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SHORT COMMUNICATION

Dual N and O isotopes of nitrate in natural plants: first insights into individual variability and organ-specific patterns

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Abstract Nitrate (NO_3^-) is an important form of nitrogen (N) available to plants. The measurements of NO_3^- concentration [NO_3^-] and isotopes ($\delta^{15}N$ and $\delta^{18}O$) in plants provide unique insights into ecosystem NO_3^- availability and plant NO_3^- dynamics. This work investigated the variability of these parameters in individuals of a broadleaved (*Aucuba japonica*) plant and a coniferous (*Platycladus orientalis*) plant, and explored the applicability of tissue NO_3^- isotopes for deciphering plant NO_3^- utilization mechanisms. The NO_3^- in washed leaves showed concentration and isotopic ratios that were much lower than that in unwashed leaves, indicating a low contribution of atmospheric NO_3^- to NO_3^- in leaves. Current leaves showed higher [NO_3^-] and isotopic ratios than mature leaves. Moreover, higher leaf [NO_3^-] and isotopic enrichments (relative to soil NO_3^-) were found under higher soil NO_3^- availability for *A. japonica*. In contrast, leaves of *P. orientalis* showed low [NO_3^-] and negligible isotopic enrichments despite high soil NO_3^-. Higher [NO_3^-] was found in both fine and coarse roots of the *P. orientalis* plant, but significant isotopic enrichment was found only in coarse roots. These results reflect that the NO_3^- accumulation and isotopic effects decreased with leaf age, but increased with soil NO_3^- supply. Leaves are therefore identified as a location of NO_3^- reduction for *A. japonica*, while *P. orientalis* did not assimilate NO_3^- in leaves but in coarse roots. This work provided the first organ-specific information on NO_3^- isotopes in plant individuals, which will stimulate further studies of NO_3^- dynamics in a broader spectrum of plant ecosystems.

Keywords Denitrifier method · Nitrate reduction · ^{15}N · ^{18}O · Soil N availability · Natural plant

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Introduction

Nitrate (NO_3^-) is a major form of nitrogen (N) supporting plant growth and development (Högberg 1997; Schimel and Bennett 2004), which also plays an important signaling role for plants (Crawford 1995; Tischner 2000). Therefore, great interest has prevailed in plant NO_3^- studies associated with plant physiology and ecosystem N availability (Gebauer et al. 1988; Atkin et al. 1993; Kahmen et al. 2008). In natural forest soils, the net nitrification rate is known to be generally low, but the actual availability of NO_3^- for plants is difficult to assess (Schimel and Bennett 2004). For example, studies of N-limited ecosystems

in which soil NO_3^- was seldom detected revealed considerable reliance of plants on NO_3^- (Nadelhoffer et al. 1996; McKane et al. 2002; Nordin et al. 2004; Tye et al. 2005). Insights from ^{15}N-labeling were straightforward, but the addition of $^{15}NO_3^-$ is often large to guarantee plant uptake, by which the soil N status and dynamics might be changed, especially for N-limited systems. Therefore, deciphering the small NO_3^- pool in a plant body might greatly help in studying the availability of NO_3^- for natural plants.

In addition to soil NO_3^- availability, the NO_3^- concentration in plant tissues is determined by the NO_3^- utilization, i.e., the uptake, translocation, and assimilation (Granstedt and Huffaker 1982; Tischner 2000). Natural plants have continued to evolve with varying characteristics of N utilization in response to changing environments and N availability (Atkin et al. 1993). A major strategy is to regulate the pool size and reduction site of NO_3^- in a way that minimizes differences in growth (Andrews 1986a, b; Lexa and Cheeseman 1997). Therefore, differentiation of shoot-localized versus root-localized NO_3^- assimilation is meaningful for elucidating plant biomass partitioning and N usage strategies (Stewart et al. 1992; Scheurwater et al. 2002). The flexibility and responsiveness of plant NO_3^- assimilation has been characterized using NRA measurements (e.g., Schmidt and Stewart 1997; Scheurwater et al. 2002; Koyama and Kielland 2011). The relative concentration of NO_3^- and reduced N in the xylem sap was also examined to evaluate the partitioning of NO_3^- assimilation in shoots and roots (Andrews 1986a; Scheurwater et al. 2002). However, most studies have been conducted on N (^{15}N)-fertilized plants, for which substantial differences exist between lab and field conditions in terms of NO_3^- utilization strategies, even for plants of the same species. Moreover, natural plant canopies retain and incorporate atmospheric NO_3^- through foliar uptake, which has been shown by ^{15}N-labeling and field-manipulation experiments (Garten et al. 1998; Sparks 2009). However, neither tissue NO_3^- concentration ($[NO_3^-]$) nor NRA can differentiate NO_3^- source(s) and assess atmospheric contributions to leaf NO_3^- utilization effectively. It remains difficult for conventional methods to clarify the extent of the leaf-surface NO_3^- entering natural plant leaves (Ammann et al. 1999; Sparks 2009). Particularly in this regard, examination of isotopic differences of NO_3^- (especially $\delta^{18}O$) in the leaf body, soil, and the leaf surface of natural plants might help greatly.

Dual N and oxygen (O) isotopes of NO_3^- have been widely used in constraining the sources and behaviors of NO_3^- in ecosystems (Kendall et al. 2007). Thereby, $\delta^{15}N$ and $\delta^{18}O$ of NO_3^- in plant tissues should be explored to interpret the processing mechanisms of NO_3^- in plants (Evans et al. 1996; Yoneyama and Tanaka 1999; Needoba et al. 2004). For most terrestrial plants relying on soil NO_3^-, given null isotopic fractionations associated with root NO_3^- uptake processes (Kohl and Shearer 1980; Mariotti et al. 1982), tissue NO_3^- isotopes are mainly influenced by isotopic fractionations associated with NO_3^- reduction (Ledgard et al. 1985; Tcherkez and Farquhar 2006). The effect was suggested to be greater under high NO_3^- availability because of the larger fractions that undergo tissue NO_3^- reduction (Evans et al. 1996; Tcherkez and Hodges 2008). Therefore, both isotopic signatures and isotopic effects of tissue NO_3^- can provide insights into the location of NO_3^- reduction (Yoneyama and Tanaka 1999) and elucidate the relative importance of leaves and/or roots in NO_3^- uptake and assimilation (Mariotti et al. 1982; Evans et al. 1996; Robinson et al. 1998). To date, $\delta^{15}N$ studies of tissue NO_3^- have been performed on some fertilized plants (e.g., Mariotti et al. 1982; Ledgard et al. 1985; Yoneyama et al. 2001) and phytoplankton (e.g., Granger et al. 2004; Needoba et al. 2004). Robinson et al. (1998) generalized the $\delta^{15}N$ correlations of the main compartments in plant bodies. Physiological and biochemical mechanisms in plant NO_3^- utilization have been strengthened by several studies from isotopic perspectives (Evans 2001; Yoneyama et al. 2003; Tcherkez and Farquhar 2006; Tcherkez and Hodges 2008). However, $\delta^{18}O$-NO_3^- in terrestrial plants has been reported only for wheat by Olleros-Izard (1983). Neither $\delta^{15}N$ nor $\delta^{18}O$ of NO_3^- has been documented in natural vascular plants. The variations of these parameters among organs with different age and in different plant individuals have not been assessed to date.

