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MINIREVIEW

Nitrogen isotope discrimination as an integrated measure of nitrogen fluxes, assimilation and allocation in plants

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Fractionation of nitrogen isotopes between a plant and its environment occurs during uptake and assimilation of inorganic nitrogen. Fractionation can also occur between roots and the shoot. Under controlled nitrogen conditions, whole-plant and organ-level nitrogen isotope discrimination ($\Delta^{15}N$) is suggested to primarily be a function of three factors: nitrogen efflux back to the substrate relative to gross influx at the root (efflux/influx), the proportion of net influx assimilated in the roots and the export of remaining inorganic nitrogen for assimilation in the leaves. Here, an isotope discrimination model combining measurements of $\delta^{15}N$ and nitrogen content is proposed to explain whole-plant and organ-level variation in $\delta^{15}N$ under steady-state conditions and prior to any significant retranslocation. We show evidence that nitrogen isotope discrimination varies in accordance with changes to nitrogen supply or demand. Increased whole-plant discrimination (greater $\Delta^{15}N$ or more negative $\delta^{15}N$ relative to the source nitrogen $\delta^{15}N$) indicates increased turnover of the cytosolic inorganic nitrogen pool and a greater efflux/influx ratio. A greater difference between shoot and root $\delta^{15}N$ indicates a greater proportion of inorganic nitrogen being assimilated in the leaves. In addition to calculations of integrated nitrogen-use traits, knowledge of biomass partitioning and nitrogen concentrations in different plant organs provides a spatially and temporally integrated, whole-plant phenotyping approach for measuring nitrogen-use in plants. This approach can be used to complement instantaneous cell- and tissue-specific measures of nitrogen use currently used in nitrogen uptake and assimilation studies.

Introduction

Over the past three decades, there has been substantial progress made towards the understanding of the physiological processes underlying nitrogen uptake and utilization in plants (Glass et al. 2002, Hirel et al. 2007, Masclaux-Daubresse et al. 2010). However, gains in plant nitrogen use efficiency have been limited, in part, because of the complexity of nitrogen uptake, acquisition and utilization. Characterization of nitrogen fluxes throughout plant development is difficult because of spatial (Hawkins and Robbins 2010, Bloom et al. 2012, Luo et al. 2013) and temporal (Delhon et al. 1995, Wang et al. 2012) heterogeneity in these fluxes. It can be technically difficult to integrate instantaneous measures of inorganic fluxes to account for this heterogeneity. Therefore, the development of an integrated measure of nitrogen fluxes would be a valuable complement to instantaneous measures of nitrogen fluxes used for characterizing nitrogen fluxes at the molecular and cellular levels.

Nitrogen isotopes at natural abundance have potential to provide integrated process information for nitrogen fluxes, assimilation and allocation (Robinson et al. 1998, Comstock 2001, Evans 2001). Change in the isotopic composition of plant tissues relative to source nitrogen (fractionation) arises through a kinetically determined process whereby, during assimilation of inorganic nitrogen, the heavier isotope (^{15}N) is 'discriminated'

against causing a relatively greater fraction of the lighter isotope (^{14}N) to be incorporated into product (Handley and Raven 1992). Isotopic compositions are expressed as $\delta^{15}N$ values (‰):

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

where $R_{product}$ is the $^{15}N/^{14}N$ isotope ratio of the sample and $R_{standard}$ is the isotope ratio of a known standard [$R_{standard}$ (air) = 0.0036765]. This value is then used to calculate nitrogen isotope discrimination ($\Delta^{15}N$) relative to the source (Farquhar et al. 1989):

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

Since $1 + \delta^{15}N_{product}/1000$ will not deviate significantly from 1 when isotope ratios are close to 0‰, discrimination ($\Delta^{15}N$) can also be closely approximated by $\delta^{15}N_{product} - \delta^{15}N_{substrate}$ and expressed as a relative change in isotope composition relative to the source (Evans 2001, Pritchard and Guy 2005, Kalcsits and Guy 2013a, 2013b).

The fundamental restriction preventing application of the basic kinetic isotope fractionation model to nitrogen is that nitrogen is usually not available from an open- or steady-state environment and is spatially, temporally and chemically heterogeneous (Nordin et al. 2001). Localized depletion in the rhizosphere can cause variation in substrate $\delta^{15}N$ that may complicate physiological interpretation of plant $\delta^{15}N$ values (Robinson 2001). There can be no discrimination if the substrate pool is fully consumed as it becomes available. Consequently, plant nitrogen isotope composition in natural environments often reflects the isotopic composition of the environmental substrate (Dawson et al. 2002). In contrast, for carbon isotope discrimination in plants, air represents an unchanging pool of carbon where isotopic composition and concentration are relatively stable. Therefore, changes in $\delta^{13}C$ reflect physiology of the plant (Farquhar et al. 1982); namely, the balance between CO_2 supply (conductance) and CO_2 demand (assimilation).

Analogous to the fractionation of carbon isotopes during photosynthesis, when the nitrogen environment is kept at steady-state (e.g. under carefully controlled hydroponic conditions), nitrogen isotope discrimination will depend on the relationship between nitrogen supply and nitrogen demand (Evans 2001, Pritchard and Guy 2005, Kalcsits and Guy 2013a, 2013b). As such, to be able to use nitrogen isotope discrimination as a tool to identify nitrogen uptake and assimilation traits using small changes in nitrogen isotopes at natural abundance, the substrate concentration must be homogenous around the roots and, for genotypic comparisons, plants must be exposed to the same homogenous environment. If these conditions are met, variability in $\delta^{15}N$ between and within individual plants will reflect differences in internal physiology rather than the $\delta^{15}N$ of the external environment.

