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Recycling of nitrogen in the xylem of *Prunus avium* trees starts when spring remobilization of internal reserves declines

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Summary Nitrogen (N) storage capacity of cherry (*Prunus avium* L.) trees grown in sand culture was preconditioned by applying contrasting N supplies for one year. During the spring of the following year, a constant amount of ^{15}N was supplied and the dynamics of N remobilization and root uptake were characterized as a function of internal N status of the trees. To calculate the flux of N through xylem, both xylem sap N concentration and whole-tree transpiration rates were measured. By comparing the cumulative flux of N through the xylem with the amount of N recovered in the new aboveground growth, we indirectly evaluated the recycling of N in the xylem, i.e., the amount of N derived from shoot–root translocation that was subsequently reloaded into the xylem.

The contrasting N storage capacities imposed during the first year affected both N remobilization and uptake from roots in the following year. Recycling of N in the xylem apparently did not occur during the remobilization of internal reserves (i.e., during the first 6–8 weeks after bud burst). However, when remobilization declined, measurement of the cumulative flux of N through the xylem overestimated the amount of N recovered in the new biomass, allowing the extent of N recycling to be evaluated. The amount of N recycling in the xylem was greater in high-N trees, which also took up less N through their roots than trees preconditioned to have a lower internal N status. This suggests that recycling of N in the xylem is a mechanism by which plants regulate N uptake by roots.

Keywords: amino acid translocation, cherry, nitrogen storage, nitrogen uptake, root-shoot-root cycling.

Introduction

Nitrogen (N) fluxes in the xylem of trees are regulated by three processes: remobilization from internal reserves, root uptake of N from the soil, and phloem–xylem recycling (Dambrine et al. 1995). Remobilization of stored N is essential for the growth of temperate, deciduous trees in spring (Millard 1996). This process provides the first source of N used for growth in the spring and can provide the major source of N used for aboveground growth each year (Neilsen et al. 1997, Weinbaum and Van Kessel 1998, Dyckmans and Flessa 2001). Although only a few studies have described the dynamics of this process in detail, it is clear that, in some species, the majority of N remobilization occurs before root uptake of N starts (e.g., *Prunus persica* (L.) Batsch, Rufat and DeJong 2001; *Sorbus aucuparia* L., Millard et al. 2001; *Malus domestica* Borkh., Dong et al. 2001; *Prunus avium* L., Grassi et al. 2002), whereas in other species the two processes are concurrent (e.g., *Betula pendula* Roth, Millard et al. 1998; *Juglans nigra* L. × *J. regia* L., Frak et al. 2002). Once remobilization ceases, root uptake provides the remainder of the N used for growth that year. The relative contributions of remobilized N and N taken up by roots to the N used for growth in any given year depends on tree age, soil fertility and other environmental factors (Millard 1996). Regulation of N uptake by roots could involve shoot–root cycling of N, because an inverse correlation has been found between the concentrations of amino acids and amides in phloem sap and nitrate uptake by the roots of *Fagus sylvatica* L. (Gessler et al. 1998), *Picea abies* (L.) Karst. (Weber et al. 1998) and *Prunus persica* (Youssefi et al. 2000) trees.

Shoot–root phloem transport of mineral elements is a normal feature of vascular plants and may serve several functions, such as control of nutrient uptake by the roots and maintenance of the cation–anion balance in the shoot (Pate 1975, Marschner et al. 1996, Weber et al. 1998). A considerable amount of the nutrients translocated to the roots can be reloaded into the xylem and translocated back to the shoot, i.e., they are recycled within the plant (Marschner 1995). Although it has been demonstrated for several herbaceous species that part of the N in the xylem sap represents a recycled fraction (Simpson et al. 1982, Cooper and Clarkson 1989, Jeschke and Pate 1991, 1992), there are few data for tree species because of the difficulty of measuring this process. Current knowledge is mainly based on detailed analyses of phloem and xylem sap in different shoot parts of individual plants and the correspond-

ing mineral element contents in the shoot parts at sequential harvests (Jeschke and Pate 1991, 1992). However, for precise assessments of phloem–xylem recycling, not only are the concentrations of solutes required, but also their flux through the conducting vessels (Marschner 1995).

