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Organic Nitrogen Uptake by Boreal Forest Plants

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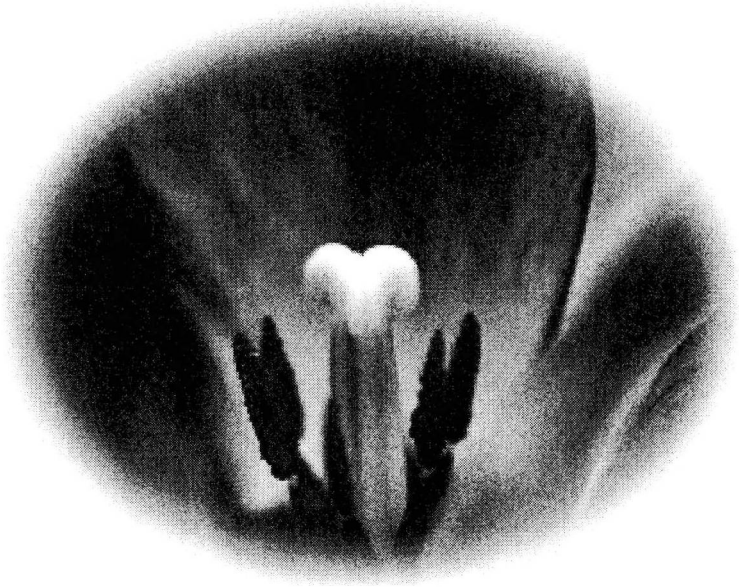
Utan er – Ingenting....

Pia. Det sägs att tre män en gång i tiden red genom öknen på kameler, ledda av en stjärna som lyste tusen gånger klarare än alla de andra. De bar på guld, myrra och rökelse. Jag bär inte på något. Men jag har en så'n stjärna. Även om jag inte har en aning om var den leder mig. Det känns som om det inte spelar någon roll, så länge den bara lyser. Du är jag, jag är du. Mi Amor.

Torgny. Vad är det egentligen du säger, vad är det du *gör*, som plockar fram det bästa hos alla i närheten? Märkligt. Jag kommer tydligt ihåg hur du sa att det, förr eller senare, skulle kännas motigt, eländigt – fördjävligt. Men du hade fel. Det gjorde det aldrig. Inte ett ögonblick. Och jag vet varför. Men jag vet inte *hur* du gjorde det. Det vet jag bara inte. Allt som står skrivet i denna lilla bok, det är ditt.

Lena. Varken du eller jag har väl egentligen någon som helst aning om vad fan det är frågan om. Men det spelar ingen roll. För vi rockar. Fett. Och de andra fattar liksom inte... Och det där bekymrade men roade? uttrycket du klär dig i när jag beter mig som en idiot... Och jag bara fortsätter... I vilket fall - Lex tackar Lois.

Vänner och medarbetare, föräldrar och bröder. På och utanför plan. Tack för allt. Ni vet själva för vad. Om inte – fråga! Drinks are on me!



Oedipa wondered whether, at the end (if it were supposed to end), she too might not be left with only compiled memories of clues, announcements, intimations, but never with the central truth itself, which must always blaze out, destroying its own message irreversably, leaving an overexposed blank when the ordinary world came back.

Thomas Pynchon, The Crying of Lot 49

Abstract

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Organic nitrogen (N) is becoming increasingly recognized as a potentially important N source for plants. Such a role have important consequences for our understanding of key processes in the nitrogen cycle, but also have implications for the way we should consider important phenomena such as global warming and nitrogen fertilization.

Despite an intense research within the area of organic N, no clear picture has emerged of its importance, neither on a plant physiological, nor on an ecosystem level. The work presented in this thesis has taken a broad approach aiming to learn more of the importance of organic N in general, and amino acids in particular for plants in boreal forest ecosystems. Thus, it includes physiological studies of the regulation of plant amino acid uptake, as well as ecophysiological studies examining the relative importance of different N sources for various plants in the field.

Briefly, the results suggest that amino acid uptake is a wide-spread ability among boreal forest plants. Amino acid uptake was found to be regulated by a number of factors, including N- and C-status of the plant and substrate access. Moreover, although mycorrhiza may have important functions for amino acid acquisition in field situations, it appears as if plant uptake capacity of organic N is unrelated to the mycorrhizal association. These results further emphasize the potential importance of organic N for the nitrogen nutrition of plants in boreal forests.

Key words: Organic N, amino acid, uptake, nitrogen, boreal forest, regulation, plant, *Pinus*.

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Plants and nitrogen – the basics

The paradox

Nitrogen is life. Or, at least, nitrogen is part of all life. All living beings need large amounts of this element, which – for instance - is an important part of the molecules many of us associate the most intimately with life – proteins and DNA.

In reality, it is quite a paradox. The growth of many plants is limited by the availability of an element that is seemingly present in unlimited amounts – nitrogen (N) (Vitousek & Howarth, 1991). For instance, more than three quarters of the air we breath consists of nitrogen gas (N_2), and when walking around in a typical boreal forest, you step around on layer after layer of living and dead organic material that contains vast amounts of nitrogen. How come plants suffer from lack of something that is so widely available?

The key to the riddle lies in accessability. Plants simply cannot take up and use all forms of nitrogen present in nature. Thus, the limitation lies, not in the amount of N in the plants environment, but rather in what forms this resource can be taken up and used by the plant, and the rates at which these forms are produced and presented to the plant.

A forgotten capacity

As being a part of a wide range of compounds, nitrogen can be found in many structures in nature, from its simplest form nitrogen gas to large and complex organic compounds, such as proteins and nucleic acids. Traditionally, however, only two forms of N have been considered as important for plant nutrition - ammonium and nitrate (Tamm, 1991). These are also the forms of N found in the plant fertilizers we normally use. And although these two inorganic compounds are no doubt very important for plant N nutrition, the soils of many ecosystems, including boreal forests, exhibit relatively low production of these inorganic compounds. Meanwhile, these soils may contain large amounts of small organic nitrogen compounds, such as amino acids – the building blocks of all proteins (*e.g.* Grov, 1963).

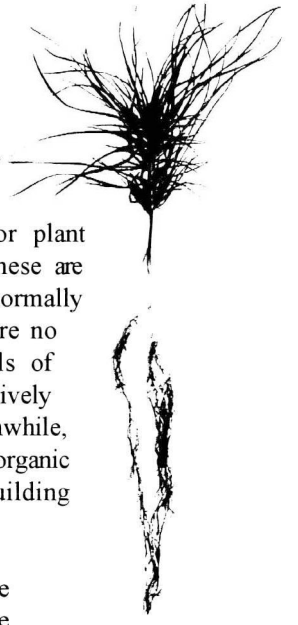

Figure 1. Scots pine (*Pinus sylvestris* L.) seedling. Subject to studies. Photograph courtesy of Jonas Öhlund

While it has been known for a long time that some plants are capable of taking up amino acids - some studies dates as far back as the 1940.s – such uptake has not, until recently, gained attention as being of any importance to plant N nutrition. An intensive research during the last decade has, however, shown that these small organic compounds may indeed play an important

role for the N nutrition of many plants. Plants may, in a way, have forgotten capacities.

Rotating N

Nitrogen flows. Meaning that N is in constant motion through different pools. From gaseous N_2 in the air, by far the largest N pool, by N-fixation into microbial and plant N, which can then be incorporated into animals. As different organisms dies, N is added to the soil as organic nitrogen in the litter. Some of the N bound in complex organic compounds is then released by physical and biological processes into smaller organic N compounds and inorganic N. Some N is leached and later deposited in the sea, some N is denitrified or volatilized and returned to the atmosphere, while some of the N is taken up by plants or microorganisms and continuously cycled between soil and biota (Figure 3).

In soil, several processes are in action converting N. In the organic material deposited to the soil as litter, N is mainly found in complex organic compounds unavailable for plant and microbial uptake. These large compounds are slowly degraded into smaller organic compounds by extracellular hydrolytic enzymes. The smaller compounds are then in part mineralized into ammonium (NH_4^+) and excreted by different microorganisms. Subsequently, ammonium can be taken up by auto- and/or heterotrophic nitrifying organisms and converted to nitrite (NO_2^-) and subsequently nitrate (NO_3^-). In some cases, nitrate can also be produced directly from organic N sources by certain heterotrophic microorganisms.

Plants have generally been considered as totally dependent on ammonium and nitrate for their nitrogen nutrition, thus being reduced to using the N left over after microbial uptake and excretion. The ever increasing amount of research showing that plants are capable of taking up amino acids indicate that this traditional view of soil N cycling must be reconsidered.