Discrimination against ^{15}N is thought to primarily occur during the assimilation of inorganic nitrogen in roots and leaves into organic nitrogen (Mariotti et al. 1982, Tcherkez 2011). Nitrate reductase and glutamine synthetase, the two enzymes required for assimilation of nitrate and ammonium, respectively, are presumed responsible for nitrogen isotope discrimination. The instantaneous discrimination factors of these enzymes establish the maximum amounts of discrimination that can occur. Although there have been large ranges in published discrimination associated with these enzymes, best estimates for nitrate reductase and glutamine synthetase are 15.4‰ (Ledgard et al. 1985) and 16.8‰ (Yoneyama et al. 1993), respectively. However, recent evidence suggests that the discrimination factor for nitrate reductase may be higher, at approximately 22‰ (Karsh et al. 2012).

Typically, whole-plant $\Delta^{15}N$ does not reflect the full discrimination factor of the enzyme, even under steady-state conditions (Evans et al. 1996, Yoneyama et al. 2001, Pritchard and Guy 2005), indicating that assimilation is not the only step affecting fractionation. Several models (Robinson et al. 1998, Comstock 2001, Evans 2001) have proposed that net discrimination arises from the partial consumption of the cytoplasmic inorganic nitrogen pool in the roots. If a portion of this pool returns to the medium or is lost to the shoot, fractionation relative to the source, or between organs, will occur (Fig. 1). At the root level, maximum discrimination occurs when the proportion of the cytoplasmic nitrogen lost outside the root, or to the shoot, approaches the amount of nitrogen imported from the substrate. When there is no loss of cytoplasmic nitrogen prior to assimilation, discrimination cannot occur because the entire nitrogen assimilatory pool will eventually be converted to organic nitrogen in the roots. At the whole-plant level, net discrimination does not occur if no inorganic nitrogen is lost from the root to the substrate, irrespective of the ultimate site of assimilation. Thus, root and whole-plant discrimination should largely be a function of supply of inorganic nitrogen relative to demand for organic nitrogen by the plant.

Since discrimination against ^{15}N occurs in the roots, cytoplasmic inorganic nitrogen will become enriched in ^{15}N (Evans et al. 1996, Yoneyama et al. 2001).

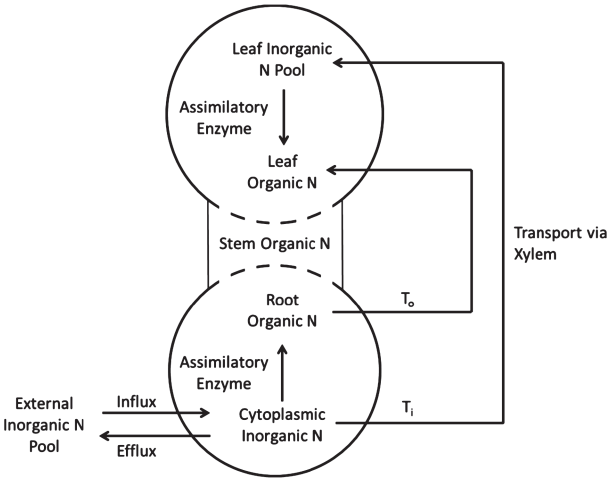

Fig. 1. Plant nitrogen fluxes during the uptake of inorganic nitrogen from the substrate pool and assimilation into either root or leaf organic sink pools. Arrows refer to unidirectional fluxes of nitrogen between pools. To, organic nitrogen translocated to the leaves; Ti, inorganic nitrogen translocated to the leaves. Stem tissue is assumed not to assimilate inorganic nitrogen and receives organic nitrogen from either the roots (via xylem) or the leaves (via phloem), or a mixture of the two.

Enriched root cytoplasmic inorganic nitrogen is also the source of inorganic nitrogen transported from the root to the shoot; therefore, leaf-assimilated nitrogen will be enriched relative to the root (Evans et al. 1996, Yoneyama et al. 2001). Consequently, cycling across the root plasma membranes and root vs shoot partitioning of nitrogen assimilation can be estimated from knowledge of dry masses, nitrogen concentrations and $\delta^{15}N$ values of roots, stems and leaves. Discrimination is determined by the nitrogen supply relative to demand where if supply increases or demand decreases, discrimination can be expected to increase and *vice versa*. The use of $\Delta^{15}N$ has potential as an integrated proxy indicator of nitrogen fluxes and assimilation partitioning in plants. In this paper, we provide the derivation of a model used to calculate multiple nitrogen-use traits, outline the primary assumptions required for this model, present evidence of environmental modification of nitrogen isotope discrimination and describe some of the work required to refine and better constrain the model.

Calculation of nitrogen-use traits from biomass, nitrogen concentration and nitrogen isotope composition

Using an isotope mass balance approach, a number of time-integrated nitrogen-use traits for plants grown under controlled hydroponic conditions can be calculated (See Table 1 for definitions of abbreviations). Assuming that there is no loss of organic nitrogen from the roots or leaves, the assimilation-averaged net flux of inorganic nitrogen across the root is equal to the total plant nitrogen divided by the root biomass:

$$\text{Net flux } \left(\text{mmol N mg}^{-1}\text{ dw}\right) = \frac{N_{total}}{Biomass_{root}} \quad (3)$$

For a rosette plant (e.g. Arabidopsis), the mass balance equation for plant $\Delta^{15}N$ is:

$$\Delta^{15}N_{plant} = \left(f_{root} \times \Delta^{15}N_{root}\right) + \left(f_{leaf} \times \Delta^{15}N_{leaf}\right) \quad (4)$$

Or in the case for woody plants or other species with a substantive stem:

$$\Delta^{15}N_{plant} = \left(f_{root} \times \Delta^{15}N_{root}\right) + \left(f_{stem} \times \Delta^{15}N_{stem}\right) + \left(f_{leaf} \times \Delta^{15}N_{leaf}\right) \quad (5)$$

where $\Delta^{15}N_i$ is discrimination and f_i is equal to the fraction of tissue nitrogen contributing to overall plant nitrogen content. Although for woody plant species, we treat the plant as having three major organs (Fig. 1),

Table 1. List of abbreviations used in the text.

