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Evaluation of the stem injection technique and subsequent ^{15}N partitioning in red alder crowns

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Abstract

Red alder (*Alnus rubra* Bong.) trees were labeled with $^{15}NO_3^-$ or $^{15}NH_4^+$ using the stem-injection method. Leaves were sampled 3 and 15 months subsequent to injection within several crown positions, including top, bottom, proximal, medial, and distal. Stem injection of both $^{15}NH_4^+$ and $^{15}NO_3^-$ at levels approaching 1% of crown N effectively labeled red alder trees. Although more variable, $^{15}NO_3^-$ may have been more efficient in initial labeling. The distribution of ^{15}N in the crown was uniform 3 months after labeling, but was diluted in the distal and top positions by the following year. In both years there was a clear increase in total N concentration toward the periphery of the tree. This increase became more pronounced with increasing crown size and crown closure. Crown position with respect to light availability may be the most important determinant in N allocation in red alder foliage.

Introduction

Increased interest in the economic, edaphic, and silvicultural benefits of alder (*Alnus* spp. Ehrhart) has helped to heighten interest in the ecology and biology of red alder (Hibbs et al., 1994; Tarrant et al., 1994). Much of this interest has centered on the fixation of N_2 in alder nodules and the high levels of foliar N in alder. Studies have sought to determine environmental influences on N_2 fixation in alder (Côté et al., 1989; Prégent and Camiré, 1985; Samuelson et al., 1990; Wheeler et al., 1981), often using changes in ^{15}N concentrations in live alder leaves as determinants (Beaupied et al., 1990; Domenach and Kurdali, 1989; Domenach et al., 1989; McNeill et al., 1994).

Improper sampling of crown foliage may lead to inaccurate estimations of N concentration or content. Gradations in foliar N have been observed in peach and apple canopies (Porpiglia and Barden, 1980; Sanchez and Righetti, 1990) and, using black alder (*Alnus glutinosa* (L.) Gaertn), Domenach and Kurdali (1989) demonstrated the danger of ignoring leaf age in N_2 fixation studies. An understanding of the variation in N within the crown is important in avoiding misrepresentation of crown N.

Studies using ^{15}N tracer techniques with seedlings or trees must also find methods to adequately label the plant. Several methods for labeling alder have been tested, including root and foliar fertilization with ^{15}N (González Prieto et al., 1995) and fixation of $^{15}N_2$ (McNeill et al., 1994). Stem injection (Horwath et al., 1992) of alder remains untried. Perhaps the main benefit of the stem-injection technique is that it labels the tree without directly affecting soil N pools. Root fertilization often results in soil contamination with ^{15}N. This presents a special problem in field studies with large trees, where transplanting is not an option. In the second season, residual ^{15}N in soil can also complicate estimations of reserve use. Foliar fertilization is limited to trees small enough to adequately and uniformly cover with the urea-^{15}N spray; large trees require prohibitive amounts of solution, much of which ultimately labels surrounding soil and vegetation.

There is some question as to the most appropriate form of N to inject into alder. Even small levels of NH_4^+ can be toxic in plant leaves (Waring and Schlesinger, 1985). Alternatively, the vast bulk of NO_3^- reductase

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activity occurs in the roots of alder, with little or no NO_3^- measurable in the stem (Blacquière and Troelstra, 1986; Pizelle and Thiery, 1986). It is unclear how well the constitutive NO_3^- reductase activity in the leaves and shoots would adjust to even a small influx of NO_3^- (Benamar et al., 1989).

The objectives of this study were to evaluate the viability of using the stem-injection procedure to label red alder (*Alnus rubra* Bong.) foliage with $^{15}NO_3^-$ and $^{15}NH_4^+$, and to assess the uniformity of foliar ^{15}N-labeling and N concentration between several different crown positions 3 and 15 month after injection.