Plant growth in boreal forests is generally limited by the supply of nitrogen (Tamm, 1991). The conventional view considers the mineralization of N into ammonium to be the bottleneck in the N nutrition of plants (*e.g.* Marschner, 1995). However, the results showing that plants have access to unmineralized N, thereby short-circuiting the conventional N-cycle, suggest that the limitation rather lies in the production of small organic compounds, such as amino acids (Jones & Kielland, 2002), as well as in the ability of different plants to efficiently compete for these compounds.

A fungal connection

In the boreal forest, as well as in most other ecosystems, the bulk of all plants exhibit a close relation with different fungi. This association is termed mycorrhiza – meaning "fungal root". There are many types of mycorrhiza, of which 3 types are of special importance in boreal forests; arbuscular, ericoid and ectomycorrhiza (Figure 2). The former is found primarily in herbs and grasses, the second in

shrubs while the latter is generally formed in tree roots. In these associations, the plant supplies the fungi with photosynthates, while the fungal part may offer the plant an increased availability to soil nutrients and, in some cases, protection from pathogens and an increased drought resistance (*e.g.* Smith & Read, 1997).

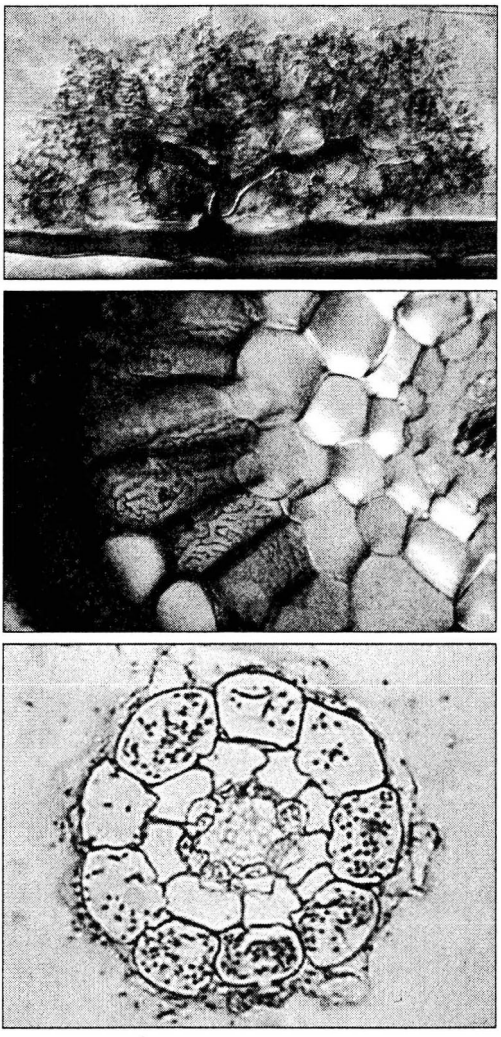

Figure 2. Images of arbuscle in plant root cell (arbuscular mycorrhiza; top), the Hartig net formed by ectomycorrhizal hyphae (middle) and ericoid mycorrhizal hyphae and cellular intrusions (bottom). Images reproduced with kind permission from Mark Brundrett and Mycologue Publications.

The importance of different mycorrhizas for plant N nutrition may vary in different situations and ecosystems, but the general consensus is that it plays an important role in strongly N-limited systems such as boreal forests. Also, it is clear that mycorrhiza have some traits important for plant nutrition in general, and plant organic N uptake in particular. First, the fungal hyphae do act as an extension of the plant root system, in this manner increasing its active surface drastically. And while ericoid mycorrhizal hyphae only extends some millimeters outward from the root surface, ecto- as well as arbuscular mycorrhizal fungi stretches far outward into the soil making up extensive hyphal networks, even connecting different plants to each others (Smith & Read, 1997).

Second, ecto- and ericoid mycorrhiza have been shown to excrete hydrolytic enzymes, in that way increasing the availability of small organic N compounds, such as amino acids and small peptides, in the soil (Bajwa, Abuarghub & Read, 1985). In these manners, the formation of mycorrhiza may have large impact on the ability of plants to utilize and compete for organic N in field situations.

The ecophysiology of N uptake

A common ability

Although plants have been known to be able of taking up and utilizing N from amino acids for a long time (Virtanen & Linkola, 1946; Melin & Nilsson, 1953; Miller & Schmidt, 1965), the physiological and ecological significance of such an uptake was not further investigated until much later. Then, several studies (Soldal & Nissen, 1978; Schobert & Komor, 1987; Schobert, Köckenberger & Komor, 1988; Jones & Darrah, 1993) surfaced suggesting that plant amino acid uptake was an active process exhibiting Michaelis-Menten kinetics and a proton gradient dependence.

More importantly, studies of a range of different arctic plants showed, not only that these plants were capable of acquiring amino acids, but also that such an uptake could account for a significant amount of the plant's N budget (Chapin III, Moilainen & Kielland, 1993; Kielland, 1994; Raab, Lipson & Monson, 1996; Raab, Lipson & Monson, 1999). Similarly, a number of studies also indicated the importance of amino acids and peptides for the N nutrition of plants associated with ecto- and ericoid mycorrhiza (Stribley & Read, 1980; Bajwa, Abuarghub & Read, 1985; Abuzinadah & Read, 1986, 1989; Finlay, Frostegård & Sonnerfeldt, 1992) and the transfer of chitin N to plants via ericoid mycorrhiza (Kerley & Read, 1995, 1997).

Subsequently, a number of studies have shown high rates of amino acid uptake by a plethora of differing plant species with differing lifestyles in a wide range of diverse ecosystems and habitats, irrespective of their associated type of mycorrhiza (*e.g.* Kielland, 1997; Raab, Lipson & Monson, 1999, Paper **IV**; Schmidt & Stewart, 1999; Falkengren-Grerup, Månsson & Olsson, 2000; Näsholm, Huss-Danell & Högberg, 2000; Streeter, Bol & Bardgett, 2000; Thornton, 2001; Henry & Jefferies, 2002). Thus, agricultural plants as well as trees, shrubs, bushes and cryptogams from as widely different habitats as the arctic and subtropial australian ecosystems have been found to take up N in the form of amino acids. Some studies have also shown uptake of heterocyclics, such as nucleobases and nucleosides, by algae as well as aquatic and terrestrial plants (Knutsen, 1972; Nakashima & Tsudzuki, 1976; Desimone *et al.*, 2002).

Together, these and similar studies show that the ability to take up amino acids is ubiquitously found throughout the plant kingdom. In addition, these data clearly point out the potentially large importance of organic N to the nitrogen nutrition of plants in general. The mere ability to take up organic N does, however, not disclose if, and in that case to what extent these compounds actually contribute to the N economy of different plants in natural situations. For this resource to be important for plant N nutrition, it has to be widely available, while plants have to be able of competing for it under field conditions.

The complex resource

With the exception of plants living in association with N-fixing organisms, plants acquire the absolute bulk of their nitrogen from the soil. And while boreal forest soils generally contain large stocks of N, plant growth in these environments is commonly limited by the restricted access to this resource (van Cleve & Alexander, 1981; Tamm, 1991).

This apparent contradiction is explained by the fact that the absolute majority, up to 95%, of N in these soils is found in complex organic compounds which are tightly bound and relatively resistant to physical and biological degradation (Mengel, 1996), leading to a slow release of soluble, plant available nitrogen. Schulten & Schnitzer (1997) summarized the content of these humic fractions as being c. 40% proteins, about 35% heterocyclics (including purines and pyrimidines), c. 19% NH_3-N and some 5 to 6% amino sugars.

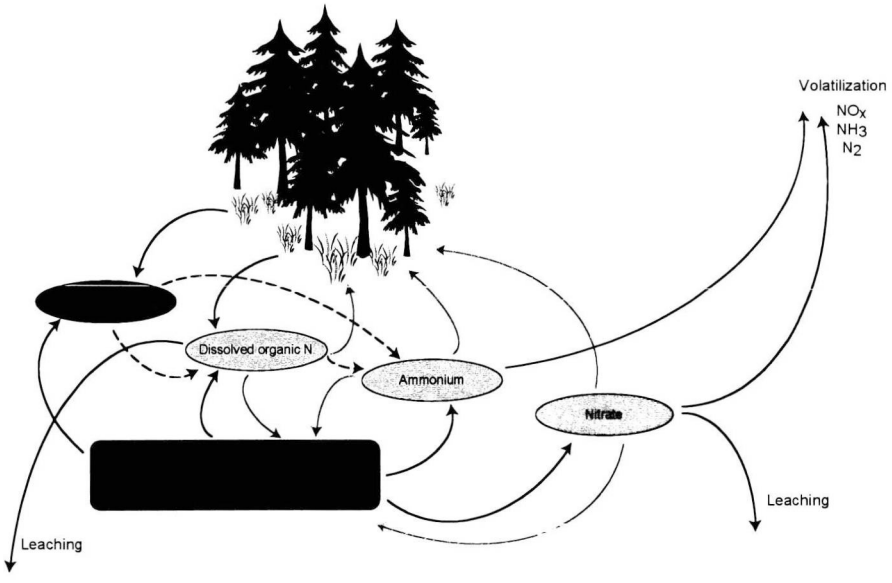

Figure 3. Simplified diagram showing the complexity of nitrogen cycling in boreal forest soils. Dashed lines indicate physical (as opposed to biological) processes. Red lines show biological uptake processes.

