FINAL REPORT

Title: Regulation of nitrate uptake by apple roots.

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Objectives:

The overall objective is to determine the physiological limitation of nitrate uptake by Fuji apple trees on M9 rootstocks in the spring and early summer. This includes:

- Measuring the influence of soil temperature on nitrate uptake by the roots
- Determining the impact of tree N status on nitrate uptake, by considering that during the period of N remobilisation from storage, xylem sap concentrations of amino acids and amides are high, causing elevated levels in the phloem sap which in turn inhibit root uptake of nitrate (see diagram 1, p 6).
- Establishing a new method for measuring the N storage capacity of apple trees by measuring the flux of amino-N translocated in their xylem sap during the period of N remobilization.
- Establishing if white fine roots (younger orders of laterals) are the main site of N uptake, by determining if inhibition of nitrate uptake in spring is correlated with the absence of living white fine roots.

Significant Findings:

- Nitrate uptake by Fuji in the spring of 2002 was not inhibited by soil temperature.
- Asparagine, Aspartic acid and Glutamine are the main N compounds, which vary in the phloem and xylem, through time and with N fertilization (both fertigation and foliar spray of urea).
- Xylem amino acid content and composition was more responsive to N than either trunk or root phloem, suggesting that the quantity and quality of N circulating in the phloem is tightly regulated at the whole plant level.
- Measurement of the flux of Asparagine, Aspartic acid and Arginine in the xylem sap from bud break to full bloom gave a good estimate of N remobilization when compared with traditional methods using ^{15}N labeled fertilizer.
- Neither the concentration nor flux of N in the xylem and phloem affected nitrate uptake, although direct phloem loading of amino acids did result in slower uptake.
- Manipulating the amount of N allocated to storage in the fall also affected the number of buds that were set and so the growth potential for the following year, thereby contributing to the tight regulation of N circulation in the phloem.

Methods employed*:*

Field Experiment

An experimental orchard was successfully planted in May 2001, at PARC, Summerland. A total of 90 Fuji trees grafted onto M9 rootstocks were planted and drip irrigation lines laid to provide two drip emitters per tree. As soon as the irrigation was applied the three nitrogen treatments for 2001 were applied. These comprised irrigation with 10, 50, or 90 ppm calcium nitrate. These treatments were applied to five replicate trees for each of four planned harvests next year (80 trees in total). Fertigation was applied between 5th June and 28th August and the drip emitters were monitored twice a week to ensure that the target concentrations of N were being maintained. The fertigation resulted in the trees receiving 2.7, 13.3 and 23.6g N tree^{-1}for the three N treatments, respectively, in a total volume of 263 litres of water tree^{-1}. The total irrigation applied to the trees between 18th May and 5th October was 434 litres tree^{-1}. At the end of the season tree growth was monitored by measuring the length of the shoot extension growth, stem diameter of each tree and no effects of the N treatments were found. In the spring of 2002, as soon as the irrigation was turned on (just after bud break) foliar sprays of urea were applied to half of the trees that received the 50ppm N treatment in 2001. The sprays were designed to provide these trees with an additional 10g N to augment the 13.3g they already received in 2001. The purpose of this treatment was to enhance their N status independently of the amount of N available for remobilization from storage.

Measurement of temperature effects on root N uptake

Nitrogen remobilization by apple in the spring occurs over a period of 25-30 days following bud burst, up to the time of full bloom, with uptake of root supplied N being low for up to 10 weeks. During this period in spring 2002 a series of four N uptake experiments were conducted. On each occasion a portion of the root system of each tree was sampled. The isolated roots were inserted in a solution of calcium nitrate, at either ambient temperature, or warmed to 5^{o}C above ambient. The uptake of N from each container was measured by depletion.

Measurement of N translocation in xylem and phloem sap

Samples of phloem exudates were collected from the roots branches and trunk. Bark pieces were lifted off and incubated in EDTA, to induce the exudation and collection of phloem amino acids.. The amino acid composition of the phloem sap was be measured by HPLC after purification by ion exchange chromatography. Samples of xylem sap were also collected from cut branches and analysed by GC-MS in order to determine which amino acids were translocated in the xylem as a consequence of remobilization. The xylem sap flux was measured using sap flow gauges fitted to the trees.

