frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 23 July 2014
doi: 10.3389/fpls.2014.00355

Nitrate dynamics in natural plants: insights based on the concentration and natural isotope abundances of tissue nitrate

Xue-Yan Liu[1,2]*, Keisuke Koba[2], Akiko Makabe[2] and Cong-Qiang Liu[1]

[1] State Key Laboratory of Environmental Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences, Guiyang, China
[2] Department of Environmental Science on Biosphere, Institute of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Japan

Edited by:
Jan Kofod Schjoerring, University of Copenhagen, Denmark

Reviewed by:
Kristian Holst Laursen, University of Copenhagen, Denmark
Benton N. Taylor, Columbia University, USA

***Correspondence:**
Xue-Yan Liu, State Key Laboratory of Environmental Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences, 46 Guanshui Road, Guiyang, Guizhou Province 550002, China
e-mail: liuxueyan@vip.skleg.cn

The dynamics of nitrate (NO_3^-), a major nitrogen (N) source for natural plants, has been studied mostly through experimental N addition, enzymatic assay, isotope labeling, and genetic expression. However, artificial N supply may not reasonably reflect the N strategies in natural plants because NO_3^- uptake and reduction may vary with external N availability. Due to abrupt application and short operation time, field N addition, and isotopic labeling hinder the elucidation of *in situ* NO_3^--use mechanisms. The concentration and natural isotopes of tissue NO_3^- can offer insights into the plant NO_3^- sources and dynamics in a natural context. Furthermore, they facilitate the exploration of plant NO_3^- utilization and its interaction with N pollution and ecosystem N cycles without disturbing the N pools. The present study was conducted to review the application of the denitrifier method for concentration and isotope analyses of NO_3^- in plants. Moreover, this study highlights the utility and advantages of these parameters in interpreting NO_3^- sources and dynamics in natural plants. We summarize the major sources and reduction processes of NO_3^- in plants, and discuss the implications of NO_3^- concentration in plant tissues based on existing data. Particular emphasis was laid on the regulation of soil NO_3^- and plant ecophysiological functions in interspecific and intra-plant NO_3^- variations. We introduce N and O isotope systematics of NO_3^- in plants and discuss the principles and feasibilities of using isotopic enrichment and fractionation factors; the correlation between concentration and isotopes (N and O isotopes: $\delta^{18}O$ and $\Delta^{17}O$); and isotope mass-balance calculations to constrain sources and reduction of NO_3^- in possible scenarios for natural plants are deliberated. Finally, we offer a preliminary framework of intraplant $\delta^{18}O$-NO_3^- variation, and summarize the uncertainties in using tissue NO_3^- parameters to interpret plant NO_3^- utilization.

Keywords: atmospheric nitrate, denitrifier method, isotopic enrichment, isotopic fractionation, nitrate reductase, oxygen isotope, plant nitrate, soil nitrogen availability

PLANT NITRATE (NO_3^-) IN A NATURAL CONTEXT

Nitrogen (N) is a key factor in the control of the primary productivity in terrestrial plant ecosystems (Vitousek and Howarth, 1991; LeBauer and Treseder, 2008). Among the N species available to plants, ammonium (NH_4^+) is dominant in the inorganic N of unfertilized soils (Schimel and Bennett, 2004) and atmospheric N deposition (Stevens et al., 2011). Some plants prefer NH_4^+ (Britto and Kronzucker, 2013) while the roots of a few plants directly absorb organic N (Chapin et al., 1993; Näsholm et al., 2009; Hill et al., 2013). However, nitrate (NO_3^-) is an important N source for all plants because of its versatile functions in both plant nutrition and physiological regulations (Raven, 2003; Wang et al., 2012). The utilization of NO_3^- (mainly uptake and reduction/assimilation) has been investigated intensively in plants through characterization of related enzymes including nitrate reductase (NR) and nitrite reductase (NiR) and their activities (NRA and NiRA, respectively) in response to different environmental conditions (Beevers and Hageman, 1969; Atkin et al., 1993; Kronzucker et al., 1995; Campbell, 1999). The framework of plant NO_3^- studies has expanded in the past few decades due to the availability of molecular techniques. A few model plants have been used in understanding the transporters responsible for NO_3^- uptake and transportation (Wang et al., 2012). Besides its function in nutrient supply, plant NO_3^- and its metabolism contain unique information related to the mediation of plant physiology, diversity, and the ecosystem N cycle (Crawford, 1995; Tischner, 2000). However, evolution has yielded diverse strategies by which plants acquire N and NO_3^- from natural environments to adapt to changes in ecosystem N availability (Chapin, 1980; Raven and Yin, 1998; Nacry et al., 2013). Therefore, there are considerable uncertainties in assessing the utilization of NO_3^- by plants in natural habitats, which cannot be explained fully by laboratory-based mechanisms because of methodological constraints. Consequently, a great need exists

for a straightforward estimation of plant NO_3^- availability and a mechanistic understanding of the processes controlling plant NO_3^- uptake and reduction. These can enhance our understanding of the role of plant NO_3^- utilization in the ecosystem N cycle and the changes of plant growth and diversity with ecosystem N status (Lambers et al., 2008; Bloom et al., 2010; Boudsocq et al., 2012).

DENITRIFIER METHOD FOR NO_3^- IN NATURAL PLANTS

Natural abundance of stable isotopes in natural plants can integrate the information related to N sources and physiological processes (Högberg, 1997; Robinson, 2001; Craine et al., 2009). The stable isotopes include $\delta^{15}N$, $\delta^{18}O$, and $\delta^{17}O$ for NO_3^-; ^{15}N:^{14}N, ^{18}O:^{16}O, and ^{17}O:^{16}O ratios expressed relative to atmospheric N_2 and standardized mean ocean water (VSMOW), respectively (Coplen, 2011). These isotopes have been broadly used for studying plant N strategies and enzymatic dynamics in natural settings (Evans, 2001; Tcherkez and Farquhar, 2006; Granger et al., 2010). Nevertheless, it is difficult to measure the concentration and isotopes ($\delta^{15}N$ and $\delta^{18}O$) of NO_3^- in plant tissues precisely using traditional methods (Liu et al., 2012a). The use of the denitrifier method for measuring low (sub-nanomole) concentrations of NO_3^- ($[NO_3^-]$) started during the mid-1980s (Lensi et al., 1985). The method has high sensitivity and is especially applicable for samples with low $[NO_3^-]$ but with high dissolved organic carbon (DOC) (Christensen and Tiedje, 1988; Binnerup and Sørensen, 1992; Aakra et al., 2000). The denitrifier method developed for both $\delta^{15}N$ and $\delta^{18}O$ analysis is based on the isotopic analysis of nitrous oxide (N_2O). The N_2O is converted from sample NO_3^- by cultured denitrifying bacteria (*Pseudomonas aureofaciens*; ATCC 13985) that lack N_2O reductase activity (Sigman et al., 2001; Casciotti et al., 2002). The method was initially performed on seawater with 20–50 nmol NO_3^-. Since then, the application has been expanded widely to accommodate isotopic analysis of NO_3^- in fresh water (e.g., groundwater, stream water, precipitation), soil and sediment water, soil extracts, as well as dissolved organic N (DON) in seawater and DON bound to diatoms as described by Koba et al. (2010a) and McIlvin and Casciotti (2011), respectively. This method has recently been used for measurements of NO_3^- in natural plants and crops (Liu et al., 2012a, 2013a; Laursen et al., 2013; Bloom et al., 2014; Mihailova et al., 2014). The established protocol facilitates the $\Delta^{17}O$ ($\Delta^{17}O = [1 + \delta^{17}O] / [1 + \delta^{18}O]^{0.5247} - 1$; Kaiser et al., 2007) analysis of leaf NO_3^- to diagnose atmosphere-derived NO_3^- in leaf uptake (Mukotaka, 2014).

The denitrifier method enables more precise measurements of subnanomole amounts of NO_3^- (Binnerup and Sørensen, 1992; Højberg et al., 1994) as compared to traditional methods that use flow injection analysis, ion chromatography, high-performance liquid chromatography, and Kjeldahl distillation. Thus, the denitrifier method overcomes the difficulties in determining NO_3^- in plant, soil, and sediment samples (Norwitz and Keliher, 1986; Anderson and Case, 1999; Alves et al., 2000). Moreover, it greatly simplifies the pretreatment procedures and reduces the risk of contamination during plant NO_3^- isotopic analysis (see the old $\delta^{15}N$ protocol in Volk et al., 1979 and Evans et al., 1996). The denitrifier method especially avoids the influence of DOC in plant extracts (Haberhauer and Blochberger, 1999) on the $\delta^{18}O$ of NO_3^- (**Figure 1**) that was previously measured as carbon monoxide with TC/EA-IRMS (Michalski, 2010).

Compared with NRA assays, concentrations and isotopic signatures of tissue NO_3^- provide more authentic evidence related to NO_3^- uptake and reduction under *in situ* N availability. *In vitro* and *in vivo* NRA measurements (Stewart et al., 1992, 1993) do not reflect the *in situ* ability of plant NO_3^- reduction. This is because firstly, the added amount of NO_3^- (often at the micromolar level) during NRA assays is uniform. Moreover it is much higher than normal NO_3^- availability and the endogenous NO_3^- in natural plants. The synthesis of the NR enzyme or the activation of NRA, however, is substrate-inducible (Beevers and Hageman, 1969; Somers et al., 1983; Campbell, 1999). Secondly, the reagents used in the assay can affect the estimation of NRA. Different analytical settings (e.g., with or without ethanol) can alter the fluxes of NO_3^- and photosynthate, resulting in different estimations (Ferrari and Varner, 1970; Aslam, 1981). Thirdly, NRA might be altered by pH adjustment and vacuum infiltration during the NRA analysis. High DOC concentrations in the plant extract also easily destroy the precision of the colorimetric determination of NO_3^- or nitrite (NO_2^-) (Alves et al., 2000).

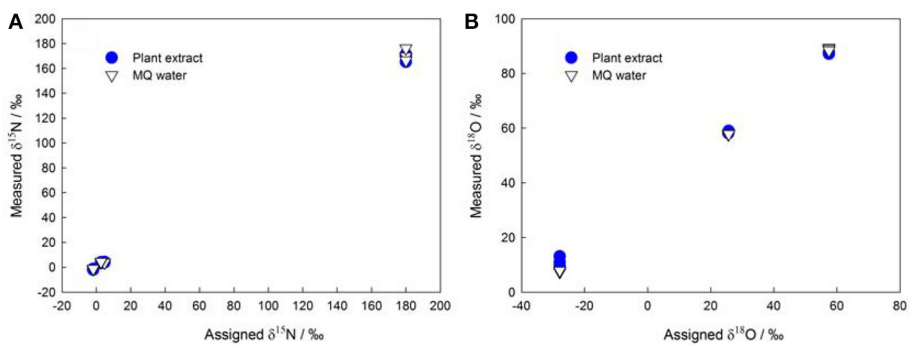

FIGURE 1 | Assigned isotopic ratios (A: $\delta^{15}N$; B: $\delta^{18}O$) of NO_3^- standards (IAEA NO_3, USGS-32, USGS-34, and USGS-35) shown against corresponding isotope values measured in MQ (Millipore) water and in plant extracts (the initial NO_3^- in plant extracts was removed using the same protocol as that described in Liu et al., 2012a).

Since natural isotope analysis does not require artificial N addition, it presents no risk of changing the soil N pools and plant N-uptake kinetics (Liu et al., 2012b). The natural abundance approach does not disturb the N pools in plants and provides information related to the NO_3^- behavior in plant tissues based on isotopic compositions and fractionations. In fact, the field application of $^{15}NO_3^-$ tracer is advantageous in terms of the total and short-term incorporation of NO_3^- into plants (e.g., McKane et al., 2002; Wanek and Zotz, 2011). However, the added tracer cannot bypass the influence of soil microbial activity, which can greatly change the picture of N uptake and preference over time (Harrison et al., 2007). Measurements of cytosolic and vacuolar NO_3^- concentrations have been conducted to explore factors controlling uptake, intracellular transport and assimilation. However, related techniques such as compartmental radiotracer (e.g., ^{13}N; Kronzucker et al., 1995), efflux analysis, nuclear magnetic resonance, cell fractionation, and NO_3^--selective microelectrodes showed high cost and low field operability (Zhen et al., 1991; Miller and Smith, 1996). The calculated [NO_3^-] is especially sensitive to the small error of the estimation of cytosolic and vacuolar volumes, the precisions of which are difficult to ascertain.