Because of the low $[NO_3^-]$ and high DOC in extracts of natural plants, it is difficult for traditional isotopic methods to measure the concentration, $\delta^{15}N$, and $\delta^{18}O$ of tissue NO_3^-. However, the newly developed denitrifier method enables measurements of both $\delta^{15}N$ and $\delta^{18}O$ with 20–50 nmol NO_3^- (Sigman

et al. 2001; Casciotti et al. 2002). Furthermore, no interference of DOC occurs during sample pretreatment and bacterial conversion of NO_3^- in plant extracts (for a detailed review and methodology, see Liu et al. 2012). The present study was targeted at: (1) the isotopic differentiation of NO_3^- in leaves and on leaf surfaces, and their implications for atmospheric NO_3^- uptake; (2) whether the concentration and isotopes of NO_3^- in plant organs respond to soil NO_3^- availability or not, and whether they are useful to identify the site of NO_3^- reduction or not.

Materials and methods

Plant samples and treatment

This study was conducted in western Tokyo, Japan, which has a temperate monsoon climate with respective annual average temperature and rainfall of 15.3 °C and 1,790 mm. The open bulk N deposition measured in a field experimental station in this area (Field Museum Tamakyuryo, 35°59′N; 137°36′E) is nearly 17.9 kg N ha^{-1} $year^{-1}$ (Takebayashi et al. 2010 and references cited). A broadleaved plant (*Aucuba japonica*) and a coniferous plant (*Platycladus orientalis* (L.) Franco) were selected. Both are evergreen vascular plants.

For the examination of the difference between NO_3^- on the leaf surface and NO_3^- in the leaf body, current leaves of both species were sampled from two single trees in a woodland area of Fuchu (35°40′N; 139°28′E). Parts of leaf samples were washed immediately with deionized water to remove NO_3^- that had adsorbed onto the leaf surface. Washed and unwashed leaves ($n = 3$ for each) were then dried at 55 °C for about 48 h to constant weights. Then they were finely ground using a ball mill (MM200; Retsch GmbH and Co. KG).

To investigate responses of leaf [NO_3^-] and isotopes to soil NO_3^- availability, two *A. japonica* stands were chosen in the woodlands of Fuchu and Mt. Takao (35°38′N; 138°14′E) in western Tokyo, Japan. Current leaves ($n = 3$), petioles ($n = 3$), mature leaves ($n = 3$), and mineral soils (0–20 cm, $n = 6$) were collected from one plant individual of *A. japonica* at each site. To investigate NO_3^- reduction between leaves and roots, samplings of current leaves ($n = 6$) and mature leaves ($n = 3$), fine roots (1–3 mm diameter, $n = 5$) and coarse roots (5–10 mm diameter, $n = 6$), and mineral soils (0–30 cm, $n = 9$) were conducted on a single tree of the coniferous plant (*P. orientalis*) at the Fuchu site. All these plant samples were washed carefully, dried at 55 °C, and ground using the method described above. Soils were collected within 0.5 m from the tree trunk. Each soil sample was composited in the field from 3 to 5 subsamples. Soils were passed through a 2 mm mesh sieve to remove roots and coarse fragments. Sieved soils were used to determine water contents. They were extracted with 2 M KCl solution within 8 h after sampling.

The main purpose of this study was demonstration of the feasibility of using [NO_3^-] and isotopic compositions ($\delta^{15}N$ and $\delta^{18}O$) in plant organs for interpreting soil NO_3^- availability and plant NO_3^- utilization. Although our sampling scheme for individual plants cannot enable us to conclusively infer the NO_3^- utilizing characteristics of the studied species, the scheme presents the advantage of showing what parts of a tree individual (leaves or roots, current or mature leaves, fine or coarse roots) are responsive to NO_3^- reduction under uniform external NO_3^- (soil NO_3^- isotopes often varied among sites). Moreover, to reveal the degree to which those parameters vary among plant individuals will motivate subsequent studies of broad plant communities.

Nitrate extraction and measurement

In 20 ml headspace vials, 0.25 g dried plant and 10 ml deionized water were mixed. Then the vials were evacuated in a vacuum desiccator for 30 min for sufficient penetration of water into interstitial NO_3^-. The vials were crimp-sealed with Teflon-backed silicone septa (20-AC-CBT3; Chromacol) before shaking for 30 min. The denitrifier *Pseudomonas aureofaciens* (ATCC# 13985) was incubated for 6–10 days in working medium of Tryptic Soy Broth (Difco Laboratories) amended with KNO_3, NH_4Cl, and KH_2PO_4 according to the method described by Casciotti et al. (2002) and by Koba et al. (2010). Before use, *P. aureofaciens* was concentrated by centrifugation, washed with NO_3^- free medium and dispensed into new NO_3^- free medium. Then samples were purged with pure N_2 for 2 h. The prepared plant extract was purged with pure N_2 gas for 1 h, then 2 ml of the NO_3^- free medium with denitrifiers was injected into the vials using disposable syringes

(1–10 ml; Terumo Corp) and needles (26 gauge; Terumo Corp). The samples were incubated overnight on a horizontal shaker to allow for complete conversion of NO_3^- to N_2O before the addition of 0.2 ml of 5 M NaOH to stop the bacterial activity and to scavenge CO_2. The septa were sealed using silicone sealant (KE-42-T; Shin-Etsu Chemical Co. Ltd.) and were inverted to prevent leakage after each injection.

Concentrations of N_2O in the headspace were measured at 25 °C using a gas chromatograph equipped with an electron capture detector (GC/ECD, GC-14B; Shimadzu Corp., Kyoto, Japan). The calibration curve between the measured N_2O (peak area) and [NO_3^-] in extracted solution was prepared using standards with known [NO_3^-] (Binnerup and Sørensen 1992; Højberg et al. 1994; Liu et al. 2012). The [NO_3^-] in blanks was less than 0.1 μmol N l^{-1}. The [NO_3^-] in soil extracts was determined colorimetrically using an autoanalyzer (TRAACS 800; Bran-Luebbe, Tokyo, Japan). The KCl extracts were frozen until isotopic analysis using the denitrifier method described above (Koba et al. 2010).

The system used for isotopic measurement consisted of an isotope-ratio mass spectrometer (Delta XP; Thermo Fisher Scientific K.K., Yokohama, Japan) coupled with Precon (Thermo Finnigan) and GC (Agilent, HP6890; Hewlett Packard Co., Palo Alto, CA, USA) equipped with Poraplot column (25 m × 0.32 mm) and GC interface III (Thermo Fisher Scientific K.K., Yokohama, Japan). Then N_2O from the vial was cryofocused twice using liquid N_2. Subsequently, purified N_2O was introduced into the GC-IRMS; N_2O and CO_2 were separated chromatographically. The calibration curve between measured isotopes of N_2O and those of NO_3^- was prepared using USGS-32, USGS-34, USGS-35, and IAEA-NO_3. Natural abundances of ^{15}N and ^{18}O were calculated as $\delta^{15}N$ and $\delta^{18}O$ values in per mil (‰) units, as

$$\delta X = \left[\left(R_{\text{sample}}/R_{\text{standard}}\right) - 1\right] \times 10^3$$

where $X = {}^{15}N$ or ^{18}O and $R = {}^{15}N/{}^{14}N$ or $^{18}O/{}^{16}O$. The analytical precision for $\delta^{15}N$ was better than 0.2 ‰ and 0.5 ‰ for $\delta^{18}O$.

Statistics

To examine differences in [NO_3^-], $\delta^{15}N$-NO_3^- and $\delta^{18}O$-NO_3^- among treatments and organs, one-way analysis of variance (ANOVA) was conducted with the sample treatment and organ as the main effects. Replicates of plant tissues from single trees were pseudo in the strict sense of ecological studies. For that reason, they can only be used to compare specific differences among organs under the same soil context. Therefore, statistical results in this study cannot elucidate features of each species, but can only reflect that of the single tree we studied. Tukey's HSD procedure was used to make pairwise comparisons. Independent sample *t* tests were conducted to assess differences in soil [NO_3^-] among sites. Linear correlation analysis was used to examine the relations between [NO_3^-] and isotopic variables. Values are means ± SD. Statistically significant difference was inferred for $P < 0.05$. Statistical analyses were conducted using software (SPSS 13.0 for Windows; SPSS Inc.).