Variables	Definitions
$\delta^{15}N$	Stable nitrogen isotope relative abundance calculated using Eqn 1 (‰)
$\Delta^{15}N_i$	Isotope discrimination for a sample or pool (*i*) expressed using Eqn 2 (‰)
f_i	The fraction of nitrogen that is *i* for a given nitrogen pool
N_i	The nitrogen content of a sample calculated from the biomass and analyzed nitrogen concentration (μmol)
$[N]_i$	The nitrogen concentration of a sample (μmol mg^{-1} dw)
Net flux	Total plant nitrogen expressed as per unit of root biomass
$N_{leaf\ pool}/N_{total}$	The fraction of plant nitrogen coming from the general leaf nitrogen pool. This includes a fraction of stem nitrogen and all leaf nitrogen
Ti/Tt	The proportion of leaf nitrogen (Tt) that was transported to the leaves as inorganic nitrogen (Ti) (expressed as a fraction)
P_{root}	The proportion of plant assimilation occurring in the roots (expressed as a fraction)
E/I	Efflux/influx across the plasma membrane of the root. Efflux is equal to the net loss of inorganic nitrogen from the root. Influx is equal to the gross influx into the root from the substrate
Δ_{enz}	The discrimination factor of the assimilatory enzyme

we assume only leaves and roots are the major sites of nitrogen assimilation. Stem $\Delta^{15}N$ can be predicted by a mass balance equation using the leaf and root $\Delta^{15}N$ values as end members depending on the source for that stem nitrogen:

$$\Delta^{15}N_{stem} = \left(\Delta^{15}N_{root} \times f_{stem\ root}\right) + \left(\Delta^{15}N_{leaf} \times f_{stem\ leaf}\right) \quad (6)$$

Eqn 5 can be rearranged to yield the fraction of stem N that is from the roots ($f_{stem\ root}$) or the leaves ($f_{stem\ leaf}$). For the fraction from leaves, and noting that $f_{stem\ root} = 1 - f_{stem\ leaf}$:

$$f_{stem\ leaf} = \frac{\left(\Delta^{15}N_{stem} - \Delta^{15}N_{root}\right)}{\left(\Delta^{15}N_{leaf} - \Delta^{15}N_{root}\right)} \quad (7)$$

To partition between leaf and root nitrogen pools and their isotopic signatures, the contributions of each to total plant nitrogen content needs to be determined. The proportion of total plant nitrogen found in the leaf pool ($N_{leaf\ pool}/N_{total}$) is then a function of plant tissue nitrogen concentration and tissue mass:

$$\frac{N_{leaf\ pool}}{N_{total}} = \frac{\left(Biomass_{leaf} \times [N]_{leaf}\right) + \left(f_{stem\ leaf} \times Biomass_{stem} \times [N]_{stem}\right)}{\left(Biomass_{plant} \times [N]_{plant}\right)} \quad (8)$$

where $[N]_i$ is the bulk tissue nitrogen concentration. $\Delta^{15}N_{plant}$ can then be described as:

$$\Delta^{15}N_{plant} = \frac{N_{leaf\ pool}}{N_{total}} \times \Delta^{15}N_{leaf} + \left(1 - \frac{N_{leaf\ pool}}{N_{total}}\right) \times \Delta^{15}N_{root} \quad (9)$$

Most root nitrogen will be organic ($\Delta^{15}N_{root\ organic}$) but some small fraction ($f_{root\ inorganic}$) will be inorganic ($\Delta^{15}N_{root\ inorganic}$). Consequently, $\Delta^{15}N_{root}$ can be expanded to:

$$\Delta^{15}N_{root} = \Delta^{15}N_{root\ organic} \times \left(1 - f_{root\ inorganic}\right) + \Delta^{15}N_{root\ inorganic} \times \left(f_{root\ inorganic}\right) \quad (10)$$

and rearranged to yield $\Delta^{15}N_{root\ organic}$:

$$\Delta^{15}N_{root\ organic} = \frac{\Delta^{15}N_{root} - \Delta^{15}N_{root\ inorganic} \times \left(f_{root\ inorganic}\right)}{\left(1 - f_{root\ inorganic}\right)} \quad (11)$$

Relative to $\Delta^{15}N_{root\ organic}$, $\Delta^{15}N_{root\ inorganic}$ is increased by the discrimination factor associated with the assimilatory enzyme (Δ_{enz}). For present purposes, however, we set $f_{root\ inorganic}$ to 0 to simplify this calculation but emphasize that this parameter could be used if $f_{root\ inorganic}$ were assayed from tissue samples in addition to isotopic composition and total nitrogen concentration.

To partition assimilation, it is assumed that all inorganic nitrogen translocated to the shoot (Ti; Fig. 1) enters the xylem in isotopic equilibrium with the root cytoplasmic pool (i.e. $\Delta^{15}N_{xylem\ inorganic} = \Delta^{15}N_{root\ inorganic}$). Organic nitrogen delivered to the shoot (To; Fig. 1) is assumed to not differ isotopically from root-assimilated organic nitrogen. Therefore, net leaf discrimination is the mass balance of $\Delta^{15}N_{xylem\ inorganic}$ and $\Delta^{15}N_{root\ organic}$ and can be calculated as:

$$\Delta^{15}N_{leaf} = \Delta^{15}N_{xylem\ inorganic} \times \left(\frac{Ti}{Tt}\right) + \Delta^{15}N_{root\ organic} \times \left(1 - \frac{Ti}{Tt}\right) \quad (12)$$

where Ti/Tt is equal to the proportion of total leaf pool nitrogen (Tt; Fig. 1) transported as inorganic nitrogen from the roots and assimilated in the leaves.