MAJOR SOURCES AND PROCESSES OF NO_3^- IN NATURAL PLANTS

Root NO_3^- uptake from the soil is achieved by active transportation (Wang et al., 2012). The extracellular NO_3^- enters the cytosol of plant cells where it is either reduced by NR to NO_2^- or stored in the vacuoles (**Figure 2**). The NO_2^- will be transported into plastids (in root) or chloroplasts (in leaf) and reduced further by NiR to reduced N (**Figure 2**). Both NRA and NiRA are well known to be substrate-inducible, meaning that the *de novo* synthesis of the enzyme results from the presence and increase of the NO_3^- in plants (Beevers and Hageman, 1969; Campbell, 1999). The induction of NRA by both soil and airborne NO_3^- is an important mechanism to elucidate the interactions among NO_3^- uptake, translocation/allocation, and reduction dynamics (Norby et al., 1989; Scheible et al., 1997a; Tischner, 2000).

The NO_3^- transported by the xylem flow, either directly from soil or partially processed by root NR, is the initial NO_3^- reaching leaves and shoots (Peuke et al., 2013). This is especially true for plants growing at some pristine sites (e.g., arctic tundra) where the atmospheric NO_3^- availability is negligible. However, in regions with substantial NO_3^- deposition, both atmospheric NO_x and NO_3^- serve as potential sources of NO_3^- in leaves (Wellburn, 1990; Raven and Yin, 1998; Sparks et al., 2001), especially for non-vascular plants such as mosses, which rely more on atmospheric nutrients (Liu et al., 2012c). Leaf NO_3^- acquisition from the atmosphere is conducted through passive diffusion mechanisms wherein uptake through the stomata is dominant (Wellburn, 1990; Raven et al., 1992; Gessler et al., 2002) (**Figure 2**). The leaf-accessible NO_3^- in the atmosphere includes an array of inorganic and organic ions and compounds (Wellburn, 1998; Teklemariam and Sparks, 2004; Vallano and Sparks, 2008). Although, previous tracer studies have described their incorporation into leaves (Hanson and Garten, 1992; Yoneyama et al., 2003; Lockwood et al., 2008), it is rather difficult to apply the natural abundance method for estimating field contributions of atmospheric NO_3^-. This can be attributed to the heterogeneity in chemical and deposition forms, and temporal and spatial distributions (Sievering et al., 2007; Sparks, 2009).

CONCENTRATION LEVELS AND IMPLICATIONS OF NO_3^- IN NATURAL PLANTS

Nitrate cannot be produced in photoautotrophic plants, except in a few legumes (Hipkin et al., 2004). The presence of NO_3^- in any part of a plant constitutes evidence of NO_3^- uptake by the plant and reflects that external NO_3^- is available; and that the rate of uptake is higher than the rate of reduction. The NO_3^- that is extractable from a plant organ is often a sum of the amounts from the extracellular pool, cytosolic pool, and vacuolar pool (**Figure 2**). These pool sizes and turnover rates are regulated by both environmental and physiological factors (Zhen et al., 1991; Miller and Smith, 1996), which determine the isotopic signatures of the extracted NO_3^-. Generally, the concentration level and distribution of NO_3^- in vascular plants and the variations among species is a complex result of two important factors: external availability (previously often evaluated through NO_3^- concentration and net nitrification rate in soil) and physiological strategies (mainly including uptake, translocation, and reduction dynamics). Moreover, the external factors also consider the availability of NO_3^- relative to NH_4^+ or other N sources because it can influence both plant NO_3^- uptake and assimilation (Boudsocq et al.,

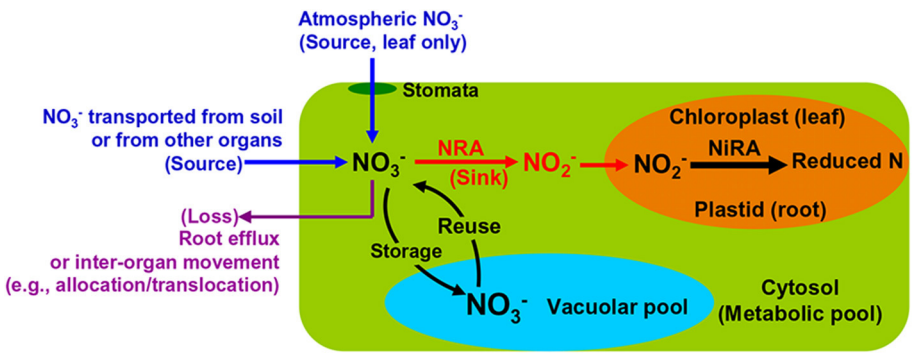

FIGURE 2 | Schematic map showing major NO_3^- sources and processes in leaves and roots of natural plants.

2012; Liu et al., 2012c; Britto and Kronzucker, 2013) while the physiological factors include the affinity of plants to different soil NO_3^- levels (Wang et al., 2012; Kalcsits and Guy, 2013).

First, the distribution of organ-specific NO_3^- concentrations among plants under different growing conditions (**Figures 3**, **4A**) showed that plants growing in natural soils might also have a high NO_3^- accumulation. In natural forests, leaf NO_3^- concentrations of some species can be as high as 1000–10000 μg-N g^{-1} dw (**Figure 4A**; Gebauer et al., 1988; Koyama et al., 2013), which was even higher than those of some crops (e.g., Bloom et al., 2014) and N-polluted natural plants (**Figure 3**). Plant NO_3^- concentrations are indicators or predictors of the soil N cycle (e.g., soil nitrification and soil NO_3^-) and forest N pollution (Stams and Schipholt, 1990; Aber et al., 1998; Fenn and Poth, 1998; Koba et al., 2003). Such concentrations show higher sensitivities than bulk N and NRA parameters in revealing species-level responses to N enrichment (Fenn et al., 1996; Jones et al., 2008; Tang et al., 2012). The increase in NO_3^- concentration in roots and or leaves with external NO_3^- was observed under both natural soil conditions and experimental N addition (e.g., Stewart et al., 1993; Lexa and Cheeseman, 1997; Wang and Schjoerring, 2012). However, the level of leaf NO_3^- and its response to soil NO_3^- variation differ among species with distinct uptake or accumulation rates. For example, the NO_3^- concentrations in plants (mostly as mosses) we recently investigated (Liu et al., 2012a,c, 2013a) were much lower than those reported by Gebauer et al. (1988) or Koyama et al. (2013) on vascular plants (**Figure 4A**) when compared within a similar soil [NO_3^-] range (e.g., 0–15 mg-N kg^{-1} soil, dw). Besides, the correlation between leaf NO_3^- and soil NO_3^- is apparent for plants with low NO_3^- concentrations (**Figure 4A**). However, synthesis or extrapolation to different plants with distinct NO_3^- accumulation abilities should be done carefully when evaluating soil N enrichment or N saturation.

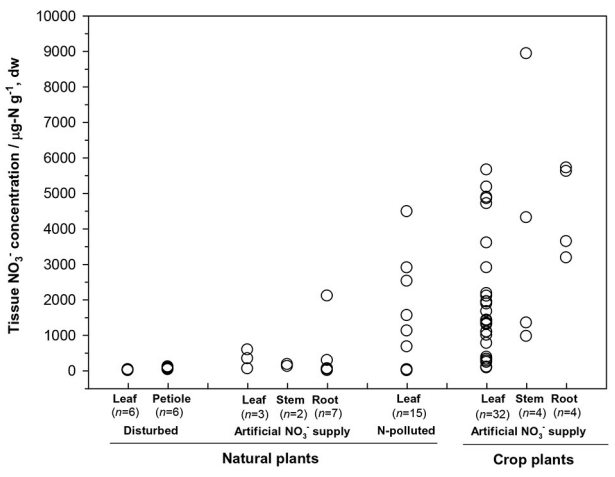

FIGURE 3 | Tissue NO_3^- concentrations in natural plants growing under disturbed conditions (acidic irrigation and liming; Gebauer et al., 1988), in N-polluted forest plants (Stams and Schipholt, 1990), in natural and crop plants with artificial NO_3^- supply (data of natural plants were cited from Gebauer et al., 1984; Stadler and Gebauer, 1992; Robe et al., 1994; Simon et al., 2014. Data of crop plants were cited from Evans et al., 1996; Yoneyama and Tanaka, 1999; Prasad and Chetty, 2008 and references cited therein).

Second, considerable differences (up to 4–5 orders) exist in the level of NO_3^- among plant organs and species (**Figures 3**, **4A**). The organ-specific patterns of NO_3^- accumulation among coexisting plants can differ with soil N availability and the plant growing stage (Gebauer et al., 1984; Stewart et al., 1993; Liu et al., 2013a). However, this has complicated the use and selection of proper organs and species to evaluate ecosystem N availability based on tissue NO_3^- analysis. McKane et al. (2002) used ^{15}N tracers in the field to show that NO_3^- uptake in the tundra plants did not passively follow external availability, but depended on specific ecophysiological traits. NO_3^- preference in *Carex* was determined by the appearance of ^{15}N tracer in *Carex* biomass, which showed that the NO_3^- preference might reflect only the $^{15}NO_3^-$-acquiring efficiency associated with root traits, but not NO_3^- assimilation given significantly lower NRA in *Carex* than in other species (Nadelhoffer et al., 1996). Therefore, additional studies should be conducted to determine the extent of organ-specific and species-specific variability of NO_3^- concentration that reflects plant NO_3^- strategy, and the heterogeneity of NO_3^- available to roots. The available data for natural plants revealed a clear increase in NO_3^- concentration with bulk N while a decrease with C/N (a clear turning at the C/N of 20–30) in different organs or tissue types (**Figure 4B**). Similarly, Zhen and Leigh (1990) reported that shoot NO_3^- accumulated as a linear function of bulk N in wheat plants once a threshold N was exceeded. These results reflected the regulation of overall physiological N demand on the NO_3^- utilization in natural plants (Imsande and Touraine, 1994). The regulation might be unidirectional because the contribution of NO_3^- to bulk N assimilation appears to be much lower than that for other N forms in plants (portrayed in **Figure 4B**). The complexity of the mutual regulations behind the inverse relation between NO_3^- and C/N might be comparable with the multi-scale inverse relation prevailing between NO_3^- and organic C observed in different ecosystems (Taylor and Townsend, 2010). So far, little direct and simple evidence has been obtained for the driving mechanisms of C and N metabolism on NO_3^- uptake, allocation, and accumulation in natural plants. A clearer relation is that even when external NO_3^- is uniform, the NO_3^- concentration is often higher in organs (especially for growing leaves) of species with higher NRA than in those with lower NRA (Gebauer et al., 1988; Cruz et al., 1991; Widmann et al., 1993; Min et al., 1998). Mutual induction between the maintenance of high NO_3^- concentration and that of NR synthesis or NRA activation were elucidated in view of C metabolism and N demand in response to availability and growing conditions (Stewart et al., 1993; Scheible et al., 1997a,b; Scheurwater et al., 2002). The lower NO_3^- concentration and NRA might be associated with lower N metabolism and demand in organs and plants with higher C/N and vice versa. Therefore, except regulation by soil NO_3^- concentration, the uptake and distribution of NO_3^- in a plant might follow the regime of organ-specific or whole-plant metabolic activities.