In this study, *Prunus avium* trees were grown in sand culture and their N storage capacity preconditioned by applying contrasting N supplies for one year. During the spring of the following year, we supplied a constant amount of ^{15}N to all of the trees. We then analyzed labeled and unlabeled N in the different organs through sequential harvests to determine the pattern of N remobilization and N uptake. Based on measurements of both xylem sap N concentration and whole-tree transpiration rates, we calculated the flux of N through the xylem. We then compared the cumulative amount of N transported in the xylem during spring with the amount of N recovered in new aboveground growth to assess: (1) the effect of remobilization of stored N on the recycling of N in the xylem; and (2) the effect of recycling of N in the xylem on N uptake by roots.

Material and methods

Experimental design

One-year-old seedlings of *Prunus avium* (80 in total, provenance northern Italy) were lifted from a nursery while dormant and placed in cold storage until planted in 14-dm^3 pots containing fine sand in May 1999. The trees were arranged under a shade cloth (about 50% of transmittance) in four randomized blocks. Nitrogen at natural abundance was applied as NH_4NO_3 once a week to provide each plant with a total of 0.6 g from May to June (about 0.06 g $week^{-1}$) and with either 1 g (LN) or 8 g of N (HN) from July to October (about 0.06 and 0.45 g $week^{-1}$, respectively). This allowed us to obtain trees with different N status but similar dimensions (Grassi et al. 2002).

Additionally, an automatic drip irrigation system (four emitters per plant) distributed 1 dm^3 of nutrient solution per pot, three times per week. This provided each plant during the whole growing season with 0.8 g P, 2.7 g K, 0.9 g Ca, 1.4 g S, 0.3 g Mg, 6 mg B, 3 mg Mn, 3 mg Zn, 1 mg Cu and 45 mg Fe EDDHA (ethylenediamine-di(*o*-hydroxyphenylacetic) acid). When required, additional water was given manually.

In January 2000, pots were carefully washed with deionized water to remove any residual N present in the sand and then transferred to a greenhouse and arranged in four replicate blocks according to their height. Each block contained 10 plants from each treatment. From late February 2000 and throughout the spring, the number of open buds on each tree was assessed every 1–2 days. The day on which the first bud opened was designated as the date of bud burst for each individual plant. From bud swelling onward, each plant was resupplied with a nutrient solution identical to that used in 1999, except that plants in both treatments received 0.45 g $week^{-1}$ of N as $^{15}NH_4{}^{15}NO_3$ enriched with ^{15}N to 7.75 atom %.

Between March 28 and June 20, 2000, 10 subsequent measurement periods of 7–14 days each were selected. In each period, transpiration of one set of four LN and four HN trees was measured, and at the end of the period xylem sap was collected and trees were harvested.

Transpiration measurements

Transpiration rates of the eight selected trees were measured for the whole measurement period as described by Grassi et al. (2002). At the beginning of each period, drip emitters were removed and each selected pot was enclosed in a plastic bag. During the period, a known amount of nutrient solution (from 0.5 to 1.5 dm^3, depending on the water requirements of the trees, which varied during the experiment) was given manually three times per week, and at the same time the plastic bags were opened for a few minutes to avoid anoxia of the root system. The amount of N supplied to the trees, however, remained constant at 0.45 g $week^{-1}$. Transpirational water loss during each measurement period (g $tree^{-1}$) was calculated as the difference between the sum of the weight of the plant and pot at the beginning of the sampling period, plus the weight of the nutrient solution that had been provided, and the weight of the plant and pot at harvest.