Albeit resistant to degradation, humic compounds are degraded by the action of extracellular hydrolytic enzymes, thereby releasing amino acids as well as other amino compounds and ammonium into soil solution (*e.g.* Attiwill & Adams, 1993). These compounds are subsequently available for plant and microbial uptake. In this context, ecto- and ericoid mycorrhizal fungi may be key players, as these fungi have exhibited an ability to excrete proteolytic enzymes (*e.g.* Bajwa,

Abuarghub & Read, 1985; Leake & Read, 1989), thereby releasing amino acids and small peptides from soil bound proteins. In addition, ericoid mycorrhiza have been shown capable of releasing chitinolytic enzymes, leading to the release of amino sugars (Kerley & Read, 1995, 1997). Ericoid mycorrhizal fungi also appear to have the capacity to release and use N from protein-polyphenol complexes (Bending & Read, 1996), which are found both in soil solution and in the humic fractions of soil (Yu *et al.*, 2002).

The breakdown of complex humic substances is, however, not the only source of soluble N. Decaying plant roots and litter as well as microbial turnover can be expected to be important sources of dissolved N, a pool of N comprised mainly of free or complexed proteins, free amino acids, amino sugars, ammonium and, in some cases, nitrate (Michalzik & Matzner, 1999; Yu *et al.*, 2002, Figure 3). Likewise, leakage and excretion from plant roots and rhizomes as well as microbes may add to the soluble pool of these compounds (Näsholm & Persson, 2001). Several studies have also indicated that disturbances to the soil environment, such as freeze-thaw events (Schimel & Clein, 1996; Lipson & Monson, 1998) and water-logging (Schmidt & Stewart, 1997) may lead to a rapid release of amino acids into soil solution by stressed, damaged or dying plant roots and soil microorganisms.

A number of reports have shown that the pool of soluble amino acids in boreal and arctic soils generally is relatively large (Grov, 1963; Ivarson & Sowden, 1969; Ktsoyev, 1978; Abuarghub & Read, 1988b; Kielland, 1994; Michalzik & Matzner, 1999; Jones & Kielland, 2002; Yu *et al.*, 2002), in several cases larger than the pool of ammonium (Abuarghub & Read, 1988a; Kielland, 1995; Näsholm *et al.*, 1998; Nordin, Högberg & Näsholm, 2001; Jones & Kielland, 2002), while pools of nitrate in boreal forest soils are generally comparatively small (*e.g.* van Cleve & Alexander, 1981). In addition, the pool of free amino sugars may be significant and constitute up to 12% of the total dissolved organic N (Michalzik & Matzner, 1999).

As amino acids are constantly released and rapidly taken up by microbes and plant roots, the amino acid pool in soil is highly dynamic, turning over rapidly. Several reports have shown half-lifes of amino acids in the range of 1.7-28.7 h in different soils (*c.f.* Lipson & Näsholm, 2001), and the amino acid pool in taiga soils have been suggested to turn over as fast as up to 20 times per day (Jones & Kielland, 2002). The large flow of amino acids suggests that there is a large, relatively constant availability of these compounds open for plant as well as microbial uptake.

An important factor when considering plant uptake of any nutrient is its delivery to root surfaces. Indeed, this factor has been pointed out as the single most important issue for root uptake in systems exhibiting poor resource buffering, in this case low levels of plant available N (Leadley, Reynolds & Chapin III, 1997). Nutrients can be delivered to root surfaces in 3 fundamental ways; mass flow, diffusion or root proliferation, of which the second is generally considered to be the most important process for nutrients in low concentrations (Nye, 1977).

While the rate of diffusion in soil may be affected by several factors such as temperature, soil water content, concentration gradients and molecular mass, electric charge can be expected to play an important role. The electrical charge of a compound will affect the adsorption of the compound to soil surfaces, which are mainly negatively charged . Hence, cationic compounds have a tendency to exhibit much lower diffusion rates than do anions or neutral compounds (Jones, 1999). Accordingly, ammonium, but foremost basic amino acids, such as arginine and lysine, exhibit significantly lower rates of diffusion than do nitrate and neutral (although zwitterionic) amino acids, such as alanine or glycine. As a result, plant capture of these basic amino acids may be expected to be lower and more dependent on root and hyphal proliferation as compared to other compounds.

Does mycorrhiza matter?

As mentioned, a large majority of all plants are in natural situations associated with mycorrhizal fungi (Harley & Harley, 1987), and in boreal ecosystems mycorrhizal mycelium generally constitute a large proportion of the soil microbial biomass (*c.f.* Chalot & Brun, 1998). Mycorrhizal connections may confer several benefits to the plant part, such as pathogen protection and drought resistance (Smith & Read, 1997). Mycorrhizal associations may also have a large impact on the plant nutrition in general and, in nitrogen limited systems such as boreal forests, for plant N nutrition in particular.

Accordingly, AM has been shown to take up nitrate (*e.g.* Johansen, Finlay & Olsson, 1996; Cliquet, Murray & Boucard, 1997; Subramanian & Charest, 1999; Hawkins, Johansen & Eckhardt, 2000), ammonium (*e.g.* Johansen, Jacobsen & Jensen, 1994; Javelle *et al.*, 1999; Hawkins, Johansen & Eckhardt, 2000) as well as amino acids (Cliquet, Murray & Boucard, 1997; Hawkins, Johansen & Eckhardt, 2000) and to subsequently transfer N from these compounds to their respective host plant. In addition, several studies have shown arbuscular mycorrhiza as capable of transferring N from organic patches to plants, although it could not be concluded in what form the acquired N was actually transferred (Hodge, Robinson & Fitter, 2000a; Hodge *et al.*, 2000a; Hodge, Campbell & Fitter, 2001).

The beneficial effect of ectomycorrhiza on plant N nutrition is long since recognized, and a large quantity of research has shown uptake and/or growth on nitrate as well as ammonium and amino acids of ectomycorrhizal fungi (*e.g.* Abuzinadah & Read, 1988; Chalot *et al.*, 1996; Tibbett *et al.*, 1998; Javelle *et al.*, 1999; Kreuzwieser *et al.*, 2000; Plassard, Bonafos & Touraine, 2000; Anderson, Chambers & Cairney, 2001; Blaudez *et al.*, 2001; Lilleskov, Hobbie & Fahey, 2002). Furthermore, the identification and characterization of transporters for ammonium (Javelle *et al.*, 2001) and amino acids (Nehls *et al.*, 1999; Wipf *et al.*, 2002a) in the fungi *Amanita muscaria* and *Hebeloma cylindosporum* has provided a functional background to the uptake of these compounds by ectomycorrhizal fungi.

And while there is doubt considering the actual benefit of ectomycorrhiza to plant uptake of nitrate (Kreuzwieser *et al.*, 2000; Plassard, Bonafos & Touraine, 2000), the importance of this fungal association to plant ammonium and amino acid uptake is well substantiated. Additionally, ectomycorrhizal, in contrast to non-mycorrhizal plants have been shown to have access to protein N, an effect of the capacity of ectomycorrhizal fungi to release proteolytic enzymes (Chalot & Brun, 1998; Nehls *et al.*, 2001). With this background, the results showing increased plant growth of ectomycorrhizal plants on organic N sources is hardly surprising (*e.g.* Abuzinadah & Read, 1986; Finlay, Frostegård & Sonnerfeldt, 1992; Turnbull, Goodall & Stewart, 1995; Turnbull *et al.*, 1996; Andersson, Ek & Söderström, 1997; Chalot *et al.*, 2002; Wallander, 2002).