Results

Washed and unwashed leaves

Unwashed leaves showed remarkably higher [NO_3^-] (0.40 ± 0.00 μmol N g dw^{-1} for *A. japonica* and 0.27 ± 0.01 μmol N g dw^{-1} for *P. orientalis*; as means ± SD) than corresponding washed leaves (0.05 ± 0.00 μmol N g dw^{-1} and 0.08 ± 0.01 μmol N g dw^{-1}, respectively) (Fig. 1a). The $\delta^{15}N$ and $\delta^{18}O$ of NO_3^- determined from unwashed leaves (*A. japonica*: 5.1 ± 0.1 ‰ for $\delta^{15}N$ and 63.3 ± 0.2 ‰ for $\delta^{18}O$; *P. orientalis*: 4.8 ± 0.0 ‰ for $\delta^{15}N$ and 67.0 ± 0.4 ‰ for $\delta^{18}O$) were apparently higher than those of washed samples (*A. japonica*: −0.4 ± 0.3 ‰ for $\delta^{15}N$ and 36.1 ± 1.5 ‰ for $\delta^{18}O$; *P. orientalis*: 0.3 ± 0.1 ‰ for $\delta^{15}N$ and 54.1 ± 0.2 ‰ for $\delta^{18}O$) (Fig. 1b, c). Furthermore, species-dependent differences in NO_3^- isotopes were observed among washed leaves ($P < 0.05$), although unwashed leaves showed similar NO_3^- isotopes between the two plants (Fig. 1b, c).

Leaves of *A. japonica* under different soil NO_3^- availability

The means [NO_3^-] in leaves and petioles of *A. japonica* were 0.05–0.07 μmol N g dw^{-1} under low soil [NO_3^-] (0.12 μmol N g dw^{-1}), reaching

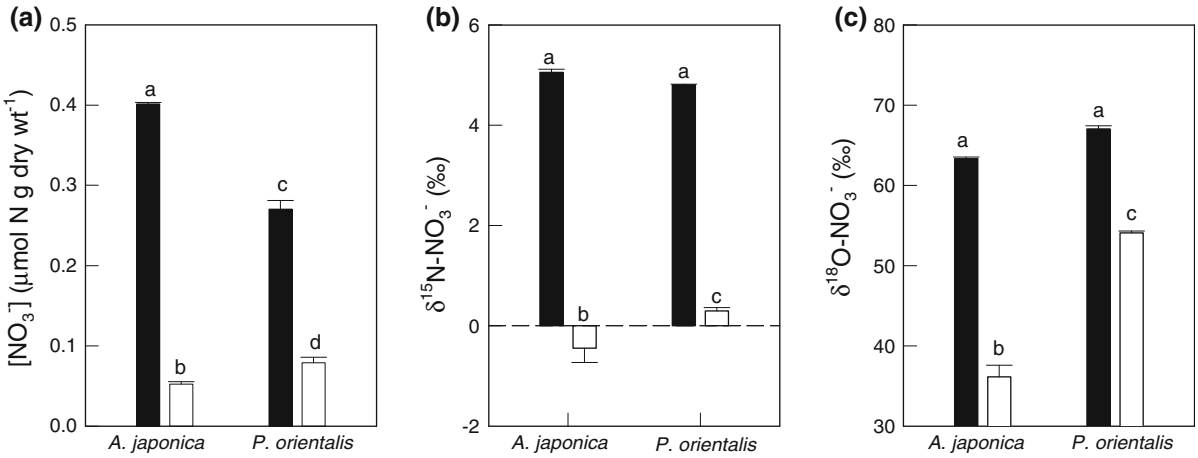

Fig. 1 (**a**) Concentration, (**b**) $\delta^{15}N$, and (**c**) $\delta^{18}O$ of NO_3^- of washed and unwashed leaves of *A. japonica* and *P. orientalis* ($n = 3$ per treatment). Values (mean ± SD) not sharing the *same letter* are significantly different ($P < 0.05$)

0.11–0.41 μmol N g dw^{-1} under higher soil $[NO_3^-]$ (0.35 μmol N g dw^{-1}) (Fig. 2a, b). In general, the $\delta^{15}N$ and $\delta^{18}O$ of leaf NO_3^- were enriched relative to soil NO_3^- isotopes (except for $\delta^{15}N$ of mature leaves). Furthermore, the isotopic enrichment was more apparent under higher soil NO_3^- availability (Fig. 2d, f). Moreover, under higher soil $[NO_3^-]$, current leaves and petioles showed significantly higher $[NO_3^-]$ and isotopes than mature leaves did (Fig. 2). Positive correlations were found between leaf $[NO_3^-]$ and isotopes (Fig. 3a). However, such differences in $[NO_3^-]$ and isotopes between current and mature leaves as well as concentration–isotope correlations were not pronounced for the plant with lower NO_3^- availability (Figs. 2, 3b).

Leaves and roots of *P. orientalis*

The site chosen for *P. orientalis* has higher soil $[NO_3^-]$ (0.45 ± 0.04 μmol N g dw^{-1}, Fig. 4a) than those of *A. japonica* (0.12–0.35 μmol N g dw^{-1}, Fig. 3a, b). However, *P. orientalis* had generally lower leaf $[NO_3^-]$ than those of *A. japonica*, and showed no substantial difference between current leaves (0.09 ± 0.00 μmol N g dw^{-1}) and mature leaves (0.08 ± 0.01 μmol N g dw^{-1}) (Fig. 4a). Leaf $\delta^{15}N$–NO_3^- of the *P. orientalis* (−0.1 ± 0.5 ‰ for current leaves and −1.9 ± 0.1 ‰ for mature leaves) did not differ from that of soil (−1.8 ± 1.4 ‰) (Fig. 4b). However, the $\delta^{18}O$–NO_3^- in leaves (52.1 ± 1.7 ‰ for current leaves and 45.8 ± 0.8 ‰ for mature leaves) was significantly greater than that of soil (7.2 ± 0.4 ‰) (Fig. 4c). Intriguingly, roots of *P. orientalis* showed much higher $[NO_3^-]$ than leaves, with the highest accumulation in the fine roots (1.04 ± 0.06 μmol N g dw^{-1}) (Fig. 4a), in which $\delta^{15}N$–NO_3^- (−5.2 ± 2.0 ‰) and $\delta^{18}O$–NO_3^- (−11.6 ± 6.0 ‰) were depleted compared with that of soil (Fig. 4b, c). Enriched δ^{15} N–NO_3^- values were only found in the coarse roots, in which both $\delta^{15}N$ and δ^{18} O showed a tendency of isotopic enrichments (relative to soil NO_3^-) with the decrease in $[NO_3^-]$. In contrast, the variation of $[NO_3^-]$ caused no substantial isotope effects on either N or O isotopes of leaf NO_3^- ($y = -0.8\ln x - 1.0$, $R^2 = 0.007$, $P = 0.83$ for δ^{15} N; $y = -2.2\ln x + 48.1$, $R^2 = 0.003$, $P = 0.87$ for $\delta^{18}O$) (Fig. 5b).