Sap collection and analysis

Between 1130 and 1430 h, xylem sap was collected from each tree just before it was harvested. A portion of the stem 30–50 cm above the collar (trees were 1.5–2 m in height) was cut and a few centimeters of bark trimmed from the distal cut end of the stem to avoid phloem contamination. The stem portion was immediately placed in a Scholander pressure chamber so that the debarked section of wood protruded. The pressure in the chamber was slowly increased to a maximum of 0.2 MPa, and the xylem sap that exuded was collected with micro-capillary tubes. Initial tests (as described by Malaguti et al. 2001) indicated that pressures up to 0.2 MPa caused no contamination by cellular components. Sap samples were stored at –70 °C until determination of total N concentration by an elemental analyzer.

Tree harvesting and analysis

Immediately after sap collection, trees were removed from their pots and the sand was carefully washed from the root system. Trees were separated into five organ types: stem (excluding current-year growth), leaves, axes (current-year growth of stem), taproot and secondary roots, which were dried, weighed and milled to pass through a 0.2-mm screen prior to ^{15}N analysis. A Tracer Mat continuous flow mass spectrometer (Finnigan MAT, Hemel Hempstead, U.K.) was used for determination of ^{15}N and total N in the different organs. The ^{15}N enrichment was used to calculate the uptake of labeled N during 2000 (Millard and Neilsen 1989). Recovery of unlabeled N in the leaves and axes provided a direct measure of N that had been taken up before 2000 and remobilized for new biomass production during the spring of 2000.

Estimation of recycling of N in the xylem

For each sampling period, the N concentration in the xylem sap was multiplied by total transpiration of the same plant to

calculate N flux through the xylem for that period. To calculate cumulative flux for each treatment, the mean N flux through the xylem estimated for all plants in a sampling period was added to the mean N flux of the previous period. This calculation assumed that the concentration of N in the xylem sap—which was determined at the end of the sampling period—was representative of the mean concentration during the whole period. From this assumption, potential errors may arise as a result of (i) possible diurnal variations in xylem sap N concentration because of variations in transpiration rate, and (ii) possible variations in xylem sap N concentration during the sampling period. However, Grassi et al. (2002) demonstrated that, for the same *P. avium* trees, these potential errors were negligible and the assumptions were equally valid for HN and LN trees.

The fraction of N that was recycled in the xylem during the experiment was estimated by comparing the cumulative amount of N transported in the xylem with the amount of N recovered in the new aboveground growth. These calculations assumed that, if the cumulative amount of N transported in the xylem overestimated the amount of N present in the new growth, this was due to a fraction of N recovered in the xylem being derived from shoot–root translocation and subsequent reloading into the xylem (Marschner et al. 1996).

Data analysis

Data for biomass of the different organs, recovery of unlabeled and labeled N, and concentration of N collected during the second year were related to the stage of development by using the number of days from bud burst as a measure of time. The data were fitted against time using polynomial functions of Table Curve 2D software (SPSS). The coefficient of determination (r^2), the statistical significance of the regression (P) and the 95% confidence limits were also calculated. The last parameter was used to compare treatments.

Results

Tree growth and N status

At the onset of growth in the second year, tree mass did not differ significantly between N treatments (Figure 1). The mean mass of both stems and taproots decreased at the beginning of the season (Figure 1), whereas no clear pattern was observed for the growth of secondary roots. Total plant biomass started to increase after the first month and, by the end of the experiment, it was nearly the same as at the beginning of the season (data not shown), with no differences between treatments ($P > 0.05$). At the end of the experiment, tree N status had no statistically significant effect on leaf growth ($P > 0.05$), but it did affect the growth of axes ($P < 0.05$, Figure 1).