Ericoid mycorrhizas are far less studied than the previously mentioned two forms. Nevertheless, their capacity to take up ammonium and amino acids is well documented (*e.g.* Stribley & Read, 1980; Xiao & Berch, 1999; Cairney *et al.*, 2000; Sokolovski, Meharg & Maathuis, 2002). Besides ectomycorrhiza, ericoid mycorrhizas also exhibit the ability to excrete proteolytic enzymes (*e.g.* Bajwa, Abuarghub & Read, 1985; Leake & Read, 1989; Bending & Read, 1996) and transfer protein N to its host plant. Similarly, ericoid mycorrhizas have also been shown competent to degrade and transfer chitin N to its host plant (Leake & Read, 1990; Kerley & Read, 1995, 1997).

Hence, although plants generally appear to have the capacity to take up amino acids by their own means, mycorrhizal associations have apparent benefits for plant acquisition of organic N. All important mycorrhizal types found in the boreal forest appear to have the capacity to take up organic N. The mycorrhizal hyphal network provides a significant extension to the plant root system, thus increasing the N-foraging capacity of the plant. In addition, ecto- and ericoid mycorrhiza also exhibit the essential ability to contribute to the degradation of complex soil organic N, in this manner making a large pool of N available for plant N nutrition. Indeed it appears as if mycorrhiza does matter.

The competition

In the N-limited environment of the boreal forest, plants have to compete for the available nitrogen with other plants but also, above all, with soil microorganisms such as bacteria and fungi. In general, microorganisms are considered outstanding in this competition, and plants have commonly been considered as relatively passive competitors for N, able only of acquiring the N in excess of microbial needs, excreted as ammonium and nitrate (Marschner, 1995, Figure 4). However, the capability of plants to take up organic N, thus short-circuiting N mineralization, calls for a re-evaluation of the competitive abilities of plants for N (Eviner & Chapin III, 1997).

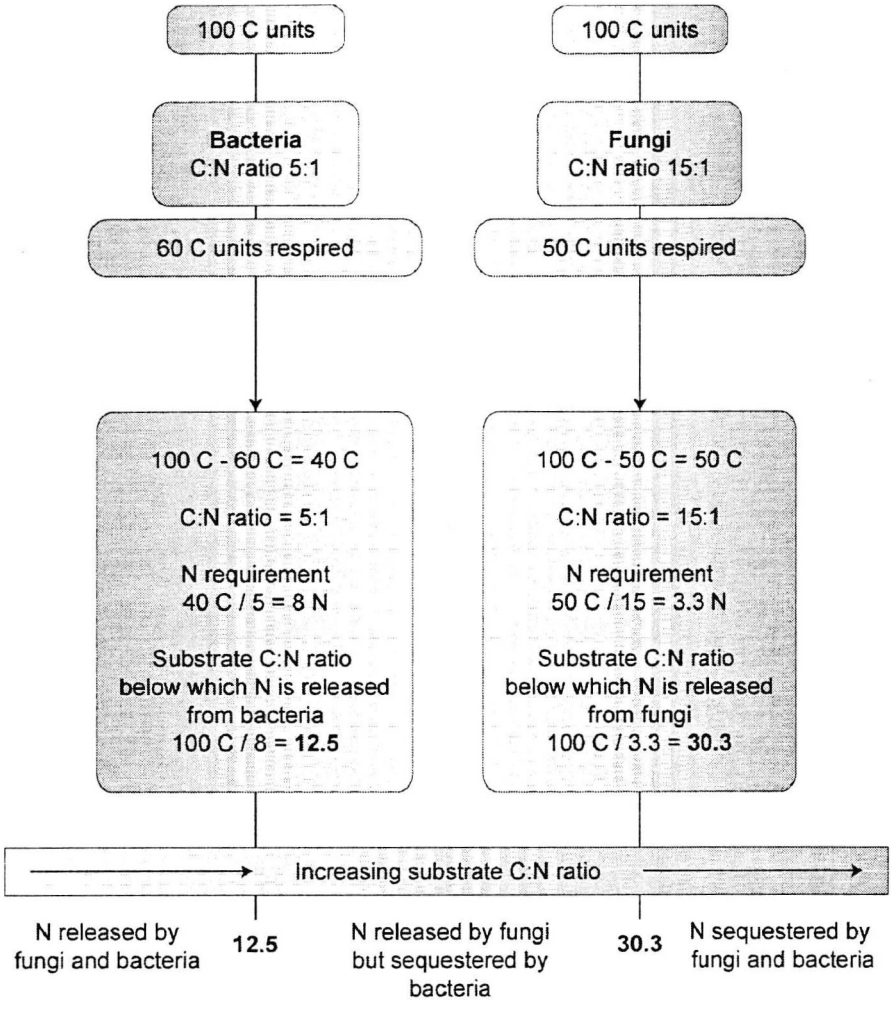

Figure 4. Schematic diagram showing the impact of soil C:N ratio on N release or sequestration by soil microorganisms. A high C:N ratio leads to N starvation of both bacteria and fungi in soil, thus increasing N competition both in between soil microbes and between plants and microbes. Low C:N ratios result in a decreased competition for N. At intermediate C:N ratios (in this example between 12.5 and 30.3), plants are met by competition primarily from soil bacteria, while fungi exhibit C limitation. N taken up in excess of microbial needs is generally exudated in the form of ammonium. Image is redrawn from Hodge *et al.* 2000b.

Short-term studies have pointed out the fierce competition of N, showing plant uptake to be some 5% of the total amount of added N (Schimel & Chapin III, 1996; Owen & Jones, 2001). These results are hardly surprising considering the large surface area comprised by microorganisms in soil. However, when trying to estimate the competitive ability of plants vs. microorganisms there are some important factors which have to be accounted for.

First, the temporal aspect of competition is highly important. Plants are long-lived organisms competing for nutrients over an extended period of time, while many soil microorganisms exhibit much shorter life-spans. Thus, the same N atom may present itself several times to plant root competition while the microbial population turns over, giving the plant numerous opportunities to take it up (Kaye & Hart, 1997; Hodge, Robinson & Fitter, 2000b). Consequently, when using sequential sampling over an extended period of time, a steadily increasing flow of N into plant tissue was observed (Hodge *et al.*, 2000a). Similarly, long-term studies also show a larger part of added N in plants than what is seen in short-term experiments (Hart *et al.*, 1993; Schimel & Chapin III, 1996; Kaye & Hart, 1997; Hodge *et al.*, 2000b). There may, however, be large differences in N turnover time in different soil microorganisms, as fungi and actinomycetes have the ability to retranslocate N as hyphae turn over, thus extending N turnover time significantly (Hodge, Robinson & Fitter, 2000b).

In addition to differences in life-span, variation in the timing of uptake and growth may alleviate competition. A number of studies have indicated a temporal separation between plant and microbial uptake of N, suggesting there may be an annual cycling of nutrients between different organisms (Mancinelli, 1984; Singh *et al.*, 1989; Jaeger III *et al.*, 1999; Lipson, Schmidt & Monson, 1999). A similar resource partitioning has also been suggested to exist in plant communities (McKane, Grigal & Russelle, 1990; McKane *et al.*, 2002).

Second, the problem of sorting out friends from foes can make competition studies hazardous. The soil microbial component consists of a wide range of bacteria and fungi, including mycorrhizal fungi which generally comprise a large part of the soil biomass (*c.f.* Chalot & Brun, 1998). As a consequence, a large part of the measured microbial N uptake seen in different studies may in fact be uptake by mycorrhizal fungi, an uptake of which large parts may subsequently end up as plant N.

When trying to assess the relative importance of organic N uptake to plant N nutrition, it is imperative to inquire whether the competitive ability of plants differ with respect to organic N compounds as opposed ammonium and/or nitrate. As organic N constitutes both an N- and a C-source for soil microorganisms they are not likely to appear in excess of microbial demand. Thus, in contrast to ammonium and nitrate which in some situations may be regarded as excess N for some soil microbes, the competition for organic N can be assumed to be more severe.

Based of the lack of detection of ^{13}C in plant roots from studies following the fate of amino acids labeled with this stable isotope after additions to soils, it has been suggested that plants may be unable to compete for organic N under field-like conditions (Hodge *et al.*, 1998, 1999b; Hodge *et al.*, 2000a). Moreover, it has been proposed that the high turnover rate of amino acids in soil, in addition to the filtering of soil solution by soil microbes on its way through the rhizosphere towards roots may render amino acids unavailable for plant uptake (Jones, 1999; Jones & Hodge, 1999).

