



RESEARCH PAPER

Uptake, metabolism and distribution of organic and inorganic nitrogen sources by *Pinus sylvestris*

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Abstract

Although an increasing number of studies show that many plant species have the capacity to take up amino acids from exogenous sources, the importance of such uptake for plant nitrogen nutrition is largely unknown. Moreover, little is known regarding metabolism and distribution of amino acid-N following uptake or of the regulation of these processes in response to plant nitrogen status. Here results are presented from a study following uptake, metabolism, and distribution of nitrogen from NO_3^- , NH_4^+ , Glu, or Ala in Scots pine (*Pinus sylvestris* L). In a parallel experiment, Ala uptake, processing, and shoot allocation were also monitored following a range of pretreatments intended to alter plant C- and N-status. Uptake data, metabolite profiles, N fluxes through metabolite pools and tissues, as well as alanine aminotransferase activity are presented. The results show that uptake of the organic N sources was equal to or larger than NH_4^+ uptake, while NO_3^- uptake was comparatively low. Down-regulation of Ala uptake in response to pretreatments with NH_4NO_3 or methionine sulphoximine (MSX) indicates similarities between amino acid and inorganic N uptake regulation. N derived from amino acid uptake exhibited a rapid flux through the amino acid pool following uptake. Relative shoot allocation of amino acid-N was equal to that of NH_4^+ , but smaller than for NO_3^- . Increased N status as well as MSX treatment significantly increased relative shoot allocation of Ala-N suggesting that NH_4^+ may have a role in the regulation of shoot allocation of amino acid-N.

Key words: Alanine, amino acid uptake, glutamic acid, regulation, transport.

Introduction

As a number of studies have shown that a wide array of plant species have the capacity to take up organic nitrogen, there is an emerging need to learn more about the fate of organic N compounds following uptake. In addition, the effects of N uptake from various N sources on metabolite fluxes and levels as well as the regulatory networks behind N uptake, metabolism, and distribution need to be further investigated. To date, efforts to elucidate the processes of N assimilation, metabolism, and distribution and their regulation have almost exclusively concerned inorganic N (Donaldson Knoepp *et al.*, 1993; Stitt *et al.*, 2002; Foyer *et al.*, 2003), even though some studies have included data on metabolism following uptake of amino acids (Tuin and Shelp, 1994; Schmidt and Stewart, 1999; Thornton, 2001).

Regardless of the apparent ubiquity of amino acid uptake among different plant species (Schmidt and Stewart, 1999; Persson and Näsholm, 2001), the actual importance of such an uptake for plant N nutrition is still uncertain. Several studies have shown that while plants actually may take up amino acids at relatively high rates, growth on these N compounds, when supplied as the only N source, may be restricted (Andersson *et al.*, 1997; Plassard *et al.*, 2000). In this perspective, it is of large interest to investigate potential bottlenecks for plant growth performance on organic N sources. Moreover, such results further illustrate the need for studies of metabolism and relative distribution following uptake of various N sources.

Uptake and assimilation of NO_3^- and NH_4^+ is under strict control by a complex network of signals originating from C- and N-metabolism as well as from plant hormones (Coruzzi and Zhou, 2001). In effect, uptake of NO_3^- and

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NH_4^+ is transcriptionally as well as post-transcriptionally regulated by the respective substrate. Moreover, expression of NO_3^- and NH_4^+ transporters is under the control of downstream metabolites such as Gln and NO_3^- transporters have also been found to be regulated by light, most likely mediated by sucrose or glucose (Glass *et al.*, 2002). In addition to transporters, central enzymes in N assimilation, for example, nitrate reductase (NR, EC 1.6.6.1), Gln synthetase (GS, EC 6.3.1.2), Glu synthase (GOGAT, EC 1.4.1.14, 1.4.7.1) as well as phosphoenolpyruvate carboxylase (PEPc; EC 4.1.1.49) have been found to be regulated by NO_3^- and various signals from downstream nitrogen and carbon metabolism (Foyer *et al.*, 2003).

The few available investigations of amino acid uptake regulation have suggested that there may be important similarities between the control of NH_4^+ , NO_3^- , and amino acid uptake. Accordingly, the latter may stand under the control of substrate access and plant N status, mediated by endogenous levels of NH_4^+ , as well as sucrose and/or glucose (Persson and Näsholm, 2002, 2003). These regulations are in line with amino acid uptake as a potentially important contributor to plant N nutrition and raise the question whether the metabolism and distribution of acquired amino acids are also regulated in response to plant C- and N-status. Due to the central and multiple roles of many amino acids, regulation of their metabolism can be expected to occur at multiple levels and in response to a plethora of factors. In order to understand the importance of organic N for plant nitrogen nutrition, it is imperative to learn more about the metabolic fate of these compounds following uptake.

In order to examine metabolism and distribution of various inorganic and organic N sources following root uptake, a study tracing labelled N from exogenously added NO_3^- , NH_4^+ , Ala or Glu in roots and needles of non-mycorrhizal Scots pine plants (*Pinus sylvestris* L.) was conducted. In order to analyse possible regulations of metabolism and distribution in response to plant C- and N-status, the uptake and fate of N from Ala was monitored following a range of pretreatments. Thus, C-status of the plants was altered by dark treatment and/or supply

of sucrose or 2-oxoglutarate, while N-status was altered by increased N supply or treatment with the glutamine synthetase (GS) inhibitor methionine sulphoximine (MSX). N label from added compounds was traced in roots and needles by GC-MS and IRMS. In parallel, N- and C-status of the plants was monitored by analyses of adenylate compounds, sugars, organic- and amino acids and by activity assays of alanine aminotransferase (AIAT).