As is the case for inorganic nitrogen in root cytoplasm, $\Delta^{15}N_{xylem\ inorganic}$ exceeds $\Delta^{15}N_{root\ organic}$ by an amount equal to Δ_{enz}. Enrichment of the inorganic nitrogen in root tissue relative to organic nitrogen has been shown by Evans et al. (1996) and Yoneyama et al. (2001). Since root inorganic nitrogen in the cytoplasm is the source for inorganic nitrogen loaded into the xylem, on an integrated basis, these two values should be similar. More work, however, is needed to confirm this. By substituting $\Delta^{15}N_{xylem\ inorganic}$ in Eqn 12 with $\Delta^{15}N_{root\ organic} + \Delta_{enz}$, Ti/Tt can then be calculated by rearrangement:

$$\frac{Ti}{Tt} = \frac{\left(\Delta^{15}N_{leaf} - \Delta^{15}N_{root\ organic}\right)}{\Delta_{enz}} \quad (13)$$

According to Eqn 13, if the difference between leaf $\Delta^{15}N$ and root $\Delta^{15}N$ is equal to Δ_{enz}, all leaf nitrogen must be assimilated in the leaves and no organic nitrogen is translocated from the roots. Likewise, if the difference is 0, no inorganic nitrogen is translocated from the roots. Assuming that in actively growing plants, there is negligible translocation of nitrogen from the shoots to roots, the proportion of total plant nitrogen that was assimilated in the leaves is a product of $N_{leaf\ pool}/N_{total}$ and Ti/Tt. The remaining fraction of plant nitrogen must then be assimilated in the roots (P_{root}):

$$P_{root} = 1 - \left(\left(\frac{N_{leafpool}}{N_{total}}\right) \times \frac{Ti}{Tt}\right) \quad (14)$$

Because Ti removes nitrogen from the cytoplasmic pool that then becomes unavailable to root assimilation

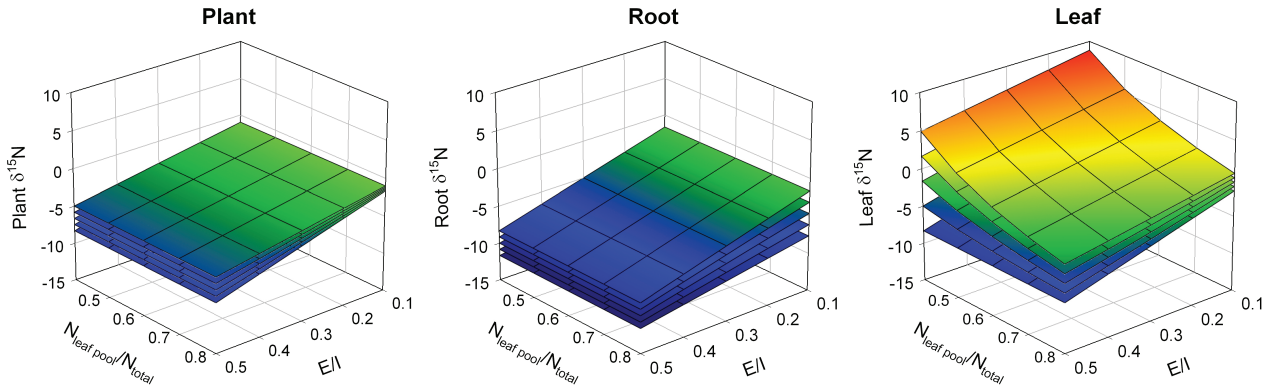

Fig. 2. Sensitivity analysis for calculating plant (A), root (B) and leaf (C) $\delta^{15}N$ relative to source $\delta^{15}N$ (0‰) as a function of efflux/influx (*E*/*I*), the proportion of nitrogen assimilated in the root (P_{root}), and the partitioning of total plant nitrogen to the leaves ($N_{leaf\ pool}/N_{total}$). Each plane represents a P_{root} value held constant while $N_{leaf\ pool}/N_{total}$ and *E*/*I* vary (P_{root} is 0.6, 0.7, 0.8, 0.9 and 1 from top to bottom for A and C, and from bottom to top for B).

or efflux (*E*), whole-plant discrimination ($\Delta^{15}N_{plant}$) is proportional to efflux over influx (*E*/*I*) and P_{root}:

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

In Eqn 15, as *E*/*I* and P_{root} approach 1, $\Delta^{15}N_{plant}$ approaches the discrimination factor of the enzyme and, likewise, as those two variables approach 0, $\Delta^{15}N_{plant}$ approaches 0. This relationship, expressed in terms of tissue $\delta^{15}N$ values, is illustrated in the first panel of Fig. 2 for ranges of *E*/*I* and P_{root} values. Note that the partitioning of nitrogen assimilation between organs will not itself alter whole-plant (first panel) or root $\delta^{15}N$ (second panel) but will affect $\delta^{15}N_{leaf}$ (third panel) and, by extension, plant stem tissue (Eqn 6), because it determines the portion of total shoot nitrogen that is root assimilated. Also note that $\delta^{15}N_{leaf}$ can be greater than the source if P_{root} and $N_{leaf\ pool}/N_{total}$ are relatively low, and especially if *E*/*I* is also low. Plant and root $\delta^{15}N$ will still be lower than the source in all cases. Although Fig. 2 represents theoretical ranges in whole-plant and organ-level nitrogen isotope discrimination, in reality, this range is much smaller. By rearranging Eqn 15, an estimate of *E*/*I* can be obtained from:

$$\frac{E}{I} = \frac{\Delta^{15}N_{plant}}{\Delta_{enz} \times P_{root}} \quad (16)$$

Measures of root and leaf assimilation activities are obtained by calculating the amount of plant nitrogen derived from either roots or leaves and expressing it as a function of the biomass of either roots or leaves:

$$\text{Root assimilation activity } (\mu\text{mol N mg}^{-1}\text{ dw}) = \frac{N_{total} \times P_{root}}{Biomass_{root}} \quad (17)$$

$$\text{Leaf assimilation activity } (\text{mmol N mg}^{-1}\text{ dw}) = \frac{N_{total} \times (1 - P_{root})}{Biomass_{leaf}} \quad (18)$$

Model assumptions

To reduce complications to the model, the following simplifying assumptions were made which will be discussed in detail. There is opportunity, however, to empirically test these assumptions that will either confirm or more likely, greater refine the understanding of nitrogen isotope discrimination in plants.