There are, however, several reports of plant organic N uptake in the field (Näsholm *et al.*, 1998; Näsholm, Huss-Danell & Högberg, 2000, 2001; Nordin, Högberg & Näsholm, 2001, Paper **V**), indicating that plants are indeed capable of competing for this resource under natural conditions. Moreover, studies of N partitioning between plant and microorganisms after additions of organic N sources indicate that the competitive ability of plants for organic N is readily comparable to the capacity to compete for ammonium or nitrate (*e.g.* Kaye & Hart, 1997; Jaeger III *et al.*, 1999; Hodge, Robinson & Fitter, 2000b).

The physiology of plant N uptake

While comparative studies of plant N uptake and competition in natural systems may provide a framework for the understanding of N dynamics in these systems, a different approach is needed for the understanding of the *underlying reasons* for the different strategies of various species in distinct systems. Thus, by taking a closer look on the physiological and cellular processes of plant N uptake and metabolism, we can attain information about the individual plant's performance in field situations.

Means of transportation

In order to be available for metabolism, any nutrient has to enter the cell by passing its plasma membrane. The primary function of the plasma membrane is to provide a barrier between cellular metabolism and the outside environment. Biological membranes are permeable to gases, such as O_2 and CO_2, small uncharged polar molecules, such as water or ethanol, and non-polar molecules. These types of compounds can pass the membrane at relatively large rates by passive diffusion (Garrett & Grisham, 1999; Lodish *et al.*, 2000).

Ions, and larger polar molecules, such as amino acids, can not passively diffuse over biological membranes at any appreciable rates. Thus, the transport of such molecules must be mediated by some kind of transport mechanism. In addition, the transport of many compounds, most notably water and sugars, capable of diffusion over membranes is accelerated by transporters. In many cases, transport has to be facilitated against a concentration gradient, a process which is energy demanding. In plants, this energy is provided by the establishment of proton (H^+)

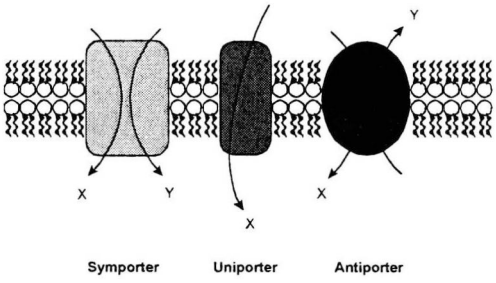

Figure 5. Simple figure showing the 3 types of carrier proteins. X indicate "primary" substrate, e.g. amino acid or sugar molecule. Y indicate "co-substrate", e.g. a proton (H^+).

gradients over the membranes via ATP hydrolysis, leading to electrochemical potentials over the membranes (Garrett & Grisham, 1999; Lodish *et al.*, 2000).

Transporters are generally classified into 3 major classes. First, *pumps* employ ATP hydrolysis to facilitate transport of small ions, such as H^+ or Na^+, against an electrochemical gradient. By doing this, pumps provide the functional means for the other two types of transporters. Second, *channels* provide a means for ions or water to enter cells downward a concentration or electrochemical gradient without interaction with the channel protein. Channels allow for simultaneous movement of several ions or molecules, consequently allowing for relatively high rates of transport, up to a million times higher than carrier proteins.

Carrier proteins transport a wide range of different compounds, although the substrate specificity for an individual carrier is generally rather restricted. The movement of compounds by carriers require a conformational change of the transporter protein, and generally allow only for the movement of one molecule of the substrate at a time. As an effect, carriers are relatively slow as compared to channels. A second effect of the conformational change is that the activity of carriers exhibit saturable kinetics which can be described by the Michaelis-Menten equation. Three types of carriers have been described; uniporters, symporters and antiporters. The former facilitates transport of its substrate down a concentration gradient, while the two latter use the energy released by transport of an ion or a molecule down an electrochemical gradient to transport its substrate upward a concentration gradient (Garrett & Grisham, 1999, Lodish *et al.*, 2000, Figure 5).

In the context of nitrogen transport and uptake, carriers are the types of transporters of highest interest, given that nitrate as well as ammonium, amino acids and heterocyclics are thought to be transported via these types of transporters.

Nitrate and ammonium transport

Nitrate uptake consists of net influx resulting from inward and outward transport of differing systems (Crawford & Glass, 1998). From physiological studies of several widely differing species, nitrate is generally considered to be transported into roots by at least 3 distinct carrier systems. First, a constitutively expressed, high-affinity transport system (cHATS) is characterized by its high affinity and relatively low maximum uptake rates (*e.g.* Aslam, Travis & Huffaker, 1992; Kronzucker, Siddiqi & Glass, 1995). Second, a high-affinity transport system

(iHATS) is induced in roots after exposure to nitrate (*e.g.* Zhou *et al.*, 1999; Min *et al.*, 2000). This system exhibits a slightly lower affinity and higher maximum uptake rates as compared to the constitutive system (Crawford & Glass, 1998).
In addition to the two high-affinity systems, a low-affinity transport system (LATS) is constitutively expressed in roots and exhibit non-saturable uptake kinetics at least up to 50 mM external nitrate concentrations, although uptake appears to be energy dependent (*e.g.* Kronzucker, Siddiqi & Glass, 1995; Huang *et al.*, 1996; Min *et al.*, 2000). All influx transport systems appear to be proton symporters, transporting two protons with every nitrate molecule (Crawford & Glass, 1998). An active outward transport of nitrate has also been shown, by what appears to be an inducible efflux transport system (*e.g.* Aslam, Travis & Rains, 1996).

In correspondence to physiological data, molecular analyses have shown that plants express an array of different nitrate transporters, belonging to either of 2 transporter families – the NRT1 or NRT2 (Williams & Miller, 2001). The NRT1 family has been suggested to contain at least 4 members in *Arabidopsis*, all functioning as low-affinity, high capacity carriers, corresponding to the function of the LATS uptake system. Such a simple correlation is, however, complicated by the fact that the *Arabidopsis* AtNRT1.2 appears to exhibit constitutive expression (Huang *et al.*, 1999), and by the apparent dual-affinity uptake kinetics of AtNRT1.1 (Liu, Huang & Tsay, 1999). In *Arabidopsis*, the NRT2 family is comprised by at least 7 carriers, while studies of barley have revealed at least 4 encoding genes. These carriers apparently function as constitutive or inducible high-affinity transporters corresponding to the function of cHATS and iHATS (Glass *et al.*, 2001; Glass *et al.*, 2002). In addition to *Arabidopsis*, transporters have been identified in a variety of species (*c.f.* Glass *et al.*, 2001). Intriguingly from a N uptake perspective, one study has also identified a nitrate transporter most likely capable of transporting the amino acid histidine in *Brassica napus* (Zhou *et al.*, 1998).

Similar to nitrate uptake, the net uptake of ammonium is the resulting difference between influx and efflux components (Britto *et al.*, 2001). Ammonium uptake is concentration dependent and display 2 distinct kinetic phases with one high-affinity saturable component and one apparently insaturable linear phase (*c.f.* von Wirén *et al.*, 2000a; Williams & Miller, 2001). Although the mechanisms behind high-affinity transport are under debate, a recent study indicate that at least one of the identified transporter (LeAMT1;1) utilizes a uniporter mechanism for transport (Ludewig, von Wiren & Frommer, 2002). These results do however not preclude that other transporters may employ proton symport (Williams & Miller, 2001; Ludewig, von Wiren & Frommer, 2002). Low-affinity ammonium uptake is thought to be mediated by channels, of which some candidates have been identified (*c.f.* Howitt & Udvarti, 2000; Williams & Miller, 2001).

Molecular studies of *Arabidopsis* have identified 6 genes corresponding to ammonium transporters, of which 5 are classified to belong to a single gene family - the AMT1 (Gazzarrini *et al.*, 1999, EMBL accession numbers AL35353 and AP000382, Gazzarrini and von Wirén, unpublished). The remaining gene -

which deviates from the others structurally and appears more related to some bacterial ammonium transporters than to AMT1 transporters - is categorized into a separate gene family dubbed the AMT2 (Sohlenkamp *et al.*, 2000). Members of both these gene families have also been identified in other plant species, such as tomato (von Wirén *et al.*, 2000b), rice (EMBL accession number AF001505, von Wirén et al., unpublished) and the legume *Lotus japonicus* (Simon-Rosin, Wood & Udvardi, 2003).

Clearly, a range of genes encoding nitrate and ammonium transporters have been identified in several different plant species. Although one of the functions of these transporters is certainly to mediate uptake of nitrogen from soil, some of these transporters may also fulfil other functions. For example, nitrate is commonly sequestered in vacuoles for storage, and most species translocate differing amounts of nitrate to the shoot. Both these processes require transporters for loading and unloading of nitrate in and out of vacuoles and xylem. Moreover, plant photorespiration releases large amounts of ammonium which has to be transported over organellar membranes in order to be recycled. Obviously, this calls for a large transport capacity of this ion, and studies of the transporter AMT2 also suggest that this transporter may function as an ammonium retriever during photorespirational events (Sohlenkamp *et al.*, 2000; Simon-Rosin, Wood & Udvardi, 2003).