The choice of Ala and Glu as model substrates was based on their relatively high content in soil solution (Yu *et al.*, 2002) and their potentially large role in transfer of N to plants from mycorrhizal fungi (Chalot and Brun, 1998).

Materials and methods

Plant material

Scots pine (*Pinus sylvestris* L.) seeds, obtained from seed orchard 410 (63°15' N), Robertsfors, Sweden, were surface-sterilized in 15% H_2O_2 for 15 min, rinsed in sterile water, and planted in 0.5 dm³ pots containing autoclaved quartz sand. Plants were grown in a greenhouse for 151 d under a 16/8 h light/dark cycle at 200–280 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The substrate was kept continuously moist and from day 21 to 137, plants were fertilized twice a week using the commercial fertilizer Superba STM (Hydro Agri, Landskrona, Sweden) at an N concentration of 5 mM (for fertilizer composition see Persson and Näsholm, 2002). From day 137 up to day 151, plants were fertilized with an N-free solution in all other respects composed as the Superba STM fertilizer provided earlier. At day 152, plants were harvested. At harvest, roots were carefully cleaned from sand, rinsed in 0.5 mM CaCl_2 followed by sterile water and then immediately transferred to their respective pretreatment solutions. All plants were non-mycorrhizal.

Pretreatments and incubations

Following harvest, the experiment consisted of two distinct portions: pretreatments aimed to alter plant C- and N-status followed by incubations in labelled N compounds. All pretreatments were performed for 36 h and are shown in Table 1. In addition to the compounds specific to some pretreatments, all solutions contained the same N-free fertilizer (referred to as 'base' in Table 1) as used in the last stage of plant growth, with all other nutrients complementary to an N concentration of 10 mM. Following pretreatments, roots were carefully rinsed in 0.5 mM CaCl_2 followed by sterile water and then immediately transferred to the experimental incubations. During pretreatments, as well as during experimental incubations, solutions were intensely bubbled with air. All pretreatments and incubations

Table 1. Incubations, pretreatments, and abbreviations used in this study

Treatment	Pretreatment	Incubation	Abbreviation
N-starved	Base	Base	No-N
NO_3^-	Base	$^{15}\text{NO}_3^-$	NO_3^-
NH_4^+	Base	$^{15}\text{NH}_4^+$	NH_4^+
Glutamic acid	Base	^{15}N -Glu	Glu
Control	Base	^{15}N -Ala	C
Light	Base, 36 h darkness	^{15}N -Ala	-L
Light/sucrose	Base, 36 h darkness, 3 mM sucrose	^{15}N -Ala	-LS
Sucrose	Base, 3 mM sucrose	^{15}N -Ala	S
2-oxoglutaric acid	Base, 1 mM 2-oxoglutaric acid	^{15}N -Ala	2-oxo
+N	Base, 1.5 mM NH_4NO_3 (3 mM N)	^{15}N -Ala	+N
MSX	Base, 1 mM methionine sulphoximine	^{15}N -Ala	MSX

were performed at pH 5.0 in 20 °C under an illumination of 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$, except for the two light-deprivation pretreatments which were performed in complete darkness. Experimental incubations lasted for 3 h and, apart from the N-free fertilizer (base), contained either of the ^{15}N -labelled compounds Ala, Glu, NH_4^+ , or NO_3^- at a concentration of 3 mM. Labelled compounds were obtained from the Cambridge Isotope Laboratories, Inc., MA, USA.

Subsequent to incubations, all plant roots were immediately washed once in a 5 mM exchange solution containing the unlabelled variety of the compound in which they had been incubated (Ala, Glu, NH_4^+ or NO_3^-) and then three times in 0.5 mM CaCl_2 , followed by three times in sterile water. Before storage, plants were carefully patted dry. Roots and shoots were separated and immediately frozen in liquid nitrogen and transferred to a -80 °C freezer for storage until analysed. All experiments were performed in triplicates ($n=3$) using a single plant for each replicate. Passive amino acid uptake was tested and found negligible in a prior study using the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, Persson and Näsholm, 2002).

Sample preparation and analyses

Prior to analyses, roots and shoots were separated and needles were removed from stems. All plant material was then (without thawing) freeze-dried under reduced pressure. When dry, samples were milled in liquid N using a ball mill, and subsequently freeze-dried again. The resulting dry, fine powder was used for all subsequent analyses.

Total ^{15}N was analysed in roots and shoots by continuous flow isotope ratio mass spectrometry (CF-IRMS) using an Europa Scientific, Europa 20–20 stable isotope analyser. Label in individual amino acids was measured using GC-MS as in Persson and Näsholm (2001). In short, amino acids were extracted from some 20–100 mg dried and milled plant material by shaking in 1.5 ml ice-cold sterile water for 20 min. 500 μl of the extract was purified by solid phase extraction using Alltech strong cation exchange (SCX) cartridges. The resulting extracts were evaporated to dryness under reduced pressure and redissolved in 40 μl dimethyl formamide (DMF) containing the internal standards γ -aminoisobutyric acid and hydroxy-L-proline. Amino acids in samples were derivatized to their *tert*-butyldimethylsilyl (tBDMS) derivatives using 10 μl *N*-methyl-*N*-*tert*-butyldimethylsilyl-trifluoroacetamide (MTBSTFA) as derivatization agent. Samples were heated at 70 °C for 20 min and allowed to cool to room temperature before GC-MS analyses. Derivatization agents were purchased by Pierce Inc., Rockford, IL, USA.

