Research paper

Gross nitrogen retranslocation within a canopy of Quercus serrata saplings

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Nitrogen (N) retranslocation within tree canopies has been intensively studied and assumed to function as a one-way process (e.g., from older to newer leaves). However, recent studies have found that both N output and input occur in individual leaves, suggesting that ‘gross’ N retranslocation exists behind ‘net’ N retranslocation. In the present study, the amount and direction of gross N retranslocation within a canopy of deciduous oak Quercus serrata Thunb. ex. Murray saplings were investigated. Labeling was conducted with leaves of Q. serrata saplings cultivated under conditions of low-N (LN) or high-N (HN) fertility. Subsequently, N movement within the canopy was traced. Leaves at two different positions in the canopy (top and lateral) were labeled to determine the direction of gross N retranslocation. To detect seasonal differences, the leaf-labeling experiment was conducted twice during the early and late phases of the growing season. In addition, to compare the quantitative importance of gross N retranslocation and root N uptake, the latter was determined by labeling Q. serrata roots. The N-labeling experiment revealed gross N retranslocation among leaves, i.e., from top to lateral, lateral to top and lateral to lateral positions. Gross N retranslocation was quantitatively more important than root uptake, especially for plants cultivated at LN fertility. Season also affected the amount of gross N retranslocation, and these effects differed between LN and HN fertilities. These findings suggest that N allocation within a canopy is controlled dynamically by both gross N output and input. The mechanisms controlling gross N output and input likely function as key determinants of N allocation within a tree canopy.

Keywords: crown, foliage, nitrogen resorption efficiency, nitrogen remobilization, stable isotope.

Introduction

Foliar nitrogen (N) content is strongly linked to leaf photosynthetic capacity (Hirose and Werger 1987, Evans 1989), and many studies have revealed patterns of N distribution among leaves in the canopy. For example, leaf N content per area is strongly linked to the within-canopy light gradient (Field 1983, Hirose and Werger 1987). Leaf age also affects leaf N content, with larger N content in younger leaves than older leaves (Hikosaka et al. 1994). In addition, leaf position within a canopy (leaf rank and height) affects N allocation, and leaf N content is larger in upper-position leaves (Frak et al. 2006, Ueda et al. 2009).

The process of N retranslocation within a canopy is important for optimizing N allocation for both deciduous and evergreen tree species. Previous studies have demonstrated that N is retranslocated from older leaves into younger leaves even without senescence (Fife and Nambiar 1984, Nambiar and Fife 1987). Because N retranslocation is affected by the development of sink organs (Nambiar and Fife 1991, Hikosaka 2005), N retranslocation changes seasonally following plant phenology (Milla et al. 2005). In addition, soil N availability affects N retranslocation during both the growing season and leaf senescence (Ono et al. 1996, Grassi et al. 2003).

Although most studies have assumed that N retranslocation is a one-way process (e.g., from older to newer leaves), leaf N content may result from the difference between gross output and gross input. Ueda et al. (2009, 2011) conducted 15N labeling from roots using saplings of Quercus serrata Thunb. ex. Murray, and demonstrated that input or output of labeled N was detected in leaves after the completion of leaf expansion without appreciable changes in total N content. These results
suggest that N retranslocation occurs even in the absence of ‘net’ N retranslocation (Ueda et al. 2009, 2011). The issue of gross N retranslocation raises a question about the function of such movement. Because N availability is limited in many terrestrial biomes (Aerts and Chapin 2000), gross N retranslocation is assumed to allow adaptation to N-limiting environments. As a first step toward understanding the function of gross N retranslocation, the direction and amount of retranslocated N should be investigated. Previous studies have applied labeled N to the roots of saplings, and all the leaves and other organs were labeled (Ueda et al. 2009, 2011). Thus, both the direction and amount of gross N retranslocation were uncertain.

The objective of this study was to determine the direction and amount of gross N movement within a tree canopy. For this purpose, 15N labeling was conducted using leaves at two positions within a canopy (top and lateral) and traced N retranslocation within a canopy of Q. serrata saplings. Similar to other Quercus species (e.g., Le Hir et al. 2006, Willaume and Pagès 2006), Q. serrata saplings have several growth flushes per growing season (Ozawa et al. 2000, Mizumachi et al. 2006). To detect seasonal differences in the direction and amount of gross N retranslocation, leaf labeling was conducted in the early phase of the growing season (during later flush leaf expansion) and during the late phase (after the later leaf flush was completed). Leaf labeling was conducted using saplings cultivated at two different N fertility levels to detect the effect of N availability on gross N retranslocation within the canopy.