The HN plants had a higher N status at the start of the second year of the experiment than the LN trees. They had a greater N concentration in both taproots (mean + SE: 14.6 + 0.6 and 6.4 + 1.0 mg g^{-1} for HN and LN trees, respectively; $P < 0.001$) and secondary roots (15.6 + 0.8 and 10.0 + 1.1 mg g^{-1} for HN and LN trees, respectively; $P < 0.01$). As a result, during the

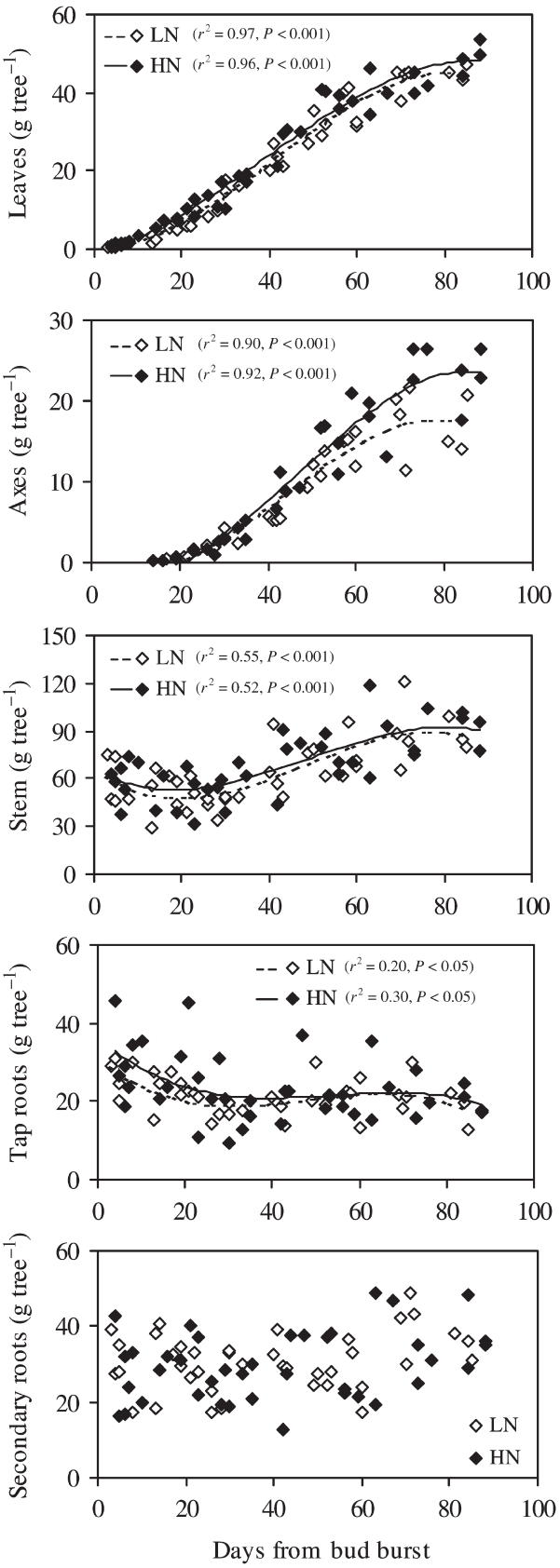

Figure 1. Effects of N supply in 1999 on the biomass of leaves, axes, stems, taproots and secondary roots during 2000. Values are for the individual high-nitrogen (HN) and low-nitrogen (LN) trees.

second year, HN trees contained almost twice as much unlabeled N as the LN trees (mean + SE: 1240 ± 44.1 mg and 646 ± 26.2 mg, respectively; $P < 0.001$) (Figure 2, left column).

N remobilization and uptake

Tree N status significantly affected both N remobilization and N uptake by roots during the second year (Figure 2). Remobilization of unlabeled N for leaf growth started immediately after bud burst in both treatments and, when considering both leaves and axes, reached 90% of the final value 50 and 35 days after bud burst for HN and LN trees, respectively. By the end of the experiment, HN trees remobilized about twice as much N as LN trees ($P < 0.001$). However, the proportion of unlabeled N that was subsequently remobilized was similar in HN and LN trees (39 and 37%, respectively). By assessing the relative changes in unlabeled N in plant organs over the second growing season, we could estimate their relative contribution of N for remobilization (Figure 2, left column). The percent-

