}N$) is well approximated by $\delta^{15}N_{sample} - \delta^{15}N_{source}$($\Delta\delta^{15}N$) (Evans 2001; Pritchard and Guy 2005; Kalcsits et al. 2014). Here, $\Delta\delta^{15}N$ was only calculated for samples from plants grown hydroponically. However, a lack of confidence in the homogeneity and temporal stability of the $\delta^{15}N$ of the sand substrate limits the expression of samples from the sand substrate to $\delta^{15}N$.

Experimental design and statistical analysis

Comparisons between species and treatments for total plant dry mass, root and shoot N concentration, and root and shoot $\delta^{15}N$ were made using analysis of variance (ANOVA). ANOVA was carried out with a General Linear Model (SAS 9.1.3; SAS Institute Cary, NC) to obtain estimates of the least squares means followed by the *F* and *T* tests for separation of means ($\alpha = 0.05$).

Results

Seedlings grown in hydroponics media

At harvest, plant biomass was more than five times greater when grown with high nitrogen compared to low nitrogen (Fig. 1). However, there were significant interactions between tree species and nitrogen supply. For aspen and pine, biomass was greater under the 1.5 mM NO_3^- treatment than 1.5 mM NH_4^+ treatment. When seedlings were grown with a low nitrogen supply, there was no significant difference in biomass due to nitrogen source for any species. Root:shoot ratios of aspen were similar between treatments. However, for pine and spruce, the root:shoot ratio decreased as the nitrogen concentration of the substrate increased (Fig. 2). For pine, the difference in the root:shoot ratio between high or low nitrogen supply was less than it was for spruce. The root:shoot ratio of aspen was less affected by changes in nitrate concentrations than the two conifer species. Whole-plant tissue nitrogen concentration for nitrate-grown aspen was almost five times greater than the nitrogen concentration of plants grown with NH_4^+ (Table 1). For the two other species, nitrogen concentration was also highest when grown on NO_3^- but the differences between nitrogen sources were not as great as they were for aspen. For pine and spruce, plants on 0.1 mM NH_4^+ had lower nitrogen concentrations than when grown with 1.5 mM NH_4^+.

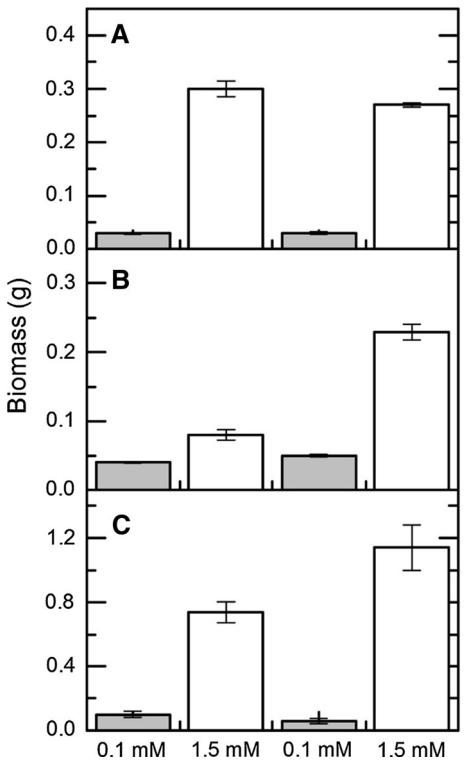

Fig. 1 Biomass ±SE ($N = 18$) of **a** aspen (*Populus tremuloides*), **b** pine (*Pinus contorta* var. *latifolia*) and **c** spruce (*Picea glauca*) seedlings grown hydroponically in a 1/10th Johnson's solution supplemented with either 0.1 or 1.5 mM nitrogen as $(NH_4)_2SO_4$ (*right*) or $Ca(NO_3)_2$ (*left*) as the sole nitrogen source