Other factors such as light and water regimes might also influence plant NO_3^- accumulation through the pathway of photosynthetic regulation (Widmann et al., 1993; Simon et al., 2014). Cárdenas-Navarro et al. (1999) found concurrent and

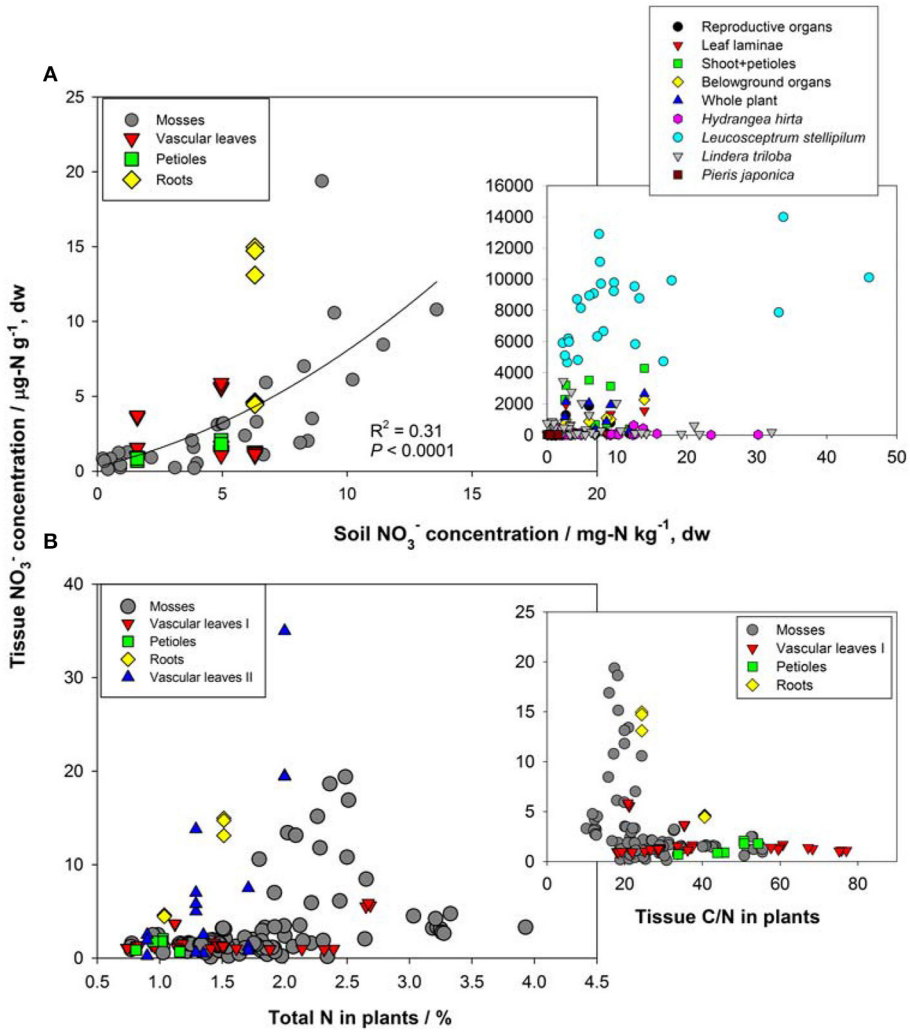

FIGURE 4 | (A) Relation between NO_3^- concentrations in soil and natural plants. Plant NO_3^- data in the left panel are shown for individual samples in Guiyang, southwestern China and western Tokyo, Japan reported by Liu et al. (2012a; 2012c, 2013a; 2013b). Plant NO_3^- data in the right panel show organ-specific and whole-plant concentrations (averages of different species) in ecosystems of Central Europe (see details in Gebauer et al., 1988), and leaf NO_3^- of different species (*H. hirta, P. japonica, L. stellipilum, L. triloba*) in a temperate forest of central Japan (Koyama et al., 2013). **(B)** Relations between total N, C/N, and tissue NO_3^- concentration in natural plants. Mosses include different species in different habitats of Guiyang, Southwestern China, and Western Tokyo, Japan (cited from Liu et al., 2012a,c). Vascular leaves I, petioles and roots were reported for a coniferous and a broadleaved plant in western Tokyo, Japan (cited from Liu et al., 2013a). Vascular leaves II included fern, oak, and pine species at the Camp Paivika and Camp Osceola forest sites in the San Bernardino Mountains of southern California, USA (cited from Fenn et al., 1996).

linearly correlated changes in whole-plant NO_3^- and water content during the day–night cycle, reflecting a homoeostasis effect of endogenous NO_3^- concentration. Besides, as discussed above, the heterogeneity of soil NO_3^- available to roots of coexisting species should not be excluded considering the differences in root morphology and spatial distribution. Given the difficulties in determining rhizospheric soil NO_3^- concentration and flux, it would be promising to measure NO_3^- concentrations in roots to evaluate NO_3^- availability to the whole plant or aboveground organs.

ISOTOPIC SYSTEMATICS OF NO_3^- IN PLANTS

Stable isotopes of NO_3^- in plants are controlled mainly by NO_3^- sources and isotopic effects involved in NO_3^- acquisition and reduction processes (Robinson et al., 1998; Comstock, 2001; Evans, 2001; Cernusak et al., 2009).

The $\delta^{15}N$ of NO_3^- in soil is reported mostly within −10 to +10‰; however, the $\delta^{15}N$ of newly-produced NO_3^- in soil is usually low because of strong isotopic effects of nitrification, on the other hand, the values can be elevated at sites with marked denitrification (Mariotti et al., 1981; Högberg, 1997; Koba et al., 1998, 2003, 2010b; Houlton et al., 2006; Takebayashi et al., 2010). Atmospheric NO_3^- has a wider $\delta^{15}N$ range (−15 – +15‰) because of its complex production pathways and sources (Heaton, 1990; Felix et al., 2012; Altieri et al., 2013). The $\delta^{15}N$ of NO_3^- is generally lower in wet than in dry deposition (Heaton et al., 1997; Elliott et al., 2009), but both often show a $\delta^{15}N$ range

overlapping with that of soil NO_3^-. The $\delta^{18}O$ of initial NO_3^- produced in soil is usually estimated using the $\delta^{18}O$ of *in situ* H_2O (normally −25 – 4‰) and atmospheric O_2 (ca. 23.5‰) in a 2:1 ratio, assuming no exchange and fractionation of oxygen (O) isotopes occurs during nitrification and the NO_3^- is produced solely through chemolithoautotrophic nitrification (Amberger and Schmidt, 1987). However, kinetic isotopic fractionation and O exchange between NO_2^- and H_2O often occur during nitrification, which can eliminate the isotopic signal of O_2 effecting lower $\delta^{18}O$ than the predicted values (Fang et al., 2012). The O of NO_3^- in atmospheric deposition is derived mainly from O_2 and O_3, which have distinctly higher $\delta^{18}O$ and $\Delta^{17}O$ signatures than those of soil NO_3^-. In contrast to the overlapping $\delta^{15}N$ for different NO_3^- sources, $\delta^{18}O$ and or $\Delta^{17}O$ provide a clear separation between soil and atmospheric NO_3^- sources. The $\delta^{18}O$ of soil NO_3^- produced by nitrification is distinctly lower (mean = −4.0‰; −7.3 to −0.9‰; Fang et al., 2012) than that of atmospheric NO_3^- (60 − 100‰). The latter has high $\Delta^{17}O$ values (around 25‰) in contrast to 0‰ of soil-derived NO_3^- (Kendall et al., 2007; Michalski, 2010; Costa et al., 2011) (**Figure 5**).

The process of NO_3^- entry into root cells and subsequent transport processes within plants *per se* cause no isotope effect because of the lack of bond breakage. However, the acquisition of NO_3^- through mycorrhizae to root cells potentially causes an isotopic difference between tissue NO_3^- in roots and NO_3^- in soil. Root NO_3^- may be enriched in heavier isotopes relative to soil NO_3^- if the NO_3^- has experienced reduction during the N assimilation of mycorrhizae associated with the roots. Mycorrhizal fungi have substantial NO_3^- reduction capacity (Ho and Trappe, 1975), but the fungal NR is present only in the presence of NO_3^- and absence of NH_4^+ (Cove, 1966). So far, the isotopic effect of NO_3^- acquisition through mycorrhizae on tissue NO_3^- in natural plants has not been estimated or differentiated. Pate et al. (1993) demonstrated that the bulk $\delta^{15}N$ differences between non-mycorrhizal and mycorrhizal species (with significant NO_3^- storage and NRA) reflected the utilization of different N sources. There appears to be little or no isotopic discrimination within the plant during or subsequent to uptake of NO_3^-. Mycorrhizal fungi are expected to show higher bulk $\delta^{15}N$ than available N sources [potentially including NO_3^-, NH_4^+, and DON (at least amino acids)] in soil and bulk N of host plants. However, the isotopic mechanism differed from that of tissue NO_3^- and the isotope effect differed among mycorrhizal types (Högberg, 1997; Craine et al., 2009; Hobbie and Högberg, 2012). Högberg et al. (1999) showed that the ECM fungus had higher bulk $\delta^{15}N$ relative to the *Pinus sylvestris* plant, and the fractionation against ^{15}N was smaller when NO_3^- was the source than when NH_4^+. It caused a marginal decrease in $\delta^{15}N$ of the N passing from the substrate through the fungus to the host, which is explained by the small size of the fungal N pool relative to the total N of the plant, i.e., the high efficiency of transfer (Emmerton et al., 2001; Hobbie and Högberg, 2012). The significant shift in $\delta^{15}N$ of fungal species was a function of fungal physiology; thus, it is difficult to constrain the N sources (using bulk $\delta^{15}N$) by mycorrhizal fungi or their plant partners in natural conditions (Emmerton et al., 2001).

The efflux of NO_3^- from root to soil or the subsequent transport of NO_3^- within plants is not expected to discriminate ^{15}N as with the entry of soil NO_3^- into root cells (Mariotti et al., 1982; Shearer et al., 1991). This can be attributed to that the diffusion of NO_3^- through the membrane carriers of plant cells does not cause bonding breakage or consumption (Werner and Schmidt, 2002; Granger et al., 2004; Needoba et al., 2004). However, isotopic differences can occur between organs if partial NO_3^- reduction occurs in roots before transportation. The transport of NR-processed NO_3^- from roots to leaves might be misunderstood as isotopic fractionations of NO_3^- transport or NO_3^- reduction in shoots. So far, isotopic fractionations ($\varepsilon = ({}^{l}k/{}^{h}k - 1) \times 1000$, where ${}^{l}k$ and ${}^{h}k$ respectively stand for the reaction rate constants for lighter and heavier isotopes) during the reduction of NO_3^- by NR in leaves were reported as 15‰ for

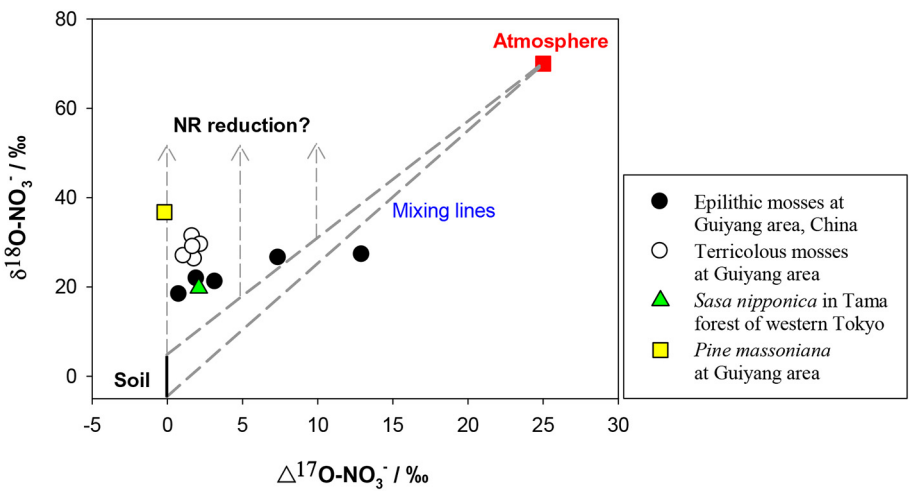

FIGURE 5 | Preliminary relation between $\delta^{18}O$ and $\Delta^{17}O$ values of NO_3^- in mosses and vascular plants. The $\delta^{18}O$ and $\Delta^{17}O$ values were considered respectively, as −5 to 5‰ and 0‰ for soil NO_3^- (black and solid line), 70 and 25‰ for atmospheric NO_3^- (red square). Dashed lines show the isotopic range of mixing between atmospheric and soil sources. Dashed lines with arrows show the vectors of $\delta^{18}O$ enrichments because of NR reduction.

both N in spinach (Ledgard et al., 1985; Tcherkez and Farquhar, 2006) and O in wheat (Olleros-Izard, 1983) (**Table 1**). Direct measurement of endogenous NO_3^- reduction in mosses after N deprivation showed similar values (Liu et al., 2012b) (**Table 1**). Although, NR isotopic fractionations have not been directly measured in roots, predictions can be made about the net enrichment of NO_3^- isotopes in roots relative to those of soil NO_3^- (Δ_{root}; expressed as $\delta_{root} - \delta_{soil}$). These values should be either negligible if substantial NO_3^- reduction did not occur (Scenario 1; $\Delta_{root} = \delta_{root} - \delta_{soil} \approx 0$), or be close to the reported ε values of NRA in leaves (ε_{NR}) (0 − 27‰; **Table 1**) if NO_3^- reduction occurred in the root (Scenario 2; $\Delta_{root} = \delta_{root} - \delta_{soil} \approx \varepsilon_{NR} > 0$) (**Figure 6**). However, if the modification of soil NO_3^- isotopes by soil microbial activities such as denitrification occurred later than root uptake, the observed isotopic values of root NO_3^- can also be slightly lower than those of soil NO_3^- despite reduction in roots (e.g., in the fine roots of a conifer investigated in Liu et al., 2013a). Furthermore, the variation of NO_3^- isotopes with soil depth directly caused isotopic differences in initial NO_3^- sources available to co-existing plants with different root depths. Therefore, considering this fact, soil reference samples should be collected corresponding to root distribution for characterizing the soil NO_3^- isotopes available to specific plants.