Homogeneous nitrogen environment

Environmental heterogeneity or localized rhizosphere depletion of inorganic nitrogen can reduce the amount of isotope discrimination observed in plants or complicate the interpretation of discrimination. For this reason, field or even media-grown plants are not suitable for assaying nitrogen isotope discrimination in plants (see Kalcsits 2013, Kalcsits and Guy 2013a, 2013b). Because of restricted mixing and long diffusion distances; sand, perlite or any other structured soil medium may result in localized ^{15}N enrichment of substrate around the roots. Plants may show reduced discrimination if diffusion becomes rate limiting. Furthermore, in either soil or liquid media, periodic addition of nitrogen to maintain a constant supply without completely replacing residual nitrogen will result in an enrichment of the solution over time.

A well-aerated hydroponics solution is best to maintain a homogeneous nitrogen environment for plants where nitrogen isotope composition is an indication of internal physiological processes, and not the external

environment. To maintain homogeneity in the nitrogen environment, frequent monitoring of inorganic nitrogen concentration is required. The volume of the solution must be large enough to ensure minimal depletion (no more than 10%) of the substrate over time and when required, the solution must be completely replaced using the same nitrogen source (chemical and isotopic composition). Similar to using too small of a volume, plants that become too large have high nitrogen demand that makes it difficult to ensure a homogeneous nitrogen environment.

Loss of organic nitrogen from the roots or the shoot does not have a large effect on nitrogen isotope composition

Organic nitrogen lost from roots can impact isotope composition if it is (1) lost in significant quantities and/or (2) the isotope composition of previously assimilated nitrogen lost from the plant strongly deviates from the bulk isotope composition of the whole plant.

Exudation of organic nitrogen from the root has been shown to occur in many plant species under many conditions and contributes to improved bioavailability of macro and micronutrients (Johnson et al. 1996, Dakora and Phillips 2002). However, the amount of nitrogen exuded is only a small fraction of the total plant nitrogen budget. For example, Hale et al. (1975) provided a range of exudation rates from roots in multiple species, and in all cases, exudation was in the μmol plant^{-1} week^{-1} range. Carvalhais et al. (2011) reported similar amino acid exudation rates in *Zea mays* (maize). In the case of most plants, this would amount to an inconsequential fraction of nitrogen lost from the plant. Robinson et al. (1998) suggested that exudation of enriched organic nitrogen causes roots to have lower δ^{15}N than leaves. In order to significantly alter isotopic mass balance in this way, organic nitrogen exudation from roots would need to be compound-specific and proportionately high enough to impact δ^{15}N. The difference in δ^{15}N between different forms of organic N rarely exceeds 10‰ (Werner and Schmidt 2002, Gauthier et al. 2013). From previous work quantifying the proportions of individual nitrogen-containing compounds exuded from roots, the impact on root δ^{15}N would be small. There is also the possibility that organic nitrogen loss could occur during root mortality or during stress events that result in loss of stability of the plasma membrane. However, under optimum, hydroponic growing conditions, root mortality or stress exposure would not be expected.

Losses of previously assimilated nitrogen from leaves also have potential to affect nitrogen isotope composition of plants. Similar to other nitrogen transformations, there are likely to be kinetic isotopic effects associated with either volatilization of NH_3 or nitrous oxide emissions from leaves that discriminate against ^{15}N. The reported discrimination factor for volatilization of NH_3 is approximately 40‰ (Mariotti et al. 1982). Although nitrogen losses from leaves as NH_3 have been observed, they are unlikely to be of large effect. Schjoerring and Mattson (2001) reported that 1–4% of shoot nitrogen was volatilized as NH_3 from a variety of crop plants and, importantly, that the majority of this loss occurred during senescence, which we have carefully avoided. Johnson and Berry (2013) modeled the isotope fractionation associated with volatilization of NH_3 from leaves of actively growing plants as a function of NH_3 compensation points in leaves and atmospheric NH_3 concentration. The fractionation associated with these losses was minimal (i.e. <1‰) when stomatal conductance was high and temperatures were below 25°C, similar to conditions in which plants would be grown using the model proposed above.

Translocation of nitrogen from shoot to root

By assigning a unidirectional flux of organic and inorganic nitrogen between roots and shoots, it provides end points for determining partitioning of leaf and root-assimilated nitrogen present in the leaves. Although this condition may not be met under many circumstances, particularly in senescent or perennial plants, it is likely valid for young, rapidly growing plants prior to any changes in phenology or exposure to abiotic or biotic stress. Translocation between the shoots and roots through the phloem has been shown to be a negligible fraction relative to the upward fluxes of inorganic and organic nitrate through the xylem (Pate 1980). Comstock (2001) included translocation from the shoot to the root in their intra-plant nitrogen isotope discrimination model. Although some organic nitrogen may move into roots via the phloem, this flux will likely be inconsequential to the mass nitrogen budget during active growth, allowing simplification of the model and calculation of the partitioning of assimilation between roots and leaves. This would have to be considered under conditions where one would expect remobilization of large pools of nitrogen (e.g. abiotic stress or phenological changes) from the shoot to the root.