**Materials and methods**

**Plant cultivation**

To avoid the direct translocation of leaf-applied labeled nitrate via xylem, a ring-porous deciduous oak (Q. serrata) was chosen as the focal species. Ring-porous species have low hydraulic connectivity of leaf-to-leaf pathways (Orians et al. 2005). Quercus serrata occurs in East Asia and is an important component of deciduous temperate forests in Japan (Ohsawa et al. 2008). Two hundred individuals of Q. serrata saplings (~40 cm in height) were acquired from the Miyagi Central Forest Association (Miyagi Prefecture, northeast Japan) in April 2010. The roots of each plant were washed to remove any soil contamination, and each sapling was transplanted to a plastic pot (1.5 l) containing washed sand. The saplings were grown in a greenhouse at Tohoku University’s experimental garden in Sendai, Miyagi Prefecture (altitude of 150 m; 38°15’N, 140°50’E). The greenhouse was covered with a plastic sheet on top to prevent rain, but the sides were open to allow free air circulation: air temperature inside the house was the same as that of outside. Saplings were watered every 2 days. The average annual temperature in 2010 in Sendai was 13.2 °C, and the maximum and minimum average monthly temperatures were 27.2 °C (August) and 2.1 °C (February), respectively (Japan Meteorological Agency).

After bud break (end of May), each tree received 200 ml of a complete nutrient solution every week. The concentration of fertilizer was adjusted to provide two different levels of N fertility following Mizumachi et al. (2004, 2006): 100 individuals were grown under low-N fertility (LN; 0.2 mM NH4NO3) and the other 100 individuals were grown under high-N fertility (HN; 2 mM NH4NO3). Other nutrients were supplied equally in the HN and LN treatments, as described by Koyama and Tokuchi (2003).

**Plant treatment with 15N**

To determine the direction of gross N retranslocation, leaves at two different positions in the canopy (top and lateral) were labeled. Top leaves were at the top of main shoot and lateral leaves were selected from the mid-crown region and were not shaded. The details of top and lateral position are described in Ueda et al. (2009).

To investigate seasonal differences in gross N retranslocation, the 15N labeling experiment was conducted twice, i.e., during the early (after first-flush leaves had completed leaf expansion in late June) and late (after the later leaf flush was complete in mid-August) phases of the growing season. During the early phase (July to August), second-flush leaves emerged. All top shoots produced second-flush leaves, but few plants had second-flush leaves in the lateral position. Top shoots in HN treatment produced more second-flush shoots than in LN treatment (3.0 ± 1.0 and 1.2 ± 1.1, respectively, P < 0.05, t-test). After the completion of leaf expansion of second-flush leaves in mid-August, there were no further flushes. In the early and late phases, each of 10 saplings for each leaf-labeling experiment (i.e., top or lateral leaves at two different N fertilities) was labeled with Na15NO3 spray (1 mM with >98 atom% 15N). The spray was applied to leaves on a top shoot or a lateral shoot, and these shoots were covered with plastic bags during labeling to prevent the labeling of other plant parts. These labeled leaves were sprayed every 2 days over a period of 2 weeks. In total, ~150 μg of labeled N was applied to each leaf. All labeled and unlabeled reference pots were watered and supplied with an appropriate nutrient solution as described above. After 2 weeks of labeling, remnant N was removed from the leaf surface by gently washing the labeled leaves with 0.5 mM CaCl2 and the leaves were then rinsed with tap water.

To detect N input into leaves from roots, N labeling from roots was also conducted. After 2 weeks of leaf labeling, each of five saplings that did not experience leaf labeling was treated with a labeled nutrient solution added to their pots as HN or LN treatments.

**Plant harvest**

Five saplings from each labeling experiment with leaves (i.e., top or lateral leaves at two different N fertilities) were harvested
just after the completion of leaf labeling (July and September, respectively). The other saplings from the experiment (labeled through top or lateral leaves, or by the roots) were harvested 4 weeks later (August and October, respectively).

Harvested leaves were washed with 0.5 mM CaCl₂ and tap water, and then rinsed with deionized water. All top and lateral leaves were collected; when lateral leaves were labeled, labeled and unlabeled lateral leaves were separated, and unlabeled lateral leaves were selected from the mid-crown region and were not shaded. Samples were dried at 60 °C for 1 week, weighed and then powdered.