In a closed system, isotopic enrichment occurs with the enzymatic consumption of substrate NO_3^- and ε_{NR} is expressed as $\Delta/\ln[NO_3^-]_{remaining}$ fitted to the Rayleigh isotope fractionation model, where Δ represents the isotopic difference of remaining NO_3^- from the initial NO_3^- ($\delta_{remaining} - \delta_{initial}$) (e.g., Granger et al., 2004, 2010). Isotopic enrichment also takes place for NO_3^- remaining in plants after deprivation of NO_3^- or N supply, because the tissue NO_3^- pool is only changed by the NRA in a closed system (e.g., Liu et al., 2012b). Thus far, no experimental work has been done to explain the variability of $^{18}\varepsilon_{NR}$ in and among vascular plants. In NO_3^--supply studies, shoots tend to have higher $\delta^{15}N$ values because of the allocation of root NR-processed NO_3^- from roots to shoots (Kalcsits and Guy, 2013) or significantly higher $^{15}\varepsilon_{NR}$ (by 3.3–6.9‰) than roots (Yoneyama and Kaneko, 1989; Evans et al., 1996; Yoneyama et al., 2001). Evidence from marine biota showed that both $^{15}\varepsilon_{NR}$ and $^{18}\varepsilon_{NR}$ can vary with growing conditions and that significantly different ε values exist among species (**Table 1**). In field conditions, NO_3^- in an organ is more likely to be an open system with continuous source inputs (uptake), sinks (reduction), and outputs (translocation) (**Figure 2**). The uptake and allocation often occur according to the reduction ability and the distribution of NR, for example, a higher concentration and more NR are likely to exist in growing leaves (Gebauer et al., 1988; Cruz et al., 1991; Widmann et al., 1993). Passive or high accumulation as in mosses (Liu et al., 2012c) can happen in some organs such as conifer roots that are unable to reduce it (Liu et al., 2013a). Therefore, δ values of tissue NO_3^- might not always follow the normal "Rayleigh type" relation, instead might increase with the increase in tissue $[NO_3^-]$ or show a non-significant correlation with $[NO_3^-]$ in the tissues (Liu et al., 2012c, 2013a). In fact, experimental studies have also shown the interplay of plant NO_3^- uptake and reduction activity. The ^{15}N discrimination during NO_3^- assimilation in several higher plants was positively correlated with the supplied and tissue NO_3^- concentrations, and negatively correlated with plant age (Kohl and Shearer, 1980; Mariotti et al., 1980, 1982; Bergersen et al., 1988; Liu et al., 2013a). Accordingly, the Rayleigh relation between NO_3^- and its isotopes is not always applicable to examine ε_{NR} values and NO_3^- reduction in organs of natural plants.

For some plants, NO_3^- is not available in soil substrates. It can only be acquired from deposition (e.g., non-vascular plants or epiphytes). Alternatively, it is not available in deposition but can only be taken up from the soil (e.g., plants growing in arctic pristine ecosystems with negligible NO_3^- deposition). In these plants, it is also feasible to diagnose leaf NO_3^- reduction using Δ_{leaf} (the net enrichment of NO_3^- isotopes in leaves relative to those of source NO_3^-) (Scenarios 3–6; **Figure 6**).

Scenario 3: If no NO_3^- was transported from soil to leaves, and leaf NO_3^- if any, was completely derived from atmosphere, but no reduction occurred, then:

$$\Delta_{leaf} = \delta_{leaf} - \delta_{atm} \approx 0.$$

Table 1 | Isotopic effects reported for NO_3^- reduction (*) or net NO_3^- assimilation in different biota.

Biota	$^{15}\varepsilon$ / ‰	$^{18}\varepsilon$ / ‰	References
Eukaryotic NR enzymes (from fungus and marine diatoms)	26.6*	24.9*	Karsh et al., 2012
Moss	12.1*	14.4*	Liu et al., 2012b
Strains of prokaryotic plankton	0.4–8.6	0.9–8.1	Granger et al., 2010
Spinach and wheat	15.0*	15.0*	Olleros-Izard, 1983; Ledgard et al., 1985; Tcherkez and Farquhar, 2006
Eukaryotic algae	5.6–20.4	5.1–21.0	Granger et al., 2004
Marine phytoplankton	2.7–15.2	–	Needoba and Harrison, 2004
	4–9 (field) 2.2–6.2 (lab)	–	Needoba et al., 2003
Tomato	11.3–12.9	–	Evans et al., 1996
Leafy vegetable	14.2–18.1	–	Yoneyama and Kaneko, 1989; Yoneyama et al., 2003
Grasses	0.0–3.3	–	Mariotti et al., 1982
Pearl Millet and soybeans	0.0-9.5	–	Mariotti et al., 1980, 1982; Bergersen et al., 1988
Red clover	1.7–6.5	–	Kohl and Shearer, 1980

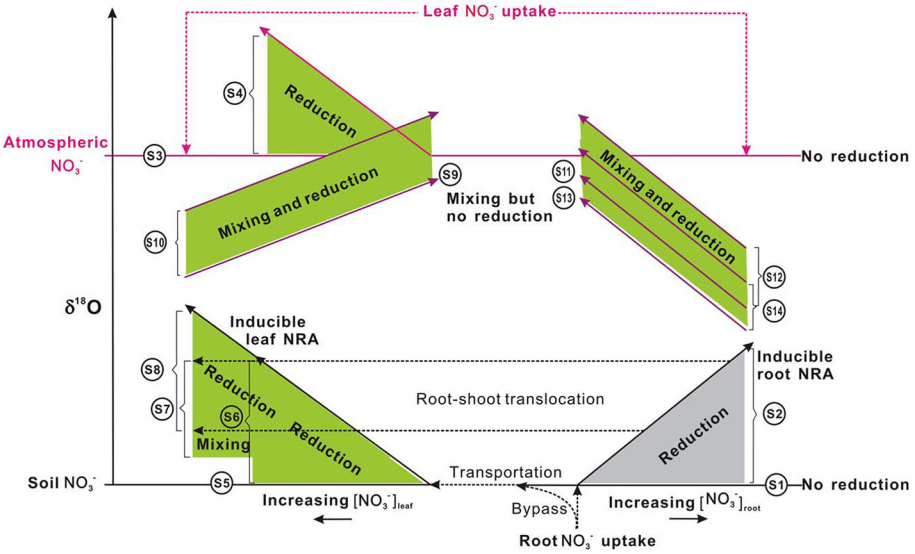

FIGURE 6 | Schematic showing $\delta^{18}O$-NO_3^- variations in plants under different uptake (from soil and or atmospheric sources: distinct in the $\delta^{18}O$ value), translocation (from soil and or root to shoot), and reduction (potentially inducible by increasing $[NO_3^-]$ or no reduction and no isotopic enrichment with NO_3^- accumulation, depending on species). Long and short solid lines with arrows respectively show the vectors of $\delta^{18}O$-NO_3^- and $[NO_3^-]$ variations. Dashed lines with arrows show the uptake, transportation, and translocation of NO_3^- from the soil to roots and or to leaves, from atmosphere to leaves, during which isotope effects were regarded as negligible. Shaded areas (gray for roots, green for leaves) show isotopic enrichment during the mixing of different sources (the $\delta^{18}O$-NO_3^- in plants should be distributed between the $\delta^{18}O$ values of sources, depending on the fraction of each source) and or the occurrence of NR reduction activities (the $\delta^{18}O$-NO_3^- in plants would be higher than the $\delta^{18}O$ of sources but the magnitude of enrichment depends on *in situ* NR dynamics; presumably less than that presented in **Table 1**). For scenarios that occurred, leaf uptake of atmospheric NO_3^- was assumed to be homogeneous. The shaded area, the spatial distance, and length of lines had no quantitative implications. S1–S12 correspond to scenarios 1–12 in the main text. Briefly, S1, no occurrence of NO_3^- reduction in roots; S2, (inducible) root NO_3^- reduction; S3, no NO_3^- was transported from soil to leaves and leaf NO_3^- was derived from the atmosphere, but no reduction occurred; S4, no NO_3^- was transported from soil to leaves and leaf NO_3^- was from atmosphere and (inducible) reduction occurred; S5, leaf NO_3^- was taken up directly from the soil, but no reduction occurred; S6, leaf NO_3^- was taken up from the soil and reduction occurred therein; S7, leaf NO_3^- is completely or partially transported from the root where it has experienced reduction, but no further reduction in the leaf; S8, leaf NO_3^- is completely or partially transported from the root where it has experienced reduction, and is further reduced in the leaf; S9, leaf NO_3^- was from both atmosphere and soil but no reduction occurred in the leaf; S10, leaf NO_3^- was from both atmosphere and soil, and reduction occurred in the leaf; S11, leaf NO_3^- is a mixture of atm-NO_3^- and root NO_3^- but no reduction occurred; S12, leaf NO_3^- is a mixture of atm-NO_3^- and root NO_3^-, and reduction occurred in the leaf; S13, leaf NO_3^- is a mixture of soil NO_3^-, atm-NO_3^-, and root NO_3^-, but no reduction occurred in the leaf; S14, leaf NO_3^- is a mixture of soil NO_3^-, atm-NO_3^-, and root NO_3^-, and reduction occurred in the leaf. The $\delta^{18}O$ differences between S13 and S11, between S12 and S14 depend on the fraction of soil NO_3^- in the mixed pool of leaves.

Scenario 4: If no NO_3^- was transported from soil to leaves and leaf NO_3^- was acquired from atmosphere; and reduction occurred, then:

$$\Delta_{leaf} = \delta_{leaf} - \delta_{atm} > 0.$$

Scenario 5: If all leaf NO_3^- was taken up directly from the soil and no reduction occurred in roots or leaves, then:

$$\Delta_{leaf} = \delta_{leaf} - \delta_{soil} \approx 0.$$

Scenario 6: If leaf NO_3^- was transported completely from the soil and reduction occurred only in the leaves, then

$$\Delta_{leaf} = \delta_{leaf} - \delta_{soil} > 0.$$

The induction of NR by atmospheric-derived NO_3^- has been shown in plants exposed to airborne N oxides (e.g., Norby et al., 1989; Wellburn, 1990). Scenarios 3–4 are expected to be true for mosses because atmospheric NO_3^- has been assumed as the sole source (Liu et al., 2012a). Nevertheless, isotopic partitioning of N sources (Liu et al., 2013b) and further $\Delta^{17}O$ analysis (**Figure 5**) suggests that moss NO_3^-, even at epilithic habitats, is actually a mixture of atmospheric NO_3^- and soil-derived NO_3^-. Thus, it is becoming clear that mosses can acquire substantial N from substrates; and moss NO_3^- is a valid atmospheric bio-monitor only for species growing on rare N-free substrates. Scenarios 5–6 demonstrated NO_3^- dynamics of vascular plants in the tundra of northern Alaska, where the $\Delta^{17}O$ of NO_3^- in plants with surprisingly high $[NO_3^-]$ was found as 0‰ (e.g., *Polygonum bistorta*). However, examining only Δ_{leaf} seems insufficient to determine NO_3^- reduction location, since, isotopic enrichments of leaf NO_3^- might result from root reduction activities before moving up to leaves (Scenario 7).

Scenario 7: If the leaf NO_3^- is completely or partly transported from the root where it has experienced reduction, but no reduction has occurred in the leaf; then an isotope mass-balance calculation can be conducted to quantify the amount of leaf NO_3^-

accumulated directly from soil and indirectly from roots:

$$\Delta_{root} = \delta_{leaf} - \delta_{soil} \approx \delta_{root} - \delta_{soil} > 0,$$
$$\Delta_{leaf} = \delta_{leaf} - \delta_{root} < 0, \text{ and}$$
$$\delta_{leaf} = (1 - f_{root}) \times \delta_{soil} + f_{root} \times \delta_{root}.$$

The reduction of NO_3^- that has experienced reduction in roots can further increase the isotopic enrichment of leaf NO_3^- relative to soil NO_3^- (Scenario 8) (**Figure 6**). This has been demonstrated by the $\delta^{15}N$ difference between roots and leaves in plants growing with NO_3^- with known $\delta^{15}N$ values (Yoneyama and Kaneko, 1989; Evans et al., 1996; Yoneyama et al., 2001). This NO_3 reduction occurs especially in plants that are capable of reducing NO_3^- in both shoots and roots (Stewart et al., 1992).