Sugars and organic acids were analysed using GC-MS. Samples were prepared from the same water extracts obtained in the amino acid analyses. 100 μl of these extracts was immediately evaporated to dryness and redissolved in 50 μl pyridine, in which the internal standard phenyl- β -D-glucoside was dissolved. In order to methoximate samples, they were once again evaporated to dryness and redissolved in 80 μl of 30 mg ml^{-1} methoxyamine in pyridine. Tubes were capped and samples were heated for 1 h at 37 °C. Sugars and organic acids were then derivatized to their trimethylsilyl (TMS) derivatives by the addition of 20 μl *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) and heating at 70 °C for 30 min. Samples were allowed to cool to room temperature prior to GC-MS analysis. ATP and ADP analyses were performed as in Gardeström and Wigge (1988).

Alanine aminotransferase (AlAT; EC 2.6.1.2) activity was assayed after extraction of 20–100 mg of freeze-dried plant material in 1 ml buffer containing 100 mM TRIS-HCl (pH 7.2), 0.5 mM EDTA, and 10 μM dithiothreitol (DTT). DTT was added from a fresh solution before each extraction. Extractions were performed at 4 °C for 5 min and followed by centrifugation at 22 000 g at 1 °C for 5 min. Enzyme activity was determined using 100 μl of the resulting supernatant in 900 μl of assay buffer. The final composition of the assay buffer (after

addition of extract) was: 100 mM TRIS-HCl (pH 7.2), 150 μM pyridoxal-5-phosphate, 50 mM Ala, 25 mM 2-oxoglutarate, 100 μM NADH, and 204 nkat lactate dehydrogenase (LDH). The reaction was started by addition of Ala and followed by monitoring the consumption of NADH by LDH after 0, 1, and 2 h at 340 nm. Control samples were run simultaneously in identical solutions where no Ala was added. All assays exhibited linearity over the measured time interval.

Uptake data, metabolite and isotopic label contents and distribution data were made subject to ANOVA, followed by Tukey's test, using StatView 5.0 statistical software.

Results

Uptake, metabolism and distribution of NO_3^- , NH_4^+ , Glu, and Ala

Uptake of the various N sources fed to plants varied significantly (Fig. 1), being highest for Glu (58.3 ± 2.5 $\mu\text{mol g}^{-1}$ DW; mean \pm SE), intermediate for Ala and NH_4^+ (33.8 ± 0.9 and 37.1 ± 0.2 $\mu\text{mol g}^{-1}$ DW, respectively; mean \pm SE) and lowest for NO_3^- (7.3 ± 0.1 $\mu\text{mol g}^{-1}$ DW, mean \pm SE). The relative distribution of labelled N to the shoot in the NO_3^- -fed plants was significantly higher (2.4%) compared with plants fed any of the other N sources (0.3%, 0.2%, and 0.1%, respectively; Fig. 1). Very low levels (close to or below the detection levels) of ^{15}N were detected in the needle amino acid pools of all plants, mostly in the Glu, Asn, and Gln pools. Only the [^{15}N]Ala-supplied plants exhibited detectable [^{15}N]Ala in needles (data not shown).

Glu-fed roots exhibited a significantly larger relative amount of label in the amino acid pool (13.6%) than did roots from the Ala-, NO_3^- -, or NH_4^+ -fed plants (6.0%, 7.0%, and 6.8%, respectively; Fig. 1). These numbers translate to a flux of 50.4 μmol of labelled N g^{-1} DW into down-stream metabolism in the Glu-fed roots, and 31.7, 6.6, and 34.5 $\mu\text{mol N g}^{-1}$ DW in the Ala-, NO_3^- -, and NH_4^+ -fed plants, respectively. Assuming an average protein amino acid weight of 130 g mol^{-1} and one N atom per amino acid, the N flux equates to a maximum protein production rate of 0.3–2.2 mg g^{-1} DW h^{-1} from exogenously acquired N. The relative distribution of label within the specific amino acid pools can be seen in Table 2. In plants exposed to Glu, NO_3^- or NH_4^+ , the bulk of the label could be found in the Gln and Glu pools, while plants fed with Ala exhibited a distinctly increased Ala/Gln label distribution ratio.

Plants supplied with the various N sources did not exhibit any significant differences in root concentrations of ATP, ADP or the sum of the two pools (Fig. 2). Roots fed Glu or NO_3^- , however, exhibited lower ATP:ADP ratios than did roots supplied with Ala or NH_4^+ . Concentrations of organic acids and sugars did not exhibit any significant differences between the various treatments, except for 2-oxoglutaric acid, which was found to be significantly higher than controls in the Glu-supplied roots (Fig. 2). Total concentration of amino acids in plant roots