Greenhouse Experiment

Seventy-two Fuji on M9 rootstock were planted in sand culture in early June 2002. The trees were laid out in a randomized block design, with 2 groups (two sets of treatments, see below) x 4 replicate blocks. During the second week of September, the trees were moved to a growth chamber in order to simulate fall conditions and induce dormancy by

a decrease in temperature and light levels. Two treatments were applied to the trees. In the first (High N) all the leaves were allowed to senesce normally, thereby allowing N to be withdrawn into storage pools, which were further augmented by continuing to apply N with the irrigation in the fall. For the second (low N) treatment the trees had 75% of their leaves removed before senescence started and received no further N with the irrigation in the fall. The aim of these treatments was to produce trees with contrasting amounts of N allocated to storage, without having affected their growth or development during the following growing season. During the second week of November the trees were transferred to a cold room and kept in the dark at 4ºC. The trees were chilled for 6 weeks and returned to the greenhouse in the third week of December, when the temperature and light regime was set to mimic spring conditions. All trees had their buds counted before returning to the greenhouse.

A total of 7 harvests were taken, the first when the trees were still dormant, the second 2 weeks after the start of budbreak and thereafter twice weekly. On two harvest dates, the bark of a set of Low N trees was peeled off and put in contact with an EDTA solution containing (Loaded) or not (Control) amino acids (100mM Asp and Gln). This treatment allowed the amino acids circulating in the phloem / xylem sap to be manipulated independently of any increase in sap N resulting from the remobilisation of stored N (see diagram 1, p 10). Two days prior harvest, the trees had their buds, new shoots and leaves counted, and were watered abundantly, to leach any residual N. They were then supplied with a solution of calcium nitrate, highly enriched with ^{15}N (from 98 to 21 atom %), given at regular interval for 45 hr, and had their sand leached again to stop any further uptake of labeled N. Each tree was weighed immediately before the first addition of labeled N and before final leaching, to determine the amount of water transpired during the period of labeling. At harvest, xylem sap was collected using a pressure bomb from all branches and pooled. Bark pieces were lifted off the rootstock and incubated with EDTA, to induce the exudation and collection of phloem amino acids. Xylem and phloem sap samples were kept frozen until analysis by GC-MS of amino N concentration and ^{15}N enrichment. The root system of each tree was sub sampled to assess: (a) free amino N in the fine roots, (b) root viability and (c) root length of fine versus coarse roots. Free amino acids were extracted from the fine roots using the method described by Schneider et al. (1996) and analyzed by HPLC. Root viability was assessed using TTC by quantification of its reduced form (Formazan) after an ethanol extraction. To measure root length, a subsample of the root system was laid-out and scanned immediately after harvest. Scanned images were then digitalized, and the total root length assessed on each sample separating out different classes of thickness.

Results and Discussion

Field Experiment

The growth pattern of both spur and shoot leaves in the spring of 2002 is shown in Fig 1a for the treatment receiving 50 ppm nitrate-N. Spur leaves had finished expanding by about 138 JD, by which time uptake of labelled N from the fertigation had provided about 10% of their N (Fig 1b), the remaining 90% being derived from remobilisation. Shoot leaves continued expanding until the end of the sampling period (165 JD), and relied on root uptake for about 30% of their N. Slow rates of N uptake by the roots were evident as early as 127 JD. The soil temperature at 10 cm depth throughout the experiment is shown in Fig 1c. The temperature below which nitrate uptake by apple roots is inhibited is in the range 5-10^{0}C (Bhat, 1982). During the experiment there was only a 5-day period (between 128-132 JD) when the minimum daily soil temperature fell below 10^{0}C, while the maximum daily temperature was greater than 10^{0}C throughout. It is unlikely, therefore, that nitrate uptake by roots was inhibited by low soil temperatures.