The Δ^{15}N of the organic fraction in the root compared to the bulk tissue Δ^{15}N

Under lower substrate concentrations in the μ*M* range, the impact of setting the fraction of inorganic nitrogen in the root to zero is expected to be small. In the

proposed model, the measured tissue $\delta^{15}N$ value was considered to be equal to the organic fraction (i.e. we set $f_{root\ inorganic}$ to 0 in Eqn 11). Since cytoplasmic inorganic nitrogen in the root will be enriched relative to the root organic nitrogen pool, the bulk root $\delta^{15}N$ will then be enriched relative to the organic $\delta^{15}N$ in the root. By setting the fraction of inorganic nitrogen in the root to 0, there is an underestimation of the isotopic difference between root- and leaf-assimilated organic nitrogen. In turn, the model underestimates Ti/Tt to some extent, with consequent effects on the calculation of P_{root} and *E*/*I*. Sampling for cytoplasmic nitrogen content or isotope composition at harvest is possible; however, single time-point measurements are not stable (Peuke et al. 2013) and may not correspond with the time-integrated information provided by measuring the isotope composition. By acquiring a time-integrated estimate of the fraction of inorganic nitrogen in the cytoplasm under specific nitrogen conditions, a more accurate estimate of root organic $\delta^{15}N$ could be used.

The fraction of inorganic nitrogen in the roots has been shown to vary from 1 to 10%, depending on growing conditions and nitrogen source (Evans et al. 1996, Yoneyama et al. 2001, Black et al. 2002). This fraction increases as substrate concentration increases (Kolb and Evans 2003). Yoneyama et al. (2001), e.g. found that the cytoplasmic inorganic nitrogen pool can account for as much as 10% of the overall nitrogen in the root when grown with either 2 or 8 m*M* NO_3^-. Evans et al. (1996) reported much lower fractions of inorganic nitrogen (approximately 1–2%) in roots of *Solanum lycopersicum* (tomato) grown on an ecologically relevant NH_4^+ concentration (50 μ*M*). Within reported ranges of between 1 and 10% of root nitrogen being inorganic nitrogen, root discrimination can be underestimated by as much as approximately 1.5‰. As the $\delta^{15}N$ of the organic nitrogen deviates from the bulk tissue $\delta^{15}N$ (i.e. a greater fraction of inorganic nitrogen in the root), both P_{root} and *E*/*I* decrease. Additionally, since the differences in the fraction of inorganic nitrogen in root tissue among genotypes are likely going to be small, applications that make relative comparisons among genotypes are valid.

For leaves, nitrogen would be considered a closed system during active, vegetative growth (i.e. no loss of nitrogen). Therefore, $\Delta^{15}N_{leaf}$ is the mass balance between inorganic ($\Delta^{15}N_{xylem\ inorganic}$) and organic ($\Delta^{15}N_{root\ organic}$) nitrogen translocated to the shoot from the root via the xylem (Eqn 11). Comstock (2001) assumed that the isotopic composition of the organic fraction assimilated in the leaves was equal to xylem inorganic nitrogen, and this must be true if all inorganic nitrogen translocated through the xylem is assimilated. However, a certain fraction will be unassimilated at any point in time and cytoplasmic inorganic nitrogen will be enriched in ^{15}N relative to leaf-assimilated organic nitrogen. In agreement, Gauthier et al. (2013) recently reported that nitrate $\delta^{15}N$ was greater than the organic nitrogen in leaves of *Brassica napus*. Therefore, knowledge of the isotopic composition of leaf inorganic nitrogen is inconsequential to the model because the mass balanced sum of leaf-assimilated organic nitrogen ($\Delta^{15}N_{leaf\text{-}assimilated}$) and inorganic nitrogen in the leaves ($\Delta^{15}N_{leaf\ inorganic}$) is equal to the $\Delta^{15}N_{xylem\ inorganic}$ and adjusting leaf $\Delta^{15}N$ to account for the inorganic fraction in the leaf would result in the underestimation of overall leaf $\Delta^{15}N$.

Assimilation in plant organs other than leaves and roots

In most plants, nitrogen assimilation primarily occurs in leaves or roots. However, small amounts of inorganic nitrogen assimilation likely occurs in stems. Currently, there is no information on glutamine synthetase activity in stem tissue, but Hunter (1985) measured nitrate reductase activity in *Glycine max* (soybean) stems and found less than 3% of the activity seen in leaves. In poplar, Black et al. (2002) found a similarly low proportion of nitrate reductase in stems. Although assimilation might also occur in stem tissue, the assimilatory volume would be small in comparison with roots and leaves. In herbaceous species where stems would likely be included in the bulk shoot tissue isotope analysis (e.g. Kalcsits and Guy 2013a) stems would likely behave similar to leaves. The $\delta^{15}N$ of the organic nitrogen assimilated in the stem would be equal to the $\delta^{15}N$ of the inorganic nitrogen source unloaded from the xylem since all inorganic nitrogen unloaded from the xylem into the stem would be assimilated. Thus, to simplify the mixing model, we assigned nitrogen assimilation to either roots or leaves, and treated stem nitrogen as originating from one or the other or both.

Carry-over of isotopically distinct nitrogen in propagules (seeds or cuttings)

Remobilization of nitrogen from seeds or vegetative cuttings has the potential to influence the isotopic composition of new growth. Greater differences in the $\delta^{15}N$ of the source relative to the propagule produce a greater carry-over effect on new growth (Kalcsits and Guy 2013b). However, if the fraction of remobilized nitrogen is small compared to the amount of newly acquired nitrogen, the impact of this carry-over nitrogen will be negligible. In experiments where seed size is large and experimental length short, carry-over nitrogen may have

a larger effect, particularly if the $\delta^{15}N$ of the carry-over nitrogen deviates from the $\delta^{15}N$ of the source nitrogen for the experiment. Using a corrective approach outlined in Kalcsits and Guy (2013b), this carry-over nitrogen can be accounted for and the $\delta^{15}N$ of the new growth adjusted accordingly.