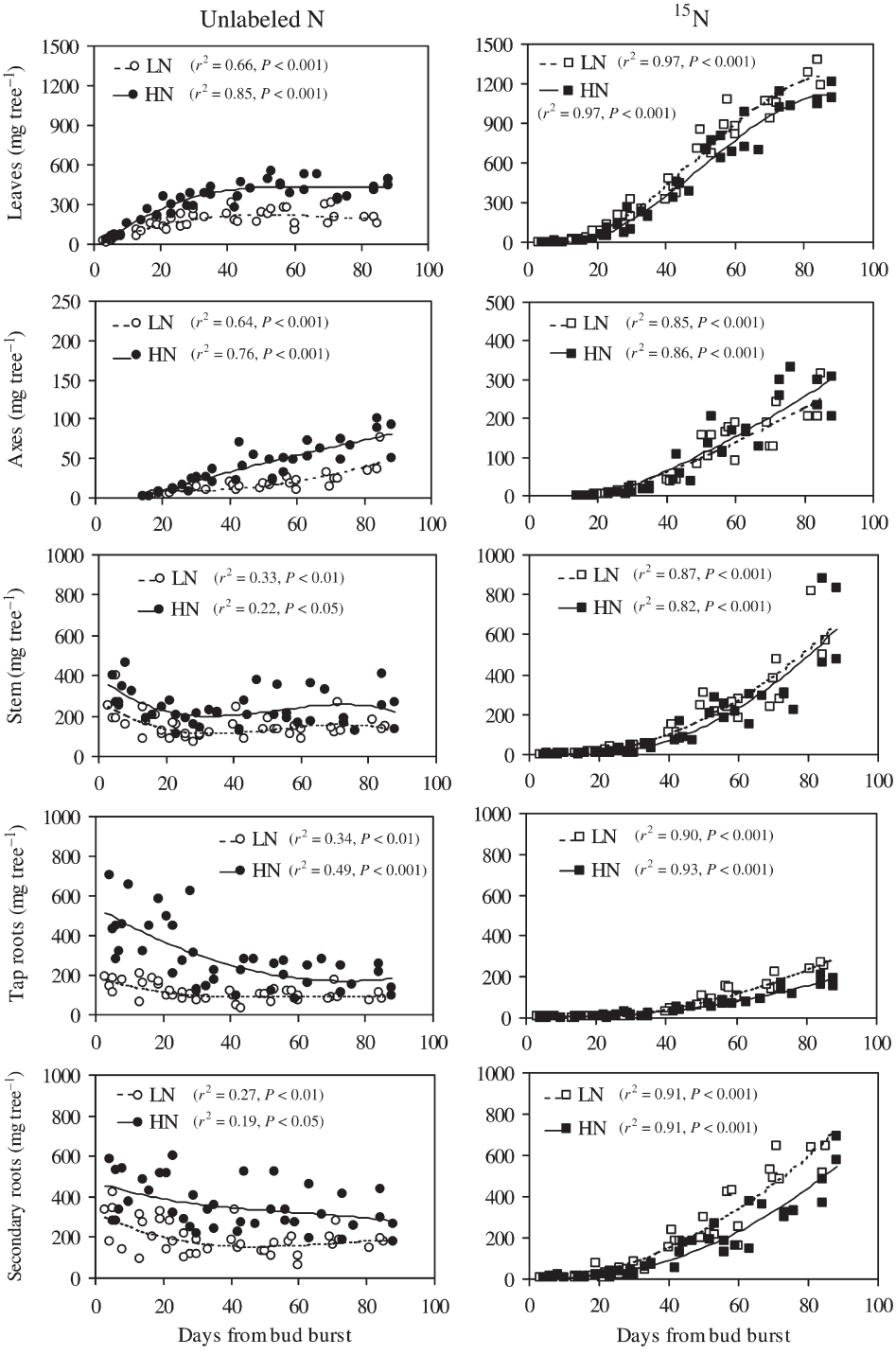

Figure 2. Effects of N supply in 1999 on the recovery of unlabeled N (i.e., N taken up before 2000, left column) and ^{15}N (i.e., N taken up from roots during 2000, right column) in the different organ types as a function of days from bud burst during 2000. Values are for the individual high-nitrogen (HN) and low-nitrogen (LN) trees.