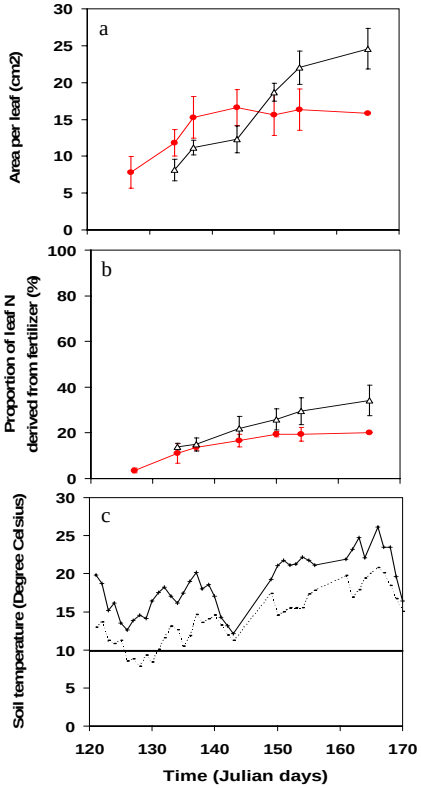

Fig 1: Area (a) and N content (b) of spur (●) and shoot (△) leaves from trees fertilized with 50 ppm N.
Soil temperature (c), maximum (plain line) and minimum (dotted line) collected at 10 cm depth.

The root incubation experiments also showed that temperature was not limiting root uptake of nitrate (Table 1). At four separate dates roots from the trees receiving either 10 ppm or 90 ppm nitrate-N were isolated from gel/soil medium they had grown into and incubated for 4 hours in a solution of calcium nitrate which was either cooled to 12°C or warmed to 25°C. Uptake of nitrate was measured by depletion from the solution at the ends of the incubation period. The data were variable, but demonstrated that at pink and petal fall uptake of nitrate was not inhibited by root temperature of 12°C compared to 25°C.

JD	Growth stage	Treatments			
		10 ppm		90 ppm	
		12 ^{o}C	25^{o}C	12^{o}C	25^{o}C
121	Pink	-	84 ± 74 (2)	59 ± 21 (3)	24 ± 3 (2)
149	Petal Fall	130 ± 57 (3)	130 ± 57 (3)	238 ± 122 (4)	231 ± 10 (2)

Table 1. The effects of growth stage, N supply and root temperature on N uptake. Values are expressed as μg N g^{-1} root dry matter hour^{-1} and are the mean and standard deviation (and the number of replicates). In each case there were less than the 5 replicates used, as there was no N depletion measured from some of the solutions.

A possible physiological limitation to N uptake in the spring could be the flux of amino acids arriving in the roots via the phloem. These could be elevated due to the high xylem fluxes during N remobilization. To assess these fluxes the xylem and phloem sap of trees were collected and their amino acid composition measured and expressed as a flux. Fig 2 shows the flux of N in the xylem which, during remobilization, was unaffected by N supply. However, after remobilization had finished the flux was due to root uptake and was affected significantly by N supply. The foliar urea sprays applied to the trees following bud burst increased the xylem flux of N once remobilization had finished, probably as a consequence of a shoot to root to shoot cycling of N in the trees.

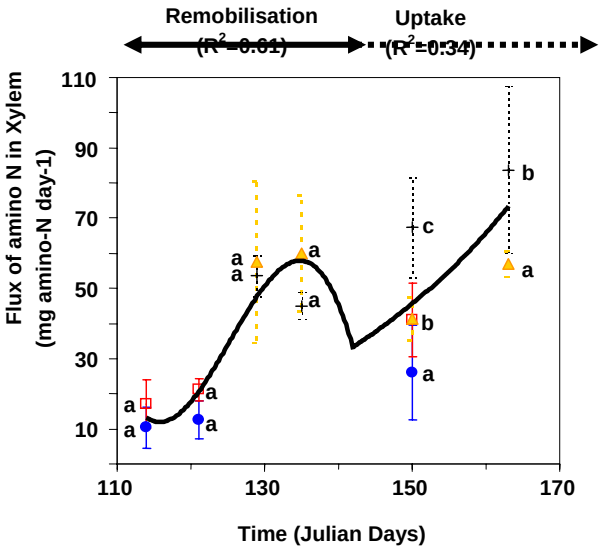

Fig. 2: Time-course of the flux of amino-N in the xylem. Data are means of 4 –5 replicates for trees fertigated with 10 (●), 90 (□), or 50 ppm –N with (+) or without (▲) foliar-urea spray. Vertical bars represent standard deviation of means. Curves fitting used knowledge of amino acid translocation in xylem from Fig 3.