Discussion

NO_3^- on and in leaves

Significantly higher $[NO_3^-]$ of unwashed leaves indicates that the leaf-surface NO_3^- is a much larger pool than that in the leaf body. The $\delta^{15}N$ and $\delta^{18}O$ of NO_3^- extracted from unwashed leaves were close to isotopic values of atmospheric NO_3^- (Kendall et al. 2007), but they did not differ between broadleaved and coniferous species (Fig. 1), showing the dominance of atmospheric NO_3^- on leaf surfaces irrespective of the

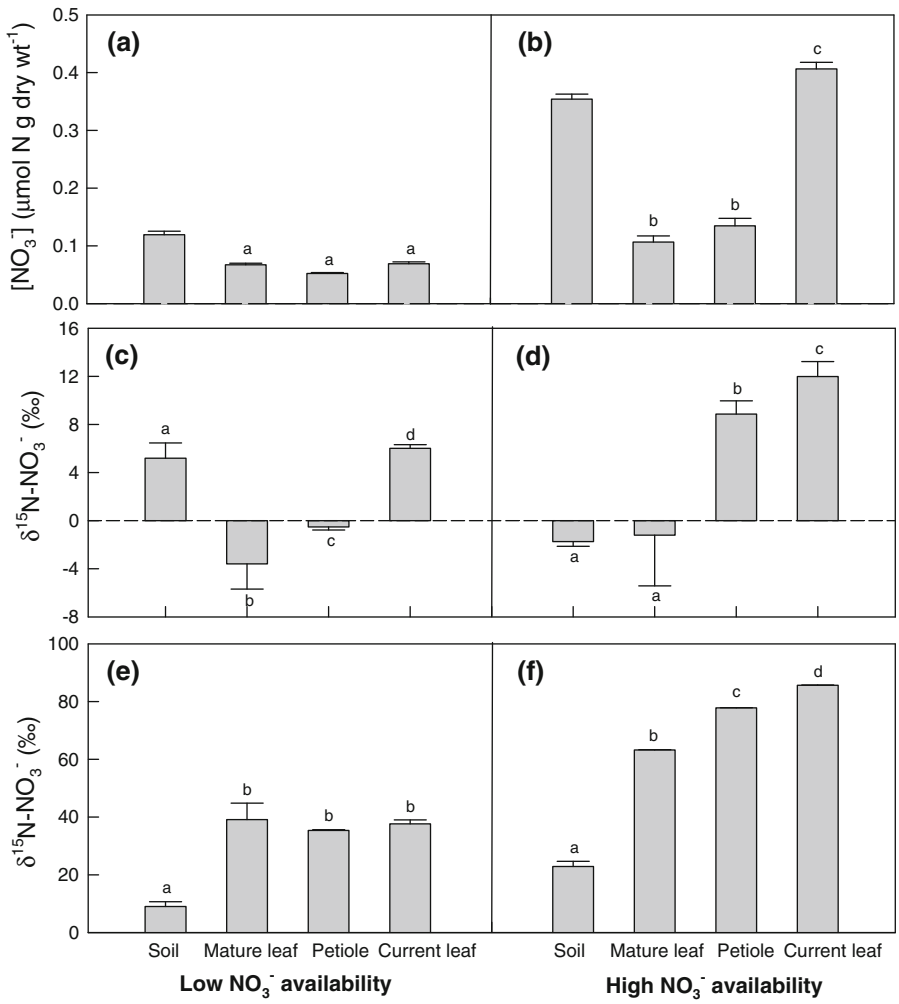

Fig. 2 Concentration (**a**, **b**), $\delta^{15}N$ (**c**, **d**), and $\delta^{18}O$ (**e**, **f**) of NO_3^- in petioles, current and mature leaves of *A. japonica* at sites with low and high soil NO_3^- availability ($n = 3$ for plant tissues, $n = 6$ for soils). Values (mean ± SD) not sharing the *same letter* are significantly different ($P < 0.05$)

species. A large body of evidence has been gained from ^{15}N tracer and field simulations for the canopy retention of atmospheric N, from which the retention was estimated variously as 0–50 % of plant N demand (for a review, see Sparks 2009). However, this does not mean that atmospheric N entered into leaves. It is difficult to quantify the leaf uptake of atmospheric NO_3^- in field conditions. In this study, the leaf-surface NO_3^- was differentiated and calculated according to the $[NO_3^-]$ between washed and unwashed leaves, accounting for averages of 87 % and 71 %, respectively, for *A. japonica* and *P. orientalis* leaves. The $\delta^{15}N$ and $\delta^{18}O$ of the leaf-surface NO_3^- is calculable using the following equations:

$$\delta^{15}N_{unwashed}\text{-}NO_3^- = f_{washed}\delta^{15}N_{washed}\text{-}NO_3^- + f_{surface}\delta^{15}N_{surface}\text{-}NO_3^-,$$

$$\delta^{18}O_{unwashed}\text{-}NO_3^- = f_{washed}\delta^{18}O_{washed}\text{-}NO_3^- + f_{surface}\delta^{18}O_{surface}\text{-}NO_3^-.$$

Results showed that the average $\delta^{15}N$ and $\delta^{18}O$ of leaf-surface NO_3^- were 6.5 ‰ and 73.8 ‰ for the *A. japonica*, and 7.0 ‰ and 68.7 ‰ for the *P. oriental*, respectively. These values showed integrated isotopic

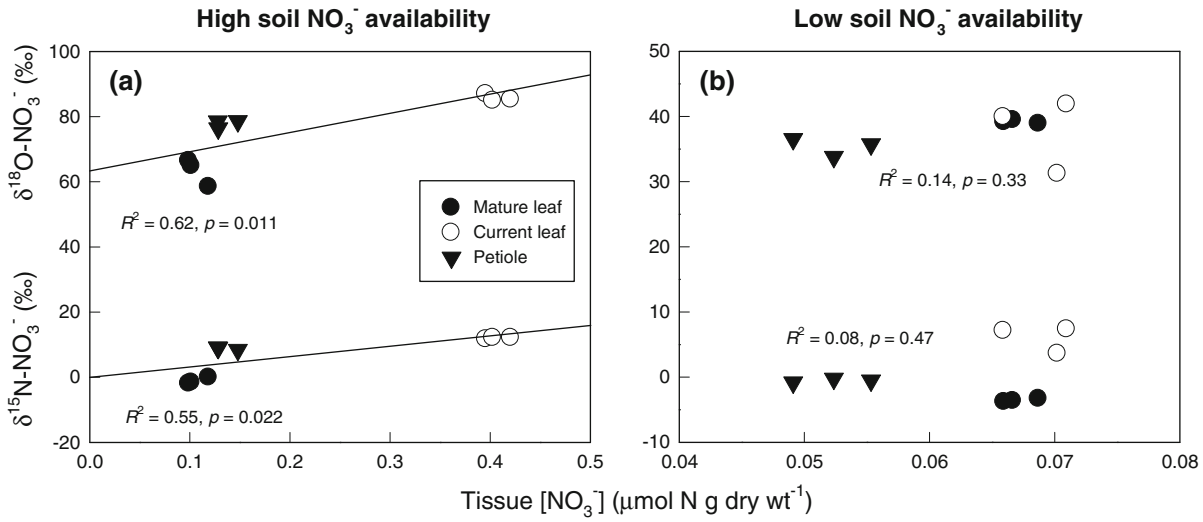

Fig. 3 Correlations of $[NO_3^-]$ with $\delta^{15}N$–NO_3^- (*open cycles*) and $\delta^{18}O$–NO_3^- (*filled cycles*) in leaves of *A. japonica* under (**a**) high and (**b**) low soil NO_3^- availability