Organic N transport

Due to the necessity of distributing different compounds between and within cells, transporters of organic compounds serve a wide range of functions in plants. One of these functions can be the uptake of organic N compounds from soil. In addition, it should not be forgotten that N taken up by mycorrhizal fungi also need to be transported over the plasma membrane of the plant cell. It has been suggested that the transfer of N between fungus and plant occurs in the form of certain amino acids (Chalot & Brun, 1998; Chalot *et al.*, 2002). Thus, even in the case of mycorrhizal uptake of N, plants are likely to need amino acid transporters in order to utilize this nitrogen.

Amino acid transport

Plants have been found to possess a plethora of amino acid transporters, and genomic analyses indicate that the *Arabidopsis* genome may encode as many as 53 different amino acid transporters (Wipf *et al.*, 2002b). This large abundance of transporters may give the impression of a large functional redundancy in amino acid transport. However, amino acids are central metabolites filling many different functions in plant cells. Moreover, amino acids constitutes the "nitrogen currency" of many plants and make up the compounds by which most of the N is translocated in plants. The central role of amino acids calls for a dynamic transport system capable of gratifying the needs of different tissues and organelles in various situations and developmental stages. Accordingly, data on the properties, regulation and expression patterns of different transporters suggest that the great abundance of transporters is indeed a necessity, rather than a redundancy (Fischer

et al., 1998; Williams & Miller, 2001). As a consequence, only a fraction of the identified transporters may be expected to take part in the acquisition of amino acids from the soil.

The *Arabidopsis* genome contain putative and identified plant amino acid transporters that can be classified in 3 superfamilies; the APC (Amino acid-Polyamine-Choline) transporter family, the ATF1 (Amino acid Transporter Family) and the MFS (Major Facilitator Superfamily) (Wipf *et al.*, 2002b). So far, however, only members of the two former have been characterized. And while only one plant transporter, the AtCAT1, from the APC superfamily has been characterized (Frommer *et al.*, 1995), an abundance of transporters have been identified and characterized from the ATF1. Plant transporters in the ATF1 belongs in either of 5 sub-families; the amino acid permeases (AAPs), the lysine-histidine transporters (LHTs), putative auxin transporters (AUXs), the proline transporters (ProTs) or the aromatic and neutral amino acid transporters (ANTs) (*e.g.* Ortiz-Lopez, Chang & Bush, 2000).

Most characterizations of amino acid transport have suggested that the transporter mechanism is driven by proton symport (*e.g.* Soldal & Nissen, 1978; Kinraide & Etherton, 1980; Li & Bush, 1991; Boorer *et al.*, 1996). Indeed, in a recent, thorough study of AtAAP transporters, these carriers are also shown to utilize proton symport, in which the uncharged forms of the amino acids are transported in association with one proton (Fischer *et al.*, 2002). These results do, however, not preclude the possibility that other transporters, fulfilling other tasks in cellular transport may utilize other transport mechanisms.

While amino acid transporters generally exhibit broad substrate specificities, some carriers have been shown to transport only a limited spectrum of amino acids (Table 1). Thus, while AAPs and ANT1 show broad substrate specificities as regarding to amino acids (Fischer *et al.*, 1995; Montamat *et al.*, 1999; Neelam *et al.*, 1999; Tegeder *et al.*, 2000; Chen *et al.*, 2001; Fischer *et al.*, 2002), ProTs, AtLHT1 and AtCAT1 apparently transport a more narrow range of substrates (Frommer *et al.*, 1995; Rentsch *et al.*, 1996; Chen & Bush, 1997; Breitkreuz *et al.*, 1999; Schwacke *et al.*, 1999; Ueda *et al.*, 2001). An implication of the broad substrate specificities of several transporters is that the *in vivo* transport activity should be largely determined by the relative amino acid concentrations at the site of transport.

The multitude of amino acid transporters found in the *Arabidopsis* genome suggests a diversity of functions for the distinct carriers. Accordingly, expression patterns of the so far investigated transporters appears to be widely varying. Although there is yet no evidence of any specific amino acid transporter being responsible for the uptake by roots, molecular and biochemical data of amino acid carriers nevertheless supply the functional background for such uptake. These data indicate that amino acid transporters exhibiting high affinity and broad substrate specificity are expressed in root tissue of plants, potentially capable of mediating an uptake from the rhizosphere. However, while transporter expression in root epidermal or cortex cells is certainly a prerequisite for a function as a rhizosphere

Table 1. Identified and characterized amino acid transporters in various plant species. At: *Arabidopsis thaliana*, Rc: *Ricinus communis*, Le: *Lycopersicon esculentum*, Vf: *Vicia faba*, Na: *Nepenthes alata*, Ps: *Pisum sativum*, Hv: *Hordeum vulgare*. Aa denotes amino acids. R: root, S: stem, SoL: source leaves, SiL: sink leaves, Fl: flowers, Fr: fruits. ?: not determined. v: vascular tissue.
References: 1: (Frommer *et al.*, 1995), 2: (Marvier *et al.*, 1998), 3: (Bick *et al.*, 1998), 4: (Neelam *et al.*, 1999), 5: (Fischer *et al.*, 1995), 6: (Schulze, Frommer & Ward, 1999), 7: (Rentsch *et al.*, 1996), 8: (Montamat *et al.*, 1999), 9: (Schwacke *et al.*, 1999), 10: (Tegeder *et al.*, 2000), 11: (Chen *et al.*, 2001), 12: (Chen & Bush, 1997), 13: (Bennett *et al.*, 1996), 14: (Fischer *et al.*, 2002), 15: (Ueda *et al.*, 2001).
In addition to transporters mentioned in table, LeProT2 and LeProT3 have been identified but not characterized in tomato (Schwacke *et al.*, 1999), as has PsAAP2 in pea (Tegeder *et al.*, 2000). Furthermore, Potato (StAAP1 and 2) and tobacco (NtAAP1) transporters are recorded in databases (*c.f.* Williams & Miller, 2001). Similarly, AtAAP7, AtAAP8 and OsProT are recorded in databases (Ueda *et al.*, 2001; Wipf *et al.*, 2002b).

Transporter	Substrate specificity	Expression						Refs
		R	S	SoL	SiL	Fl	Fr	
AtAAP1	Neutral aa	-	-	+	-	+	+	14
AtAAP2	Acid, neutral aa	v	v	v	-	-	-	14
AtAAP3	Acid, neutral, basic aa	+	-	+	-	-	-	5
AtAAP4	Acid, neutral aa	-	+	+	-	-	-	5
AtAAP5	All aa except aromatic	+	+	+	-	+	+	5
AtAAP6	Acid, neutral aa	+	-	-	+	-	-	14
AtProT1	Pro	+	+	+	+	+	+	7
AtProT2	Pro	+	+	+	+	+	-	7
AtANT1	Neutral and aromatic aa	-	+	+	?	+	?	11
AtAUX1	Putative auxin, aa transporter	+	?	?	?	?	?	13
AtLHT1	Lys, His	+	+	+	+	+	+	12
AtCAT1	Basic aa	v	+	v	-	+	+	1
RcAAP1	All aa + dipeptides	+	?	-	-	?	?	2
RcAAP2	Not determined	+	?	-	-	?	?	3
RcAAP3	Acid, neutral, basic aa	+	?	+	+	?	?	4
LeProT1	Pro, GABA	-	-	-	-	+	-	9
VfAAP2	Acid, neutral aa	-	+	+	+	+	-	8
VfAAPa	Not determined	-	+	+	-	+	-	8
VfAAPb	Not determined	-	-	-	-	+	+	8
VfAAPc	Not determined	-	-	+	+	+	-	8
NaAAP1	Not determined	Mostly in pitchers and petioles						6
NaAAP2	Not determined	Mostly in pitchers and petioles						6
NaAAP3	Not determined	Mostly in pitchers and petioles						6
PsAAP1	Acid, neutral, basic aa	+	+	+	-	+	+	10
HvProT	Pro	+	?	-	?	?	?	15

amino acid scavenging system, it is by no means a guarantee for such a function as these transporters may serve a variety of purposes, such as cell-to-cell transfer and xylem loading.