Over 90% of N in the xylem sap occurred in only three amino compounds (Table 2), with asparagine being predominant. In contrast, phloem sap from the trunk contained significant amounts of threonine (compared with the xylem) and little aspartic acid, but the predominant compound was again asparagine. In the root phloem over 80% of the N was in asparagine (Table 2). Having established the main amino compounds in the xylem and phloem, their flux was studied. Table 3 shows that there were significant changes through time in the flux of N in the xylem (particularly as asparagine) as a consequence of remobilization, which also affected the phloem sap in the trunk. N supply (both as fertigation and foliar urea sprays) increased the flux of N in the xylem. There was also evidence of a trend for an increase in the concentration of asparagine in root phloem when 50 or 90 ppm nitrate-N was applied compared with the 10 ppm treatment (Table 2). These data are preliminary and further analyses are required. However, they do suggest that changes in the xylem sap flux of N due to remobilization and/or N supply also result in changes to phloem translocation of N, particularly asparagine.

Table 2: Composition of the three types of sap sampled at ½-inch green (JD 114), expressed as the percentage of total mass of amino N recovered. Note that only amino acids containing more than 2% of total amino N are included in the table. Data are means of 4 replicates. Means with different letter within a row are different at P<0.05. Level of significance of differences between sap type are given at P<0.05 (*), P<0.01 (**) or P<0.001 (***). Amino acids accounting for more than 10 % of sap amino N are highlighted in bold.

Amino acid	**Sap type**			Significance
	Phloem		**Xylem**	
	Root	Trunk	Branch	
Alanine	3.2 a	1.2 b	0.2 b	**
Asparagine	**81.4 a**	**33.5 b**	**43.7 c**	***
Aspartic acid	2.6 a	2.4 a	**21.2 b**	**
GABA	1.1 a	3.8 b	0.7 a	**
Glutamic acid	1.9 a	4.1 b	0.9 a	**
Glutamine	1.1 a	**31.2 b**	**27.6 b**	**
Proline	1.2 a	3.2 b	0.3 a	***
Serine	1.6 a	5.4 b	0.5 a	**
Threonine	3.6 a	**11.3 b**	3.8 a	*

Table 3: Responses to N treatments and time of amino-N in xylem and phloem saps. Significance of the changes through time or due to N treatments are given at P<0.001 (***), P<0.01 (**), P<0.05 (*), P<0.1 (~) or P>0.1 (ns). Percentages indicate he proportion of the change in amino-N that was accounted for by each responsive amino acid

	Flux of N in Xylem (branch)	Concentration of N in Phloem (Roots)	Flux of N in Phloem (Trunk)
Change through time	*** Asn (70%) Asp (15%) Gln (<5%)	~ Asn (98%)	* Gln (40%) Asn (30%) Thr (15%)
Change due to Fertigation treatments	*** Asn (55-65%) Gln (15-25%)	~ Asn (90%)	ns
Change due to application of foliar-urea	** Asn (60%) Gln (30%)	ns	ns

Measurement of remobilization by sap N flux

Previously, we examined how compounds which are translocated during remobilization might allow the flux of N to be calculated, by measuring the xylem sap flow and the concentration of the relevant compounds (Neilsen et al., WTFRC report 2001). We can now report that further analysis of these data has confirmed this hypothesis (Guak et al., 2003). Analysis of xylem sap collected during the period of remobilization in an experiment similar to the current one showed that there were large quantitative and qualitative differences in the composition of the xylem sap through time. During remobilization Asparagine, Aspartic acid, Arginine and Glutamine accounted for 28%, 19%, 28% and 19% of the total N recovered in the saps, and that after remobilization had finished these values changed to 25%, 36%, 29% and 5%, respectively. The flux rate for N in each of the four main amino acid and amides in the xylem for each sampling period was calculated. Values of the sap flux rate for each sampling time were obtained from the regression made between daily sap flux and time. The calculated flux of N in the xylem as Asparagine, Aspartic acid and Glutamine peaked around full bloom at JD 130 (Fig. 3). Thereafter their flux rate declined. In contrast, the flux rate of Arginine peaked at full bloom, decreased until fruit set (JD 158) but then increased again to give a value at JD 206 that was three times the flux found at full bloom.