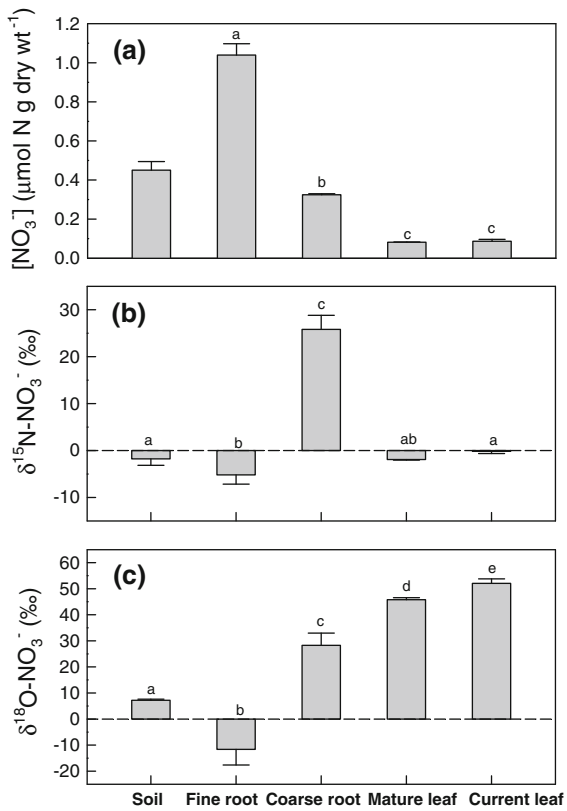

Fig. 4 (**a**) $[NO_3^-]$, (**b**) $\delta^{15}N$–NO_3^-, and (**c**) $\delta^{18}O$–NO_3^- in soil (0–30 cm, $n = 9$), fine roots ($n = 5$), coarse roots ($n = 6$), mature leaves ($n = 3$), and current leaves ($n = 6$) of *P. orientalis*. Values (mean ± SD) not sharing the *same letter* are significantly different ($P < 0.05$)

information of atmospheric NO_3^- available for leaf uptake, which might be different from those obtained from throughfall and deposition samples. Given the difficulties in measuring wet and dry deposition, NO_3^- analysis of unwashed leaves constitutes a quick and simple method to assess the in situ canopy retention of NO_3^- deposition. Moreover, because of the additional fractionation by NRA causing ^{15}N-enrichment of the residual NO_3^- pool, leaf NO_3^- isotopes are expected to be similar or enriched compared to those of atmospheric NO_3^- if the leaf cellular NO_3^- is dominated by atmospheric-derived NO_3^-. Oppositely, NO_3^- in washed leaves was isotopically depleted compared to unwashed leaves and wet deposition, reflecting a much low contribution of atmospheric NO_3^- to NO_3^- in the leaves. Furthermore, $\delta^{15}N$ values might be overlapped for NO_3^- on and in leaves (from atmospheric and soil respectively) because of ^{15}N fractionation of assimilation, whereas $\delta^{18}O$ is still straightforward because of much lower $\delta^{18}O$ of soil NO_3^- than that of atmospheric-derived NO_3^- (Kendall et al. 2007). Similar with our recognition, the direct uptake of N from deposition was estimated as 1–5.8 % of N in field coniferous leaves (Garten et al. 1998). Dail et al. (2009) also found that less than 5 % (3–6 %) of ^{15}N was recovered in living foliage and wood after a 2-year $^{15}NH_4^{15}NO_3$ addition to the canopy, showing very low N contributions directly from N deposition.

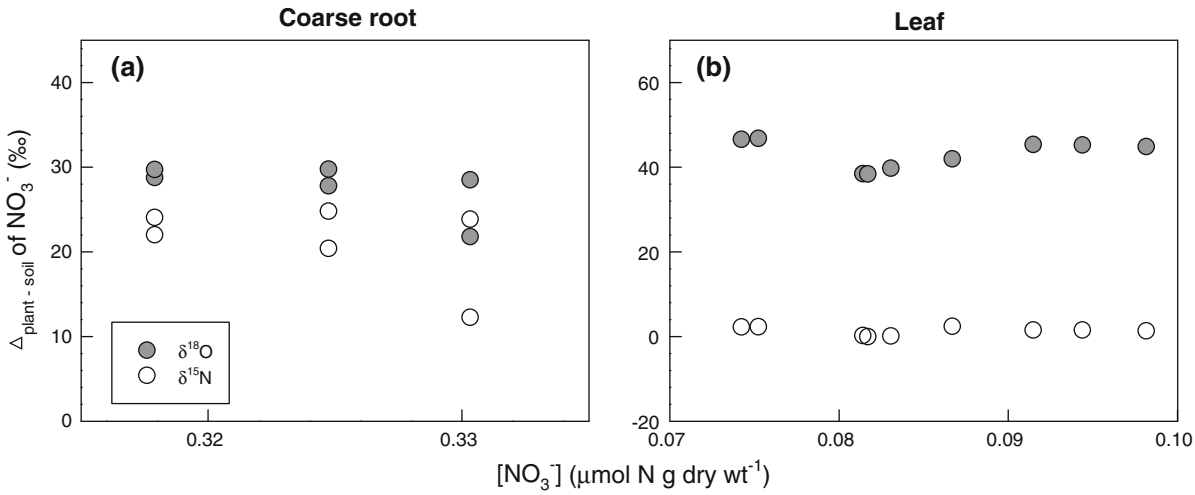

Fig. 5 N and O isotopic enrichments (relative to those of soil NO_3^-) of NO_3^- in (**a**) leaves, (**b**) coarse roots of *A. japonica* plotted against corresponding tissue $[NO_3^-]$

Leaf $[NO_3^-]$ and isotopes of *A. japonica*-in response to soil NO_3^- availability

Enhanced leaf $[NO_3^-]$ under higher soil $[NO_3^-]$ (Fig. 2a, b) reflected increased foliar NO_3^- uptake with soil NO_3^- availability (Fenn and Poth 1998). Higher $\delta^{15}N$ and $\delta^{18}O$ of leaf NO_3^- than those of soil NO_3^- resulted from isotopic fractionations during leaf NO_3^- reduction. The isotopic fractionation during plant NO_3^- utilization has been shown to be determined by the fractions of NO_3^- assimilation. If larger fractions of NO_3^- were assimilated, greater isotopic enrichment in residual substrate NO_3^- would be found (Mariotti et al. 1982; Robinson et al. 1998; Evans 2001). Experimentally, the $\delta^{15}N$ fractionation of plant bulk N was shown to be smaller under lower external $[NO_3^-]$. Stronger ^{15}N enrichment in residual NO_3^- in supplied solutions can occur simultaneously because more NO_3^- is assimilated at lower NO_3^- supplies (Kohl and Shearer 1980; Mariotti et al. 1982; Yoneyama and Kaneko 1989; Evans et al. 1996). Different from previous studies, we measured residual NO_3^- in plant tissues. Results showed greater isotopic enrichment in leaf NO_3^- under higher soil $[NO_3^-]$ (Fig. 2). Results of this measurement indicated that the isotopic fractionating mechanism observed for tissue NO_3^- differed slightly from that observed from measuring $\delta^{15}N$ of plant bulk N and residual NO_3^- in supplied solutions (Evans et al. 1996). Regarded in terms of the mechanism, higher soil NO_3^- availability induced higher uptake (the rate of uptake often exceeded assimilation; therefore, tissue NO_3^- can always be detected in plant organs; Evans 2001), NRA, and allocation of more NO_3^- according to the distribution of NR (Bloom et al. 1992; Crawford 1995; Tischner 2000). Under high soil NO_3^- availability, higher isotopic signatures of tissue NO_3^- resulted from larger fractions of tissue NO_3^- assimilation, but the residual $[NO_3^-]$ in plants with high soil $[NO_3^-]$ remained high because of higher uptake. Also for that reason, residual $[NO_3^-]$ in plant tissues was observed being responsive to soil NO_3^- availability. Under low soil NO_3^- availability, the plant had low NO_3^- uptake and lower fractions of NO_3^- assimilation, and therefore lower isotopic enrichment. A similar relation between N availability and N demand in controlling isotopic discriminations has also explained the observed intra-plant $\delta^{15}N$ variation in plants with different genotypes (Evans et al. 1996; Högberg et al. 1999). Our study revealed that leaf $[NO_3^-]$ (uptake), the fraction of NO_3^- assimilation (relative to uptake) and associated isotopic effects increased with soil NO_3^- availability (Tischner 2000; Evans 2001; Yoneyama et al. 2001). For the first time, the effect of NO_3^- availability on tissue NO_3^- isotopes in natural plants and the responsiveness of leaf $\delta^{18}O$–NO_3^- were documented.