Methods

This study was conducted in the Cascades Range at the H.J. Andrews Experimental Forest, Willamette National Forest, Oregon (44°09′ N, 122°22′ W). Red alder and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) were planted on the clear-cut site 7 y prior to the study. Ten single-stemmed alder trees, fairly uniform in size (about 5 m height and 15 cm diameter at breast height), growth habit (live crown of about 3.5 m in diameter beginning at about 20 cm in height), and spacing (4.2–4.2 m), were injected with an artificial sap solution containing $^{15}NO_3^-$ or $^{15}NH_4^+$ using the method described by Horwath et al. (1992). The sap solution, composed of 5.0 mmol L^{-1} KCl and 0.4 mmol L^{-1} malic acid, was adjusted to pH 5.4 with HCl or KOH (Dickson et al., 1985). $^{15}NO_3^-$ was injected as KNO_3 at 66.0 atom % ^{15}N and $^{15}NH_4^+$ as $(NH_4)_2SO_4$ at 69.2 atom % ^{15}N. Five trees received the KNO_3 and five received the $(NH_4)_2SO_4$. The injection procedure consisted of drilling a hole (0.635 cm diameter) at the base of the tree downward at a 45° angle through about 75% of the diameter of the tree. A 0.635-cm diameter plastic tube stoppered with a rubber septum was inserted 1 cm into the hole. Air was removed from the hole by injecting sap solution through the septum, thereby forcing the air through an open-ended needle also inserted through the septum. When the air was expelled both needles were removed and a final needle connected by a tube to the sap reservoir was inserted. The sap reservoir consisted of a 1-L bottle hung above the drilled hole and filled with 250 mL of sap solution containing either 12 mmol L^{-1} $(NH_4)_2SO_4$ or 27 mmol L^{-1} KNO_3. When most of the sap solution was taken up by a given tree, the reservoir was refilled with N-free sap solution and allowed to drain.

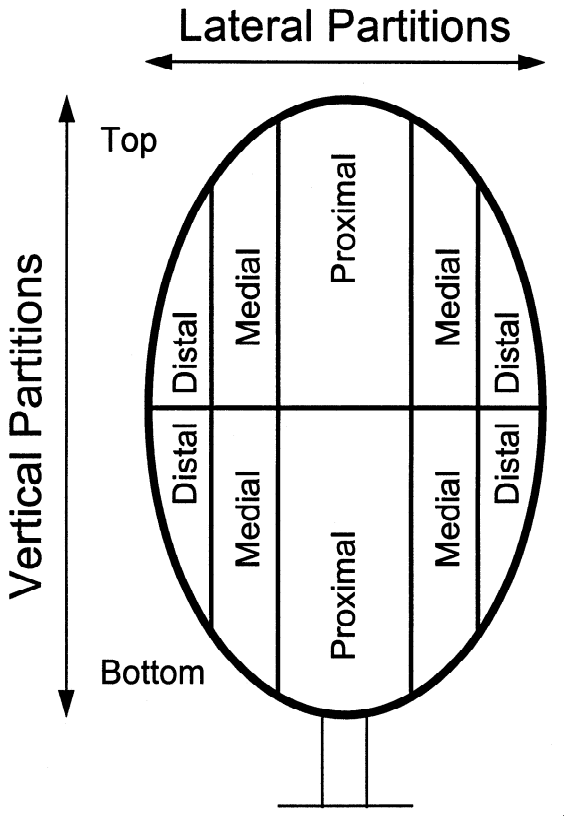

Figure 1. Crown partitions used for leaf sampling and statistical comparisons of lateral and vertical distribution of N concentration and ^{15}N distribution.

Prior to the injection of each tree, leaves were collected from the entire crown and combined into a composite sample. The trees were injected on 6 July 1994. The length of time the trees assimilated the initial 250 mL of sap solution varied from 15 min to 2.5 h. Most trees did not fully drain the secondary, N-free sap solution until the following day. After the stem injection, the crowns were sampled twice: the first time on 20 September 1994 and the second on 18 September 1995. The post-injection sampling involved dividing the crowns into six partitions (Figure 1). A composite sample of 20–40 leaves was collected from each partition during each sampling. The oven-dried samples were ground to pass through a 40-mesh sieve and analyzed for N concentration and atom % ^{15}N on a Europa Scientific ANCA-MS automated mass spectrometer or a Europa Scientific 20/20 automated mass spectrometer (Europa Scientific Ltd., Crewe, UK). The precision levels at natural abundance of the ANCA-MS and 20/20 are ±0.0003 and 0.00007 atom % ^{15}N, respec-

tively. Duplicate samples were run within and between the mass spectrometers to ensure consistency. Results of the duplicate runs were tested for differences using a paired t-test at the 0.05 level of significance. Without exception, samples run on each machine and repeated on the same machine were consistent.