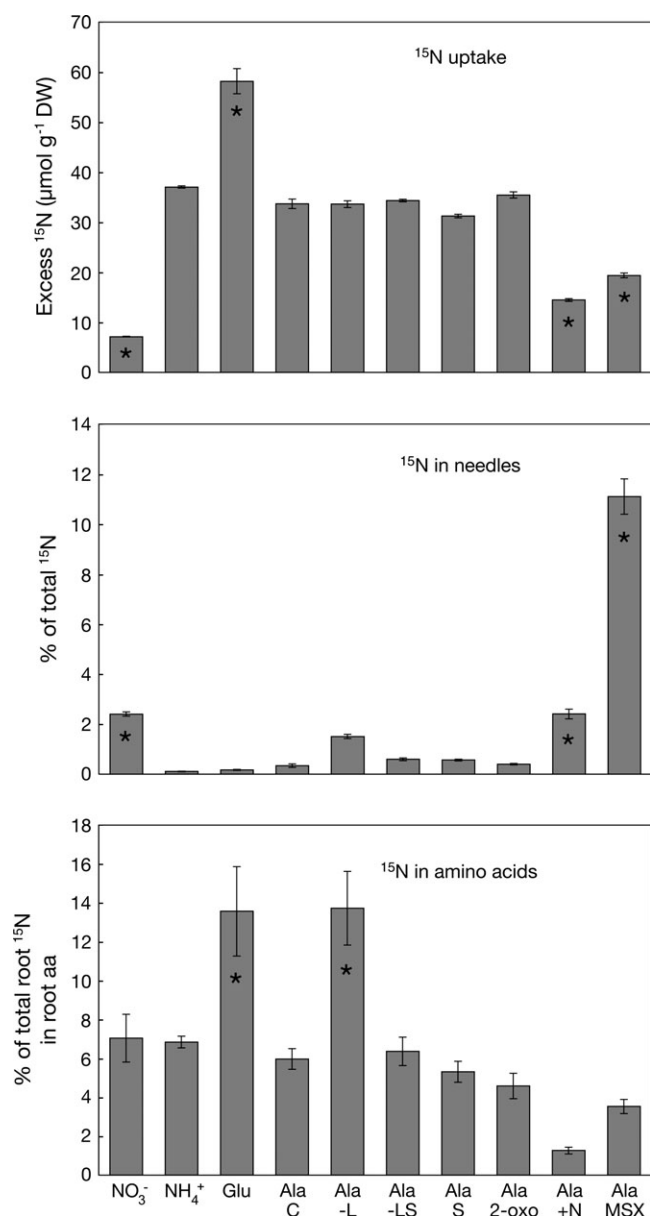


Fig. 1. Top panel displays total uptake of N sources. Middle panel shows relative distribution of total ^{15}N into needles (as calculated by ^{15}N needles/ ^{15}N needles + ^{15}N roots). Bottom panel shows the relative amount of ^{15}N in amino acids of total root ^{15}N content. All values presented are means \pm SE ($n=3$). Bars labelled with an asterisk exhibit significant differences from Ala controls ($P < 0.01$). Abbreviations as in Table 1.

was significantly higher in the Glu-fed plants compared with all other N source treatments, as a consequence of significantly higher concentrations of all specific amino acids except Ala and Asp (Fig. 3). AIAT activity was found to be high in relation to N uptake (1.1 ± 0.1 to 1.5 ± 0.1 $\text{mmol g}^{-1}\text{ DW h}^{-1}$; mean \pm SE) but did not vary as a result of exposure to any of the N sources (Fig. 2). N-starved controls (No-N; Table 1) exhibited no significant differences from Ala-fed plants in any of the measured variables (data not shown).

Effects of pretreatments on uptake and utilization of Ala

Uptake of Ala was significantly lower in plants pretreated with NH_4NO_3 (+N) or MSX, compared with all other plants (Fig. 1). +N and MSX pretreated plants also exhibited a significantly increased relative distribution of labelled N into the needles compared with controls (2.4%, 11.1%, and 0.3%, respectively; Fig. 1). In addition, the relative needle distribution of labelled N in the MSX plants was significantly higher than in the +N plants. Although [^{15}N]Ala was detected in needles of all Ala-fed plants, the levels were *c.* 10-fold higher in the MSX needles compared with all other needles (data not shown).

The flux of N through the amino acid pool into downstream metabolism varied between 14.3 (+N) and 33.8 μmol (2-oxo) of labelled N $\text{g}^{-1}\text{ DW}$. The relative amount of label in the amino acid pool of the roots was significantly higher in the -L plants compared with controls (13.7% versus 6.0% of total labelled N, respectively; Fig. 1). The relative distribution of label in the specific amino acids varied according to Table 2. These data show that, while the +N plants exhibited significantly higher incorporation of label into Asn, the MSX plants displayed significantly higher relative amounts of label in Ala and Glu, while incorporation of label into Gln was significantly lower, compared with controls.

Pretreatments did not result in significant changes of the ATP, ADP or ATP+ADP root concentrations, except for MSX-treated roots, which showed a decrease of ATP levels compared with controls. ATP to ADP ratios were significantly lower than control in the S, +N and MSX plants (Fig. 2). While malate concentrations were significantly higher in the -LS plants than in control plants, the other two organic acids and the sugar concentrations did not exhibit any significant differences in pretreated roots versus controls. Total amino acid concentrations, as well as concentrations of all specific amino acids except GABA and Gln was significantly higher in the -L roots as compared to controls (Fig. 3). In the +N roots, Ala concentrations were lower than in controls. AIAT activity was significantly increased by 6.5 times in the -L plants and by 2.9 times in the MSX plants, compared with controls (Fig. 2).

Discussion

Uptake of the various N sources varied significantly (Fig. 1), being highest for Glu, about 60% of Glu uptake for Ala and NH_4^+ , while NO_3^- uptake amounted to only about 12% of Glu uptake. As the N compound concentrations used in this study (3 mM) are to be considered high in relation to normal field situations (Yu *et al.*, 2002), uptake may be regarded as indicative of maximum uptake and seem to corroborate earlier studies showing a relatively low NO_3^- uptake capacity by conifer species

Table 2. ^{15}N distribution in the respective amino acids of roots after 3 h incubation in various ^{15}N compounds

Values shown are means \pm SE ($n=3$) and are presented as percentages of total ^{15}N in the amino acid pool. Minor aa denotes minor amino acids and comprises Gly, Val, Leu, Ile, Pro, Ser, Thr, Phe, Lys, and Tyr. Different letters indicate significantly different values ($P < 0.01$). Treatment abbreviations as in Table 1.