**Analysis of total N and ¹⁵N concentration**

The concentrations of total N and ¹⁵N were determined at the Stable Isotope Facility at the University of California, Davis. Labeled N content per leaf was calculated as

\[
\text{Leaf } ^{15}\text{N} = N_{\text{sample}} \times (^{15}\text{N}_{\text{sample}} - ^{15}\text{N}_{\text{reference}}) / 100
\]  

where leaf ¹⁵N (µg/leaf) is the amount of excess labeled N that had accumulated per individual leaf, Nsample is the total N content per sample leaf, ¹⁵Nsample is the atom% ¹⁵N of the sample leaf and ¹⁵Nreference is the atom% ¹⁵N in the leaves of reference saplings.

Because changes in leaf mass and leaf area affect the calculation of N retranslocation (van Heerwaarden et al. 2003), gross N resorption efficiency was calculated on an individual leaf basis as follows:

\[
^{15}\text{NRE} = 100 \times (\text{leaf}^{15}\text{N}_{\text{pre}} - \text{leaf}^{15}\text{N}_{\text{after}}) / \text{leaf}^{15}\text{N}_{\text{pre}}
\]  

where ¹⁵NRE (%) is the gross N resorption efficiency of the labeled leaf, leaf¹⁵Npre is the excess labeled N content per labeled leaf just after labeling and leaf¹⁵Nafter is the excess labeled N content per labeled leaf 4 weeks after the completion of labeling.

Because all leaves on each chosen shoot were labeled, the value of gross N input into unlabeled leaves was calculated as N retranslocation from one shoot. The contribution of retranslocated N from labeled shoots to the total N content of unlabeled leaves [N derived from labeled shoot, Ndfl(%)] was calculated following Martinez-Alcántara et al. (2011):

\[
\text{Ndfl} = (^{15}\text{N}_{\text{unlabeled}} - ^{15}\text{N}_{\text{reference}}) \times 100 / (^{15}\text{N}_{\text{labeled}} - ^{15}\text{N}_{\text{reference}})
\]  

where ¹⁵Nunlabeled is the atom% ¹⁵N in unlabeled leaves 4 weeks after the completion of leaf labeling and ¹⁵Nlabeled is the average atom% ¹⁵N in labeled leaves just after labeling was finished.

Using Ndfl, N input into unlabeled leaves from labeled shoots was estimated as follows:

\[
N_{\text{input}} = N_{\text{unlabeled}} \times \text{Ndfl} / 100
\]  

where Ninput (µg/leaf) is the amount of gross N input into the unlabeled leaf from labeled shoots and Nunlabeled is the amount of N per unlabeled leaf 4 weeks after the completion of leaf labeling.

Because each sapling has several lateral shoots, the contribution of retranslocated N from all lateral shoots within a sapling (Ndfl-total) was estimated as follows:

\[
\text{Ndfl-total}(\%) = \text{Ndfl} \times B_{\text{total}} / B_{\text{shoot}}
\]  

where Btotal is the biomass of the total lateral leaves per sapling and Bshoot is the biomass of the lateral leaves per labeled shoot.

In addition, the amount of leaf N derived from root N uptake was calculated using Eq. (1).

**Statistical analyses**

All analyses were performed using IBM SPSS statistics 19.0 (IBM, Tokyo, Japan). Differences in leaf mass and total N content per individual leaf were compared using three-way analysis of variance (ANOVA) with leaf position, N fertility and sampling date as factors and a significance cutoff of P = 0.05. When the effect of sampling date was significant and interactions were not significant, Tukey’s honestly significant difference (HSD) post hoc test was applied to detect differences among sampling dates. ¹⁵N content per leaf and ¹⁵NRE were also compared using three-way ANOVA with shoot position, N fertility and season as factors. When there was a significant interaction between N fertility and season, two-way ANOVA with shoot position and N fertility as factors and N fertility and season as factors. When there was a significant interaction between N fertility and season, two-way ANOVA with shoot position and N fertility as factors and N fertility and season as factors. Differences in the amount of N retranslocated from each direction were compared using two-way ANOVA with N fertility and season as factors. Differences in the contributions of retranslocated N to total N in leaves were compared using three-way ANOVA with direction, N fertility and season as factors.

**Results**

**Effects of leaf position, N fertility and season on individual leaf biomass and N mass**

Leaf position (top and lateral) and N fertility (HN and LN) significantly affected individual leaf mass (Tables 1 and 2). Individual leaf mass was larger in top-position than in lateral-position leaves and in HN treatments than in LN treatments (Table 1). On the other hand, sampling date did not significantly
Values indicate means ± 1 SD (n = 5).

affect individual leaf mass (Table 2), suggesting that leaf expansion was completed before the experiment started. No significant interactions were detected among the three factors (i.e., leaf position, N fertility and sampling date).

