Variations in the amino acid composition of xylem sap of *Betula pendula* Roth. trees due to remobilization of stored N in the spring

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ABSTRACT

Seasonal patterns of N translocation in the xylem sap of *Betula pendula* were studied, to determine whether specific amino acids were recovered in spring as a consequence of N remobilization. Seedlings were grown in sand culture and provided with $^{15}$NH$_4$-NO$_3$ (at 2.2 atom percent excess) for one growing season. The following winter dormant trees were transplanted into fresh sand and given N at natural abundance thereafter. Destructive harvests were taken during bud burst and leaf growth to determine the pattern of $^{15}$N remobilization and N uptake, along with isolation of xylem sap for analysis of their amino acid profiles and $^{15}$N enrichment by GC-MS. $^{15}$N remobilization occurred immediately following bud burst, while N derived from root uptake did not appear in the leaves until 12 d after bud burst. During N remobilization there was a 10-fold increase in the concentration of N in the xylem sap, due predominantly to increases in citrulline and glutamine. The $^{15}$N enrichment of these two amino acids demonstrated the increase in their concentration in the xylem sap following bud burst was due to N remobilization. These results are discussed in relation to measuring N remobilization and storage capacity of trees in the field.

Key-words: Betula pendula; bud burst; citrulline; glutamine; N remobilization; xylem sap.

INTRODUCTION

Much of the nitrogen (N) used by deciduous trees for leaf growth in the spring comes from the remobilization of stored reserves (Millard 1996). Storage of N occurs during the winter in specific wood and bark storage proteins that are degraded during spring shoot growth (Nsimba-Lumaki & Penmans 1986; Sauter & van Cleve 1992; Wetzel et al. 1989; Coleman et al. 1991). Roots of young trees have also been reported to store N during the winter (Tromp 1983; Millard & Proe 1991). Measuring the contribution of N storage and remobilization to the seasonal growth of trees is difficult (Nambiar & Fife 1991; Millard 1996). Whole-tree N budgets for deciduous trees are often imprecise, because they attempt to quantify remobilization by measuring N withdrawal from senescing leaves (e.g. Ryan & Bormann 1982; Staff 1982; Côté & Camiré 1987), without allowing for N uptake in the autumn contributing directly to storage (Millard & Thomson 1989; Millard & Proe 1991; Wendler & Millard 1995). As an alternative to whole-tree N budgets, $^{15}$N has often been used to quantify N uptake and storage (Millard 1996). However, the use of isotopes is limited to sand culture experiments with small trees, since field studies cannot allow for uptake of native soil N at natural abundance simultaneously with fertilizer $^{15}$N, thereby underestimating internal cycling of N (Millard 1996).

An alternative method to quantify N remobilization by trees may be to study their N translocation patterns in the spring. Xylem has been shown to be the main pathway for long distance translocation of organic-N compounds towards growing meristems (Moreno & Garcia-Martinez 1983; Sauter & van Cleve 1992). Several studies have shown a peak in concentration of N in xylem sap during bud burst and leaf growth (Glavac & Jochheim 1993; Schneider et al. 1994). Several authors have suggested that remobilization is responsible for a large part of these N fluxes in the xylem in the spring (Sauter & van Cleve 1992; Schneider et al. 1994). An alternative hypothesis is that the increase in N concentration is due to uptake and assimilation in the roots and translocation, while the decrease in concentration is due to increased transpiration rates during leaf growth diluting N in the xylem sap.

We have grown the deciduous tree *Betula pendula* in sand culture for 2 years. During the first year the trees were supplied with only $^{15}$N enriched to 2.2 atom percent excess. In the second year the trees received N at natural abundance and during the spring and summer destructive harvests were taken along with collection of xylem sap in order to (1) determine whether there are seasonal patterns of N translocation in the xylem sap, (2) quantify the duration of $^{15}$N translocation in the xylem in relation to leaf growth in the spring, and (3) determine whether there are specific amino acids recovered in the xylem sap as a consequence of N remobilization.
MATERIALS AND METHODS