Crown size and leaf area index were not measured. Therefore, statistical tests involving ^{15}N were conducted using transformed data to control for variation within groups resulting from differences in crown sizes. Namely, for a given tree, the atom % ^{15}N of each partition was divided by the highest atom % ^{15}N measured in that tree, resulting in a fraction between zero and one. This transformation maintained the relationships of atom % ^{15}N allocated to the various partitions of the crown, while controlling for the effect of crown size and initial transpiration rate on the level of ^{15}N enrichment. When referring to comparisons involving ^{15}N we will use the terms 'relative ^{15}N allocation' or 'relative ^{15}N distribution.'

Because the individual trees were the experimental units and the various partitions within a crown most likely correlated with each other, a repeated measures analysis of variance was considered most appropriate for this data set. The repeated measures analysis of variance corrected for correlation and was able to test for interaction between treatment and crown position, treatment-level responses, and partition-level responses. Possible treatment-level responses included a difference between treatments at each crown partition ('by-partition' treatment-level response) and a difference between treatments averaged across crown partitions ('across-partition' treatment-level response). The possible partition-level response was the difference between crown partitions, averaged across treatments. If the repeated measures analysis of variance indicated a difference at the partition-level, contrasts were used to determine if specific partitions differed from each other. Because the number of experimental units was limited ($\leq$5 per treatment), the analysis was split into two basic comparisons: lateral and vertical. Unless otherwise stated, a p-value of 0.05 or less was deemed significant. By-partition treatment-level responses are only reported if they were significant.

Results

Three injected trees were dropped from the data analysis due to procedural errors. The remaining trees included three treated with $^{15}NO_3^-$ and four treated with $^{15}NH_4^+$. Both the $^{15}NO_3^-$ and $^{15}NH_4^+$ treatments clearly raised atom % ^{15}N levels above the pre-injection levels of the alder trees, which were near natural abundance (Figure 2). Unfortunately, statistical tests could not be run on these data because we could not correct for the confounding effects of crown size on enrichment. Although the NO_3^- treatment appeared to result in both higher and more variable enrichment in September 1994, these data should be viewed with caution given the lack of information on crown size. By the September 1995 sampling, ^{15}N enrichment of the crowns in both treatments had decreased. The decrease in ^{15}N enrichment in the NO_3^- treatment appeared to be substantially greater.

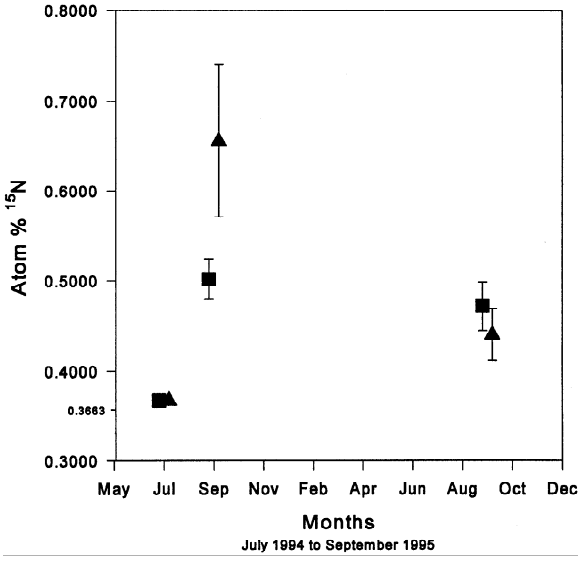

Figure 2. Atom % ^{15}N enrichment over time for $^{15}NH_4^+$ (■) and $^{15}NO_3^-$ (▲) treatments, uncorrected by crown size. Error bars are one standard error. Error bars for July 1994 are smaller than data points.