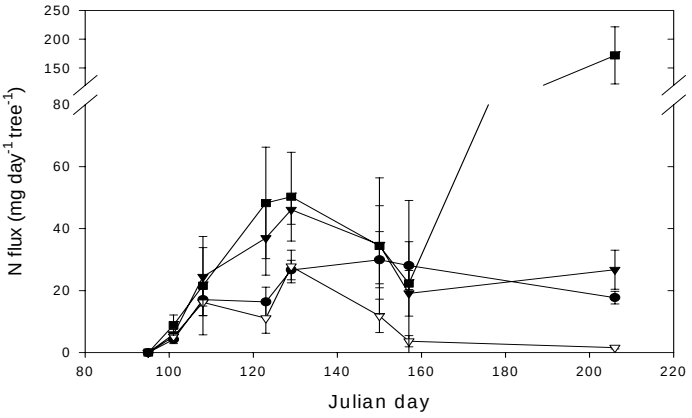

Figure 3. Time course for the flux of N in the xylem of *M. domestica* trees. Values are for Asp (●), Gln (▽), Asn (▼)and Arg (■) and are means and standard errors of 12 replicates.

The amount of N translocated in the xylem as a consequence of remobilization was calculated by fitting curves to the data for the flux of N in each of the amino acids and

amides in Fig 3. The area under the curves for Aspartic acid, Asparagine and Glutamine up to JD 158 were then integrated and summed. Integration up to JD 158 was chosen because by then the recovery of unlabelled N in the new growth above–ground had shown that N remobilization had finished. Figure 4 shows a comparison of the amount of N remobilized up to JD 158, as measured by the recovery of unlabelled N in new above – ground growth (data not shown), with the amount calculated by the flux of N in Aspartic acid, Asparagine and Glutamine, with and without Arginine, in the xylem for the same period. The slope of the two regression lines showed that sap amino-N flux including Arginine overestimated remobilization (slope = 1.54), compared with the flux without Arginine (slope = 1.01). These data, therefore, confirm that Arginine is unlikely to be involved in N remobilization and so the flux probably represents root uptake of N.

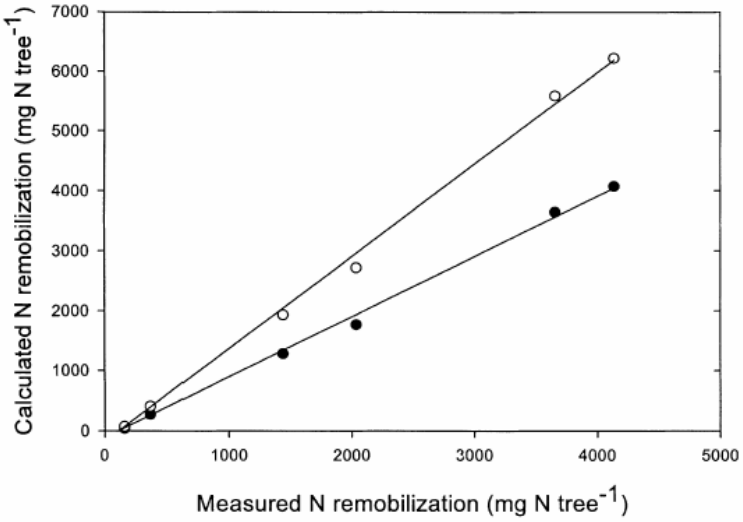

Figure 4. Comparison of the amount of N remobilized by apple trees measured by the recovery of unlabelled N in their new growth during the remobilization period (JD 96 to JD 158), with the amount calculated by the flux of Asp, Gln and Asn (filled circles) or Asp, Gln,Asn and Arg (open circles) in their xylem sap.