Treatment	Ala	GABA	Asp	Glu	Asn	Gln	Minor aa
NO_3^-	6.9 \pm 0.3 c	0.4 \pm 0.5	7.8 \pm 2.5	14.1 \pm 2.4 cd	8.1 \pm 7.3 a	62.5 \pm 8.0 a	1.8 \pm 0.8 b
NH_4^+	3.7 \pm 0.3 c	1.2 \pm 0.2	6.6 \pm 0.6	8.9 \pm 0.7 d	1.5 \pm 0.8 a	75.2 \pm 2.2 a	3.0 \pm 0.8 b
Glu	3.4 \pm 0.5 c	3.7 \pm 0.9	0.3 \pm 0.1	21.1 \pm 1.6 abcd	2.5 \pm 0.4 a	67.3 \pm 3.0 a	2.2 \pm 0.2 b
Ala (C)	25.5 \pm 1.1 b	6.0 \pm 1.0	7.2 \pm 1.4	17.1 \pm 1.0 bcd	4.4 \pm 1.2 a	34.3 \pm 1.1 b	5.4 \pm 0.9 ab
Ala (-L)	23.3 \pm 0.4 b	3.6 \pm 0.4	8.1 \pm 0.4	19.9 \pm 0.3 abcd	4.2 \pm 0.6 a	35.3 \pm 0.5 b	5.7 \pm 0.1 a b
Ala (-LS)	23.1 \pm 0.8 b	6.9 \pm 1.1	7.2 \pm 0.3	22.4 \pm 0.5 abc	2.3 \pm 1.4 a	31.8 \pm 1.8 b	6.3 \pm 0.3 ab
Ala (S)	21.7 \pm 0.9 b	4.1 \pm 1.0	9.3 \pm 0.2	23.2 \pm 1.2 abc	4.5 \pm 0.9 a	31.5 \pm 1.5 b	5.7 \pm 0.7 ab
Ala (2-oxo)	19.7 \pm 0.3 b	5.2 \pm 0.4	6.3 \pm 3.2	27.7 \pm 0.9 ab	3.3 \pm 0.7 a	31.8 \pm 2.4 b	6.1 \pm 0.5 ab
Ala (+N)	24.5 \pm 2.6 b	7.2 \pm 2.1	6.5 \pm 1.3	16.5 \pm 3.0 bcd	35.1 \pm 14.1 b	11.9 \pm 9.2 bc	12.4 \pm 3.6 a
Ala (MSX)	39.7 \pm 3.6 a	4.2 \pm 2.8	6.7 \pm 2.0	32.5 \pm 5.3 a	5.9 \pm 2.2 a	2.6 \pm 2.0 c	8.4 \pm 0.3 ab

(Kronzucker *et al.*, 1997; Wallander *et al.*, 1997; Min *et al.*, 2000). These data provide an additional indication of the potential importance of amino acids to plant N nutrition, similar to a large array of previous studies (Raab *et al.*, 1999; Thornton, 2001; Persson and Näsholm, 2002). These results do not, however, give any information concerning the relative importance of the various N compounds to plant N nutrition in the field, as the study does not take into consideration important factors such as soil compound concentrations and fluxes, competition and mycorrhizal associations (Lipson and Näsholm, 2001; Persson *et al.*, 2003). The comparatively high uptake of Ala and Glu seen in this study may, however, be of importance in the transfer of N from ectomycorrhizal associations, as Ala and Glu have been suggested to be compounds transferred from mycorrhizal fungi to host plants (Chalot and Brun 1998).

Earlier studies of amino acid uptake by Scots pine have suggested that uptake may be regulated by root concentrations of NH_4^+ (Persson and Näsholm, 2002, 2003). These findings are further supported by the data from the present study (Fig. 1), where uptake is significantly decreased after exposure to the GS inhibitor MSX. In addition, 36 h pretreatments with NH_4NO_3 resulted in a decrease of Ala uptake. As NH_4NO_3 pretreated plants also exhibited higher total N content compared with all other plants, this corroborates earlier results showing decreased amino acid uptake as a result of high N status (Persson and Näsholm, 2002). Caution should, however, be taken when interpreting data from MSX treatments, as MSX is an amino acid analogue and may compete with amino acid uptake. To avoid competitive effects, roots were carefully washed after pretreatments, and MSX was not included during the subsequent uptake experiments. MSX can also be expected to exert a toxic effect after prolonged treatments, due to the effect on central metabolic pathways. Metabolic data, however, do not show any major differences compared with plants exposed to other pretreatments, suggesting a negligible toxic effect in the present study. In contrast to the effect of N pretreatments, the present study does not

support earlier results showing an up-regulation of amino acid uptake following preincubations in sucrose (Fig. 1; Persson and Näsholm, 2003). Moreover, light deprivation did not result in any changes of uptake in the present study. The lack of effect of these treatments on amino acid uptake may be explained by a failure of these pretreatments to alter sugar pools or fluxes sufficiently, since none of these pretreatments resulted in significant changes in tissue sugar concentrations (Fig. 2).