Transport of other organic N compounds
Uptake of exogenous N-heterocyclics has been shown in algae, aquatic plants, plant pollen and roots (Knutsen, 1972; Nakashima & Tsudzuki, 1976; Kamboj & Jackson, 1985; Desimone *et al.*, 2002). Thus, it is not surprising that plants, in addition to amino acid transporters, also have been shown to possess carriers mediating transport of a range of other organic N compounds. Transporters of N-heterocyclics from 3 distinct families have been identified in plants.

First, AtPUP1 (purine permease) belongs to a large family of transporters (AtPUP1-15 can be identified using genomic analysis). AtPUP1 was shown to be expressed throughout the plant except in roots and was shown to transport *e.g.* adenine and cytosine, but also the cytokinin zeatin and alkaloids such as caffeine with high affinity (Gillissen *et al.*, 2000). Transport occurred against a concentration gradient, was pH-dependent and sensitive to the prescence of protonophores, indicating that AtPUP1 is a secondary active transporter.

AtUPS1 (ureide permease) is a member of a family of transporters containing at least 5 members in *Arabidopsis*. AtUPS1 mediates uptake of oxo-derivatives of N-heterocyclics, such as allantoin, uric acid and xantine, the former with a K_m of about 50 µM. As in the case of PUP1, uptake exhibited the characteristics of a secondary active transporter. However, AtUPS1 also contain a "Walker A" motif in its large cytosolic central domain, indicating that the protein may constitute a primary pump of its substrates. The expression pattern of AtUPS1 suggests that it may be active in root retrieval of N-heterocyclics from the rhizosphere (Desimone *et al.*, 2002).

The maize transporter protein ZmLPE1 (leaf permease) belongs to the large plant transporter family NAT (Nucleobase-Ascorbate Transporter). Kinetic analyses of ZmLPE1 indicate that it is a highly specific, high-affinity transporter for uric acid and xantine, probably functioning as a proton symporter (Argyrou *et al.*, 2001). In addtion to these transporters, a putative nucleoside transporter, AtENT1, has also been cloned in *Arabidopsis* (Li & Wang, 2000). This transporter has, however, not been characterized in depth.

It has been known for some time that plants are capable of transporting peptides (Sopanen, Sinervo & Ahokas, 1985; Jamai, Chollet & Delrot, 1994). Subsequently, peptide transporters have been identified in *Arabidopsis* (Steiner *et al.*, 1994; Rentsch *et al.*, 1995; Song *et al.*, 1996), *Hordeum vulgare* (West *et al.*, 1998) and in the carnivorous plant *Nepentes alata* (Schulze, Frommer & Ward, 1999). The PTRs (peptide transporters) seemingly function as carriers of small peptides (up to 6 aa in length) and show broad substrate specificity. Kinetic parameters vary depending on the size and composition of the peptide, but the transporters exhibit high affinity to short hydrophobic peptides. Interestingly, the AtPTR2-B has been shown capable to transport nitrate in addition to peptides.

One study indicated relatively high expression of the AtPTR2-B throughout the plant (Song *et al.*, 1996), although this was contradicted in another study (Frommer, Hummel & Rentsch, 1994).

The transport of heterocyclics and peptides may serve many functions, for example in seed germination, where large amounts of N is translocated from seed protein stores. The prescence of these transporters does, however, provide a functional capability for a controlled plant uptake of these N-compounds from external sources.

Mycorrhizal N uptake

As mentioned earlier, N uptake from nitrate, ammonium and amino acids by arbuscular mycorrhiza has been shown (*e.g.* Johansen, Jacobsen & Jensen, 1994; Hawkins, Johansen & Eckhardt, 2000). The mechanisms behind this uptake is, however, poorly studied. Similarly, studies of the molecular and biochemical background of N uptake by ericoid mycorrhiza is lacking. In contrast, the functional background of N uptake by ectomycorrhizal fungi has recently been examined in a series of studies.

Ammonium transport by the ectomycorrhizal (EM) fungus *Paxillus involutus* has been characterized using ^{14}C-labeled methylamine, an ammonium analogue (Javelle *et al.*, 1999). This study indicated a proton-gradient dependent transport exhibiting a half-saturation constant (K_m) of 180 µM and a V_{max} of 22.8 mmol g^{-1} dry weight h^{-1}. Studies using methylamine should be considered with caution, however, as the uptake of this compound may exhibit differences from uptake of native ammonium (Sohlenkamp *et al.*, 2000). In addition to the studies of *Paxillus*, two ammonium transporters, HcAMT2 and HcAMT3, have been identified in the EM fungus *Hebeloma cylindrosporum* (Javelle *et al.*, 2001). These transporters were characterized by functional complementation of yeast and found to serve as carriers with overlapping affinities (AMT2 exhibiting high-affinity and AMT3 with lower affinity) towards methylamine.

Amino acid transport by the EM fungus *Paxillus involutus* (Chalot *et al.*, 1996) exhibited a broad substrate specificity towards amino acids and a K_m between 7 and 27 µM depending on amino acid. Transport was shown to be pH-dependent and sensitive to protonophores. Similarly, an amino acid transporter with exhibiting high affinity towards a wide range of amino acids was identified and dubbed AmAAP1 in *Amanita muscaria* (Nehls *et al.*, 1999). In *Hebeloma cylindosporum* , the general amino acid transporter HcGAP1 was found to mediate uptake of a broad spectrum of amino acids using a secondary active uptake mechanism (Wipf *et al.*, 2002a). The two latter transporters were speculated to function in amino acid uptake and retrieval from the soil.

Controlling N uptake

Due to the central role of nitrogen in plant metabolism, uptake and assimilation of N are processes subject to a complex and strict regulation by a range of factors connected to plant N and C status (*e.g.* Lejay *et al.*, 1999; Coruzzi & Bush, 2001; Glass *et al.*, 2002; Stitt *et al.*, 2002, Figure 6). Although recent research has improved the knowledge of N uptake regulation, an integrated picture is still lacking, especially concerning the regulation of organic N uptake.

Among the 7 so far identified high-affinity nitrate transporters in *Arabidopsis*, the AtNRT2;1 and AtNRT2;2 have been suggested to constitute the inducible transporter system, while the AtNRT2;3 through 2;6 display features corresponding to cHATS (Glass *et al.*, 2001). Studies of *Hordeum* NRT2s indicate that the HvNRT2;1 through NRT2;4 function as inducible HATS. The iHATS is recognized as being inducible following nitrate resupply after N-starvation (Aslam, Travis & Huffaker, 1992; Kronzucker, Siddiqi & Glass, 1995). Although such an induction also applies to the cHATS, the induction of iHATS has been shown to be in the range of 30 times higher (*c.f.* Crawford & Glass, 1998). In addition to up-regulation in response to N resupply, the iHATS is subsequently down-regulated after a short time of induction and by improved N status (Glass *et al.*, 2002).

The induction of nitrate uptake has been shown to be accompanied by a concurrent increase in NRT2 transcript. The rapid increase in transcript levels has, by the use of nitrate reductase (NR) mutants, been shown to be a direct effect of nitrate, as opposed to down-stream metabolites (Lejay *et al.*, 1999; Vidmar *et al.*, 2000b). The iHATS down-regulation has been proposed to be consequence of transcriptional down-regulation by glutamine (Zhou *et al.*, 1999; Vidmar *et al.*, 2000a) as well as to post-transcriptional down-regulation by endogenous ammonium (Kronzucker, Glass & Siddiqi, 1999; Fraisier *et al.*, 2000). Furthermore, some studies imply that light, possibly mediated by sucrose, up-regulates both transcript levels of NRT2 as well as nitrate uptake rates in *Arabidopsis*, tomato and tobacco (Lejay *et al.*, 1999; Ono, Frommer & von Wiren, 2000; Matt *et al.*, 2001).

Turning to ammonium uptake, AtAMT1;1 transcript increases dramatically as an effect of nitrogen starvation (Gazzarrini *et al.*, 1999; Rawat *et al.*, 1999). Moreover, high-affinity uptake has been shown to correlate strongly with transcript levels of AMT1;1 in *Arabidopsis thaliana* (Rawat *et al.*, 1999). In similar to a study of the tomato LeAMT1;1 (von Wirén *et al.*, 2000b), this study also suggested that levels of glutamine or a downstream metabolite was responsible for transcriptional control of AtAMT1;1. In *Arabidopsis*, AMT1;2 and AMT1;3 are also found in roots. While AMT1;2 does not exhibit changes in transcript levels due to N-starvation, AMT1;3 show a slight increase under these conditions (Gazzarrini *et al.*, 1999). In tomato, LeAMT1;2 is expressed in roots and show an increase in transcript abundance in response to resupply of N (von Wirén *et al.*, 2000b). The low-affinity ammonium transport system appears not to be subject to regulation (*c.f.* Glass *et al.*, 2001).