Experimental design

Two-year-old Betula pendula Roth. seedlings (55 in total) were lifted from a nursery while dormant and placed in cold storage until planted in pots (55 cm diameter × 45 cm deep) containing fine sand on 2 May 1994. The trees had previously received a moderate N supply. The pots were arranged in five randomized blocks in a greenhouse and watered with 300 cm$^3$ of a nutrient solution containing 8·0 mol N m$^{-3}$ as $^{15}$NH$_4$$^{15}$NO$_3$ enriched with $^{15}$N to 2·2 atom percent excess. Other nutrients supplied were as described by Millard & Proe (1991). Trees were watered every 2 d throughout the summer and autumn. A natural photoperiod was used and the greenhouse was ventilated to provide temperatures close to ambient. During leaf senescence abscised leaves were removed from the surface of the sand twice weekly. Throughout the winter the trees were kept frost-free (≥ 2 °C) and moist. In December 1994 the trees were carefully removed from the pots and all sand washed off the roots before being planted into fresh sand in a new pot. Thereafter, the trees were kept moist with distilled water until 22 February 1995 when they were re-supplied with nutrient solution identical to that used in 1994, except that the NH$_4$NO$_3$ had $^{15}$N at natural abundance. Whilst the trees were still dormant the total number of buds on each tree were counted. Throughout the spring the number of open buds on each tree was assessed daily. For each individual tree the day the first open bud was noted was designated as the date of onset of bud burst. The number of days until all the buds on an individual tree were open was designated as the duration of bud burst.

Tree harvesting and analysis

Destructive harvests of B. pendula were taken on 27 February, 6, 15, 22 and 29 March, 5, 12 and 26 April and 10 and 30 June. With a total of 55 trees, one per block had been allocated as spare, in case an individual died. None of these spare trees was needed. At each harvest one tree of the appropriate species was selected at random from each block and removed. The sand from the pot was sieved and remobilized for leaf growth during the spring of 1995.

Collection and analysis of xylem saps

During tree harvesting, two or three twigs between 20 and 25 cm in length and still with a terminal bud or leaves intact were removed. Some 10 cm of bark was removed from the cut end of the twigs or stem to avoid contamination with phloem sap. The sample was then placed in a Scholander pressure chamber so that only a section of wood with the bark removed protruded. The end of the twig was recut and the pressure in the vessel increased until xylem sap was exuded and collected by micro capillary tubes. All the xylem sap collected from one tree was pooled and weighed (typically between 5 and 35 mg). An aliquot of sap was taken for determination of ATP by the Luciferine/luciferase assay (Strehler & Totter 1952) using the Sigma Luciferine–Luciferase reagent kit, to ensure no contamination with phloem sap. Sap samples were stored at −70 °C until analysis of the concentration and $^{15}$N enrichment of their individual amino acids by gas chromatography linked to mass spectrometry (GC-MS). Xylem sap (10–15 mg) and nor-valine (50 mm$^3$ of an aqueous solution containing 6·34 μg nor-valine cm$^{-3}$) as internal standard were added to a Reacti-Vial (Pierce & Warriner, Chester, England) and taken to dryness at 40 °C under a stream of nitrogen. The derivatization reagent (50 mm$^3$) comprising 495 volumes of N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide, 1 volume of tert-butyldimethylchlorosilane and 4 volumes of acetonitrile was added to the dried material and heated at 150 °C for 1 h to convert the free amino acids to their t-butyldimethylsilyl derivatives (t-BDMS). The analyses of the derivatives were carried out using GC-MS in the single ion recording (SIR) mode. The instrumentation used was a VG TRIO 1 quadrupole mass spectrometer linked to a Fisons Series 8000 gas chromatograph fitted with an AS800 autosampler (Fisons Instruments UK, Crawley, Sussex).

Separation of the derivatives was effected using a fused silica SP Sil 8 CB column, 30 m × 0·32 mm id × 0·25 μm phase thickness (Chrompask[UK] Ltd, London). The column was operated with a temperature programme of 100 °C for 8 min, increased to 250 °C at 5 °C min$^{-1}$, held for 2 min, increased to 290 °C at 15 °C min$^{-1}$ and held for 5 min. The sample was introduced to the column using a split injection system (10:1 split) and the injector temperature was 250 °C. The interface line temperature between the gas chromatograph and the mass spectrometer was 290 °C. The mass spectrometer was operated under electron impact ionization mode with an ionization energy of 70 eV and a source temperature of 250 °C. Data were obtained with a scan rate of 0·02 s decade$^{-1}$ using SIR. The masses monitored corresponded to M-57 and M-57 + 1. The complete list of ions monitored is given in Table 1. Enrichments of $^{15}$N in individual amino acids were calculated from the ratio of M-57 and M-57 + 1 ions in natural abundance and enriched amino acids (Campbell 1974). For the estimation of amino acid concentrations, response factors were calculated for each amino acid by analysing a solution containing known weights of amino acids before
and after the analysis of each batch of samples. Quality control was assured by analysing standard solutions of amino acids.