Moreover, larger pool size and isotopic enrichments of NO_3^- were found in current leaves than in mature leaves (Fig. 2), reflecting intra-plant differences of NO_3^- uptake and assimilation. With the same

NO_3^- availability, NO_3^- uptake and allocation are expected to be higher in organs with higher N demand and reduction ability (such as current leaves), reflecting the specific demand-driven reduction mechanism (Imsande and Touraine, 1994; Tischner 2000). Older leaves usually have lower NR levels. Therefore, they contain less NO_3^- and lower isotopic signatures. With higher NRA, current leaves assimilated larger fractions of NO_3^- relative to the uptake. Thereby greater isotopic enrichment (compared with the soil NO_3^- source) occurred in current leaves than in mature leaves (Fig. 2). Furthermore, [NO_3^-] in petioles and in current and mature leaves of *A. japonica* showed positive correlation with $\delta^{15}N$ and $\delta^{18}O$ values (Fig. 3). Different from the tendency of isotopic enrichments with the decrease in [NO_3^-] in the same organ (e.g. Fig. 5) or $\delta^{15}N$ enrichments (relative to plant $\delta^{15}N$) in NO_3^- solutions supplied to the same plant (Evans et al. 1996), the positive correlation here reflected different abilities among these organs to uptake and assimilate NO_3^- (i.e., the regulation of soil NO_3^- on tissue [NO_3^-], whereas organ-specific NRA on the fraction of NO_3^- reduction and isotopic signatures). All these results suggest that soil NO_3^- can be reduced in the leaves of *A. japonica*, and that the pool size and isotopic effects of tissue NO_3^- increase with soil NO_3^- availability and organ-specific N demand.

Reduction of NO_3^- in coarse roots of *P. orientalis*

The *P. orientalis* plant showed lower leaf [NO_3^-] than that of *A. japonica* although the former had higher soil [NO_3^-] (Figs. 2b, 4a). This difference reflects the genotypical characteristics of NO_3^- accumulation, which differ among different plant taxa (Högberg et al. 1999; Malagoli et al. 2000; Evans 2001). Low leaf [NO_3^-] and non-significant $\delta^{15}N$ enrichment (relative to the soil NO_3^-) suggested low assimilation in leaves of the *P. orientalis* (Fig. 4), and/or a low NO_3^- use by this coniferous species with a preference for NH_4^+ or organic N (Fig. 5a). Moreover, [NO_3^-] and isotopes in current and mature leaves did not mutually differ, confirming low uptake and reduction even in current leaves of the *P. orientalis*. Accordingly, leaves were not the main site of NO_3^- assimilation in the investigated *P. orientalis*.

Higher root [NO_3^-] indicated a higher ability of NO_3^- accumulation in roots than in leaves of the *P. orientalis* (Fig. 4a). Previously, higher efficiency of NO_3^- utilization in roots than in leaves was also reported for other coniferous species (e.g., Ponderosa pine; Bassirirad et al. 1997). However, root NO_3^- assimilation is not always coupled to a plant's NO_3^- uptake. Reduction activities in roots can be unaffected by NO_3^- uptake and soil [NO_3^-] (Breteler and Nissen 1982; Andrews 1986a, b). In this case, a greater proportion of absorbed NO_3^- would remain unassimilated in roots. Thereby, root NO_3^- isotopes would not be enriched compared with those of soil NO_3^- (Robinson et al. 1998; Comstock 2001). According to this principle, the fine root appears not as a location of NO_3^- reduction in the *P. orientalis* because of lower isotopic ratios than those of soil NO_3^- ($\Delta\delta^{15}N\text{-nitrate}_{root-soil} = -3.4$ ‰, $\Delta\delta^{18}O\text{-nitrate}_{root-soil} = -18.8$ ‰; Figs. 4b, c). Principally, the transportation of NO_3^- into and out of root cells would not discriminate ^{15}N (e.g., by pearl millet, Mariotti et al. 1982; Shearer et al. 1991) because of the lack of bonding breakage during the diffusion of NO_3^- in a hydrated form through the membrane carriers (Werner and Schmidt 2002; Granger et al. 2004; Needoba et al. 2004). For example, $\delta^{15}N$ depletion of −5 ‰ was observed in plant bulk N relative to substrate NO_3^- supplied hydroponically (Kohl and Shearer 1980), which was interpreted as being caused by the efflux of ^{15}N-enriched residual NO_3^- from roots, not by the uptake process. However, the residual NO_3^- in fine roots should have assembled those of soil NO_3^- even if the reduction of NO_3^- and the accumulation or excretion of non-converted NO_3^- did not occur (Evans 2001; Tcherkez and Hodges 2008; Cernusak et al. 2009). Probably, the observed lower NO_3^- isotopes in fine roots than soil NO_3^- resulted from the uptake of ^{15}N-depleted NO_3^- from other parts of soil profiles.

Significant isotopic enrichment occurred in NO_3^- of coarse roots (Fig. 4), suggesting the reduction of NO_3^- in coarse roots of the studied *P. orientalis* (cypress species). Similarly, root NO_3^- assimilation was found to predominate in some coniferous species such as *Vigna unguiculata* and *Zea mays* (Andrews 1986a, b). Higher NRA in roots than in leaves is also normal in coniferous species such as *Pinus sylvestris* and *Larix deciduas* (Seith et al. 1994; Malagoli et al. 2000). Recently, we found that NO_3^- is a major N source for Hinoki cypress (*Chamaecyparis obtusa*) at N-rich sites (Takebayashi et al. 2010). However, our evidence shows only that coarse roots did reduce a large part of the NO_3^- imported into them. Because

of distinct differences between coarse roots and aboveground biomass, the evidence does not necessarily reflect that they are the major site of NO_3^- assimilation in this plant. Based on the organ-specific pattern of NO_3^- isotopes, the utilization of NO_3^- entering into the *P. orientalis* can be explained as a two-pool model: (1) NO_3^- is partly exported to coarse roots where it is assimilated and stored as organic N, and (2) the largest fraction of NO_3^- is exported to aboveground tissues, although the assimilation has not occurred temporarily. Measuring NO_3^- in xylem sap is necessary to interpret conclusively whether NO_3^- is reduced in roots, not in leaves.