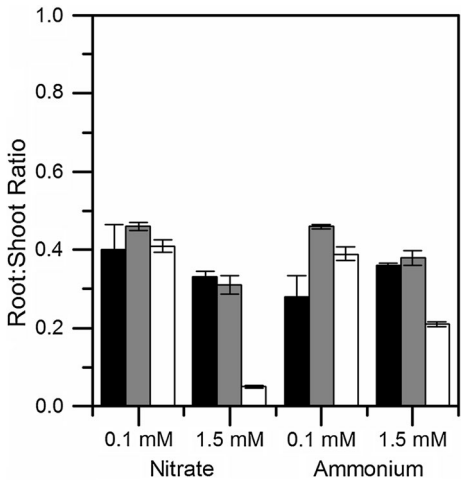

Fig. 2 Root:shoot biomass ratios ±SE ($N = 18$) of trembling aspen (*Populus tremuloides*) (*black bars*), lodgepole pine (*Pinus contorta* var. *latifolia*) (*gray bars*) and white spruce (*Picea glauca*) (*white bars*) seedlings grown hydroponically in a 1/10th Johnson's solution supplemented with either 0.1 or 1.5 mM nitrogen as $(NH_4)_2SO_4$ or $Ca(NO_3)_2$ as the sole nitrogen source

The $\delta^{15}N$ values for hydroponically grown plants were negative relative to the source and ranged from −5.94 to

Table 1 Whole-plant tissue nitrogen concentration (mg N/g d.w.) ±SE ($N = 6$) of aspen (*Populus tremuloides*), pine (*Pinus contorta* var. *latifolia*) and spruce (*Picea glauca*) seedlings grown hydroponically in a 1/10th Johnson's solution supplemented with either 0.1 or 1.5 mM nitrogen as $(NH_4)_2SO_4$ or $Ca(NO_3)_2$ as the sole nitrogen source

Nitrogen concentration (mM)	Nitrogen source	*P. tremuloides*	*P. contorta*	*P. glauca*
0.1	$(NH_4)_2SO_4$	8.8 ± 1.7	7.8 ± 0.4	10.5 ± 0.6
	$Ca(NO_3)_2$	42.5 ± 6.9	17.9 ± 1.2	24.4 ± 0.9
1.5	$(NH_4)_2SO_4$	9.8 ± 0.1	12.4 ± 0.4	20.3 ± 1.4
	$Ca(NO_3)_2$	47.6 ± 1.4	19.2 ± 2.3	22.7 ± 1.6

−1.65 ‰, (Fig. 3) indicating consistent nitrogen isotope discrimination under hydroponic conditions. For nitrate-grown plants, discrimination was greatest in pine and lowest in aspen and spruce. Aspen was the only tree species that showed large root-to-leaf $\delta^{15}N$ differences when grown on either NO_3^- or NH_4^+, although nitrate-grown plants had a greater leaf–root $\delta^{15}N$ difference than ammonium-grown plants (Table 2). No other treatments or species showed significant enrichment of the shoot $\delta^{15}N$ relative to the root $\delta^{15}N$.

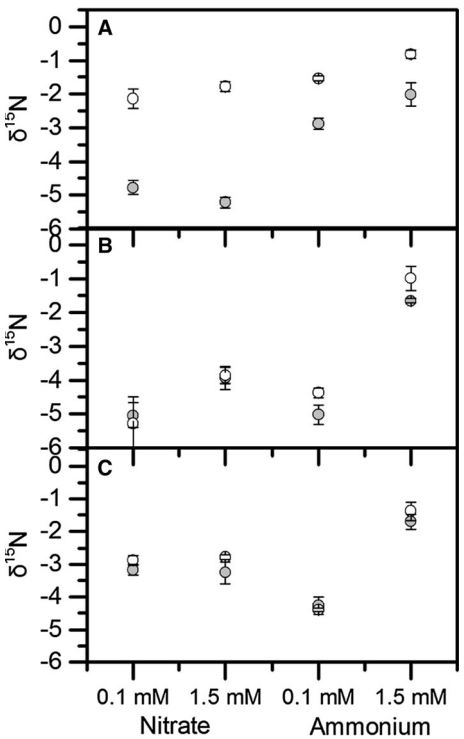

Fig. 3 Nitrogen isotope composition ($\Delta\delta^{15}N$) ±SE ($N = 6$) of roots (*filled circles*) and leaves (*empty circles*) of **a** aspen (*Populus tremuloides*), **b** pine (*Pinus contorta* var. *latifolia*) and **c** spruce (*Picea glauca*) seedlings relative to the source $\delta^{15}N$ ($Ca(NO_3)_2 = -1.23$ ‰ and $(NH_4)_2SO_4 = 0.48$ ‰) grown hydroponically in a 1/10th Johnson's solution supplemented with either 0.1 or 1.5 mM nitrogen as $(NH_4)_2SO_4$ or $Ca(NO_3)_2$ as the sole nitrogen source