Scenario 8: If the leaf NO_3^- is completely or partially transported from roots where it has experienced reduction; and if it is further reduced in the leaf. In this case, a partitioning similar to scenario 7 can be done by considering the Δ_{leaf} in the isotope mass-balance calculation:

$$\Delta_{root} = \delta_{root} - \delta_{soil} > 0 \text{ and}$$
$$\delta_{leaf} = [(1 - f_{root}) \times \delta_{soil} + f_{root} \times \delta_{root}] + \Delta_{leaf}.$$

Plant NO_3^- in scenarios 1–8 was derived either from the soil or atmosphere (**Figure 6**). A supplemental diagnosis of NR dynamics was to examine the covariance of $\Delta\delta^{18}O$:$\Delta\delta^{15}N$ ratios (Δ is the isotopic enrichment of plant NO_3^- relative to source NO_3^-; $\Delta = \delta_{plant} - \delta_{source}$). This diagnosis helped determine whether the N–O bond breakage attributable to NO_3^- reduction was the single process driving NO_3^- ^{15}N and ^{18}O enrichments. Theoretically, the dissociation of an O atom from NO_3^- predicted that NO_3^- isotopes would be fractionated in an O-to-N ratio of ca. 0.6 (Brown and Drury, 1967). However, the NR often had the same O-to-N isotopic imprint on substrate NO_3^- in experimental studies. Consequently, the 1:1 trend was considered ubiquitous for biological NO_3^- reduction (Granger et al., 2004, 2010). However, for leaves of vascular plants that acquire NO_3^- from both atmosphere and soil, it is difficult to constrain leaf NO_3^- reduction based only on the Δ_{leaf} ($\delta_{leaf} - \delta_{source}$) and ε_{NR}, because the mixing of atmospheric NO_3^- can raise the δ values (especially $\delta^{18}O$). Liu et al. (2013a) observed that the $\delta^{18}O$:$\delta^{15}N$ ratios in roots of a conifer generally followed the 1:1 rule; although leaf NO_3^- showed distinctly higher $\delta^{18}O$:$\delta^{15}N$ ratios (2.5:1) because of the mixing of atmospheric NO_3^-.

As described above, the fraction of atmospheric-derived NO_3^- (F_{atm}) in leaves can be estimated using $\Delta^{17}O$ mass-balance calculation ($F_{atm} = \Delta^{17}O_{leaf} / \Delta^{17}O_{atm} < 1$). Thereafter, the leaf NO_3^- sources and NR dynamics can be further constrained.

Scenario 9: If leaf NO_3^- was absorbed from both the atmosphere and soil, but no reduction occurred in the leaf, then the fraction of atmospheric-derived NO_3^- calculated using $\delta^{18}O$ or $\delta^{15}N$ (f_{atm}) is expected to be similar to F_{atm}, as

$$\delta_{leaf} = (1 - f_{atm}) \times \delta_{soil} + f_{atm} \times \delta_{atm},$$
$$\text{and} \quad f_{atm} \approx F_{atm} < 1.$$

Scenario 10: If leaf NO_3^- was absorbed from both the atmosphere and soil, and reduction occurred in the leaf, then:

$$\delta_{leaf} = [(1 - f_{atm}) \times \delta_{soil} + f_{atm} \times \delta_{atm}] + \Delta_{leaf},$$
$$f_{atm} \approx F_{atm} < 1,$$
$$\text{and } \Delta_{leaf} = \delta_{leaf} - [(1 - F_{atm}) \times \delta_{soil} + F_{atm} \times \delta_{atm}] > 0.$$

Scenario 11: If leaf NO_3^- is a mixture of atm-NO_3^- and root NO_3^-, but no reduction occurred, then:

$$\delta_{leaf} = (1 - f_{atm}) \times \delta_{root} + f_{atm} \times \delta_{atm}$$
$$\approx [(1 - f_{atm}) \times (\delta_{soil} + \Delta_{root}) + f_{atm} \times \delta_{atm}],$$
$$f_{atm} \approx F_{atm} < 1,$$
$$\text{and } \Delta_{root} = \delta_{root} - \delta_{soil}$$
$$\approx [(\delta_{leaf} - F_{atm} \times \delta_{atm})/(1 - F_{atm})] - \delta_{soil} > 0.$$

Scenario 12: If leaf NO_3^- is a mixture of atm-NO_3^- and root NO_3^-; and if the reduction occurred in the leaf, then:

$$\delta_{leaf} = [(1 - f_{atm}) \times \delta_{root} + f_{atm} \times \delta_{atm}] + \Delta_{leaf}$$
$$\approx [(1 - f_{atm}) \times (\delta_{soil} + \Delta_{root}) + f_{atm} \times \delta_{atm}] + \Delta_{leaf},$$
$$f_{atm} \approx F_{atm} < 1,$$
$$\Delta_{root} = \delta_{root} - \delta_{soil} > 0,$$
$$\text{and } \Delta_{leaf} = \delta_{leaf} - [(1 - f_{atm}) \times \delta_{root} + f_{atm} \times \delta_{atm}] > 0.$$

Scenario 13: If leaf NO_3^- is a mixture of soil NO_3^-, atm-NO_3^-, and root NO_3^-, but no reduction occurred in the leaf, then:

$$\delta_{leaf} = (1 - f_{atm} - f_{soil}) \times \delta_{root} + f_{atm} \times \delta_{atm} + f_{soil} \times \delta_{soil},$$
$$f_{atm} \approx F_{atm} < 1,$$
$$\text{and } \Delta_{root} = \delta_{root} - \delta_{soil} > 0.$$

Scenario 14: If leaf NO_3^- is a mixture of soil NO_3^-, atm-NO_3^-, and root NO_3^-, and if reduction occurred in the leaf, then:

$$\delta_{leaf} = \left[(1 - f_{atm} - f_{soil}) \times \delta_{root} + f_{atm} \times \delta_{atm} + f_{soil} \times \delta_{soil}\right] + \Delta_{leaf},$$
$$f_{atm} \approx F_{atm} < 1,$$
$$\Delta_{root} = \delta_{root} - \delta_{soil} > 0,$$
$$\text{and } \Delta_{leaf} = \delta_{leaf} - \left[(1 - f_{atm} - f_{soil}) \times \delta_{root} + f_{atm} \times \delta_{atm} + f_{soil} \times \delta_{soil}\right] > 0.$$

The parameters in the scenarios 9–14 (f_{atm}, F_{atm}, Δ_{root}, Δ_{leaf}) above, provide theoretical constraints on possible NO_3^- sources and reduction dynamics in leaves of field plants. As explained above, $\delta^{15}N$ values of NO_3^- often overlapped for soil and atmospheric sources, although $\delta^{18}O$ and or $\Delta^{17}O$ can provide a clear differentiation between them (Kendall et al., 2007; Michalski,

2010). Consequently, the scenarios above are better suited to the $\delta^{18}O$ (depicted in **Figure 6**) than $\delta^{15}N$ analysis, particularly when leaf NO_3^- was a mixing pool for different sources. The other solution to diagnose atmospheric NO_3^- mixing and reduction is the $\Delta^{17}O$-$\delta^{18}O$ correlation, which has been used to trace NO_3^- sources and dynamics in aquatic environments (Tsunogai et al., 2011). Although preliminary, the $\Delta^{17}O$ values in mosses showed clearly higher F_{atm} than vascular plants, especially in epilithic mosses. Although, the $\Delta^{17}O$ in terricolous mosses and vascular leaf samples was as low as 0.0–2.2‰, even at high NO_3^- concentration levels (**Figure 5**), suggesting a 0.0–8.8% of atmospheric contribution to leaf NO_3^- pool. The NRA should be responsible for $\delta^{18}O$ enrichment relative to the mixing values if plant-absorbed NO_3^- has not been influenced by denitrification in soil. Such characterization cannot be warranted by correlation between $\delta^{15}N$ and $\delta^{18}O$, or between tissue [NO_3^-] and isotopes (e.g., Liu et al., 2012c).

UNCERTAINTIES IN TISSUE NO_3^--ISOTOPE METHODS AND FUTURE WORKS

Although, the sampling time of plant materials can be controlled, diurnal and seasonal variations in tissue NO_3^- and its isotopes should be verified in future works. Until now, no experimental work has directly examined NR enzymatic isotope kinetics in roots and leaves of higher plants. Moreover, it is difficult to mimic *in situ* NR isotope effects in field conditions. Isotope effects associated with NO_3^- uptake and efflux remain unverified for roots. They were measured recently as 1–3‰ in growing cells of marine diatoms, and different O and N fractionations for both uptake and efflux were thought to cause the net $^{18}\varepsilon$:$^{15}\varepsilon$ of NO_3^- assimilation above 1 (Karsh et al., 2014). The routes of transformation and entry of inorganic and organic NO_3^- sources from the atmosphere into leaf cells and subsequent cellular actions have not been clarified, especially for non-aqueous processes. Consequently, the sources and supply rates of atmospheric NO_3^- and their isotope signals should be explored further. Thus far, the $\Delta^{17}O$ information of leaf NO_3^- was sparse, and is mostly available for leaves with high NO_3^- levels. It should be verified whether the atmospheric contribution is higher in low-[NO_3^-] leaves or not. It is promising to measure NO_3^- isotopes in xylem flow and twig samples for NO_3^- transportation and translocation. Results of such studies can potentially provide useful insights into intraplant NO_3^- transportation and translocation, although the sampling methods of xylem flow are mostly destructive and in-twig NO_3^- might be very low. For these reasons, more field works on tissue NO_3^- at the organ, stand, and species levels should be done along with source isotope analysis. The scenarios proposed above provide the first conceptual constraint for both sources and NO_3^- isotope effects in field plants. In conclusion, the concentration and isotopic analyses of NO_3^- in plant tissues together provide new insights for elucidating plant NO_3^- sources and strategies. These strategies will be valuable for exploring the communication of plant N utilization with environmental N pollution and altering ecosystem N cycles.

ACKNOWLEDGMENTS

This work was supported by a Grant for Projects for the Protection, Preservation and Restoration of Cultural Properties in Japan by the Sumitomo Foundation, Grants-in-Aid for Creative Scientific Research (Nos. 21310008), the Program to Create an Independent Research Environment for Young Researchers from the Ministry of Education, Culture, Sports, Science and Technology, Japan, the NEXT Program (GS008) from the Japan Society for the Promotion of Science (JSPS), and JSPS KAKENHI Grant Number 26252020. Xue-Yan Liu was also supported by the National Natural Science Foundation of China (No. 41021062, 41273026) and the JSPS postdoctoral program for foreign researchers (No. 09F09316). We appreciate Drs. Muenoki Yoh, Lina Koyama, and Arata Mukotaka for the fruitful discussions.

REFERENCES

Aakra, Å., Hesselsøe, M., and Bakken, L. R. (2000). Surface attachment of ammonia-oxidizing bacteria in soil. *Microbial. Ecol.* 39, 222–235. doi: 10.1007/s002480000006

Aber, J., McDowell, W., Nadelhoffer, K., Magill, A., Berntson, G., Kamakea, M., et al. (1998). Nitrogen saturation in temperate forest ecosystems: hypotheses revisited. *Bioscience* 48, 921–934. doi: 10.2307/1313296

Altieri, K., Hastings, M., Gobel, A., Peters, A., and Sigman, D. (2013). Isotopic composition of rainwater nitrate at Bermuda: the influence of air mass source and chemistry in the marine boundary layer. *J. Geophys. Res. Atmos.* 118, 304–311. doi: 10.1002/jgrd.50829

Alves, B. J. R., Zotarelli, L., Resende, A. S., Polidoro, J. C., Urquiaga, S., and Boddey, R. M. (2000). Rapid and sensitive determination of nitrate in plant tissue using flow injection analysis. *Commun. Soil Sci. Plant Anal.* 31, 2739–2750. doi: 10.1080/00103620009370623

Amberger, A., and Schmidt, H.-L. (1987). Natiirliche Isotopengehalte yon Nitrat als Indikatoren fiir dessen Herkunft. *Geochim. Cosmochim. Acta*. 51, 2699–2705. doi: 10.1016/0016-7037(87)90150-5

Anderson, K. A., and Case, T. E. (1999). Evaluation of plant nitrate extraction techniques and effect on commonly used analytical methods of detection. *Commun. Soil. Sci. Plant Anal.* 30, 1479–1495. doi: 10.1080/00103629909370301

Aslam, M. (1981). Reevaluation of anaerobic nitrite production as an index for the measurement of metabolic pool of nitrate. *Plant Physiol.* 68, 305–308. doi: 10.1104/pp.68.2.305

Atkin, O. K., Villar, R., and Cummings, W. R. (1993). The ability of several high arctic plant species to use nitrate nitrogen under field conditions. *Oecologia* 96, 239–245. doi: 10.1007/BF00317737

Beevers, L., and Hageman, R. H. (1969). Nitrate reduction in higher plants. *Annu. Rev. Plant Physiol.* 20, 495–522. doi: 10.1146/annurev.pp.20.060169.002431

Bergersen, F. J., Peoples, M. B., and Turner, G. L. (1988). Isotopic discriminations during the accumulation of nitrogen by soybeans. *Aust. J. Plant Physiol.* 15, 407–420. doi: 10.1071/PP9880407

Binnerup, S. J., and Sørensen, J. (1992). Nitrate and nitrite microgradients in barley rhizosphere as detected using a highly sensitive denitrification bioassay. *Appl. Environ. Microb.* 58, 2375–2380.