A comparison of first-year relative allocation of ^{15}N in lateral partitions (proximal/medial/distal) revealed no significant interaction between treatment and lateral partition effects (p = 0.51), and no significant differences between treatments (p = 0.49) or between lateral partitions (p = 0.53). A similar comparison of vertical partitions (top/bottom) was suggestive of an interaction between treatment and vertical partition responses (p = 0.06), although there were no significant differences between treatments (p = 0.47) or between vertical partitions (p = 0.33). The interaction appeared to result from a higher relative distribution of ^{15}N in the bottom partitions of NO_3^--labeled compared with NH_4^+-labeled

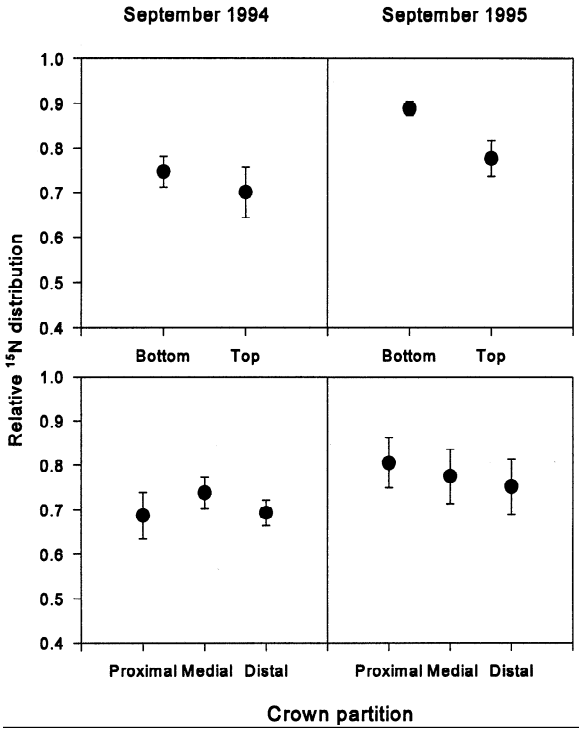

Figure 3. Relative distribution of ^{15}N ($atom\%_{partition}/atom\%_{high}$) in vertical and lateral partitions in September 1994 and September 1995. Error bars are one standard error.

trees, and a lower relative distribution of ^{15}N in the top partitions of NO_3^--labeled versus NH_4^+-labeled trees.

In September 1995 there was no significant interaction between treatment and relative distribution of ^{15}N in lateral partitions (p = 0.84) or in vertical partitions (p = 0.70). Additionally, there was no treatment effect in relative distribution of ^{15}N in lateral partitions (p = 0.47) or in vertical partitions (p = 0.97). Lateral distribution of ^{15}N between partitions was not significantly different (p = 0.07) but was suggestive statistically and visually (Figure 3) of an emerging pattern, warranting further analysis. A contrast between proximal and distal crown partitions suggested a lower relative distribution of ^{15}N in the distal partition (p = 0.08). A second contrast, between the proximal and medial partitions, revealed no difference in relative distribution of ^{15}N (p = 0.29). A comparison of vertical partitions revealed a significantly higher distribution of ^{15}N in the bottom half of the tree crowns (p = 0.03; Figure 3).

In the first year, relative distributions of ^{15}N in lateral partitions were not significantly correlated with one another (p values $\geq$ 0.17), nor were they correlated with one another in the vertical partitions (p = 0.52). By September 1995, however, the allocation of ^{15}N in each of the lateral partitions was positively correlated with the allocation in the other partitions (p values $\leq$ 0.01), although relative distributions of ^{15}N in vertical partitions were not significantly correlated with each other (p = 0.17).

Nitrogen concentration generally showed the opposite trends as those of relative ^{15}N distribution. The first sampling in September 1994 revealed no significant interaction between treatment and horizontal partitions (p = 0.98), and no significant differences in N concentration between treatments (p = 0.18). However, there were differences in N concentration between partitions. Namely, although the distal partitions did not differ significantly in N concentration from the medial partitions (p = 0.12), they exhibited higher N concentrations than the proximal partitions (p = 0.002). There were no significant interactions between treatment and vertical partitions (p = 0.89), no significant treatment effects (p = 0.08), and N concentration in top and bottom partitions was not significantly different (p = 0.16). The low p-value associated with the treatment response was caused by slightly lower values in the NO_3^- trees.