The conditions used in the preparation of t-BDMS derivatives resulted in the deamination of citrulline to ornithine. The absence of ornithine in the xylem sap was verified by preparing termethylsilyl (TMS) derivatives of the amino acids in randomly selected samples and analysing them by GC. No chromatographic peaks corresponding to the TMS derivative of ornithine could be found. Therefore, throughout this work citrulline was estimated as ornithine. Under the GC-MS conditions employed, the precision of isotope ratio analysis was ±0.3 atom percent excess.

### RESULTS

#### Bud burst and leaf growth

The pattern of the onset of bud burst is shown in Fig. 1. The first tree to break bud was noted on 16 February, but it was a further 30 d until each tree had at least one bud burst. There were 110 ± 11 buds per tree and the duration of bud burst within an individual (i.e. time between the first and last bud bursting) ranged from 3 to 22 d with a mean value of 10 ± 0.5 d (Fig. 2). Because of the variation between individual trees in both the onset and duration of bud burst, all subsequent data were expressed with the days from bud burst (D) for that individual tree as the time variable. Leaf growth (as measured by an increase in leaf mass) started at 15 D (Fig. 3). By the end of the experiment the trees had produced a leaf area of 3414 ± 352 cm² tree⁻¹ and leaf dry mass of 17.3 ± 1.37 g tree⁻¹.

#### Pattern of N remobilization

Recovery of ¹⁵N in the leaves was used as a measure of N remobilization which started before any unlabelled N taken up by the roots was recovered in the leaves (12 D). Remobilization of N had finished by 50 D (Fig. 4). Parameters derived from fitting equations to the data are given in Table 2 and show that a maximum of 201.7 mg ¹⁵N was remobilized for leaf growth (representing 37% of total leaf N).

The N remobilized for leaf growth could have been stored during the winter in any of the perennial tissues. To determine which tissues were used to store N, the ¹⁵N contents of both the bark and wood of the various age class of stems and the roots were measured in trees harvested at the time of bud burst and again after remobilization had finished (65 ± 1.7 D). The amount of N remobilized for leaf growth was calculated as the difference in the ¹⁵N contents between these two harvests (Table 3). The net loss of ¹⁵N from perennial tissues was similar to the recovery of remobilized N in their leaves (Fig. 4). Trees stored N throughout

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**Table 1.** Masses of ions monitored for the determination of the concentration of ¹⁵N enrichment of amino acids in xylem saps

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Mass of ions (amu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asn</td>
<td>417, 418, 419</td>
</tr>
<tr>
<td>Asp</td>
<td>418, 419</td>
</tr>
<tr>
<td>Glu</td>
<td>432, 433</td>
</tr>
<tr>
<td>Gln</td>
<td>431, 432, 433</td>
</tr>
<tr>
<td>Cit</td>
<td>474, 475, 476</td>
</tr>
<tr>
<td>Ser</td>
<td>390, 391</td>
</tr>
<tr>
<td>Gly</td>
<td>218, 219</td>
</tr>
<tr>
<td>His</td>
<td>440, 441, 442</td>
</tr>
<tr>
<td>Gaba</td>
<td>274, 275</td>
</tr>
<tr>
<td>Ala</td>
<td>232, 233</td>
</tr>
<tr>
<td>Thr</td>
<td>404, 405</td>
</tr>
<tr>
<td>Pro</td>
<td>286, 287</td>
</tr>
<tr>
<td>Tyr</td>
<td>466, 467</td>
</tr>
<tr>
<td>Val</td>
<td>288, 289</td>
</tr>
<tr>
<td>Ile</td>
<td>302, 303</td>
</tr>
<tr>
<td>Leu</td>
<td>302, 303</td>
</tr>
<tr>
<td>Phe</td>
<td>336, 337</td>
</tr>
<tr>
<td>Lys</td>
<td>431, 432, 433</td>
</tr>
</tbody>
</table>

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all their perennial tissues, with greater amounts in the bark than the wood of the stems. The root system stored 52% of the total 15N remobilized for leaf growth (Table 3).