Interspecific differences in nitrogen source preference for sand-grown seedlings

Seedlings grown in sand were similar compared to those in hydroponics. At the end of the experiment, aspen biomass was the greatest and spruce, the lowest (Fig. 4). Nitrogen source affected the growth of each tree species differently, as demonstrated by a significant interaction between species and nitrogen source. Aspen was the largest when grown with only NO_3^- and the smallest when grown with only NH_4^+ with an average of 0.59 and 0.49 g biomass, respectively. In contrast, the biomass of both spruce and pine was not different among nitrogen sources. The nitrogen concentration of seedlings grown in sand media ranged from 12 to 25 mg N/g d.w. (Table 3) indicating that nitrogen supply was not a limitation to growth for any species. Nitrogen concentrations were inversely proportional to biomass accumulation ($r = -0.67$, $P = 0.0001$). However, total nitrogen content was still greater in treatments with higher biomass than treatments with higher nitrogen concentration (data not shown).

When plants were grown in a sand media, whole-plant $\delta^{15}N$ was not significantly depleted relative to the substrate. In some cases, there was net enrichment of the whole-plant $\delta^{15}N$ relative to the substrate $\delta^{15}N$ (Fig. 5). Shoots of plants grown under NO_3^-were enriched relative to the root in aspen and pine (Fig. 5). When grown with NO_3^-, aspen and pine had a difference in $\delta^{15}N$ between the shoot and the root of 2.26 and 1.01 ‰, respectively. When these two species were grown with NH_4^+, there was no enrichment of the shoot relative to the root. However, in spruce, there was no difference between the shoot and root $\delta^{15}N$ under any nitrogen treatments. Similar to what we observed in hydroponics, aspen had the greatest difference in $\delta^{15}N$ between the shoot and the root.

Discussion

There were differences among tree species in nitrogen isotope composition. Whole-plant and organ-level nitrogen isotope composition among species was consistent with the

Table 2 Differences in $\delta^{15}N$ ±SE ($N = 6$) between the leaves and roots of aspen (*Populus tremuloides*), pine (*Pinus contorta* var. *latifolia*) and spruce (*Picea glauca*) seedlings grown hydroponically in a 1/10th Johnson's solution supplemented with either 0.1 or 1.5 mM nitrogen as $(NH_4)_2SO_4$ or $Ca(NO_3)_2$ as the sole nitrogen source

Nitrogen concentration (mM)	Nitrogen source	*P. tremuloides*	*P. contorta*	*P. glauca*
0.1	$(NH_4)_2SO_4$	1.34 ± 0.40[a]	0.65 ± 0.76	−0.12 ± 0.46
	$Ca(NO_3)_2$	2.63 ± 0.84[a]	−0.24 ± 0.69	0.28 ± 0.04[a]
1.5	$(NH_4)_2SO_4$	1.20 ± 0.57[a]	0.66 ± 0.64	0.31 ± 0.49
	$Ca(NO_3)_2$	3.40 ± 0.46[a]	0.07 ± 0.18	0.47 ± 0.17[a]

[a] Significant difference between root and leaf $\delta^{15}N$ ($\alpha = 0.05$)

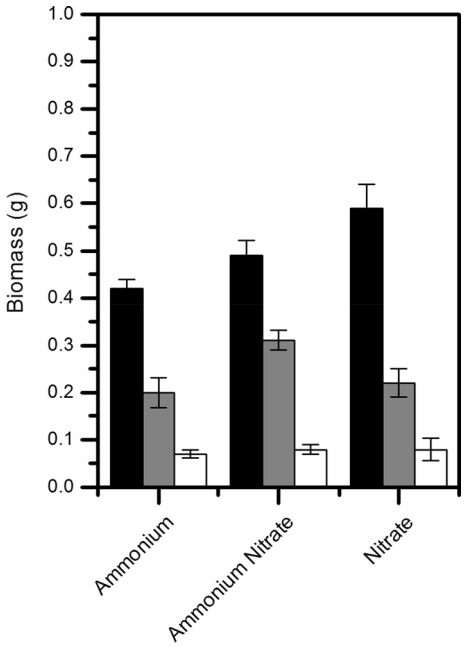

Fig. 4 Biomass ±SE ($N = 6$) of aspen (*Populus tremuloides*) (*black bars*), pine (*Pinus contorta* var. *latifolia*) (*gray bars*) and spruce (*Picea glauca*) (*white bars*) seedlings grown in sand culture with 1/10th Johnson's nutrient solution supplemented with 0.1 mM nitrogen as $Ca(NO_3)_2$, $(NH_4^-)_2SO_4$, or a 50:50 mix of the two