This is the first time that such a method has been attempted for field-grown trees. N remobilization is a source-driven process, and so all N allocated to storage in apple trees in the autumn is remobilized the following spring. Therefore calculation of the flux of remobilized N should allow the N storage capacity of a tree to be determined. This could be used to determine the impact of management on the ability of trees to store N. Even if the results are only relative, the method should allow a good comparison between trees from different treatments and offer significant advantages over the traditional methods using isotopes and destructive tree harvests.

Greenhouse experiment

During the period of N remobilization in the spring the concentration of amino compounds in both the xylem and the phloem increases, as shown by the data above. To determine if theses fluxes inhibit root uptake they were manipulated in two ways. First, by manipulating leaf senescence and N supply in the fall to produce Low N and High N trees that differed in their allocation of N to storage, and secondly by direct loading of amino acids into the phloem. Manipulation of the amount of N stored affected the concentration of N in both the xylem and the phloem the following spring (Fig 5). The pattern of xylem N concentration was similar to those found in previous studies and clearly established that remobilization lasted for 35 days following budbreak (Fig 5a). The time-course of amino N concentration in the phloem closely followed that measured in the xylem, the highest

concentrations being found during the period of remobilisation (Fig 5b). Because remobilization occurs before rapid root uptake of N (Fig 3), the N content of the leaves just after remobilization had finished gave an indication of the total amount of N remobilized (Fig 5c). Significantly more N was remobilized by High N compared with Low N trees, showing that the treatment to manipulate the N storage capacity of the trees had been effective. However, the data from the ^{15}N uptake experiments showed that throughout the study there were no significant differences in N uptake per tree, between High N and Low N trees (Fig 6). This suggests that at the whole tree level uptake was unaffected by the magnitude of the concentration of N in the xylem and phloem.

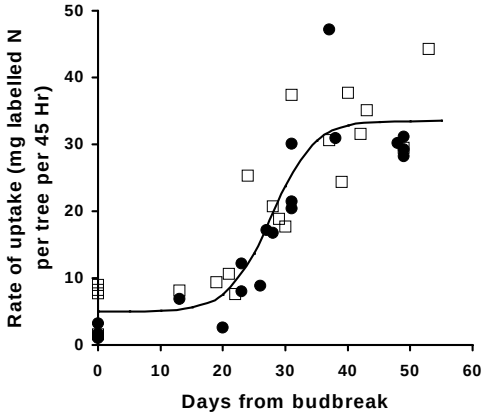

Figure 6: Time course of the rate of nitrate uptake by whole tree measured on high N (●) and Low N (□) trees.

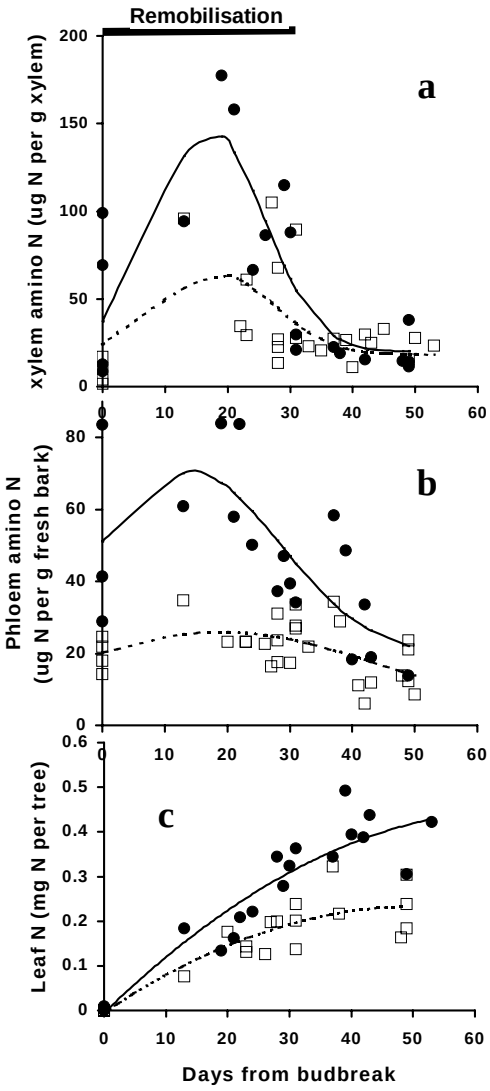

Fig. 5: Time course of amino N concentration in xylem (**a**) and phloem (**b**) saps, and N content of leaves (**c**) collected from collected from High N (●) and low N (□) trees.