The relative distribution of acquired N into needles was higher in plants exposed to NO_3^- , compared with the other N sources (Fig. 1). As the uptake of NO_3^- was smaller than the uptake of the other nitrogen sources, this did not result in a larger total N allocation to shoots. Pretreatments with NH_4NO_3 , and particularly MSX, resulted in drastic increases of the relative needle distribution of labelled N from Ala (Fig. 1). The MSX plants also exhibited a significant increase in absolute ^{15}N levels in the needles (data not shown). These data suggest that translocation of N to the shoot may be controlled by factors connected to N metabolism. These data do not allow any elaboration on the underlying mechanisms behind the increased needle allocation of ^{15}N in MSX-treated plants.

Although the relative amount of needle N translocation in this study was generally low compared with earlier studies performed on other species (Schmidt and Stewart, 1999; Näsholm *et al.*, 2000; Thornton, 2001), the data from the MSX treatments show that Scots pine has the capacity rapidly to allocate significant proportions of N from organic N uptake into the shoot. Due to the generally low amount of label found in the amino acid pool of needles in combination with the apparent rapid flux of N through the amino acid pool, the major transport form of N could not be established. However, the increased needle ^{15}N allocation following pretreatment with MSX was paralleled by a considerable increase in needle ^{15}N Ala, while ^{15}N Asp, ^{15}N Glu, ^{15}N Asn, or ^{15}N Gln levels remained very low or absent. Similarly, ^{15}N Ala was detected in needles from all plants exposed to this compound, while not in needles of plants exposed to $^{15}\text{NO}_3^-$, $^{15}\text{NH}_4^+$, or ^{15}N Glu. Most of

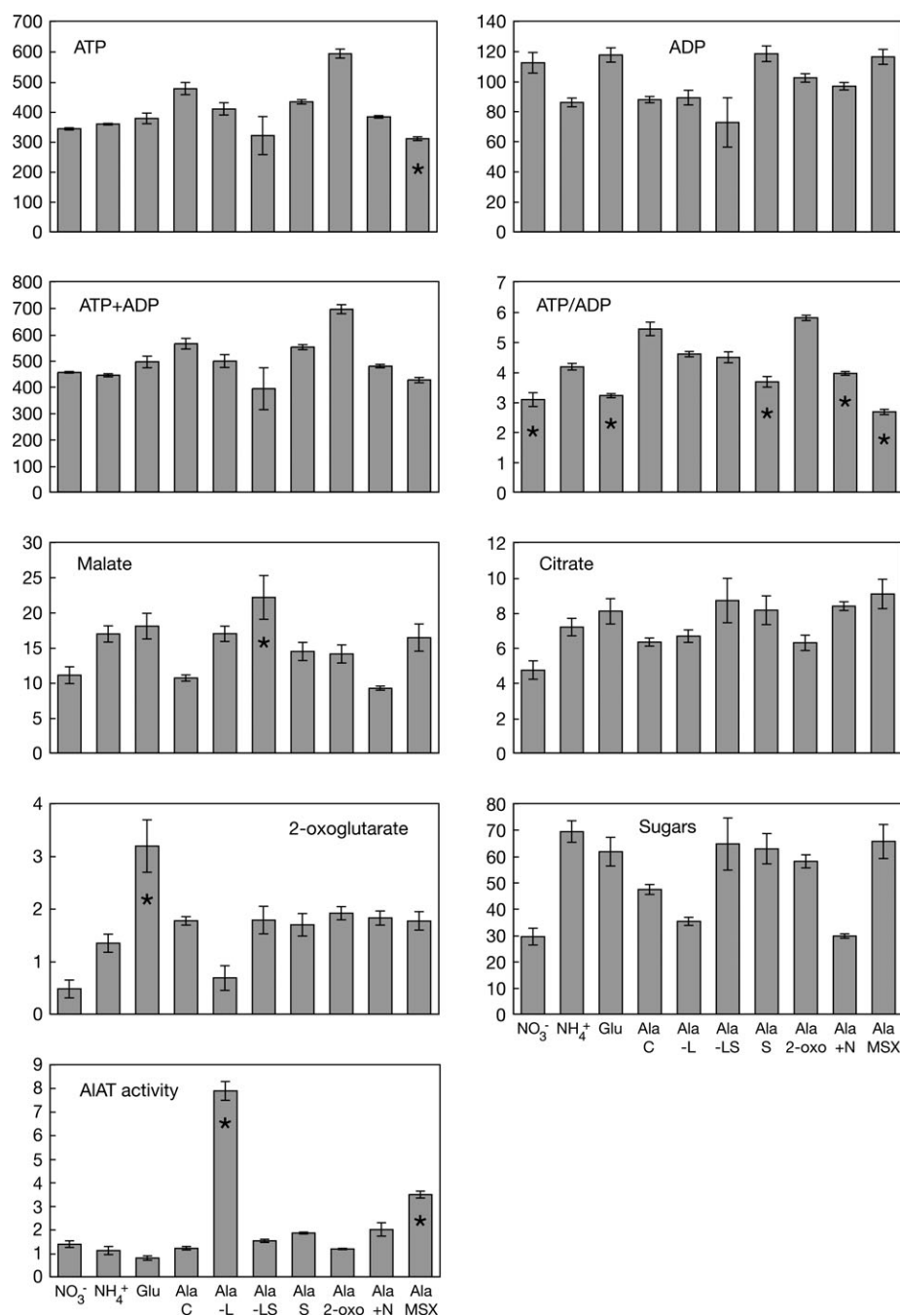


Fig. 2. Root concentrations and ratios of various metabolites as well as AlAT activity. Please note the different scales in panels. Units used are: For ATP, ADP and ATP+ADP; nmol g⁻¹ DW. For malate, citrate, 2-oxoglutarate, and sugars; μmol g⁻¹ DW. AlAT activity is presented as mmol g⁻¹ DW h⁻¹. All values presented are means ±SE (*n*=3). Bars labelled with an asterisk are significantly different from Ala controls (*P* < 0.01). Abbreviations as in Table 1.