Bloom, A. J., Burger, M., Asensio, J. S. R., and Cousins, A. B. (2010). Carbon dioxide enrichment inhibits nitrate assimilation in wheat and *Arabidopsis*. *Science* 328, 899–903. doi: 10.1126/science.1186440

Bloom, A. J., Burger, M., Kimball, B. A., and Pinter, P. J. Jr. (2014). Nitrate assimilation is inhibited by elevated CO_2 in field-grown wheat. *Nat. Clim. Change* 4, 477–480. doi: 10.1038/nclimate2183

Boudsocq, S., Niboyet, A., Lata, J. C., Raynaud, X., Loeuille, N., Mathieu, J., et al. (2012). Plant preference for ammonium versus nitrate: a neglected determinant of ecosystem functioning? *Am. Nat.* 180, 60–69. doi: 10.1086/665997

Britto, D. T., and Kronzucker, H. J. (2013). Ecological significance and complexity of N-source preference in plants. *Ann. Bot.* 112, 957–963. doi: 10.1093/aob/mct157

Brown, L. L., and Drury, J. S. (1967). Nitrogen-isotope effects in the reduction of nitrate, nitrite, and hydroxylamine to ammonia. I In sodium hydroxide solution with Fe(II). *J. Chem. Phys.* 46, 2833–2837. doi: 10.1063/1.1841123

Campbell, W. H. (1999). Nitrate reductase structure, function and regulation: bridging the gap between biochemistry and physiology. *Annu. Rev. Plant Physiol.* 50, 277–303. doi: 10.1146/annurev.arplant.50.1.277

Cárdenas-Navarro, R., Adamowicz, S., and Robin, P. (1999). Nitrate accumulation in plants: a role for water. *J. Exp. Bot.* 50, 613–624. doi: 10.1093/jxb/50.334.613

Casciotti, K. L., Sigman, D. M., Hasting, G. M., Böhlke, J. K., and Hilkert, A. (2002). Measurement of the oxygen isotopic composition of nitrate in seawater and freshwater using the denitrifier method. *Anal. Chem*. 74, 4905–4912. doi: 10.1021/ac020113w

Cernusak, L. A., Winter, K., and Turner, B. L. (2009). Plant $\delta^{15}N$ correlates with the transpiration efficiency of nitrogen acquisition in tropical trees. *Plant Physiol*. 151, 1667–1676. doi: 10.1104/pp.109.145870

Chapin, F. S. III. (1980). The mineral nutrition of wild plants. *Annu. Rev. Ecol. Syst.* 11, 233–260. doi: 10.1146/annurev.es.11.110180.001313

Chapin, F. S. III., Moilanen, L., and Kielland, K. (1993). Preferential use of organic nitrogen for growth by a non-mycorrhizal arctic sedge. *Nature* 361, 150–153. doi: 10.1038/361150a0

Christensen, S., and Tiedje, J. M. (1988). Sub-parts-per-billion nitrate methods: use of an N_2O-producing denitrifier to convert NO_3^- or $^{15}NO_3^-$ to N_2O. *Appl. Environ. Microbiol.* 54, 1409–1413. Available online at: http://aem.asm.org/content/54/6/1409.abstract

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

Coplen, T. B. (2011). Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratio measurement results. *Rapid. Commun. Mass Spectrom.* 25, 2538–2560. doi: 10.1002/rcm.5129

Costa, A. W., Michalski, G., Alexander, B., and Shepson, P. B. (2011). Analysis of atmospheric inputs of nitrate to a temperate forest ecosystem from $\Delta^{17}O$ isotope ratio measurements. *Geophys. Res. Lett.* 38, L15805. doi: 10.1029/2011GL047539

Cove, D. J. (1966). The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. *Biochim. Biophys. Acta.* 113, 51–56. doi: 10.1016/S0926-6593(66)80120-0

Craine, J. M., Elmore, A. J., Aidar, M. P. M., Bustamante, M., Dawson, T. E., Hobbie, E. A., et al. (2009). Global patterns of foliar nitrogen isotopes and their relationships with climate, mycorrhizal fungi, foliar nutrient concentrations, and nitrogen availability. *New Phytol.* 183, 980–992. doi: 10.1111/j.1469-8137.2009.02917.x

Crawford, N. M. (1995). Nitrate: Nutrient and signal for plant growth. *Plant Cell* 7, 859–868. doi: 10.1105/tpc.7.7.859

Cruz, C. M., Soares, M. I. M., Martins-Loução, M. A., and Lips, S. H. (1991). Nitrate reduction in seedlings of carob (*Ceratonia siliqua* L.). *New Phytol*. 119, 413–419. doi: 10.1111/j.1469-8137.1991.tb00041.x

Elliott, E. M., Kendall, C., Boyer, E. W., Burns, D. A., Lear, G., Golden, H. E., et al. (2009). Dual nitrate isotopes in actively and passively collected dry deposition: utility for partitioning NO_x sources contributing to landscape nitrogen deposition. *J. Geophys. Res. Biogeosci*. 114:G04020. doi: 10.1029/2008JG000889

Emmerton, K. S., Callaghan, T. V., Jones, H. E., Leake, J. R., Michelsen, A., and Read, D. J. (2001). Assimilation and isotopic fractionation of nitrogen by mycorrhizal fungi. *New Phytol*. 151, 503–511. doi: 10.1046/j.1469-8137.2001.00178.x

Evans, R. D. (2001). Physiological mechanisms influencing plant nitrogen isotope composition. *Trends Plant Sci.* 6, 121–126. doi: 10.1016/S1360-1385(01)01889-1

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

Fang, Y. T., Koba, K., Makabe, A., Zhu, F. F., Fan, S. Y., Liu, X. Y., et al. (2012). Low $\delta^{18}O$ values of nitrate produced from nitrification in temperate forest soils. *Environ. Sci. Technol*. 46, 8723–8730. doi: 10.1021/es300510r

Felix, J. D., Elliott, E. M., and Shaw, S. (2012). The nitrogen isotopic composition of coal-fired power plant NO_x: the influence of emission controls and implications for global emission inventories. *Environ. Sci. Technol*. 46, 3528–3535. doi: 10.1021/es203355v

Fenn, M. E., and Poth, M. A. (1998). "Indicators of nitrogen status in California forests," in *Proceedings of the International Symposium on Air Pollution and Climate Change Effects on Forest Ecosystems,* eds A. Bytnerowicz, M. Arbaugh, and S. Schilling (Riverside, CA: GTR-166).

Fenn, M. E., Poth, M. A., and Johnson, D. W. (1996). Evidence for nitrogen saturation in the San Bernardino Mountains in southern California. *For. Ecol. Manage.* 82, 211–230. doi: 10.1016/0378-1127(95)03668-7

Ferrari, T. E., and Varner, J. E. (1970). Control of nitrate reductase activity in barley aleurone layers. *Proc. Natl. Acad. Sci. U.S.A*. 65, 3, 729–736. doi: 10.1073/pnas.65.3.729

Gebauer, G., Melzer, A., and Rehder, H. (1984). Nitrate content in *Rumex obtusifolius* L. I. Differences in organs and diurnal changes. *Oecologia* 63, 136–142. doi: 10.1007/BF00379795

Gebauer, G., Rehder, H., and Wollenweber, B. (1988). Nitrate, nitrate reduction and organic nitrogen in plants from different ecological and taxonomic groups of Central Europe. *Oecologia* 75, 371–385. doi: 10.1007/BF00376940

Gessler, A., Rienks, M., and Rennenberg, H. (2002). Stomatal uptake and cuticular adsorption contribute to dry deposition of NH_3 and NO_2 to needles of adult spruce (*Picea abies*) trees. *New Phytol.* 156, 179–194. doi: 10.1046/j.1469-8137.2002.00509.x

Granger, J., Sigman, D. M., Needoba, J. A., and Harrison, P. J. (2004). Coupled nitrogen and oxygen isotope fractionation of nitrate during assimilation by cultures of marine phytoplankton. *Limnol. Oceanogr.* 49, 1763–1773. doi: 10.4319/lo.2004.49.5.1763

Granger, J., Sigman, D. M., Rohde, M. M., Maldonado, M. T., and Tortell, P. D. (2010). N and O isotope effects during nitrate assimilation by unicellular prokaryotic and eukaryotic plankton cultures. *Geochim. Cosmochim. Acta* 74, 1030–1040. doi: 10.1016/j.gca.2009.10.044

Haberhauer, G., and Blochberger, K. (1999). A simple cleanup method for the isolation of nitrate from natural water samples for O isotope analysis. *Anal. Chem.* 71, 3587–3590. doi: 10.1021/ac9814243

Hanson, P. J., and Garten, C. T. (1992). Deposition of $H^{15}NO_3$ vapour to white oak, red maple and loblolly pine foliage: experimental observations and a generalized model. *New Phytol.* 122, 329–337. doi: 10.1111/j.1469-8137.1992.tb04238.x

Harrison, K. A., Bol, R., and Bardgett, R. D. (2007). Preferences for different nitrogen forms by coexisting plant species and soil microbes. *Ecology* 88, 989–999. doi: 10.1890/06-1018

Heaton, T. H. E. (1990). $^{15}N/^{14}N$ ratios of NO_x from vehicle engines and coal-fired power stations. *Tellus* 42, 304–307. doi: 10.1034/j.1600-0889.1990.00007.x-i1

Heaton, T. H. E., Spiro, B., and Roberston, S. M. C. (1997). Potential canopy influences on the isotopic composition of nitrogen and sulphur in atmospheric deposition. *Oecologia* 109, 600–660. doi: 10.1007/s004420050122

Hill, P. W., Marsden, K. A., and Jones, D. L. (2013). How significant to plant N nutrition is the direct consumption of soil microbes by roots? *New Phytol.* 199, 948–955. doi: 10.1111/nph.12320

Hipkin, C. R., Simpson, D. J., Wainwright, S. J., and Salem, M. A. (2004). Nitrification by plants that also fix nitrogen. *Nature* 430, 98–101. doi: 10.1038/nature02635

Ho, I., and Trappe, J. M. (1975). Nitrate reducing capacity of two vesicular-arbuscular mycorrhizal fungi. *Mycologia* 67, 886–888. doi: 10.2307/3758349

Hobbie, E. A., and Högberg, P. (2012). Nitrogen isotopes link mycorrhizal fungi and plants to nitrogen dynamics. *New Phytol*. 196, 367–382. doi: 10.1111/j.1469-8137.2012.04300.x

Högberg, P. (1997). ^{15}N natural abundance in soil-plant systems. *New Phytol.* 137, 179–203. doi: 10.1046/j.1469-8137.1997.00808.x

Högberg, P., Högberg, M. N., Quist, M. E., Ekblad, A., and Näsholm, T. (1999). Nitrogen isotope fractionation during nitrogen uptake by ectomycorrhizal and non-mycorrhizal *Pinus sylvestris*. *New Phytol.* 142, 569–576. doi: 10.1046/j.1469-8137.1999.00404.x

Højberg, O., Johansen, H. S., and Sørensen, J. (1994). Determination of ^{15}N abundance in nanogram pools of NO_3^- and NO_2^- by denitrification bioassay and mass spectrometry. *Appl. Environ. Microb*. 60, 2467–2472.

Houlton, B., Sigman, D., and Hedin, L. (2006). Isotopic evidence for large gaseous nitrogen losses from tropical rainforests. *Proc. Natl. Acad. Sci. U.S.A.* 103, 8745–8750. doi: 10.1073/pnas.0510185103

Imsande, J., and Touraine, B. (1994). N demand and the regulation of nitrate uptake. *Plant Physiol.* 105, 3–7.