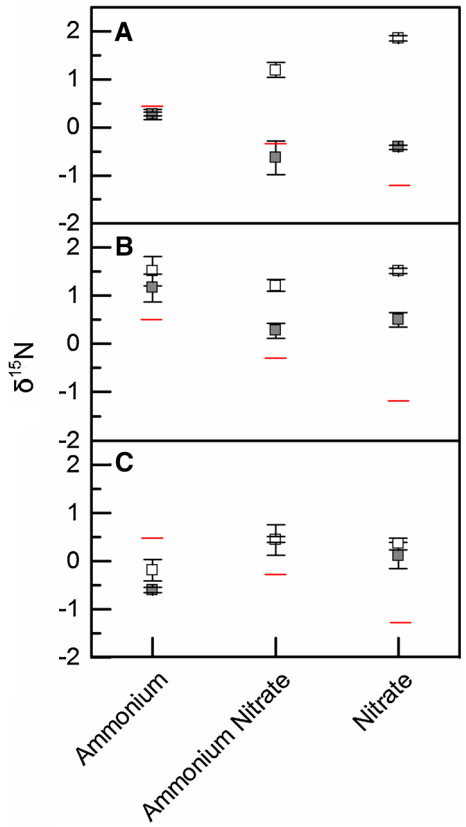

Fig. 5 Nitrogen isotope composition ($\delta^{15}N$) ±SE ($N = 6$) of roots (*filled circles*) and leaves (*empty circles*) of **a** aspen (*Populus tremuloides*), **b** pine (*Pinus contorta* var. *latifolia*) and **c** spruce (*Picea glauca*) seedlings grown in sand culture with 1/10th Johnson's nutrient solution supplemented with 0.1 mM nitrogen as $Ca(NO_3)_2$, $(NH_4^-)_2SO_4$, or a 50:50 mix of the two. *Red lines* indicate the starting $\delta^{15}N$ of the substrate

Table 3 Whole-plant tissue nitrogen concentration (mg N/g d.w.) ±SE ($N = 6$) of aspen (*Populus tremuloides*), pine (*Pinus contorta* var. *latifolia*) and spruce (*Picea glauca*) seedlings grown in sand culture with 1/10th Johnson's nutrient solution supplemented with 0.1 mM nitrogen as $Ca(NO_3)_2$, $(NH_4)_2SO_4$, or a 50:50 molar concentration mix of the two

Nitrogen source	*P. tremuloides*	*P. contorta*	*P. glauca*
$(NH_4)_2SO_4$	20.4 ± 0.9	17.2 ± 1.3	22.4 ± 3.4
50:50 mix	13.9 ± 0.6	16.8 ± 0.7	25.2 ± 4.1
$Ca(NO_3)_2$	15.1 ± 0.6	14.8 ± 0.6	26.6 ± 3.4

previous experiments that measured nitrogen fluxes and partitioning of assimilation (Min et al. 1998, 2002). Whole-plant depletion of $\delta^{15}N$ relative to the source was only observed in hydroponically grown plants. In general, there was no observed nitrogen isotope discrimination in plants grown in a sand substrate. Even still, there was interspecific variation in organ-level nitrogen isotope composition observed in both hydroponics and sand culture. In aspen, shoots were consistently enriched in $\delta^{15}N$ relative to the roots in both hydroponics and sand culture.

Enrichment of $\delta^{15}N$ of aspen leaves relative to root $\delta^{15}N$ grown with 1.5 mM NO_3^- indicated that there is some translocation of unassimilated inorganic nitrogen from the root to the shoot through the xylem. Enrichment of the cytoplasmic inorganic nitrogen pool occurs when there is fractionation during assimilation of NO_3^- in the root (Comstock 2001; Evans 2001; Robinson 2001; Kalcsits and Guy 2013a, b; Kalcsits et al. 2014). Although enrichment can occur in leaves as a result of the loss of NH_4^+ or N_2O from leaves (Gessler et al. 2000; Johnson and Berry 2013), this usually only occurs during periods of abiotic stress or senescence. Therefore, it cannot account for the enrichment in actively growing seedlings under controlled favorable growing conditions. Nitrate assimilation has been widely shown to occur in the leaves of angiosperm plants but does not normally occur in gymnosperms. Min et al. (1998) reported that nitrate reductase activity was high in aspen leaves but not detectable in the needles of pine. In contrast, nitrate reductase was active in roots of both species. Nitrate reductase was more greatly expressed in roots of aspen than pine (Min et al. 1998). The known patterns of nitrate reductase activity reported in Min et al. (1998) follow the general patterns in root to shoot differences in $\delta^{15}N$ observed in these experiments. Using compartmental analysis, NO_3^- fluxes to the leaves accounted for approximately 30 % of nitrate uptake in aspen compared to the leaves of conifer species that showed limited translocation of nitrate to the leaves (Min et al. 1999). Therefore, little or no impact on foliar isotopic composition was expected from translocated NO_3^- in gymnosperms, since all of the substrate is consumed in the root (Comstock 2001).