By the second sampling in September 1995 there was still no interaction between treatment and lateral partitions (p = 0.73), and no significant across-partition treatment difference (p = 0.16). There was a significant by-partition treatment-level response, however. The N concentrations in the proximal partitions of the NO_3^--labeled trees were significantly lower than those in the proximal partitions of the NH_4^+-labeled trees (p = 0.008; Figure 4). Similarly, there was no significant interaction between treatment and vertical partitions (p = 0.22) and no across-partition treatment difference (p = 0.26), but there was a significant by-partition treatment-level response. The bottom partitions of the NO_3^- trees were significantly lower in N concentration than those of the NH_4^+-labeled trees (p = 0.03; Figure 4). At the partition level, the distal partitions showed a higher N concentration than both the medial (p = 0.03) and the proximal (p = 0.008) partitions (Figure 4). The bottom partitions were significantly lower in N concentration than the top partitions (p = 0.02).

Discussion

Both $^{15}NO_3^-$ and $^{15}NH_4^+$ treatments were effective in labeling red alder trees. Horwath et al. (1992) reported that adding greater than 5–10% of the crown N through

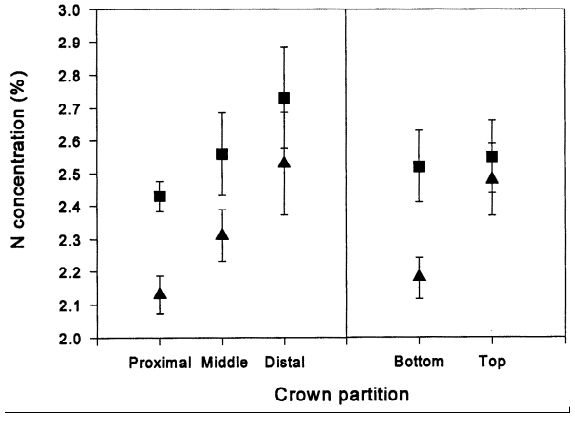

Figure 4. Nitrogen concentration (as percent of dry weight) in crown partitions in the NH_4^+ (■) and NO_3^- (▲) treatments in September 1995. Error bars are one standard error.

stem injection resulted in toxic effects in their experiments. We calculated an estimate of crown N using allometric equations (David Hibbs, personal communication, Oregon State University), and determined that the quantity of N injected into the tree was about 1% of the crown N. This amount, although low, adequately labeled the crown and had no readily discernable negative side effects.

The apparent differences in atom % ^{15}N values in Figure 2 could have resulted from a dilution effect due to differences in crown size. The high variation in the $^{15}NO_3^-$ treatment in September 1994 may have been due to one tree planted at a wider spacing than the others. This tree had a larger crown, resulting in greater dilution of the added $^{15}NO_3^-$. When this tree was excluded, the variation in the $^{15}NO_3^-$ treatment was reduced and the atom % ^{15}N increased. González-Prieto et al. (1995) reported that root fertilization of black alder with $^{15}NO_3^-$ resulted in higher, but more variable foliar labeling than fertilization with $^{15}NH_4^+$. Ultimately, because of a lack of quantitative information on crown size, no definitive statement can be made regarding the relative efficiency of labeling between treatments. In the September 1995 sampling, the crown averages of ^{15}N concentration in the $^{15}NO_3^-$-trees were more comparable to those of the $^{15}NH_4^+$-labeled trees. The initially higher labeling by the $^{15}NO_3^-$ treatment appeared to be coupled with a greater dilution in the second year. The reason for this is unknown, although it may be the result of faster growth by trees in the NO_3^- treatment (greater dilution) or a lower ratio in the use of reserve-N (labeled) to fixed-N (unlabeled) by the trees in the NO_3^- treatment.

We considered the lack of significant differences in ^{15}N distribution among partitions and of significant correlations in ^{15}N distribution between partitions in the first year (3 months after injection) to be indicative of uniform distribution of ^{15}N throughout the crown. Additionally, there was no treatment response in the distribution of crown N in the first year, which was not surprising. The amount of N added by either treatment was so small that the total N patterns of the crown should not have been affected.