Jones, M. E., Paine, T. D., and Fenn, M. E. (2008). The effect of nitrogen additions on oak foliage and herbivore communities at sites with high and low atmospheric pollution. *Environ. Pollut.* 151, 434–442. doi: 10.1016/j.envpol.2007.04.020

Kaiser, J., Hastings, M. G., Houlton, B. Z., Rockmann, T., and Sigman, D. M. (2007). Triple oxygen isotope analysis of nitrate using the denitrifier method and thermal decomposition of N_2O. *Anal. Chem.* 79, 599–607. doi: 10.1021/ac061022s

Kalcsits, L. A., and Guy, R. D. (2013). 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*. *Phys. Plant*. 149, 249–259. doi: 10.1111/ppl.12038

Karsh, K. L., Granger, J., Kritee, K., and Sigman, D. M. (2012). Eukaryotic assimilatory nitrate reductase fractionates N and O isotopes with a ratio near unity. *Environ. Sci. Technol.* 46, 5727-5735. doi: 10.1021/es204593q

Karsh, K. L., Trull, T. W., Sigman, D. M., Thompson, P. A., and Granger, J. (2014). The contributions of nitrate uptake and efflux to isotope fractionation during algal nitrate assimilation. *Geochim. Cosmochim. Acta* 132, 391-412. doi: 10.1016/j.gca.2013.09.030

Kendall, C., Elliott, E. M., and Wankel, S. D. (2007). "Tracing anthropogenic inputs of nitrogen to ecosystems," in *Stable Isotopes in Ecology and Environmental Science, 2nd Edn.*, eds R. M. Michener and K. Lajtha (Oxford: Blackwell; Oxford Press), 375–449. doi: 10.1002/9780470691854.ch12

Koba, K., Hirobe, M., Koyama, L., Kohzu, A., Tokuchi, N., Nadelhoffer, K., et al. (2003). Natural N-15 abundance of plants and soil N in a temperate coniferous forest. *Ecosystems* 6, 457–469. doi: 10.1007/s10021-002-0132-6

Koba, K., Inagaki, K., Sasaki, Y., Takebayashi, Y., and Yoh, M. (2010a). "Nitrogen isotopic analysis of dissolved inorganic and organic nitrogen in soil extracts," in *Earth, Life and Isotopes*, eds N. Ohkouchi, I. Tayasu, and K. Koba (Kyoto: Kyoto University Press), 17–37.

Koba, K., Isobe, K., Takebayashi, Y., Fang, Y. T., Sasaki, Y., Saito, W., et al. (2010b). $\delta^{15}N$ of soil N and plants in a N-saturated, subtropical forest of southern China. *Rapid Commun. Mass Spectrom.* 24, 2499–2506. doi: 10.1002/rcm.4648

Koba, K., Tokuchi, N., Yoshioka, T., Hobbie, E., and Iwatsubo, G. (1998). Natural abundance of nitrogen-15 in a forest soil. *Soil Sci. Soc. Amer. J.* 62, 778–781. doi: 10.2136/sssaj1998.03615995006200030034x

Kohl, D. H., and Shearer, G. (1980). Isotopic fractionation associated with symbiotic N_2 fixation and uptake of NO_3^- by plants. *Plant Physiol.* 66, 51–56. doi: 10.1104/pp.66.1.51

Koyama, L., Hirobe, M., Koba, K., and Tokuchi, N. (2013). Nitrate-use traits of understory plants as potential regulators of vegetation distribution on a slope in a Japanese cedar plantation. *Plant Soil* 362, 119–134. doi: 10.1007/s11104-012-1257-9

Kronzucker, H. J., Glass, A. D. M., and Siddiqi, M. Y. (1995). Nitrate induction in spruce: an approach using compartmental analysis. *Planta* 196, 683–690. doi: 10.1007/BF01106761

Lambers, H., Raven, J. A., Shaver, G. R., and Smith, S. E. (2008). Plant nutrient-acquisition strategies change with soil age. *Trends Ecol. Evolut.* 23, 95–103. doi: 10.1016/j.tree.2007.10.008

Laursen, K. H., Mihailova, A., Kelly, S. D., Epov, V. N., Bérail, S., Schjoerring, J. K., et al. (2013). Is it really organic? Multi-isotopic analysis as a tool to discriminate between organic and conventional plants. *Food Chem.* 141, 2812–2820. doi: 10.1016/j.foodchem.2013.05.068

LeBauer, D. S., and Treseder, K. K. (2008). Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. *Ecology* 89, 371–379. doi: 10.1890/06-2057.1

Ledgard, S. F., Woo, K. C., and Bergersen, F. J. (1985). Isotopic fractionation during reduction of nitrate and nitrite by extracts of spinach leaves. *Aust. J. Plant Physiol.* 12, 631–640. doi: 10.1071/PP9850631

Lensi, R., Gourbiere, F., and Josserand, A. (1985). Measurement of small amounts of nitrate in an acid soil by N_2O production. *Soil Biol. Biochem.* 17, 733–734. doi: 10.1016/0038-0717(85)90056-2

Lexa, M., and Cheeseman, J. M. (1997). Growth and nitrogen relations in reciprocal grafts of wild-type and nitrate reductase-deficient mutants of pea (*Pisum sativum* L. var. Juneau). *J. Exp. Bot.* 48, 1241–1250. doi: 10.1093/jxb/48.6.1241

Liu, X. Y., Koba, K., Liu, C. Q., Li, X. D., and Yoh, M. (2012c). Pitfalls and new mechanisms in moss isotopic bio-monitoring of atmospheric nitrogen deposition. *Environ. Sci. Technol.* 46, 12557–12566. doi: 10.1021/es300779h

Liu, X. Y., Koba, K., Makabe, A., Li, X. D., Yoh, M., and Liu, C. Q. (2013b). Ammonium first: natural mosses prefer atmospheric ammonium but vary utilization of dissolved organic nitrogen depending on habitat and nitrogen deposition. *New Phytol.* 199, 407–419. doi: 10.1111/nph.12284

Liu, X. Y., Koba, K., Takebayashi, Y., Liu, C. Q., Fang, Y. T., and Yoh, M. (2012a). Preliminary insights into $\delta^{15}N$ and $\delta^{18}O$ of nitrate in natural mosses: A new application of the denitrifier method. *Environ. Pollut.* 162, 48–55. doi: 10.1016/j.envpol.2011.09.029

Liu, X. Y., Koba, K., Takebayashi, Y., Liu, C. Q., Fang, Y. T., and Yoh, M. (2013a). Dual N and O isotopes of nitrate in natural plants: first insights into individual variability and organ-specific pattern. *Biogeochemistry* 114, 399–411. doi: 10.1007/s10533-012-9721-4

Liu, X. Y., Koba, K., Yoh, M., and Liu, C. Q. (2012b). Nitrogen and oxygen isotope effects of tissue nitrate associated with nitrate acquisition and utilization in the moss *Hypnum plumaeforme*. *Funct. Plant Biol.* 39, 598–608. doi: 10.1071/FP12014

Lockwood, A. L., Filley, T. R., Rhodes, D., and Shepson, P. B. (2008). Foliar uptake of atmospheric organic nitrates. *Geophys. Res. Lett.* 35:L15809. doi: 10.1029/2008GL034714

Mariotti, A., Germon, J. C., Hubert, P., Kaiser, P., Letolle, R., Tardieux, A., et al. (1981). Experimental determination of nitrogen kinetic isotope fractionation: some principles; illustration for the denitrification and nitrification processes. *Plant Soil* 62, 413–430. doi: 10.1007/BF02374138

Mariotti, A., Mariotti, F., Amarger, N., Pizelle, G., Ngambi, J., Champigny, M. L., et al. (1980). Eractionnements i.sotopiques de l'azoie lors des processus d'absorption des nitrates el de fixation de l'azote atmospherique par les plantes. *Phvsiologie Vegetale* 18, 163–181.

Mariotti, A., Mariotti, F., Champigny, M. L., Amarger, N., and Moyse, A. (1982). Nitrogen isotope fractionation associated with nitrate reductase-activity and uptake of NO_3^- by pearl millet. *Plant Physiol.* 69, 880–884. doi: 10.1104/pp.69.4.880

McIlvin, M. R., and Casciotti, K. L. (2011). Technical updates to the bacterial method for nitrate isotopic analyses. *Anal. Chem.* 83, 1850–1856. doi: 10.1021/ac1028984

McKane, R. B., Johnson, L. C., Shaver, G. R., Nadelhoffer, K. J., Rastetter, E. B., Fry, B., et al. (2002). Resource-based niches provide a basis for plant species diversity and dominance in arctic tundra. *Nature* 415, 68–71. doi: 10.1038/415068a

Michalski, G. (2010). Purification procedure for $\delta^{15}N$, $\delta^{18}O$, $\Delta^{17}O$ analysis of nitrate. *Int. J. Environ. Anal. Chem.* 90, 586–590. doi: 10.1080/03067310902783593

Mihailova, A., Pedentchouk, N., and Kelly, S. D. (2014). Stable isotope analysis of plant-derived nitrate – novel method for discrimination between organically and conventionally grown vegetables. *Food Chem.* 154, 238–245. doi: 10.1016/j.foodchem.2014.01.020

Miller, A. J., and Smith, S. J. (1996). Nitrate transport and compartmentation in cereal root cells, *J. Exp. Bot.* 47, 843–854. doi: 10.1093/jxb/47.7.843

Min, X., Siddiqi, M. Y., Guy, R. D., Glass, A. D. M., and Kronzucker, H. J. (1998). Induction of nitrate uptake and nitrate reductase activity in trembling aspen and lodgepole pine. *Plant Cell Environ.* 21, 1039–1046. doi: 10.1046/j.1365-3040.1998.00340.x

Mukotaka, A. (2014). *A Study of Nitrogen Oxides Dynamics Between Urban Atmosphere and the Phyllosphere Using Triple Oxygen Isotopes*. Ph. D. Dissertation, Tokyo Institute of Technology, Tokyo, 2–47.

Nacry, P., Bouguyon, E., and Gojon, A. (2013). Nitrogen acquisition by roots: physiological and developmental mechanisms ensuring plant adaptation to a fluctuating resource. *Plant Soil* 370, 1–29. doi: 10.1007/s11104-013-1645-9

Nadelhoffer, K. J., Shaver, G., Fry, B., Johnson, L., and McKane, R. (1996). ^{15}N natural abundances and N use by tundra plants. *Oecologia* 107, 386–394. doi: 10.1007/BF00328456

Näsholm, T., Kielland, K., and Ganeteg, U. (2009). Uptake of organic nitrogen by plants. *New Phytol.* 182, 31–48. doi: 10.1111/j.1469-8137.2008.02751.x

Needoba, J. A., and Harrison, P. J. (2004). Influence of low light and a light:dark cycle on NO_3^- uptake, intracellular NO_3^-, and nitrogen isotope fractionation by marine phytoplankton. *J. Phycol.* 40, 505–516. doi: 10.1111/j.1529-8817.2004.03171.x

Needoba, J. A., Sigman, D. M., and Harrison, P. J. (2004). The mechanism of isotope fractionation during algal nitrate assimilation as illuminated by the $^{15}N/^{14}N$ of intracellular nitrate. *J. Phycol.* 40, 517–522. doi: 10.1111/j.1529-8817.2004.03172.x

Needoba, J. A., Waser, N. A., Harrison, P. J., and Calvert, S. E. (2003). Nitrogen isotope fractionation in 12 species of marine phytoplankton during growth on nitrate. *Mar. Ecol. Prog. Ser.* 255, 81–91. doi: 10.3354/meps255081

Norby, R. J., Weerasuriya, Y., and Hanson, P. J. (1989). Induction of nitrate reductase activity in red spruce needles by NO_2 and HNO_3 vapor. *Can. J. For. Res.* 19, 889–896. doi: 10.1139/x89-135

Norwitz, G., and Keliher, P. N. (1986). Study of organic interferences in the spectrophotometric determination of nitrite using composite diazotization-coupling reagents: *Analyst* 111, 1033–1037. doi: 10.1039/an9861101033

Olleros-Izard, T. (1983). *Kinetische Isotopeneffekte der Arginase und Nitratreduktase Reaktion: ein Betrag zur Aufkl˙arung der entsprechenden Reaktionmechanismen*. Ph. D. thesis, Technischen Universität München, Freising-Weihenstephan.