age of remobilized N coming from shoot, taproots and secondary roots was 20, 54 and 26%, respectively, in HN plants; and 25, 35 and 40%, respectively, in LN plants. Therefore, for both treatments, the role of roots as a storage site for N was predominant, whereas the contribution of the stem was relatively small.

During the second vegetative season, more ^{15}N (i.e., N taken up from roots during 2000) accumulated in the leaves, taproots and secondary roots of LN plants than of HN plants (Figure 2, right column). At the whole-plant level, N taken up by roots was slightly but significantly ($P < 0.05$) higher in LN trees than in HN trees (Figure 3).

Calculation of N recycling in the xylem

Figure 4 shows the total amount of N measured in the new aboveground growth (leaves and axes) and the calculated cumulative N transported in the xylem in relation to days from bud burst, for both treatments. During the first 40–50 days after bud burst (i.e., during the remobilization period), the data sets were similar for HN and LN trees. By comparing the calculated cumulative N flux in the xylem (y) with the total N measured in the new aboveground growth (x) only during the remobilization period (i.e., till 50 and 35 days after bud burst for HN and LN trees, respectively), regressions close to the 1:1 line were found for both HN and LN trees ($y = 1.10x + 2.10$, $r^2 = 0.96$, for HN plants; $y = 1.04x - 14.08$, $r^2 = 0.83$, for LN plants). This further confirmed that, for both treatments, the method for calculating N flux in the xylem was valid during the remobilization period. However, after that period, the cumulative N flux in the xylem progressively overestimated the amount of N recovered in the new aboveground growth. This discrepancy, which we considered as an indirect estimate of the recycling of N in the xylem, was higher in HN plants than in LN plants (Figure 5). By the final harvest (about 90 days after bud burst), the cumulative N transported in the xylem (value of the regression by the final harvest + 95% confidence limit) was 3801 + 191 mg tree^{-1} and 3122 + 182 mg tree^{-1} for the HN and LN trees, respectively (Figure 4). Over the same period, the total flux of N involved in shoot–root recycling (i.e., the difference between cumulative amount of N transported in the xylem and the amount of N in the new aboveground growth) was calculated to be 1879 + 168 mg tree^{-1} and 1356 + 181 mg tree^{-1} for the HN and LN trees, respectively (Figure 5). Thus the recycling of N was a major contributor to the flux of N in the xylem when remobilization declined.

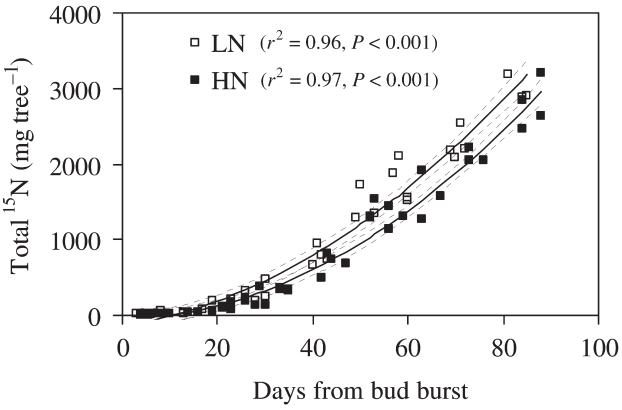

Figure 3. Recovery of ^{15}N at the whole-plant level as a function of days from bud burst during 2000. High-nitrogen (HN) and low-nitrogen (LN) treatments refer to the amount of N received in 1999. Dotted lines indicate the 95% confidence limits.