these plants, however, also exhibited similar amounts of [¹⁵N]Asp, [¹⁵N]Glu, [¹⁵N]Asn, and [¹⁵N]Gln in needles. Thus, although Asp, Glu, Asn, or Gln are generally considered the major N translocation compounds in plants, other compounds may function as carriers in the N translocation in Scots pine. Moreover, the possibility of ¹⁵NO₃⁻ and ¹⁵NH₄⁺ being translocated prior to incorporation into amino acids can not be excluded.

Ala can be metabolized in a number of ways (Fig. 4). These data indicate that Ala-N, following uptake, is rapidly transferred to Glu by the action of AlAT (EC 2.6.1.2), and then further transferred to other amino acids by a range of aminotransferases (Fig. 4; Table 2; Buchanan *et al.*, 2000). It is also possible that some N is directly transferred into other amino acids without passing the Glu pool, for example, by the action of Ala-2-oxo acid transaminase

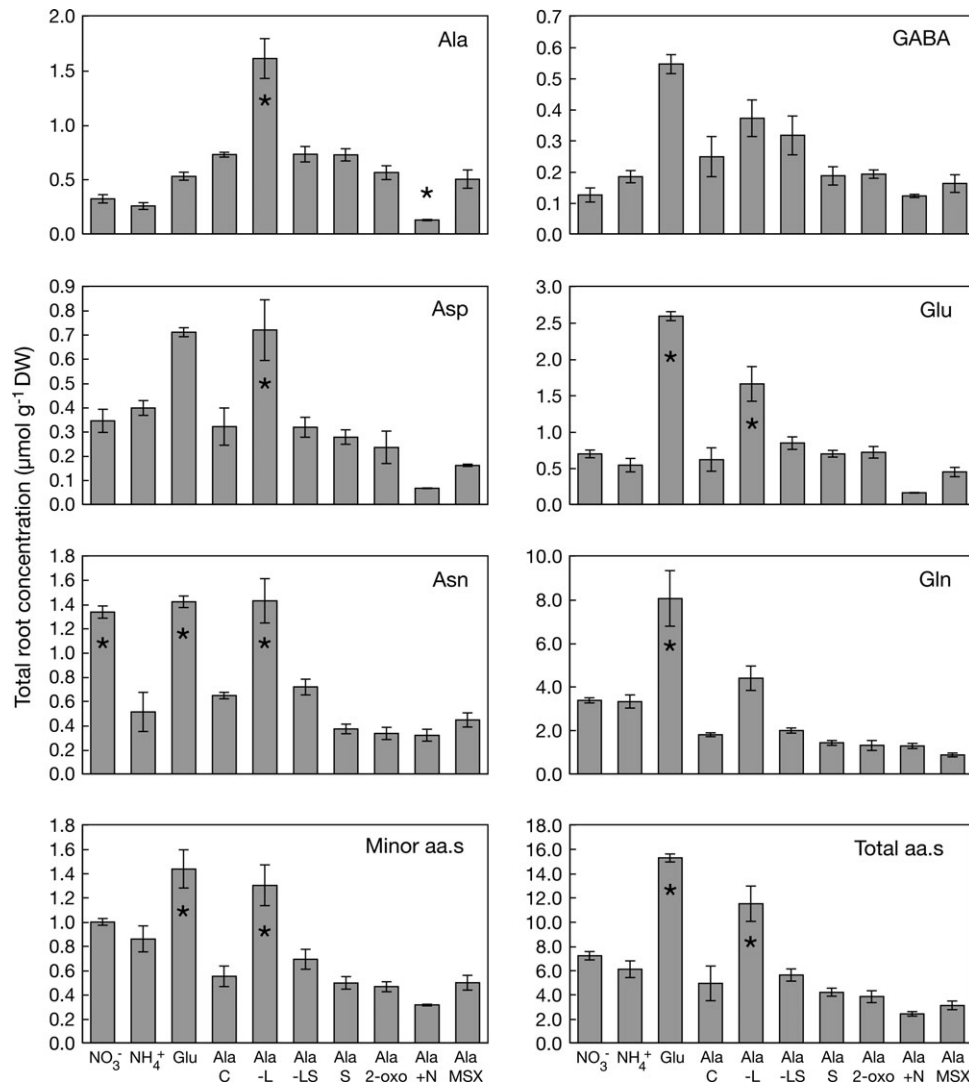


Fig. 3. Total (labelled+unlabelled) root concentrations of various amino acids. Minor aa denotes minor amino acids and includes: Gly, Val, Leu, Ile, Pro, Ser, Thr, Phe, Lys, and Tyr. Values presented are means \pm SE ($n=3$). Note the different scales in different panels. Bars labelled with an asterisk are significantly different from Ala controls ($P < 0.01$). Abbreviations as in Table 1.

(EC 2.6.1.12), although the large amount of label found in the Glu pool suggests such transfer to be of less importance. The 2-oxoglutarate pretreated plants also showed a (non-significant) tendency for increased transfer of N into Glu (27% of label in Glu versus 17% in controls; Table 2), suggesting the importance of 2-oxoglutarate supply for this reaction.