Composition of xylem saps

Before analysing all of the xylem saps collected from the experiment, the recovery of N in the sap was checked. A bulked sample of xylem sap was collected from field-grown trees in July 1995. Some 30–40 twigs were sampled and the sap combined to give some 500 mg of sap. The bulked sample was analysed by GC-MS for the amino acid composition, and by CHN analyser. The total amino acid N recovered by the GC-MS analysis was 58.4 μg N g⁻¹ xylem sap, whereas the CHN analyser measured 55 μg N g⁻¹ sap. There was no detectable nitrate recovered in the samples and since the GC-MS analysis accounted for all the N-containing compounds, this method was used for all subsequent analyses of xylem sap.

The total N concentration in xylem sap following bud burst is shown in Fig. 5. The N concentration in sap started to rise immediately after bud burst, peaked around
22 D and then fell until around 40 D. This peak in N concentration occurred during the period of N remobilization (Fig. 4). The maximum mean concentration of N recovered in xylem at any one harvest was 382 ± 33·5 mg N g⁻¹ sap at the harvest taken on 29 March. At this time some 70% of the N was recovered in two amino acids, citrulline and glutamine (Table 4). When the concentration of N had fallen there were qualitative differences in the composition of the sap. The amount of N recovered in each amino acid fell by varying amounts, ranging from 61% for alanine to 98% for histidine, valine, isoleucine and phenylalanine. During N remobilization citrulline accounted for 37% of the N in the sap, but by 65 D this had risen to 54%. The comparable values for glutamine were 33% and 9%, respectively. These qualitative differences in xylem sap composition suggest that the decrease in N concentration 22–40 D can not have been due solely to increased transpiration rates causing a dilution of solutes in the sap.

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Table 2. Parameters derived from fitting equations to the data for recovery of labelled ¹⁵N (Fig. 4, top) and unlabelled N (Fig. 4, bottom) in leaves in relation to days from bud burst of each tree

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Maximum Point of Time for recovery value inflection (mg tree⁻¹)</th>
<th>Point of Time for recovery of 95% of N (D)</th>
<th>Time for recovery of 95% of N (D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labelled ¹⁵N</td>
<td>201·7</td>
<td>26·5</td>
<td>48·0</td>
</tr>
<tr>
<td>Unlabelled N</td>
<td>350·1</td>
<td>43·4</td>
<td>85·3</td>
</tr>
</tbody>
</table>

Table 3. Storage of N in the stems and roots of B. pendula trees during the winter. Values are given for the ¹⁵N content (mg tree⁻¹) of perennial woody tissues in trees from the first harvest at the onset of bud burst and in trees harvested after remobilization had finished (on 26 April). Values are the mean ± SE of five replicates. The difference between the values represents the remobilisation of N for leaf growth

Table 4. Comparison of the recovery of N in amino acids in the xylem sap of B. pendula trees harvested during the period of N remobilization and after remobilization had finished. Values are given as mg amino acid N g⁻¹ xylem sap and are the mean ± SE of five replicates


**Xylem sap amino acids and N remobilization**

The increased concentration of citrulline and glutamine in xylem saps was due to N remobilization. Figure 5 shows the ¹⁵N enrichment of these amino acids recovered in xylem saps. During the period of N remobilization by B. pendula (up to 50 D) citrulline had a mean ¹⁵N enrichment of 1·9 ± 0·1 atom percent excess. Once N remobilization had finished (50 D, Fig. 4) the concentration of...
citrulline in the xylem saps had decreased (Table 4) and the $^{15}$N enrichment fell to a mean of $0.7 \pm 0.07$ atom percentage excess. A similar pattern of $^{15}$N labelling was also found for glutamine. However, the decrease in $^{15}$N enrichment after 50 D was not so pronounced in glutamine as for citrulline. These results demonstrate that the increased N concentration recovered in xylem saps around 10–40 D (Fig. 4) were due predominantly to remobilization of stored N rather than translocation of currently assimilated N.

**DISCUSSION**

We have shown that *B. pendula* exhibits a seasonal pattern of N translocation in the xylem similar to those reported for *Fagus sylvatica* (Glavac & Jochheim 1993), *Populus canadensis* (Sauter & van Cleve 1992; Schneider et al. 1994), *Picea abies* (Dambrine et al. 1995) and *Actinidia chinensis* (Ferguson et al. 1983). Furthermore we have established that the spring peak in xylem sap N concentration found immediately after bud burst corresponds with the period of N remobilization from storage. During this period two N containing compounds predominated in the xylem sap, citrulline and glutamine.