Preliminary framework of NO_3^- isotopes in natural plants

The variation of tissue NO_3^- isotopes relative to soil and atmospheric NO_3^- sources was discussed further in the N–O isotopic framework (Fig. 6; typical values of atmospheric NO_3^- were cited from Kendall et al. 2007). Overall, the distribution of plant NO_3^- isotopes tended to differ between belowground and aboveground organs (Fig. 6). Root NO_3^- isotopes differed from those of soil NO_3^-, but they were distributed roughly along the 1:1 line (Fig. 6), yielding a slope of 1.3 in the $\delta^{15}N$–$\delta^{18}O$ plot. This distribution reflected that root NO_3^- isotopes were controlled mainly by the NO_3^- assimilation or other NO_3^--consuming processes, during which O isotope effects were similar to those of N isotope (Granger et al. 2004). The $\delta^{15}N$ variations of leaf NO_3^- were explainable in relation to soil NO_3^- availability, leaf age, and genotypical features. The NO_3^- isotopes in current leaves are generally more enriched than those of mature leaves. For that reason, they are more advantageous for identification of the reduction of NO_3^- in leaves of plant species.

Different from $\delta^{15}N$, the distribution of $\delta^{18}O$–NO_3^- in aboveground organs was clearly inclined to

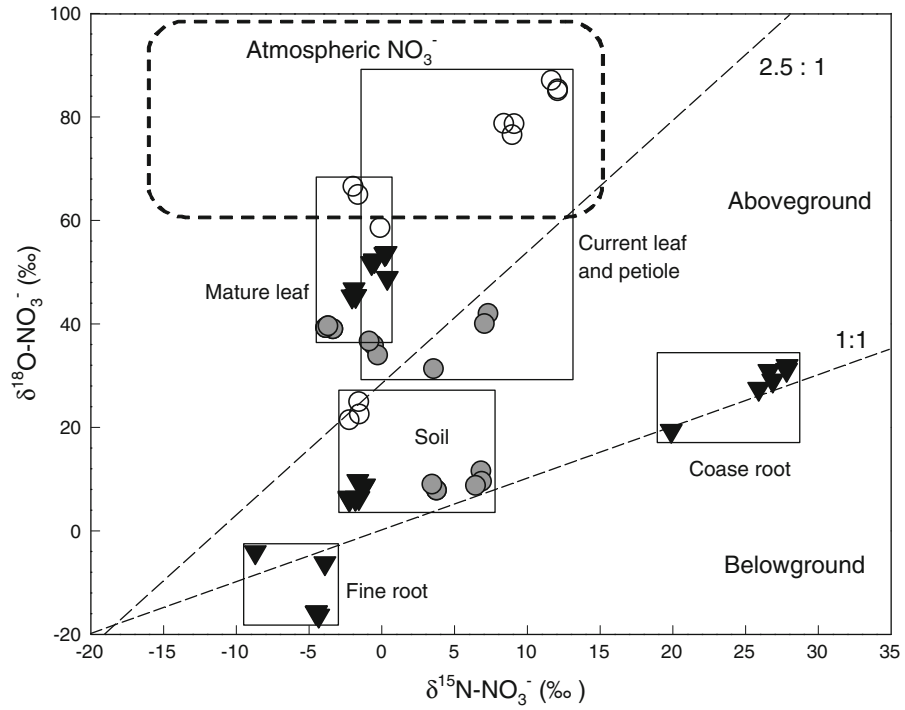

Fig. 6 Distributions of $\delta^{15}N$ and $\delta^{18}O$ of NO_3^- in plant organs and soils (*rectangles* in *solid lines*). The typical range of atmospheric NO_3^- (*rounded rectangle* in the *dashed line*) was cited from Kendall et al. (2007). *Open* and *filled circles* respectively represent data of *A. japonica* at high and low soil NO_3^- availability; *filled inverted triangles* for *P. orientalis*. Regression analyses were conducted separately on belowground organs ($y = 1.27x - 4.79$, $R^2 = 0.96$, $P < 0.0001$) and aboveground organs ($y = 2.37x + 49.07$, $R^2 = 0.54$, $P < 0.0001$). The 1:1 line is expected during NO_3^- assimilation based on a previous study of phytoplankton (Granger et al. 2004), whereas the 2.5:1 line was based on our data of aboveground organs to show a possible mixing of atmospheric NO_3^- with soil NO_3^-

the range of atmospheric NO_3^-, yielding a slope of 2.4 in the $\delta^{15}N$–$\delta^{18}O$ plot (Fig. 6). This slope reflected the mixing of atmospheric NO_3^- into plant leaves. However, because the dominance of NO_3^- uptake from soils was evidenced by leaf [NO_3^-] and $\delta^{15}N$ signatures (Figs. 2, 4) as well as lower isotopes of NO_3^- in leaves than on leaf surfaces (Fig. 1), it can be concluded that a low incorporation of atmospheric NO_3^- did not influence the responses of leaf NRA and [NO_3^-] to soil NO_3^- substantially. However, because of distinctly higher $\delta^{18}O$ of atmospheric $NO_3^-$and the isotopic effects of NO_3^- reduction, $\delta^{18}O$ of leaf NO_3^- are expected to be elevated significantly even though the uptake of atmospheric NO_3^- was very low. The framework therefore revealed the complexity of $\delta^{18}O$ variation during NO_3^- utilization in leaves of natural plants. Measurement of NO_3^- in leaf extracts for $\Delta^{17}O$ is necessary (Michalski et al. 2004).

Conclusions

This report described the first dataset of $\delta^{15}N$ and $\delta^{18}O$ of NO_3^- in natural vascular plants. The NO_3^- pools in a leaf body and on a leaf surface can be differentiated isotopically, showing the retention of atmospheric NO_3^- on leaf surfaces and low incorporation into the leaf body. Isotopic ratios of leaf NO_3^- of the *A. japonica* were found to be more enriched, generally, than those of soil NO_3^- sources, reflecting the isotopic effects of NO_3^- reduction. The isotope effects of leaf NO_3^- reduction generally follow the pattern of increasing with external NO_3^- availability, but decreasing with leaf age. Therefore, leaf NO_3^- concentration and isotopes can respond to soil NO_3^- availability, and current leaves are more sensitive than mature leaves as indicators for identifying the leaf as a NO_3^- reduction site. However, leaf NO_3^- of the studied *P. orientalis* was generally low; its isotopic ratios resembled those of soil NO_3^-. Isotopic enrichment of tissue NO_3^- and correlation with [NO_3^-] provided keys to interpretation of NO_3^- reduction, through which the reduction site of NO_3^- in the *P. orientalis* was identified as the coarse roots, not leaves. The preliminary isotopic framework of plant NO_3^- suggests that future works should differentiate isotopic effects of leaf NO_3^- reduction from the uptake of high-$\delta^{18}O$ NO_3^- from the atmosphere.