For phenotyping purposes, to provide the most comprehensive snapshot of nitrogen-use, the length of the vegetative phase must be optimized. Phenological changes such as flowering or seed filling should be avoided because of the potential for remobilization of nitrogen from root and shoot tissue to new sink tissue complicating the isotope mass balance model. However, by accounting for previously assimilated nitrogen and intra-plant variation in nitrogen isotope composition, there is opportunity to explore how changes in isotope composition during the transition from vegetative to flowering and seed-filling stages of plants can indicate source/sink relationships of nitrogen within the plant during these processes.

The $\delta^{15}N$ of remobilized organic nitrogen is equal to bulk organic $\delta^{15}N$

There is strong evidence for metabolite-specific variation in $\delta^{15}N$ (Werner and Schmidt 2002, Tcherkez 2011, Gauthier et al. 2013, Peuke et al. 2013). However, the mass balance isotope ratios of these metabolites must still be equal to the mass balance isotope ratio of the bulk tissue. There is strong variation in primary nitrogen-containing compounds being remobilized in the plant (Schurr 1998). For example, ^{15}N-enriched glutamine was observed as the primary transport form of organic nitrogen in the phloem of castor bean leaves (Peuke et al. 2013). A large portion of this nitrogen will be remobilized to developing sink leaves near the meristem. During growth, as a leaf makes the transition from a developing sink leaf (receiving enriched ^{15}N-glutamine) to a source leaf (exporting enriched ^{15}N-glutamine), the bulk isotope balance of the leaf should be approximately equal to the mass balance isotope composition of the organic and inorganic nitrogen received from the root. We acknowledge that there may be variation in $\delta^{15}N$ of leaves throughout the plant depending on sink/source activity and the timing and source of remobilized and assimilated organic nitrogen. Still though, the bulk tissue analysis used in the proposed model is an integrated measure and is therefore not directly comparable to the instantaneous, compound-specific variation in nitrogen isotope composition between organic nitrogen compounds observed in other studies. Using other similar bulk tissue measurements, Kolb and Evans (2002) and Kalcsits and Guy (2013b) both observed no fractionation during the remobilization of nitrogen occurring in spring flush. To better understand within tissue variability in nitrogen isotope discrimination (i.e. cell type or molecular levels), care must be taken to account for spatial and temporal heterogeneity that affects nitrogen fluxes and consequently, also affects measured $\delta^{15}N$ of those individual nitrogen-containing compounds. The problem of making comparisons between integrated isotopic composition of whole-plant tissue and instantaneous measurements of the isotope composition of organic nitrogen compounds within that tissue will need to be addressed in the future to better understand the relationships between source/sink nitrogen movement and compound-specific nitrogen isotope fractionation processes within the plant.

Changes in environmental nitrogen supply or demand affect whole-plant and organ-level nitrogen isotope discrimination

Nitrogen isotope composition in plants has been shown to vary depending on the surrounding environment (Table 2). The relative change of nitrogen isotope discrimination among treatments is thought to be a function of nitrogen supply relative to nitrogen demand. Changes in nitrogen form or concentration of the substrate supply can influence nitrogen isotope discrimination. Yoneyama et al. (2001), Pritchard and Guy (2005) and Kalcsits and Guy (2013a) observed increased discrimination under increased nitrogen supply to *Oryza sativa* L., *Picea glauca* (Moench) Voss and *Arabidopsis thaliana* L., respectively. Increased whole-plant discrimination is an indication of an increase in the efflux/influx ratio which is expected to occur under increased nitrogen supply. Greater nitrogen isotope discrimination was observed when *Populus balsamifera* L. were grown under ammonium compared to nitrate (Kalcsits and Guy 2013b). Increased discrimination and proposed increases in the efflux/influx ratio may be a result of symptoms of ammonium toxicity producing futile cycling of ammonia across the plasma membrane of root cells (Kronzucker et al. 2001, Britto and Kronzucker 2006). Ariz et al. (2011) nicely demonstrated a relationship between ammonium toxicity and isotope discrimination in hydroponically grown plants. Furthermore, Coskun et al. (2013) showed that greater efflux of ammonia occurs under symptoms of ammonium toxicity. The equilibrium isotope effect present between ammonium and ammonia could have an effect of the nitrogen isotope composition of the internal inorganic nitrogen pool in the root, particularly under conditions of ammonium toxicity. Further work is needed to determine the role of ammonia efflux from

Table 2. Summary of the impact of changes to nitrogen supply or demand on nitrogen isotope composition in multiple plant species. For treatment description, 'high' and 'low' represent relative treatment levels for nitrogen supply or expected demand under the described conditions.

	Species	Treatment description	Low (‰)	High (‰)	Reference
Supply					
	Arabidopsis thaliana L.	Hydroponics			Kalcsits and Guy (2013a)
		Low – 100 μ*M* NO_3^-	−3.05	−3.61	
		High – 1000 μ*M* NO_3^-			
	Hordeum vulgare L.	Hydroponics			Kolb and Evans (2003)
		Low – 0.5 m*M* NO_3^-	−0.9	−2.9	
		High – 2 m*M* NO_3^-			
	Oryza sativa L.	Pot-grown			Yoneyama et al. (2001)
		Low – 2 m*M* NO_3^-	0.65	−3.35	
		High – 8 m*M* NO_3^-			
	Multiple species	Hydroponics			Ariz et al. (2011)
		Low – 0.5 m*M* NH_4^+	∼ −4	∼ −9	
		High – 6 m*M* NH_4^+			
Demand					
	H. vulgare L.	Hydroponics			Handley et al. (1997)
		Low – salt stress	∼−1.0	∼0.5	
		High – control			
	Populus trichocarpa Torr. & A. Gray	Hydroponics			Buschhaus (2007)
		Low – ambient CO_2	−3.79	−3.46	
		High – elevated CO_2			
	Thalassiosira weissflogii Hustedt	Culture			Needoba et al. (2004)
		Low – low light	−8.47	−5.18	
		High – standard light			
	Triticum aestivum L.	Hydroponics			Yousfi et al. (2009)
		Low – 17 ds m^{-1}	−0.17	3.14	
		High – no salinity			

roots in determining nitrogen isotope discrimination in plants.