However, the Low N treatment affected more than the amount of N allocated to storage in the fall.

Table 3: The effect of the manipulation of N storage on tree morphology in the following spring after the end of N remobilisation.

	Treatment		LSD (P<0.05)	Treatment effect
	Low N	High N		
Number of buds set (tree-1)	133	149	21	P=0.12
Number of Shoots (tree-1)	38	57	23	P=0.10
Number of leaves (tree-1)	174	269	77	P=0.02
Leaf area (cm2 per leaf)	16	17	5	P=0.75
number of leaves per shoot (shoot-1)	4.8	4.8	1	P=0.96

The High N trees tended to set more buds in the fall, so that more buds broke in the spring (Table 3). This resulted in more shoots in the High N compared with Low N trees, but with a similar number of leaves per shoot and the same area per leaf (Table 3). As a consequence, the rate of N uptake per unit leaf area depended on tree N storage status. High N tree had a significantly (P=0.03) slower rate compared to that of the Low N trees. Furthermore, rate of uptake per unit leaf area remained similar during and after the period of n remobilisation for High N trees, whereas it increased through time for Low N trees, being higher after than during remobilization. This could be due to the onset of new root growth concurrent with remobilization finishing and the fact that (1) the majority of N uptake is by new roots and / or (2) the actual concentration of amino N in the fine roots decrease when root growth starts. This is being assessed at present, by analyzing the root system of the harvested trees to establish new root growth patterns, together with their viability and their amino N content. This data is not yet available.

Direct loading of amino acids into trunk vessels was carried out twice, during and after remobilization. The loading significantly increased the concentration of N in the xylem on both occasions but affected that of the phloem marginally only during the period of remobilisation (Table 4). On both occasions the phloem loading tended to slow the rate of N uptake by the trees. This effect was more pronounced during the period of remobilisation, when both xylem and phloem N concentrations were affected (Table 4). To assess the impact of phloem loading on the sites of N uptake, i.e. the (fine) roots, it will be necessary to analyze the amino acid content of the fine roots and these data are not yet available.

Table 4: The effect of amino acid loading into trunk vessels on the concentration of amino N circulating in the xylem and the phloem, and on tree uptake capacity**.**

Timing of remobilisation	During		After		LSD (P<0.05)	Loading effect
Treatment	Control	Loaded	Control	Loaded		
Xylem amino N (ug N g-1 xylem)	146	484	38	285	352	P=0.03
Phloem amino N (ug N g-1 fresh bark)	26	33	11	12	6	P=0.07
Rate of uptake (mg labelled N tree-1 per 45 hr)	37	21	36	29	18	P=0.08

Significance to the industry

Apple trees are often not responsive to the levels of N supplied to many orchards in the Pacific Northwest. This has been demonstrated in a series of experiments at PARC, over a number of years. One of the main reasons for this is that at planting the trees are replete with N from the nursery, and subsequently store and internally cycle N for their growth each year. While orchards will require some N fertilizer, understanding both the processes of N storage and remobilization, and any physiological limitation to nitrate uptake will enable precise advice about the timing of applications to ensure that N applications are used with the greatest efficiency and avoid costly wastage of fertilizer due to applications when the trees are unable to take up the N. The results from this project have shown the futility of applying N fertilizers in the spring and highlighted the opportunities for manipulating both the N status of the trees and influencing the development of buds by manipulating the N status of the tree in the fall.

Overall Budget:

Project title: Regulation of nitrate uptake by apple roots.

PI: Peter Millard

Project duration: 2001-2003

Project Total: $ 38,000*

	Year 1 (2001)	Year 2 (2002)	Year 3 (2003)
Post-doctoral fellow (April 2002-Mar 2003)		15,000	5000
Plot establishment [1]	5,000		
Supplies [2]	5,000	5,000	3000
Sample analysis [3]	10,000	20,000	8,000
Total	10,000*	20,000*	8,000*

[1] Includes the cost of the trees, irrigation equipment and other monitoring equipment.