Species previously reported to show nitrogen isotope enrichment of leaves relative to roots include *Arabidopsis thaliana* (Kalcsits and Guy 2013a, b), *Fagus sylvatica* (Peuke et al. 2006) *Hordeum vulgare* (Kolb and Evans 2003), and *Lycopersicon esculentum* (Evans et al. 1996) grown with NO_3^- as the only nitrogen source and *Populus popularis* with NH_4NO_3 (Luo et al. 2013). In Luo et al. (2013), a fast growing poplar species, *Populus alba* × *Populus glandulosa*, showed increased isotopic fractionation between roots and leaves compared to *Populus popularis*. Fractionation could be a function of either growth rate or an increased capacity to assimilate nitrogen in its leaves. To support this, Li et al. (2012) reported increased nitrate reductase activity in leaves of *Populus alba* × *Populus glandulosa* compared to *Populus popularis*. However, without more research, it is difficult to discern whether increased leaf to root differences in $\delta^{15}N$ can be directly attributed to interspecific differences in growth rates or an increased capacity to assimilate inorganic nitrogen in leaves or roots. For ammonium-grown plants, there was enrichment in the $\delta^{15}N$ of the leaves only for hydroponically grown aspen. Evans et al. (1996) reported no enrichment of leaves of tomato plants grown under only NH_4^+ nutrition. However, Kalcsits and Guy (2013a, b) observed enrichment of shoots relative to roots in *Populus balsamifera* L. This observed enrichment in another poplar species parallels the enrichment of leaves relative to roots in hydroponically grown aspen observed here indicating that this response is likely species dependent.

Overall, whole-plant isotope discrimination indicates little or no net efflux of NO_3^- or NH_4^+ when seedlings were grown in sand at low nitrogen supply; but, as already indicated, sand culture may also limit the detection of isotope discrimination. In contrast, isotope discrimination under tightly controlled hydroponic conditions revealed substantial NO_3^- efflux at both low (0.1 mM) and high (1.5 mM) nitrogen concentrations. At both NO_3^- concentrations, whole-plant isotope discrimination ranged from ~3 ‰ in aspen to ~6 ‰ in pine. Using the equation for calculating *E*/*I* from Kalcsits et al. (2014) to calculate *E*/*I*:

$$\frac{E}{I} = \frac{\Delta^{15}N_{plant}}{\Delta_{enz} \times P_{root}} \quad (3)$$

efflux/influx ranged from 0.22 to 0.41, for aspen and pine, respectively. These estimates are similar to efflux/influx values reported by Min et al. (2002) using ^{13}N compartmental flux analysis. As suggested by Evans et al. (1996), there is little or no intra-plant isotope fractionation when nitrogen assimilation is restricted entirely to the root. This is the case under most if not all conditions in the conifers, and in aspen when grown on NH_4^+ only. Intra-plant fractionation in nitrate-grown aspen seedlings was more pronounced with shoot $\delta^{15}N$ being 2.6–3.4 ‰ greater than $\delta^{15}N$. Heavy isotope enrichment of aspen shoots when supplied with NO_3^- is consistent with the known involvement of the leaves in NO_3^- assimilation.

In sand culture, there was no discrimination observed in leaves or roots relative to the substrate. This may be a consequence of a more heterogeneous or non-steady-state nitrogen environment compared to hydroponics. Whole-plant fractionation can occur under both NO_3^- and NH_4^+ nutrition in a steady-state nitrogen environment. Expression of this isotope effect is dependent upon external nitrogen being well-mixed and near steady-state with respect to concentration and $\delta^{15}N$ over time. In a solid substrate, fractionation will be influenced by factors that include localized nutrient depletion, heterogeneity in nutrient availability and isotopic composition and cation exchange capacity versus a hydroponics solution. Water holding capacity of pore space within the sand substrate may limit complete solution replacement with each successive fertilizer application. Since plants discriminate against ^{15}N, the substrate will, therefore, become gradually enriched. If complete replacement does not occur, there will be gradual

enrichment of the sand substrate over time that may, in part, explain an increase in plant $\delta^{15}N$ of sand-grown plants relative to hydroponically grown plants. Here, we assayed the hydroponics solution but the sand culture was not assayed in the same way. However, daily fertilizer applications and flushing of the sand media with fresh nutrient solution would prevent large changes in the substrate $\delta^{15}N$.