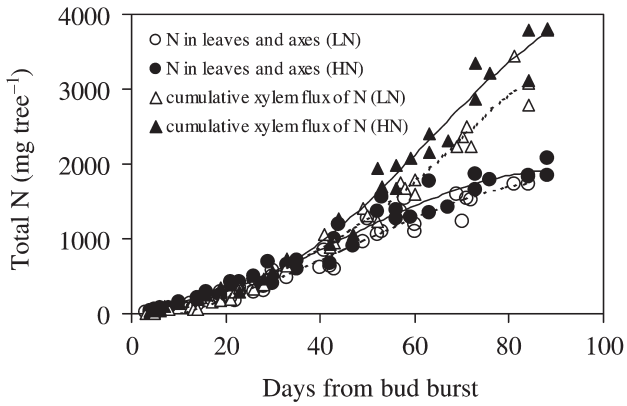

Figure 4. Cumulative flux of N through xylem of high-nitrogen (HN) and low-nitrogen (LN) trees and the amount of N measured in the leaves and axes of HN and LN trees grown in 2000. All the regressions have an $r^2 > 0.95$ ($P < 0.001$).

Discussion

Tree growth and N remobilization

The lack of an effect of N treatment on tree growth during the first year of the experiment was probably a result of applying treatments from July to October. It is likely that, during this period, most of the extra N taken up by HN trees compared with

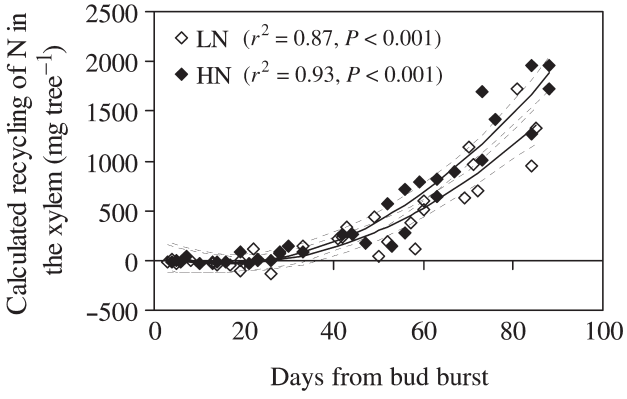

Figure 5. Recycling of N in the xylem (calculated as the difference between the cumulative flux of N through the xylem and the amount of N measured in the new leaves and axes) as a function of days from bud burst during 2000. High-nitrogen (HN) and low-nitrogen (LN) treatments refer to the amount of N received in 1999. Dotted lines indicate the 95% confidence limits.

LN trees was partitioned to the perennial organs, thereby not affecting the current-year growth (Tagliavini et al. 1998, Quartieri et al. 2002).

Fertilization increases the amount of N stored, but does not affect the efficiency with which stored N is subsequently remobilized for spring growth (Millard 1996). Accordingly, in our study, the contrasting N treatments applied during the first year of the experiment resulted in markedly different tree N status, and consequently in different amounts of remobilized N. However, the proportion of N present in the dormant trees that was subsequently remobilized in spring was similar in both HN and LN trees.

Different tree organs can be used as storage tissues for N during winter. In evergreen trees, the main site for N storage is old needles or leaves (Fife and Nambiar 1984, Millard and Proe 1992), whereas in deciduous trees, N is typically stored in woody roots and old stems (Munoz et al. 1993, Millard et al. 1998, Frak et al. 2002). In the present study, the role of roots as a storage site for N in *P. avium* appeared to be more important than that of the stem, irrespective of N treatment. Furthermore, taproots appeared to be the predominant storage site for the extra N that HN trees took up in late summer 1999.