Citrulline has been reported as the predominant amino acid in 13 species of tree studied by Barnes (1963), including *B. nigra* and *Alnus rugosa*. Sheldrake & Northcote (1968) also reported citrulline as a predominant amino acid in the xylem sap of *B. populifolia* collected before bud burst. A potential role for citrulline as a N storage compound in *Betula* was suggested by Näsholm & McDonald (1990) who found that leaf concentrations of citrulline increased in *B. pendula* trees when their N supply increased. They suggested that leaf amino acid concentrations were related to those in xylem sap and were therefore indicative of a small excess of N with respect to current usage for protein synthesis (Nashölm & McDonald 1990). Citrulline may also act as a short-term storage compound in *A. glutinosa* (Baker et al. 1997) as well as being stored in stem wood during the winter (Tonin et al. 1990). While citrulline was found in our study to be the main amino acid translocated during N remobilization by *B. pendula*, it is not used as a N storage compound during the winter. The N stored in the bark and roots of *B. pendula* during winter has been shown by electrophoresis to be in a protein (P. Millard, unpublished results).

Glutamine has also been reported as the predominant form of organic N in the xylem saps of a range of tree species (Barnes 1963; Tromp & Ovaa 1969; Sauter 1981; Schneider et al. 1994). Increases in glutamine concentrations in xylem saps from *P. canadensis* during the spring were correlated with the turnover of storage proteins in the ray paranchyma cells by Sauter & van Cleve (1992). These authors pointed out that since glutamine accounted for only 1.3% of the amino acids in the storage protein but 75% of the amino-N in the sap, protein turnover must result in glutamine synthesis before release into the xylem vessels (Sauter & van Cleve 1992). Citrulline synthesis following turnover of storage proteins must likewise occur in *B. pendula* since citrulline, as an ureide, is not an amino acid found in plant proteins (Thompson 1980).

Dambrine et al. (1995) suggested that internal cycling is responsible for a large part of the nutrient fluxes in the xylem sap recovered from the crown of *Picea abies*. We have demonstrated that both the citrulline and glutamine translocated immediately after bud burst contained N which had been assimilated predominantly during the previous year. This is the first direct experimental evidence to link patterns of amino acid translocation in the xylem with N remobilization. During the period of N remobilization (as measured by recovery of $^{15}$N in the growing leaves), the mean enrichment of citrulline (1.9 atom percent excess) was close to that of the N supplied to the tree roots the previous year (2.2 atom percent excess). These data show that some 86% of the N translocated as citrulline during the period 0–55 D had been assimilated the previous year.

Miller (1984) used nutrient budgets to calculate that 47% of the N required for growth of *B. pendula* over a

**Figure 6.** The $^{15}$N enrichment of citrulline and glutamine in xylem saps in relation to bud burst.
4–5 years rotation came from the internal cycling of N. This figure agrees well with the value of 40% we measured for leaf growth in our saplings. However, it is well established that remobilization of N becomes quantitatively more significant as trees grow (Miller & Miller 1987), because as they develop rates of N uptake decrease but as they grow their storage capacity increases (Miller 1986). It is likely that the budget Miller (1984) used in his calculations underestimated the contribution of internal cycling in providing N for leaf growth (Millard 1996).

An alternative to the construction of whole-tree N budgets for measuring the amount of N remobilized for growth has been to apply $^{15}$N to the soil before bud burst and use recovery of the isotope in leaves to determine uptake of N from the soil; leaf N prior to recovery of any isotope was then used as an estimate of N remobilization (Millard 1994). Such a technique requires that the N content of the foliage be measured precisely, which can be difficult for large trees. Another approach suggested by Ledgard & Brier (1991) was analysis of xylem saps of plants previously labelled with $^{15}$N. Our data show that this second approach could be feasible for trees without the use of $^{15}$N. Since the peak in amino acid translocation after bud burst was due to remobilization, all the additional data needed to calculate the amount of N translocated is a measure of the sap flow rates. In potted trees sap flow rate can be measured gravimetrically (Améglio et al. 1993). In larger trees it might be possible to use either heat pulse or heat sink methods (Valancogne & Nasr 1989) in conjunction with xylem sap amino acid analysis to calculate N remobilization. Such approaches merit further study. We know that all N stored during the winter is remobilized in spring (Millard 1996), so such approaches might enable the N storage capacity of a tree to be measured non-destructively for the first time.

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