[2] Includes the cost of ^{15}N labeled fertilizer, nutrient solutions and laboratory chemicals for the extraction and purification of amino acids in xylem and phloem saps and extracts of root tips

[3] Includes the cost of ^{15}N analyses by IRMS, HPLC analysis of xylem and phloem saps and root extracts, GC-MS analyses of labeled xylem and phloem saps and video analyses of roots.

* Note that other costs each year have been borne by the M. I. I. funds that have been available for this work, and the TOTAL costing given above refer to the financial contribution made by the Commission.

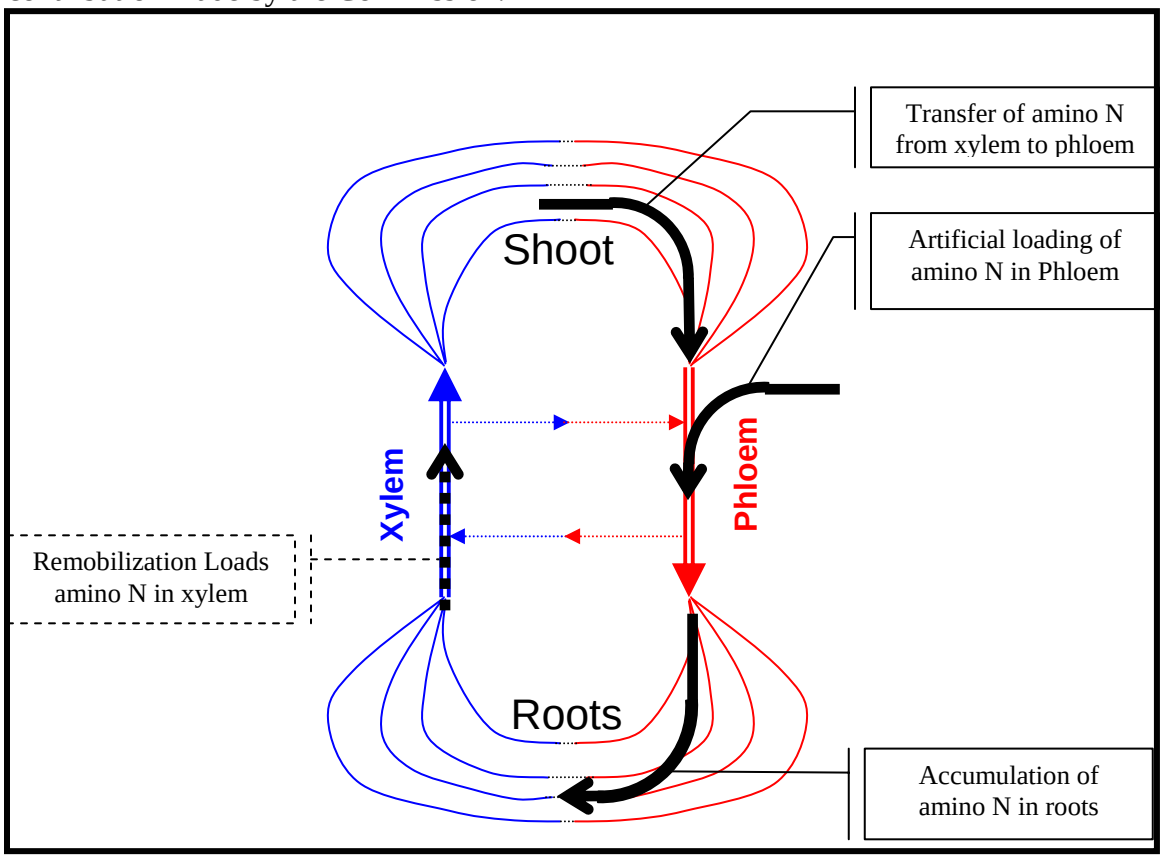

Diagram 1: Schematic representation of the circulation of amino N in the vascular system (xylem and phloem).