Total N content per leaf was significantly affected by leaf position and N fertility (Table 2). The total N content per leaf was larger in top position than in lateral position and in plants subjected to HN treatment as compared with LN treatment (Table 1). In addition, total N content per leaf was significantly affected by sampling date (Table 2). The total N content per leaf was higher in July than in August, September and October. These results suggest that the total N content per leaf decreased from July to August but did not change from August to October in both top- and lateral-position leaves under either HN or LN conditions.

Nitrogen absorption from the leaf surface and output from labeled leaves

$^{15}$N content per labeled leaf was larger during the early growing phase than in the late growing phase (Figure 1a, Table 2), suggesting that the foliage N absorption rate was higher in the early phase. In contrast, neither leaf position nor N fertility significantly affected $^{15}$N content per labeled leaf (Figure 1a, Table 2).

The gross N resorption efficiency from labeled leaves 4 weeks after the completion of leaf labeling was 16–67% (Figure 1b). The interaction between N fertility and season, and interaction among N fertility, season and position were significant (Table 2). The interaction between N fertility and season suggests that the effect of N fertility on gross N output differed between the early and late growing phases. In the early phase, two-way ANOVA revealed that gross N resorption efficiency did not significantly differ between the HN and LN treatments ($P = 0.53$), and also the effect of position and interaction between N fertility and position were not significant ($P = 0.66$ and $P = 0.09$, respectively). On the other hand, in the late growing phase, gross N resorption efficiency was higher in the LN than in the HN treatment ($P < 0.01$), and the effect of position and the interaction between N fertility and position were not significant ($P = 0.06$ and $P = 0.27$, respectively, Figure 1b).

The amount of gross N input into leaves from labeled shoots

Labeled N retranslocation was detected from top to lateral, from lateral to top and from lateral to lateral shoots on different branches (Figure 2), suggesting the presence of gross N retranslocation among simultaneously flushed leaves. Among the five directions of retranslocation examined here (roots to top, lateral to top, roots to lateral, lateral to lateral, and top to lateral), the amount of retranslocation was highest in the top-to-lateral direction (Figure 2). Gross N retranslocation from top to lateral was up to 13 times larger than lateral to lateral, and up to 18 times larger than lateral-to-top retranslocation (Figure 2a–c). Gross retranslocation from top to lateral was not significantly affected by season or N fertility (Figure 2a), suggesting that gross N retranslocation from the top-to-lateral position did not significantly differ between the early and late growing seasons under either HN or LN conditions. On the other hand, for gross N retranslocation from lateral shoots (lateral to lateral...
Table 2. Results of three-way ANOVA for data shown in Table 1 and Figures 1 and 3.

<table>
<thead>
<tr>
<th>Table 1</th>
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<td>Leaf number (leaves per shoot)</td>
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and lateral to top), significant interactions were detected between season and N fertility (Figure 2b and c). In the early phase, both lateral-to-lateral and lateral-to-top retranslocations were larger under LN conditions, whereas these values were higher under the HN condition in the late phase (Figure 2b and c). Root-uptake N was higher under HN than under LN conditions in both lateral and top leaves (Figure 2d and e). Although root-uptake N was higher in the early phase than in the late phase for lateral leaves, values did not differ between seasons in top leaves (Figure 2d and e).

**Contribution of retranslocated N to total leaf N content**

Both in lateral and top leaves, the contributions of gross N retranslocation from other leaves (top leaves and total lateral leaves) to total leaf N content were larger than that of root uptake (Figure 3, Table 2). The contributions of gross N retranslocation were up to 50 times and 1000 times larger than those of root uptake in HN and LN treatments, respectively (Figure 3). In addition, significant interactions were detected between N fertility and season (Table 2). The contribution of gross N retranslocation was larger in LN than in HN during early phase, while it was smaller in LN than in HN during late phase (Figure 3).

**Discussion**

The present study demonstrated that gross N retranslocation is bidirectional, i.e., from top-to-lateral, lateral-to-top and lateral-to-lateral position among simultaneously flushed leaves. Nitrogen allocation within a canopy was controlled dynamically by both gross N output and input, and gross N retranslocation was controlled by season (phenology) and N fertility (Figures 1–3, Table 1).
Figure 1. (a) Labeled N content per leaf after leaf labeling was completed, and (b) $^{15}$NRE from labeled leaves. Values indicate means ± 1 SD ($n=5$).