Another possible reason that may explain the differences in $\delta^{15}N$ between hydroponic and sand media is bacterial transformation of the nitrogen source (Bedard-Haughn et al. 2003; Högberg et al. 2014) that may result in the increase of substrate $\delta^{15}N$. Denitrification or volatilization can have a discrimination factor of between 40 and 60 ‰ (Bedard-Haughn et al. 2003).Therefore, even small amounts of nitrogen lost from the system can lead to enrichment of the solution. In this experiment, frequent fertilization would have replaced the solution but if small amounts of enriched solution were left behind, the substrate $\delta^{15}N$ can vary over time. Similar to how insufficient replacement of nitrogen could affect the $\delta^{15}N$ of the source nitrogen, bacterial transformation that results in volatilization of soil nitrogen pools can affect $\delta^{15}N$ of nitrogen in sand culture and should be something considered for future experiments in non-hydroponic conditions.

Localized depletion around the rhizosphere may also reduce the amount of nitrogen isotope discrimination observed because of the enrichment of the rhizosphere nitrogen relative to the bulk media pool. The magnitude of enrichment should be a function of the size of the nitrogen pool, diffusion rates between the rhizosphere and bulk substrate and discrimination potential of the plant growing in the media. Although more controlled than the natural environment, non-hydroponic growth media have potential to affect the discrimination observed because of heterogeneity in the nitrogen environment within the growth media. The greater discrimination observed in the hydroponic system likely reflects the constant circulation and frequent re-supply of a large volume of fresh media.

Biomass and nitrogen accumulation in the plant can be outward indicators of nitrogen source preference. Consistent with the previous results from other species within the genus *Populus* (Kalcsits and Guy 2013b), aspen was larger when grown with NO_3^- as the sole nitrogen source compared to NH_4^+ as the sole nitrogen source. Furthermore, Min et al. (1998) reported greater efficiencies in NO_3^- acquisition in this species than in the conifers. Conifers are generally considered to prefer NH_4^+ over NO_3^- (Kronzucker et al. 1997) and when grown hydroponically or in sand culture, growth was greater when grown with either NH_4^+ only or a mix of NO_3^- and NH_4^+. It should be noted that the results obtained with seedlings in these experiments may not reflect nitrogen-use patterns of older trees since older trees rely more heavily on stored nitrogen rather than pure nitrogen acquisition. However, given the known ecology of the species used in this study, the three conifer species used are more adapted to acidic, boreal soils and later succession stages that have higher proportions of ammonium than nitrate and aspen is more adapted to earlier succession soils that contain higher amounts of nitrate (Kronzucker et al. 1997). There were no outward indications of nitrogen deficiency at low substrate concentrations or with different nitrogen sources indicating that nitrogen supply was sufficient to support adequate plant health. Tissue nitrogen concentrations were not much different between treatments, except in aspen (Table 1) where nitrogen concentrations were greater than when grown with nitrate. Using two other poplar species, Luo et al. (2013) also reported increased tissue nitrogen concentrations in root and leaves under higher nitrogen availability. Adequate tissue nitrogen concentrations at 0.1 mM NO_3^- suggest that nitrogen was not limiting. A constant supply of 1.5 mM NO_3^- surpasses the availability of NO_3^- typical of natural forest ecosystems (Prescott et al. 2003). While biomass and nitrogen concentrations may provide an effective assay for nitrogen source preference, nitrogen isotope composition can complement this to provide more information on the physiological reasons underpinning these preferences.

In this experiment, there were significant differences observed in nitrogen isotope discrimination among species that were grown either hydroponically or in sand. Discrimination was limited in sand culture relative to hydroponics conditions indicating the absence of steady-state nitrogen conditions. A steady-state nitrogen environment is more easily maintained in hydroponics. However, the growing environment is less similar to the conditions found in the field. Although maximum discrimination was not observed in sand culture, enrichment of leaf $\delta^{15}N$ relative to root $\delta^{15}N$ was still observed. Intra-specific differences in nitrogen isotope discrimination are consistent with expected partitioning of assimilation in tree seedlings based on previous radioisotope compartmental analysis and enzymatic assays where greater leaf–root differences in nitrogen isotope composition indicate more NO_3^- assimilation in leaves of aspen compared to pine.