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References

Ammann M, Siegwolf RTW, Pichelmayer F, Suter M, Saurer M, Brunold C (1999) Estimating the uptake of traffic derived NO_2 from ^{15}N abundance in needles of Norway spruce. Oecologia 118:124–131

Andrews M (1986a) Nitrate and reduced-N concentrations in the xylem sap of *Stellaria media*, *Xanthium strumarium* and six legume species. Plant, Cell Environ 9:605–608

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

Atkin OK, Villar R, Cummings WR (1993) The ability of several high arctic plant species to use nitrate nitrogen under field conditions. Oecologia 96:239–245

Bassirirad H, Griffin KL, Reynolds JF, Strain BR (1997) Changes in root NH_4^+ and NO_3^- absorption rates of loblolly and ponderosa pine in response to CO_2 enrichment. Plant Soil 190:1–9

Binnerup SJ, Sørensen J (1992) Nitrate and nitrite microgradients in barley rhizosphere as detected by a highly sensitive denitrification bioassay. Appl Environ Microbiol 58: 2375–2380

Bloom AJ, Sukrapanna SS, Warner RL (1992) Root respiration associated with ammonium and nitrate absorption and assimilation by barley. Plant Physiol 99:1294–1301

Breteler H, Nissen P (1982) Effect of exogenous and endogenous nitrate concentration on nitrate utilization by dwarf beans. Plant Physiol 70:754–759

Casciotti KL, Sigman DM, Hasting GM, Böhlke JK, Hilkert A (2002) Measurement of the oxygen isotopic composition of nitrate in seawater and freshwater using the denitrifier method. Anal Chem 74:4905–4912

Cernusak LA, Winter K, Turner BL (2009) Plant $\delta^{15}N$ correlates with the transpiration efficiency of nitrogen acquisition in tropical trees. Plant Physiol 151:1667–1676

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

Crawford NM (1995) Nitrate: Nutrient and signal for plant growth. Plant Cell 7:859–868

Dail DB, Hollinger DY, Davidson EA, Fernandez I, Sievering HC, Scott NA, Gaige E (2009) Distribution of ^{15}N tracers applied to the canopy of a mature spruce-hemlock stand, Howland, Maine, USA. Oecologia 160:589–599

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

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

Fenn ME, Poth MA (1998) Indicators of nitrogen status in California forests. In: Bytnerowicz A, Arbaugh M, Schilling S (technical coordinators), Proceedings of the International Symposium on Air Pollution and Climate Change Effects on Forest Ecosystems, February 5–9, 1996, Riverside, GTR-166

Garten CT Jr, Schwab AB, Shirshac TL (1998) Foliar retention of 15N tracers: implications for net canopy exchange in low- and high-elevation forest ecosystems. Forest Ecol Manage 103:211–216

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

Granger J, Sigman DM, Needoba JA, Harrison PJ (2004) Coupled nitrogen and oxygen isotope fractionation of nitrate during assimilation by cultures of marine phytoplankton. Limnol Oceanogr 49:1763–1773

Granstedt RC, Huffaker RC (1982) Identification of the leaf vacuole as a major nitrate storage pool. Plant Physiol 70:410–413

Högberg P (1997) ^{15}N natural abundance in soil-plant systems. New Phytol 137:179–203

Högberg P, Högberg MN, Quist ME, Ekblad A, Näsholm T (1999) Nitrogen isotope fractionation during nitrogen uptake by ectomycorrhizal and non-mycorrhizal *Pinus sylvestris*. New Phytol 142:569–576

Højberg O, Johansen HS, 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 Microbiol 60:2467–2472

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

Kahmen A, Wanek W, Buchmann N (2008) Foliar $\delta^{15}N$ values characterize soil N cycling and reflect nitrate or ammonium preference of plants along a temperate grassland gradient. Oecologia 156:861–870

Kendall C, Elliott EM, Wankel SD (2007) Tracing anthropogenic inputs of nitrogen to ecosystems. In: Michener RM, Lajtha K (eds) Stable isotopes in ecology and environmental science, 2nd edn. Blackwell, Oxford, pp 375–449

Koba K, Inagaki K, Sasaki Y, Takebayashi Y, Yoh M (2010) In: Ohkouchi N, Tayasu I, Koba K (eds) Earth. Kyoto University Press, Life and Isotopes, pp 17–37

Kohl DH, Shearer G (1980) Isotopic fractionation associated with symbiotic N_2 fixation and uptake of NO_3^- by plants. Plant Physiol 66:51–56

Koyama L, Kielland K (2011) Plant physiological responses to hydrologically mediated changes in nitrogen supply on a boreal forest floodplain: a mechanism explaining the discrepancy in nitrogen demand and supply. Plant Soil 342: 129–139

Ledgard SF, Woo KC, Bergersen FJ (1985) Isotopic fractionation during reduction of nitrate and nitrite by extracts of spinach leaves. Aust J Plant Physiol 12:631–640

Lexa M, Cheeseman JM (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

Liu XY, Koba K, Takebayashi Y, Liu CQ, Fang YT, Yoh M (2012) 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

Malagoli M, Dal Canal A, Quaggiotti S, Pegoraro P, Bottacin A (2000) Differences in nitrate and ammonium uptake between Scots pine and European larch. Plant Soil 221:1–3

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

McKane RB, Johnson LC, Shaver GR, Nadelhoffer KJ, Rastetter EB, Fry B, Giblin AE, Kielland K, Kwiatkowski BL, Laundre JA, Murray G (2002) Resource-based niches provide a basis for plant species diversity and dominance in arctic tundra. Nature 415:68–71

Michalski G, Meixner T, Fenn M, Hernandez L, Sirulnik A, Allen E, Thiemens M (2004) Tracing atmospheric nitrate deposition in a complex semiarid ecosystem using $\Delta^{17}O$. Environ Sci Technol 38:2175–2181

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

Needoba JA, Sigman DM, Harrison PJ (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

Nordin A, Schmidt IK, Shaver GR (2004) Nitrogen uptake by arctic soil microbes and plants in relation to soil nitrogen supply. Ecology 85:955–962

Olleros-Izard T (1983) Kinetische Isotopeneffekte der Arginase und Nitratreduktase Reaktion: ein Betrag zur Aufklärung der entsprechenden Reaktionmechanismen. PhD thesis, Technischen Universität München, Freising-Weihenstephan

Robinson D, Handley LL, Scrimgeour CM (1998) A theory for $^{15}N/^{14}N$ fractionation in nitrate-grown vascular plants. Planta 205:397–406

Scheurwater I, Koren M, Lambers H, Atkin OK (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

Schimel JP, Bennett J (2004) Nitrogen mineralization: challenges of a changing paradigm. Ecology 85:591–602

Schmidt S, Stewart GR (1997) Waterlogging and fire impacts on nitrogen availability and utilization in a subtropical wet heathland (wallum). Plant, Cell Environ 20:1231–1241

Seith B, Setzer B, Flaig H, Mohr H (1994) Appearance of nitrate reductase and glutamine synthetase in different organs of the Scots pine *Pinus sylvestris* seedlings as affected by light, nitrate and ammonium. Physiol Plant 91:419–426

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

Sigman DM, Casciotti KL, Andreani M, Barford C, Galanter M, Böhlke JK (2001) A bacterial method for the nitrogen

isotopic analysis of nitrate in seawater and freshwater. Anal Chem 73:4145–4153

Sparks JP (2009) Ecological ramifications of the direct foliar uptake of nitrogen. Oecologia 159:1–13

Stewart GR, Joly CA, 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

Takebayashi Y, Koba K, Sasaki Y, Fang YT, 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

Tcherkez G, Farquhar GD (2006) Viewpoint: isotopic fractionation by plant nitrate reductase, twenty years later. Funct Plant Biol 33:531–537

Tcherkez G, Hodges M (2008) How stable isotopes may help to elucidate primary nitrogen metabolism and its interaction with photorespiration in C_3 leaves. J Exp Bot 59: 1685–1693

Tischner R (2000) Nitrate uptake and reduction in higher and lower plants. Plant, Cell Environ 23:1005–1024

Tye AM, Young SD, Crout NMJ, West HM, Stapleton LM, Poulton PR, Laybourn-Parry J (2005) The fate of ^{15}N added to high Arctic tundra to mimic increased inputs of atmospheric nitrogen released from a melting snowpack. Glob Change Biol 11:1640–1654

Werner RA, Schmidt HL (2002) The in vivo nitrogen isotope discrimination among organic plant compounds. Phytochemistry 61:465–484

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

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

Yoneyama T, Matsumaru T, Usui K, Engelaar WMHG (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

Yoneyama T, Ito O, Engelaar WMGH (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