Changes in other environmental conditions can also affect nitrogen isotope discrimination. Yousfi et al. (2009) observed increased nitrogen isotope discrimination in *Triticum aestivum* L. grown under salt stress with a reduced assimilatory demand indicating an increase in the efflux/influx ratio between the root and substrate. Similarly, Needoba et al. (2004) reported increased discrimination in *Thalassiosira weissflogii* Hustedt grown under low light conditions. In contrast, under conditions expected to increase nitrogen demand, *Populus trichocarpa* Torr. & A. Gray grown under elevated carbon dioxide had reduced isotope discrimination compared to plants grown under ambient carbon dioxide conditions (Buschhaus 2007). Changes in environmental conditions that affect both nitrogen demand and overall plant growth can influence nitrogen isotope discrimination through changes to the turnover rate of the cytoplasmic inorganic nitrogen pool in the roots.

Although theoretically expected, consistent ^{15}N depletion of the plant product has only been shown in a handful of studies (Robinson et al. 1998, 2000, Yoneyama et al. 2001, Kolb and Evans 2003, Pritchard and Guy 2005, Kalcsits and Guy 2013a, 2013b). Because nitrogen assimilation is a discriminatory step, whole-plant $\delta^{15}N$ should never be greater than substrate $\delta^{15}N$. In some cases, plant $\delta^{15}N$ has exceeded the source (Evans et al. 1996, Yoneyama et al. 2001).

The difference between root and leaf nitrogen isotope discrimination is an indication of the proportions of leaf nitrogen that is either transported from the root as organic or inorganic nitrogen. There have been few studies that have shown enrichment of leaves relative to roots (Evans et al. 1996, Kolb and Evans 2003, Kalcsits and Guy 2013a, 2013b). As enriched inorganic nitrogen (enriched in ^{15}N by the discrimination factor of the enzyme) transported from the root is assimilated in the leaves, two distinct isotopic signatures for root and leaf-derived organic nitrogen can be estimated. Therefore, through a mass balance calculation, the proportions of each source of nitrogen can be determined for the leaves. There are conditions in which root isotopic composition can exceed leaf nitrogen composition. In situations where there is a gradual drawdown of substrate nitrogen in hydroponics, root nitrogen which gets remobilized and turned over more quickly (source) than nitrogen in the leaves (sink) will be acquired at a later time from a more isotopically enriched substrate than leaf nitrogen. This has been observed in poplar

where significant depletion of the substrate over the course of the experiment produced roots that were isotopically enriched relative to the leaves (Kalcsits, unpublished results). Under conditions where substrate isotope composition is stable over time, shoots will be isotopically enriched relative to the roots. This can be modified by changing nitrogen supply or demand. In Kalcsits and Guy (2013a), *A. thaliana* L. supplied with high nitrate showed an increase in the isotopic difference between the roots and the shoot indicating a greater proportion of assimilation occurring in the leaves. Similarly, *P. balsamifera* L. grown with nitrate had a greater root–shoot difference in isotope composition than ammonium indicating that more nitrogen was assimilated in the leaves when plants were grown with nitrate (Kalcsits and Guy 2013b).

Future research directions

The model proposed here describing how nitrogen isotope composition is affected by changes in internal physiology under steady-state external conditions relies upon well grounded, but unrefined assumptions. Where discussed, these assumptions will need to be empirically tested and the model further refined to better understand temporal, spatial and compound-specific variations in nitrogen isotope composition within plants. Four particular opportunities for further work include: (1) identifying better constrained isotope discrimination factors for nitrate reductase and glutamine synthetase; (2) acquiring time-average estimates of inorganic and organic nitrogen fluxes through xylem into the leaves; (3) quantifying the movement of nitrogen from the shoot to the roots; (4) using compound-specific isotope mass spectrometry to better constrain chemical heterogeneity in nitrogen isotope composition between plant compounds across time and space. Once a better understanding of how these factors affect nitrogen isotope composition is achieved, the model can be further refined to provide more accurate estimations of nitrogen fluxes and assimilation partitioning in plants.

The value of this model lies in phenotyping for nitrogen use. By controlling environmental nitrogen conditions, an assay of integrated physiological nitrogen uptake and assimilation can be performed to identify variation in nitrogen-use patterns within or between species. However, these results may still have applications to field-based measurements of nitrogen isotope composition in plants, particularly under agricultural conditions, where nitrogen availability is greater and generally, more uniform than natural ecosystems. As nitrogen in natural environments is temporally, spatially and chemically heterogeneous, interpretation of nitrogen isotope composition of plants relative to the source from which it was acquired is limited. However, with appropriate nitrogen control and experimental design, there is potential to use the proposed model at field scales by making relative comparisons for discrimination and intra-plant isotope composition between genotypes when grown under field conditions. These conditions require more monitoring and more replication to address isotopic variability that is more inherent in natural environments.

Conclusion

The increasing amount of research on fractionation processes in plants and the use of nitrogen isotope composition in natural and controlled systems have potential to indicate nitrogen cycling processes within plants. Here, we present the derivation of a model explaining tissue-level nitrogen isotope discrimination in plants. We provide evidence that nitrogen isotope discrimination is affected by changes in nitrogen supply or demand in accordance with expectations. However, we do acknowledge this is a proxy measurement and there is opportunity for modification and/or refinement of the model based on empirical testing of the model simplifications. With appropriate experimental controls, this approach has potential to be used as a complementary phenotyping approach for integrated nitrogen uptake efficiency and partitioning of assimilation between leaves and roots of plants that are often difficult to acquire using more traditional assays.

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