Author contribution statement LAK participated in data analysis and interpretation and drafted the manuscript. XJM participated in experimental design and conducted the experiments. RDG conceived and oversaw the study, participated in analysis and interpretation, and edited the final version of the manuscript.

Acknowledgments This work was funded by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant to RDG. LAK was supported by a NSERC Vanier Canada Graduate Scholarship.

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Andrews M (1986) The partitioning of nitrate assimilation between root and shoot of higher plants. Plant Cell Environ 9:511–519

Ariz I, Cruz C, Moran JF, González-Moro MB, García-Olaverri C, González-Murua C, Aparicio-Tejo PM (2011) Depletion of the heaviest stable *N* isotope is associated with NH_4^+/NH_3 toxicity in NH_4^+-fed plants. BMC Plant Biol 11:83

Bauer D, Biehler K, Fock H, Carrayol E, Hirel B, Migge A, Becker TW (1997) A role for cytosolic glutamine synthetase in the remobilization of leaf nitrogen during water stress in tomato. Physiol Plant 99(2):241–248

Bedard-Haughn A, Van Groenigen JW, Van Kessel C (2003) Tracing ^{15}N through landscapes: potential uses and precautions. J Hydrol 272(1):175–190

Bergersen FJ, Peoples MB, Turner GL (1988) Isotopic discriminations during the accumulation of nitrogen by soybeans. Funct Plant Biol 15(3):407–420

Britto DT, Siddiqi MY, Glass AD, Kronzucker HJ (2001) Futile transmembrane NH4+ cycling: a cellular hypothesis to explain ammonium toxicity in plants. PNAS 98(7):4255–4258

Cawse PA (1967) The determination of nitrate in soil solution by ultraviolet spectrometry. Analyst 92:311–315

Comstock J (2001) Steady-state isotopic fractionation in branched pathways using plant uptake of NO_3^- as an example. Planta 214(2):220–234. doi:10.1007/s004250100602

Dijkstra P, Williamson C, Menyailo O, Doucett R, Koch G, Hungate BA (2003) Nitrogen stable isotope composition of leaves and roots of plants growing in a forest and a meadow. Isotopes Environ Health Stud 39:29–39

Evans RD (2001) Physiological mechanisms influencing plant nitrogen isotope composition. Trends Plant Sci 6(3):121–126

Evans RD, Bloom AJ, Sukrapanna SS, Ehleringer JR (1996) Nitrogen isotope composition of tomato (*Lycopersicon esculentum* Mill. cv. T-5) grown under ammonium or nitrate nutrition. Plant Cell Environ 19(11):1317–1323. doi:10.1111/j.1365-3040.1996.tb00010.x

Gessler A, Rienks M, Rennenberg H (2000) NH_3 and NO_2 fluxes between beech trees and the atmosphere—correlation with climatic and physiological parameters. New Phytol 147:539–560

Glass ADM, Britto DT, Kaiser BN et al (2002) The regulation of nitrate and ammonium transport systems in plants. J Exp Bot 53:855–864

Handley LL, Raven JA (1992) The use of natural abundance of nitrogen isotopes in plant physiology and ecology. Plant Cell Environ 15:965–985

Högberg P, Johannisson C, Högberg MN (2014) Is the high ^{15}N natural abundance of trees in N-loaded forests caused by an internal ecosystem N isotope redistribution or a change in the ecosystem N isotope mass balance? Biogeochem 117:351–358

Husted S, Schjoerring JK, Nielsen KH, Nemitz E, Sutton MA (2000) Stomatal compensation points for ammonia in oilseed rape plants under field conditions. Agric For Meterol 105(4):371–383

Johnson JE, Berry JA (2013) The influence of leaf-atmosphere NH3 (g) exchange on the isotopic composition of nitrogen in plants and the atmosphere. Plant Cell Environ 36(10):1783–1801

Johnson CM, Stout PR, Broyer TC, Carlton AB (1957) Comparative chlorine requirements of different plant species. Plant Soil 8:337–353