Pate, J. S., Stewart, G. R., and Unkovich, M. (1993). ^{15}N natural abundance of plant and soil components of a Banksia woodland ecosystem in relation to nitrate utilization, life form, mycorrhizal status and Ns-fixing abilities of component species. *Plant Cell Environ.* 16, 365–373. doi: 10.1111/j.1365-3040.1993.tb00882.x

Peuke, A. D., Gessler, A., and Tcherkez, G. (2013). Experimental evidence for diel $\delta^{15}N$-patterns in different tissues, xylem and phloem saps of castor bean (*Ricinus communis* L.). *Plant Cell Environ.* 36, 2219–2228. doi: 10.1111/pce.12132

Prasad, S., and Chetty, A. A. (2008). Nitrate-N determination in leafy vegetables: Study of the effects of cooking and freezing. *Food Chem.* 106, 772–780. doi: 10.1016/j.foodchem.2007.06.005

Raven, J. A. (2003). Can plants rely on nitrate? *Trends Plant Sci.* 8, 314–315. doi: 10.1016/S1360-1385(03)00125-0

Raven, J. A., Wollenweber, B., and Handley, L. L. (1992). A comparison of ammonium and nitrate as nitrogen sources for photolithotrophs. *New Phytol.* 121, 19–32. doi: 10.1111/j.1469-8137.1992.tb01088.x

Raven, J. A., and Yin, Z. H. (1998). The past, present and future of nitrogenous compounds in the atmosphere, and their interactions with plants. *New Phytol.* 139, 205–219. doi: 10.1046/j.1469-8137.1998.00168.x

Robe, W. E., Griffiths, H., Sleep, D., and Quarmby, C. (1994). Nitrogen partitioning and assimilation: methods for the extraction, separation and mass spectrometric analysis of nitrate, amino acid and soluble protein pools from individual plants following ^{15}N labelling. *Plant Cell Environ.* 17, 1073–1079. doi: 10.1111/j.1365-3040.1994.tb02031.x

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

Robinson, D., Handley, L. L., and Scrimgeour, C. M. (1998). A theory for $^{15}N/^{14}N$ fractionation in nitrate-grown vascular plants. *Planta* 205, 397–406. doi: 10.1007/s004250050336

Scheible, W. R., Gonzalez-Fontes, A., Morcuende, R., Lauerer, M., Muller-Rober, B., Michel Caboche, M., et al. (1997b). Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. *Plant Cell.* 9, 783–798. doi: 10.1105/tpc.9.5.783

Scheible, W. R., Lauerer, M., Schulze, E. D., Caboche, M., and Stitt, M. (1997a). Accumulation of nitrate in the shoot acts as signal to regulate shoot-root allocation in tobacco. *Plant J.* 11, 671–691. doi: 10.1046/j.1365-313X.1997.11040671.x

Scheurwater, I., Koren, M., Lambers, H., and Atkin, O. K. (2002). The contribution of roots and shoots to whole plant nitrate reduction in fast- and slow-growing grass species. *J. Exp. Bot.* 53, 1635–1642. doi: 10.1093/jxb/erf008

Schimel, J. P., and Bennett, J. (2004). Nitrogen mineralization: challenges of a changing paradigm. *Ecology* 85, 591–602. doi: 10.1890/03-8002

Shearer, G., Schneider, J. D., and Kohl, D. H. (1991). Separating the efflux and influx components of net nitrate uptake by *Synechococcus*-R2 under steady-state conditions. *J. Gen. Microbiol.* 137, 1179–1184. doi: 10.1099/00221287-137-5-1179

Sievering, H., Tomasewski, T., and Torizzo, J. (2007). Canopy uptake of atmospheric N deposition at a conifer forest: part I -canopy N budget, photosynthetic efficiency and net ecosystem exchange. *Tellus B* 59, 483–492. doi: 10.1111/j.1600-0889.2007.00264.x

Sigman, D. M., Casciotti, K. L., Andreani, M., Barford, C., Galanter, M., and Böhlke, J. K. (2001). A bacterial method for the nitrogen isotopic analysis of nitrate in seawater and freshwater. *Anal Chem.* 73, 4145–4153. doi: 10.1021/ac010088e

Simon, J., Li, X. Y., and Rennenberg, H. (2014). Competition for nitrogen between European beech and sycamore maple shifts in favour of beech with decreasing light availability. *Tree Physiol.* 34, 49–60. doi: 10.1093/treephys/tpt112

Somers, D. A., Kuo, T. M., Kleinhofs, A., Warner, R. L., and Oaks, A. (1983). Synthesis and degradation of barley nitrate reductase. *Plant Physiol.* 72, 949–952. doi: 10.1104/pp.72.4.949

Sparks, J. P. (2009). Ecological ramifications of the direct foliar uptake of nitrogen. *Oecologia* 159, 1–13. doi: 10.1007/s00442-008-1188-6

Sparks, J. P., Monson, R. K., Sparks, K. L., and Lerdau, M. T. (2001). Leaf uptake of nitrogen dioxide (NO_2) in a tropical wet forest: implications for tropospheric chemistry. *Oecologia* 127, 214–221. doi: 10.1007/s004420000594

Stadler, J., and Gebauer, G. (1992). Nitrate reduction and nitrate content in ash trees (*Fraxinus excelsior* L.): distribution between compartments, site comparison and seasonal variation. *Trees* 6, 236–240. doi: 10.1007/BF00224342

Stams, A. J. M., and Schipholt, I. J. L. (1990). Nitrate accumulation in leaves of vegetation of a forested ecosystem receiving high amounts of atmospheric ammonium sulfate. *Plant Soil* 125, 143–145. doi: 10.1007/BF00010754

Stevens, C. J., Manning, P., van den Berg, L. J. L., de Graaf, M. C. C., Wamelink, G. W. W., Boxman, A. W., et al. (2011). Ecosystem responses to reduced and oxidised nitrogen inputs in European terrestrial habitats. *Environ. Pollut.* 159, 665–676. doi: 10.1016/j.envpol.2010.12.008

Stewart, G. R., Joly, C. A., and Smirnoff, N. (1992). Partitioning of inorganic nitrogen assimilation between the roots and shoots of *cerrado* and forest trees of contrasting plant communities of South East Brasil. *Oecologia* 91, 511–517. doi: 10.1007/BF00650324

Stewart, G. R., Pate, G. S., and Unkovich, M. (1993). Characteristics of inorganic nitrogen assimilation of plants in fire-prone Mediterranean-type vegetation. *Plant Cell Environ.* 16, 351–363. doi: 10.1111/j.1365-3040.1993.tb00881.x

Takebayashi, Y., Koba, K., Sasaki, Y., Fang, Y. T., and Yoh, M. (2010). The natural abundance of ^{15}N in plant and soil-available N indicates a shift of main plant N resources to NO_3 from NH_4 along the N leaching gradient. *Rapid Commun. Mass Spectrom.* 24, 1001–1008. doi: 10.1002/rcm.4469

Tang, M. H., Porder, S., and Lovett, G. M. (2012). Species differences in nitrate reductase activity are unaffected by nitrogen enrichment in northeastern US forests. *For. Ecol. Manage.* 275, 52–59. doi: 10.1016/j.foreco.2012.03.006

Taylor, P. G., and Townsend, A. R. (2010). Stoichiometric control of organic carbon-nitrate relationships from soils to the sea. *Nature* 464, 1178–1181. doi: 10.1038/nature08985

Tcherkez, G., and Farquhar, G. D. (2006). Viewpoint: Isotopic fractionation by plant nitrate reductase, twenty years later. *Funct. Plant Biol.* 33, 531–537. doi: 10.1071/FP05284

Teklemariam, T. A., and Sparks, J. P. (2004). Gaseous fluxes of peroxyacetyl nitrate (PAN) into plant leaves. *Plant Cell Environ.* 27, 1149–1158. doi: 10.1111/j.1365-3040.2004.01220.x

Tischner, R. (2000). Nitrate uptake and reduction in higher and lower plants. *Plant Cell Environ.* 23, 1005–1024. doi: 10.1046/j.1365-3040.2000.00595.x

Tsunogai, U., Daita, S., Komatsu, D. D., Nakagawa, F., and Tanaka, A. (2011). Quantifying nitrate dynamics in an oligotrophic lake using $\Delta^{17}O$. *Biogeosciences* 8, 687–702. doi: 10.5194/bg-8-687-2011

Vallano, D. M., and Sparks, J. P. (2008). Quantifying foliar uptake of gaseous nitrogen dioxide using enriched foliar $\delta^{15}N$ values. *New Phytol.* 177, 946–955. doi: 10.1111/j.1469-8137.2007.02311.x

Vitousek, P. M., and Howarth, R. W. (1991). Nitrogen limitation on land and in the Sea: How can it occur? *Biogeochemistry.* 13, 87–115. doi: 10.1007/BF00002772

Volk, R. J., Pearson, C. J., and Jackson, W. A. (1979). Reduction of plant tissue nitrate to nitric oxide for mass spectrometric N analysis. *Anal. Biochem.* 97, 131–135. doi: 10.1016/0003-2697(79)90336-1

Wanek, W., and Zotz, G. (2011). Are vascular epiphytes nitrogen or phosphorus limited? A study of plant ^{15}N fractionation and foliar N:P stoichiometry with the tank bromeliad *Vriesea sanguinolenta*. *New Phytol.* 192, 462–470. doi: 10.1111/j.1469-8137.2011.03812.x

Wang, L., and Schjoerring, J. K. (2012). Seasonal variation in nitrogen pools and $^{15}N/^{13}C$ natural abundances in different tissues of grassland plants. *Biogeosciences* 9, 1583–1595. doi: 10.5194/bg-9-1583-2012

Wang, Y. Y., Hsu, P. K., and Tsay, Y. F. (2012). Uptake, allocation and signaling of nitrate. *Trends Plant Sci.* 17, 458–467. doi: 10.1016/j.tplants.2012.04.006

Wellburn, A. R. (1990). Why are atmospheric oxides of nitrogen usually phytotoxic and not alternative fertilizers? *New Phytol.* 115, 395–429. doi: 10.1111/j.1469-8137.1990.tb00467.x

Wellburn, A. R. (1998). Atmospheric nitrogenous compounds and ozone - is NO_x fixation by plants a possible solution? *New Phytol.* 139, 5–9. doi: 10.1046/j.1469-8137.1998.00178.x

Werner, R. A., and Schmidt, H. L. (2002). The *in vivo* nitrogen isotope discrimination among organic plant compounds. *Phytochemistry* 61, 465–484. doi: 10.1016/S0031-9422(02)00204-2

Widmann, K., Gebauer, G., Rehder, H., and Ziegler, H. (1993). Fluctuations in nitrate reductase activity, and nitrate and organic nitrogen concentrations of succulent plants under different nitrogen and water regimes. *Oecologia* 94, 146–152. doi: 10.1007/BF00317316

Yoneyama, T., Ito, O., and Engelaar, W. M. H. G. (2003). Uptake, metabolism and distribution of nitrogen in crop plants traced by enriched and natural ^{15}N: progress over the last 30 years. *Phytochem. Rev.* 2, 121–132. doi: 10.1023/B:PHYT.0000004198.95836.ad

Yoneyama, T., and Kaneko, A. (1989). Variations in the natural abundance of ^{15}N in nitrogenous fractions of *komatsuna* plants supplied with nitraie. *Plant Cell Physiol*. 30, 957–962.

Yoneyama, T., Matsumaru, T., Usui, K., and Engelaar, W. M. H. G. (2001). Discrimination of nitrogen isotopes during absorption of ammonium and nitrate at different nitrogen concentrations by rice *Oryza sativa* L. plants. *Plant Cell Environ.* 24, 133–139. doi: 10.1046/j.1365-3040.2001.00663.x

Yoneyama, T., and Tanaka, F. (1999). Natural abundance of ^{15}N in nitrate, ureides, and amino acids from plant tissues. *Soil Sci. Plant Nutr.* 45, 751–755. doi: 10.1080/00380768.1999.10415840

Zhen, R. G., Koyro, H. W., Leigh, R. A., Tomos, A. D., and Miller, A. J. (1991). Compartmental nitrate concentrations in barley root cells measured with nitrate-selective microelectrodes and by single-cell sap sampling. *Planta* 185, 356–361. doi: 10.1007/BF00201056

Zhen, R. G., and Leigh, R. A. (1990). Nitrate accumulation by wheat (*Triticum aestivum*) in relation to growth and tissue concentrations. *Plant Soil* 124, 157–160. doi: 10.1007/BF00009253

Conflict of Interest Statement: 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.

Received: 30 January 2014; accepted: 03 July 2014; published online: 23 July 2014.
*Citation: Liu X-Y, Koba K, Makabe A and Liu C-Q (2014) Nitrate dynamics in natural plants: insights based on the concentration and natural isotope abundances of tissue nitrate. Front. Plant Sci. **5**:355. doi: 10.3389/fpls.2014.00355*
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science.
Copyright © 2014 Liu, Koba, Makabe and Liu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.