Figure 2. The amount of gross N input from labeled shoot to unlabeled leaves: (a) top to lateral, (b) lateral to lateral, (c) lateral to top, and root uptake into (d) lateral and (e) top leaves. Values indicate means ± 1 SD ($n=5$). * $p<0.05$, ** $p<0.01$, ns, not significant.

Gross N resorption efficiency was higher in LN treatments than in HN treatments in the late growing phase (Figure 1b, Table 2). Gross N retranslocation from leaves compensates for N demand within a sapling under LN treatment more than HN treatment because of smaller N input from roots. In the case of net N retranslocation, LN fertility also increases N retranslocation during leaf senescence (Millard and Thomson 1989, Vizoso et al. 2008) and during the growing season (Millard and Neilsen 1989). However, gross N resorption efficiency was not different between LN and HN treatments during the early phase in this study (Figure 1b). In the beginning of leaf expansion, N storage over winter is the primary N source (Tromp 1983, Millard and Proe 1991, Millard 1996, Millard et al. 1998, Ueda et al. 2009), and thus the effect of current N fertility is small. In the present study, saplings had been grown at the same level of N fertility prior to bud break. Because N absorbed after bud-break accumulates during the growing season, the effect of N fertility in the current growing season was larger in the late phase than in the early phase. The difference in N fertility in the current growing season is expected to affect retranslocation in the following spring. During the early phase, the decrease in total N content per leaf was also detected, i.e., net N retranslocation (Tables 1 and 2). This might be the retranslocation into second-flush leaves. Net N retranslocation from one flush into the next has been reported for other tree species (Fife and Nambiar 1984, Grelet et al. 2003).

In contrast, the contribution of gross N input from simultaneously flushed leaves (0.3–9% of the total N) was smaller than N output (16–67%; Figures 1 and 3). Gross output may have been overestimated, because nitrate (i.e., the mobile form of N) was directly applied to the leaf surface. However, Ueda et al. (2011) reported that almost 70% of labeled N absorbed from roots during leaf expansion was also retranslocated over 5 months. In their study, NRE was underestimated because labeled N was applied to the roots of saplings, and thus other plant parts including roots and other leaves were labeled (Ueda et al. 2011). Indeed, Ueda et al. (2009) also demonstrated that labeled N content per leaf doubled during the 3 months after labeling was finished, suggesting $^{15}$N input from other plant organs. Therefore, gross NRE in the present study is within the possible range expected from these previous studies, and gross N input from simultaneously flushed leaves was too small to compensate for the loss of gross output. In this study, gross N input from N pools such as woody compartments and roots were not taken into account. In many deciduous woody species, woody tissues and roots are the main sites of N storage for leaf growth (reviewed by Millard and Grelet 2010), and woody tissues and roots also act as sinks for N that is retranslocated from leaves (Chapin and Kedrowski 1983, Nambiar and Fife 1991, Marty et al. 2009). Grassi et al. (2003) quantified that ~45–50% of total N passed through xylem of Prunus avium L. was root–shoot
retranslocation 3 months after bud-burst, and suggested that whole-plant level N retranslocation is substantial. The difference between gross N output and input among simultaneously flushed leaves suggests that the amount of gross N retranslocation between leaves and other organs is larger than that among simultaneously flushed leaves.

The contribution of gross N retranslocation was larger than that of root uptake in both lateral and top leaves, especially in LN treatments even without considering gross N input from other organs (Figure 3, Table 2). The amount of gross N retranslocation differed among directions (top to lateral, lateral to top, lateral to lateral, Figure 2a–c). In addition, the effects of season and N fertility differed among directions (Figure 2a–c). This unequal N retranslocation even among simultaneously flushed leaves should function to control N allocation within a canopy. Using retranslocated N rather than root uptake N to control N allocation within a plant may be an important adaptive strategy to terrestrial biomes where N availability dramatically fluctuates. Although gross N retranslocation between simultaneously flushed leaves contributed <9% to the total N content during a period of 4 weeks (Figure 3), the contribution of retranslocated N should be substantial throughout the growing season, as the growing season spans more than half of the year in many temperate forest ecosystems.

Because only saplings of one species under manipulated conditions were examined, future studies should focus on other tree species and trees at additional ontogenetic stages.

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**Conflict of interest**

None declared.

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