Kalcsits L, Guy RD (2013a) Whole plant and organ level nitrogen isotope discrimination indicates modification of partitioning of assimilation, fluxes and allocation of nitrogen in knockout lines of *Arabidopsis thaliana*. Physiol Plant 149:249–259

Kalcsits LA, Guy RD (2013b) Quantifying remobilization of pre-existing nitrogen from cuttings to new growth of woody plants using ^{15}N at natural abundance. Plant Methods 9:27

Kalcsits LA, Buschhaus HA, Guy RD (2014) Nitrogen isotope discrimination as an integrated measure of nitrogen fluxes, assimilation and allocation in plants. Physiol Plant 151(3):293–304

Karsh KL, Granger J, Kritee K, Sigman DM (2014) Eukaryotic assimilatory nitrate reductase fractionates N and O isotopes with a ratio near unity. Environ Sci Technol 46:5727–5735

Kolb KJ, Evans RD (2003) Influence of nitrogen source and concentration on nitrogen isotopic discrimination in two barley genotypes (*Hordeum vulgare* L.). Plant Cell Environ 3:1431–1440

Kronzucker HJ, Siddiqi MY, Glass AD (1997) Conifer root discrimination against soil nitrate and the ecology of forest succession. Nature 385(6611):59–61

Li H, Li M, Luo J, Cao X, Qu L, Gai Y, Luo ZB (2012) N-fertilization has different effects on the growth, carbon and nitrogen physiology, and wood properties of slow-and fast-growing Populus species. J Exp Bot 63(17):6173–6185

Luo J, Li H, Liu T, Polle A, Peng C, Luo ZB (2013) Nitrogen metabolism of two contrasting poplar species during acclimation to limiting nitrogen availability. J Exp Bot 64(14):4207–4224

Min X, Siddiqi MY, Guy RD, Glass ADM, Kronzucker HJ (1998) Induction of nitrate uptake and nitrate reductase activity in trembling aspen and lodgepole pine. Plant Cell Environ 21(10):1039–1046

Min X, Siddiqi MY, Guy RD, Glass ADM, Kronzucker HJ (1999) A comparative study of fluxes and compartmentation of nitrate and ammonium in early-successional tree species. Plant Cell Environ 22(7):821–830

Min X, Siddiqi MY, Guy RD, Glass ADM, Kronzucker HJ (2002) A comparative study of fluxes and compartmentation of nitrate and ammonium in early-successional tree species. Plant Cell Environ 22:821–830

Peuke AD (2010) Correlations in concentrations, xylem and phloem flows, and partitioning of elements and ions in intact plants. A summary and statistical re-evaluation of modelling experiments in *Ricinus communis*. J Exp Bot 61:635–655

Peuke AD, Gessler A, Rennenberg H (2006) The effect of drought on C and N stable isotopes in different fractions of leaves, stems and roots of sensitive and tolerant beech ecotypes. Plant Cell Environ 29(5):823–835. doi:10.1111/j.1365-3040.2005.01452.x

Peuke AD, Gessler A, Tcherkez G (2013) Experimental evidence for diel δ15N-patterns in different tissues, xylem and phloem saps of castor bean (*Ricinus communis* L.). Plant Cell Environ 36:2219–2228

Prescott CE, Hope GD, Blevins LL (2003) Effect of gap size on litter decomposition and soil nitrate concentrations in a high-elevation spruce fir forest. Can J For Res 33(11):2210–2220

Pritchard ES, Guy RD (2005) Nitrogen isotope discrimination in white spruce fed with low concentrations of ammonium and nitrate. Trees Struct Funct 19:89–98

Robinson D (2001) $\delta^{15}N$ as an integrator of the nitrogen cycle. Trends Ecol Evol 16(3):153–162. doi:10.1016/S0169-5347(00)02098-X

Schjoerring JK, Husted S, Mack G, Mattson M (2002) The regulation of ammonium translocation in plants. J Exp Bot 53:883–890

Solorzano L (1969) Determination of ammonia in natural waters by the phenol-hypochlorite method. Limnol Oceanogr 1969(14): 799–801

Tcherkez G, Hodges M (2008) How stable isotopes may help to elucidate primary nitrogen metabolism and its interaction with (photo) respiration in C(3) leaves. J Exp Bot 59:1685–1693

Waser NA, Yu Z, Yin K, Nielsen B, Harrison PJ, Turpin DH, Calvert SE (1999) Nitrogen isotopic fractionation during a simulated diatom spring bloom: importance of N-starvation in controlling fractionation. Marine Ecol Prog Ser 179:291–296