These results show an increased flux of N into Asn, relative to Gln following pretreatment with NH_4NO_3 . Similarly, recent results indicate that expression of the Asn synthetase (EC 6.3.1.1) gene *ASN2* in *Arabidopsis* is increased under conditions yielding high cellular levels of NH_4^+ (Wong *et al.*, 2004). Moreover, increases of N allocation into Asn instead of Gln (as seen in the +N roots) has been suggested to be an effect of carbon limitation, and the lower C:N ratio of Asn. Thus, Asn synthase expression (EC 6.3.5.4) has been reported to increase as a

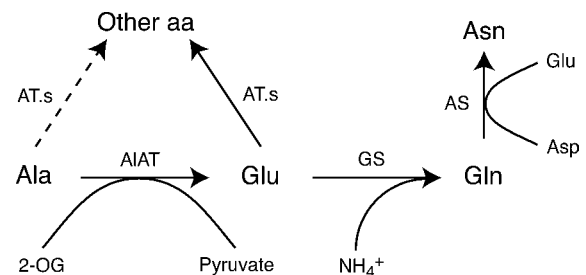


Fig. 4. Simplified diagram of Ala-N metabolism in this study. AT.s, aminotransferases; AlAT, alanine aminotransferase; GS, glutamine synthetase; AS, asparagine synthase; 2-OG, 2-oxoglutarate. Dashed line denotes pathway of relatively small importance in the current study.

consequence of decreased C fluxes (Oliveira *et al.*, 2001). Consequently, the +N roots do show a (non-significant) tendency of decreased sugar concentrations as well as significant decreases in ATP to ADP ratios (Fig. 2). While

similar patterns can be seen in the NO_3^- -fed plants without an effect on incorporation of N into Asn, such a discrepancy may be due to the shorter time-frame of exposure in the NO_3^- -incubated plants (3 h versus 39 h).

The high flux of N through the amino acid pool indicates that roots had the capacity to metabolize all acquired N rapidly. Similar results have been seen in studies of organic N uptake and metabolism by different plants, where glycine-N is rapidly further metabolized following uptake (Schmidt and Stewart, 1999; Thornton, 2001). Moreover, studies of Glu and Asp metabolism after uptake show that C from these compounds is rapidly found in organic and amino acids, as well as in CO_2 and macromolecular compounds (Tuin and Shelp, 1994; Korschuh and Thorpe, 1997). Thus, it appears as if the short-term capacity to metabolize exogenously acquired amino acids does not constitute a major bottleneck for growth on organic N, at least not for major amino acids, such as Ala, Asp, Glu, Arg, Gly, and Ser.

The actual fate of N after passage through the amino acid pool can not be pin-pointed using these data, although the flux of N through the amino acid pool (c. $0.3\text{--}2.2\text{ mg g}^{-1}\text{ DW h}^{-1}$) is in the range of protein turnover rates in studies of *Dactylis glomerata* and *Phaseolus vulgaris* ($0.8\text{--}1.15\text{ mg g}^{-1}\text{ DW h}^{-1}$, respectively; (Zerihun *et al.*, 1998; Scheurwater *et al.*, 2000). In addition, some amino acid N can be expected to enter nucleobase metabolism as well as production of secondary metabolites etc.

Alanine aminotransferase catalyses the reversible transfer of an amino group from Ala to Glu, using 2-oxoglutarate as an acceptor in the Glu-forming reaction, and pyruvate as acceptor in the Ala-forming direction (Fig. 4). Several isoforms capable of performing the AIAT reaction have been identified and suggested to have a variety of cellular functions (Liepman and Olsen, 2004). Our measurements show a notably increased AIAT activity in $-L$ roots, accompanied by a significantly lower 2-oxoglutarate to Glu ratio (data not shown). Earlier studies have seen a similar up-regulation of AIAT activity as a function of NH_4^+ addition and/or hypoxia, conditions likely to cause a need to replenish 2-oxoglutarate (Vanlerberge *et al.*, 1991; Otter *et al.*, 1992; Muench *et al.*, 1998).

Consequently, the increased AIAT activity seen in this study may be a response to replenish 2-oxoglutarate from Glu and pyruvate, rather than a regulation in order to increase transfer of N from added Ala into Glu. The lack of a similar response in the NO_3^- -fed plants (which also displayed lower 2-oxoglutarate to Glu ratios) may be related to other factors, such as the regulatory role of NO_3^- in organic acid production from carbon sources (Galvez *et al.*, 1999; Stitt *et al.*, 2002) and/or the shorter time allowed for response in the latter treatment as compared to the former (3 h versus 39 h).

Although AIAT activity was also increased in MSX-treated roots, this increase was not related to a concurrent decrease in 2-oxoglutarate concentrations. However, the increased NH_4^+ concentrations expected in these roots is likely to signal a need for 2-oxoglutarate for assimilation via the GS-GOGAT pathway (even though the added MSX inhibits such an assimilation). Thus, in this study, increased NH_4^+ levels and reduced relative 2-oxoglutarate concentrations may induce increased AIAT activity in roots in order to replenish 2-oxoglutarate from Glu and pyruvate.

In conclusion, it appears as if the capacity of Scots pine to take up, metabolize, and reallocate N from organic sources is equal to or larger than the capacity to utilize N from inorganic sources. N from Ala and Glu is rapidly transferred into a wide range of other amino acids and subsequently further metabolized. Moreover, uptake, metabolism and translocation of Ala appear to be affected by the N status of the plant. Thus, neither short-term uptake, nor metabolism or shoot translocation appear to be limiting factors for the utilization of Ala or Glu as the sole sources of N.

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