The initial decrease in biomass of taproots (Figure 1), and partly of the stem, indicates that a significant flow of carbon compounds occurred from perennial organs to the developing leaves and axes, and possibly to new roots, during the period of intense N remobilization. However, quantitative differences in the translocation of carbon compounds and N were observed. For example, taproots of HN trees, where most N for remobilization was stored, showed a 55% decrease in total N during the first 50 days after bud burst (i.e., when 90% of N remobilization occurred). This decrease was accompanied by a ~35% decrease in biomass and a ~20% decrease in N concentration. A similar pattern was observed for LN trees, suggesting that the flow of nitrogen from the perennial to the developing organs during spring was, in relative terms, quantitatively more important than the concomitant flow of carbon compounds.

Remobilization and recycling of N in the xylem

Several studies of herbaceous species have demonstrated that an important fraction, and in many cases the majority, of N in xylem sap is represented by N recycled from shoots to roots and then reloaded in the xylem (Simpson et al. 1982, Cooper and Clarkson 1989, Jeschke and Pate 1991, 1992, Jeschke and Hartung 2000). The occurrence of this cycling implies that a cumulative measure of N flux through xylem should overestimate the amount of N effectively recovered in the new biomass.

We found close agreement in both HN and LN trees between calculated cumulative N flux through the xylem and N measured in the new aboveground growth (leaves and axes) during the remobilization period (i.e., in the first 35–50 days after bud burst, Figure 4). This suggests that no shoot-to-root recycling and reloading of N into xylem occurred during this period. Because the direction of transport in the phloem is from source to sink (Pate 1975), our results are consistent with the concept that roots are sources and not sinks for N during this period. In contrast, the overestimation of cumulative N flux through xylem compared with the amount of N recovered in new aboveground growth provides indirect evidence that recycling of N occurred after the remobilization period. At the same time, the roots started to show a progressive increase in N concentration (data not shown), indicating that they became a sink for N in this period.

The amount of N involved in xylem–phloem recycling appeared to be greater in HN trees than in LN trees (Figure 5). Although the difference was relatively small, and could have been partly affected by small errors in calculating the flux of N in the xylem, it is consistent with the slightly higher transpiration rates (Grassi et al. 2002) and the higher xylem N concentrations observed in HN trees than in LN trees after the remobilization period (data not shown). Furthermore, these results are in agreement with previous studies in which N-replete trees had significantly higher N concentrations, in both the phloem and the xylem, than low-N status trees (Lee et al. 1992, Muller and Touraine 1992, Rodgers and Barneix 1993, Padgett and Leonard 1996, Youssefi et al. 2000).

Recycling of N in the xylem and capacity for N uptake

The processes regulating N uptake by tree roots are not fully understood. Besides external conditions like N concentration in the soil solution and root zone temperature (Gur et al. 1979), some internal tree factors, such as the availability of shoot-derived carbohydrates in the root (Malaguti et al. 2001) and the recycling of N, may act as regulatory mechanisms for N uptake. The well-documented inhibitory effect of amino acids in the root phloem tissue on N uptake (Muller and Touraine 1992, Rodgers and Barneix 1993, Padgett and Leonard 1996) led to the suggestion that the pool of amino-N cycling in the plant influences N uptake, by acting as a signal of the N status of the tree. Thus, when a large amount of N cycling occurs, greater inhibition of N uptake is expected (Cooper and Clarkson 1989, Imsande and Touraine 1994, Schenk 1996, Gessler et al. 1998, Rennenberg et al. 1998, Weber et al. 1998, Youssefi et al. 2000). Our results agree with this hypothesis, because HN trees had a greater recycling of N in the xylem and a lower N uptake than LN trees (cf. Figures 3 and 5).

In conclusion, comparison between the cumulative flux of N in the xylem and N content in the new aboveground growth allows a quantitative estimate of shoot-to-root recycling of N. Although the contribution of this process to total N flux in the xylem is negligible during the period of spring remobilization of stored N, it increases exponentially after that period. In our experiment, 3 months after bud burst about 45–50% of total N passed through xylem was apparently derived from shoot-to-root recycling. Furthermore, our results seem to confirm that the recycling of N in the xylem is inversely related to the N status of the tree, and suggest that this process is a mechanism by which plants regulate N uptake by roots.

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