Although N concentration did not vary by treatment, it did vary by crown position. Crown position may be the best determinant of N concentration for several interconnected reasons, including changing light exposure, photosynthetic rates, and transpiration rates. As light exposure increases in the crowns of fruit trees and even perennial herbs, photosynthesis rates have also been shown to increase, creating a greater sink for N (DeJong, 1982; Hirose et al., 1988; Kull and Niinemets, 1993; Weinbaum et al., 1989). Additionally, transpiration can be expected to be higher around the periphery of the tree where leaves are more likely to be exposed to high temperatures and wind. Higher mass flow to these peripheral areas may largely responsible for higher N concentrations.

Red alder is a very shade-intolerant tree and has been shown to decrease the size and density of leaves with increased shading (Bormann and Gordon, 1984; DeBell and Giordano, 1994; Helgerson et al., 1988). Dawson and Funk (1981) attempted to show a relationship between leaf size and N concentration in alder, but failed to account for vertical or lateral crown position. They found no significant differences in N concentration between size classes. When vertical and lateral crown positions were considered in the present study, and size class was not included as a factor, N concentration was found to increase toward the periphery of the tree.

The second year of the study was notable for a decrease in the uniformity in ^{15}N distribution and for a further differentiation in the N concentration between partitions. In September 1995, relative ^{15}N distribution was somewhat lower in the distal than in the proximal partitions. Additionally, although all correlations between lateral partitions were significant and positive, the correlation between the proximal and distal partitions was slightly lower than the other correlations. Both responses may be explained by a greater incorporation of soil and fixed N at lower atom % ^{15}N abundance levels into the distal partitions of the canopy than an incorporation of labeled N from the reserves into

the distal partitions (Sanchez, 1990). Leaves grown in the latter third of the season, and most abundantly in the distal and top partitions of the trees, use little or no reserve N (Domenach and Kurdali, 1989). Further, as discussed above, leaves of any age growing in these partitions are likely to be more photosynthetically active (Porpiglia and Barden, 1980) and a greater sink for N (DeJong, 1982; Kull and Niinemets, 1993; Weinbaum et al., 1989). Thus, although older leaves in the distal and top partitions initially had similar ^{15}N signals to leaves in the interior crown, the distal and top leaves eventually exhibited a diluted ^{15}N signal later in the season with increased fixed N and soil N uptake over and above that of interior leaves.

The increased concentration of N in peripheral partitions was measured in both top and distal partitions in the second year. As the stand canopy closed during the second year of the experiment, increased competition for light may have led to greater differentiation within both lateral and vertical canopy groupings (DeJong and Doyle, 1985). Nitrogen concentrations in the top and bottom partitions, not different under adequate light conditions in the first year of the experiment, differentiated as the canopy closed. Distal and medial partitions, not different before canopy closure, were significantly different under more shaded conditions.

An unexpected development in the second year was the significant difference in N concentrations attributable to the NO_3^- and NH_4^+ treatments. A toxicity response was considered as an explanation. Such a small addition to total crown N should not have affected the relative N response even in the first year, however. Thus, it seems especially unlikely that it would elicit a response in the second year. Only a slight, if any, indication of a treatment difference in resorption or retranslocation efficiency was observed in the isotope measurements, where the difference should have been most apparent. It seems unlikely that this was the cause. The observed differences between treatments were consistently in the form of lower N concentrations within the bottom or inside crown positions of the NO_3^- trees (Figure 4). This is suggestive of greater growth in the NO_3^- trees, resulting in lower N concentrations in shaded partitions of those trees. Yet again, it is difficult to attribute a growth response, positive in the case of the NO_3^- treatment or negative with the NH_4^+ treatment, to such a small input of N. Thus, although an actual treatment response should not be ruled out, it is possible that the observed treatment difference was an artifact of the small sample size or even of stand dynamics which favored faster growth of the NO_3^- trees.

Conclusions

Stem injection of both $^{15}NH_4^+$ and $^{15}NO_3^-$ at levels approaching 1% of crown N effectively labeled red alder trees. $^{15}NO_3^-$ may have been more efficient in initial labeling, although second-year effects are difficult to interpret. Nitrogen concentration appeared to increase toward the periphery of the tree. This increase became more pronounced with increasing crown size, crown closure, and consequent shading. Although the importance of leaf age should not be discounted in determining the source of N (Domenach and Kurdali, 1989), crown position with respect to light availability may be the most important determinant in overall N allocation in red alder foliage.

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