Although a wide range of amino acid transporters have been isolated and characterized, the knowledge of their regulation is rather poor. Amino acid uptake has been reported to be inversely correlated to plant N-status, and to be down-regulated by endogenous ammonium in *Pinus sylvestris* (Paper **II**). Moreover, in a study of *Chlorella* cells, uptake of amino acids was observed to be upregulated by the addition of glucose (Cho *et al.*, 1981). In another study, however, the addition of glucose or sucrose resulted in decreased uptake by sugarbeet leaf cells (Chiou & Bush, 1998). An investigation of *Lolium perenne* glycine uptake, glucose and sucrose were found to have a stimulating effect on uptake (Thornton, 2001), similar to the results found in paper **III**.

Such a discrepancy between results may be due to the wide array of transporters found in plants and their presumably different functions and regulations (Fischer *et al.*, 1998; Wipf *et al.*, 2002b). This is further illustrated by the concurrent up-regulation of ProTs and down-regulation of AAPs seen as a result of dessication in roots of *Hordeum* and *Arabidopsis* (Rentsch *et al.*, 1996; Ueda *et al.*, 2002). In addition to amino acid transporters, the expression levels of an allantoin transporter was observed to be up-regulated in response to substrate access and nitrogen starvation, while ammonium nutrition resulted in decreased transcript levels, possibly indicating a function of the transporter in the N nutrition of *Arabidopsis* (Desimone *et al.*, 2002).

Data on the regulation of N transporters in mycorrhizal fungi are scarce. However, the ammonium transporters HcAMT2 and HcAMT3 in the ectomycorrhizal fungus *Hebeloma cylindosporum* appears to be up-regulated by N-starvation (Javelle *et al.*, 2001). Similarly, amino acid transporter transcripts in *Amanita muscaria* seems to accumulate in response to N-depletion, although a basal expression remains even under rich N conditions (Nehls *et al.*, 1999).

Some reasons behind the difficulties of elucidating the regulation of N uptake may lie in the wide array of different transporters present in plant genomes, some possibly fulfilling other functions than N uptake from the rhizosphere. Possibly, different responses between global (whole plant) and local responses to N availability and uptake, as illustrated by root growth regulation by nitrate (Zhang *et al.*, 1999), may also add to the complex picture of N uptake regulation.

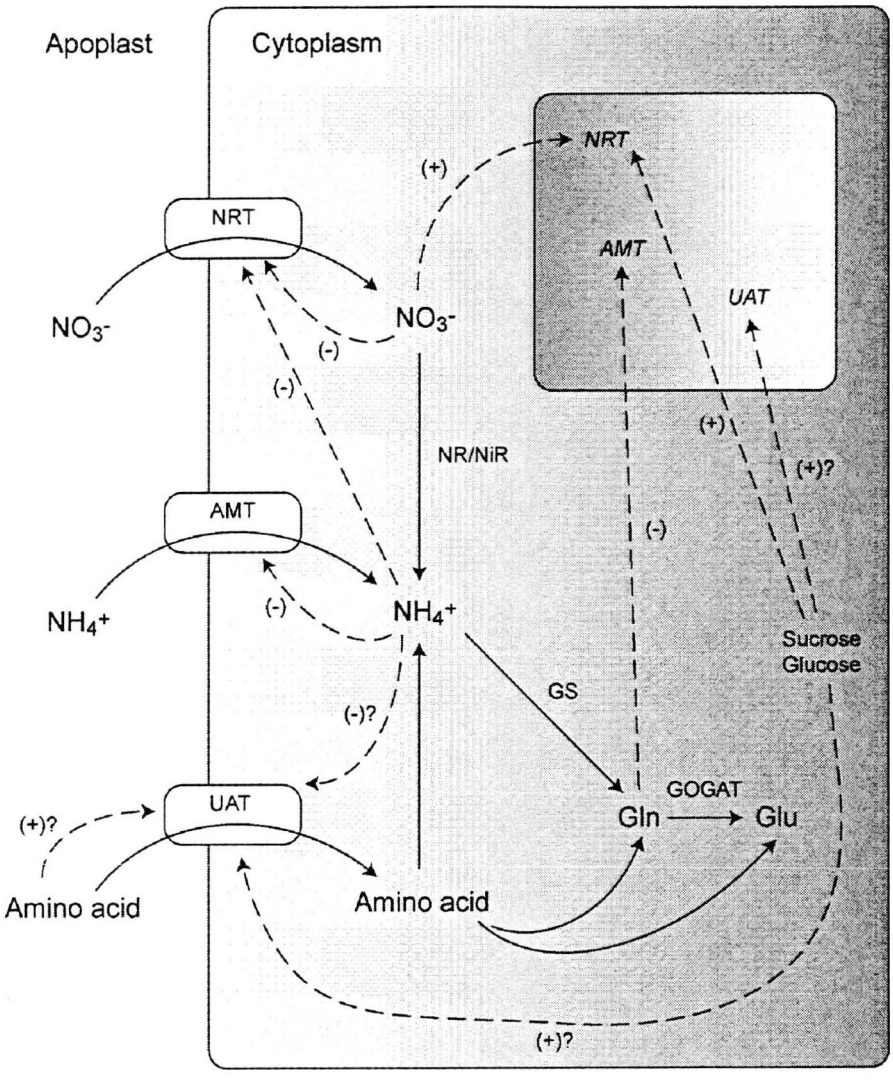

Figure 6. Schematic, simplified diagram over the regulation of nitrogen uptake mechanisms in root cells. Regulation of nitrate and ammonium transport has partly been compiled from Glass *et al.* 2002. Figure represents data obtained from studies on several plant species.

Abbreviations: NRT: high-affinity nitrate transporter, AMT: high-affinity ammonium transporter, UAT: unidentified amino acid transporter (when written in italics, these abbreviations represent transcripts of genes encoding the respective transporters). NR: nitrate reductase, NiR: nitrite reductase, GS: glutamine synthetase, GOGAT: glutamate synthase.

Plus-signs indicate an induction of activity or transcript abundance, while minus-signs indicate a suppression of activity or transcript abundance. Question-marks indicate a putative regulation.

Some methodological reflections

In order to elucidate the role of organic N for boreal forest N nutrition, we have employed a broad methodological approach, utilizing laboratory as well as field studies. This approach provides the relatively high precision obtained in the controlled conditions of the laboratory, while also bestowing the advantages of "real-world" hypothesis testing and relevance offered by field experiments.

In order to assess and follow uptake events we have chosen to use a combination of mass spectrometric techniques and isotopic labeling. Specifically, we have generally used compounds labeled with the stable isotopes ^{13}C and ^{15}N at all C- and N-positions (universally labeled), of which uptake has been analyzed using gas chromatography-mass spectrometry (GC-MS) or isotope ratio mass spectrometry (IRMS) (Box 1; Figure 7).

Box 1. Stable isotopes, gas chromatography and mass spectrometry

Stable isotopes
Elements can occur in several different varieties with different mass, depending on the content of neutrons in the atomic nucleus. Carbon is naturally present in 3 different forms: ^{12}C (98.9%), ^{13}C (1.1%) and ^{14}C (traces). Of these isotopes, the two former are stable, while ^{14}C is radioactive with a half-life of c. 5600 years. Nitrogen is present in the two forms ^{14}N (99.6%) and ^{15}N (0.4%). These varieties are naturally present in all materia in the relative amounts seen within brackets. By adding compounds "labeled" with an isotopic composition differing from the natural, these compounds and can be followed using *mass spectrometry*.

Gas chromatography
By the use of chromatography, different compounds can be separated by means of their differing physical properties, such as boiling point or polarity. In gas chromatography, compounds are separated mainly with respect to their boiling point and have to be converted into gas form in order to be analyzed. Compounds with high boiling points, such as amino acids, have to be converted into more volatile compounds by *derivatization*, in our case by adding chemical groups to reactive parts of the amino acids.

Mass spectrometry
A mass spectrometer is an instrument which measures the masses of individual molecules after converting them to ions, i.e. electrically charged molecules. The molecules can also be disintegrated, giving rise to fragmentation patterns specific for individual compounds. Thus, many compounds can be identified by analyzing their fragmentation pattern. As a mass spectrometer can differ between compounds with differing molecular masses, it can "see" the difference between identical compounds with differing isotopic composition, such as compounds labeled with stable isotopes.

While a "normal" MS measures a wide range of masses, an *isotope ratio mass spectrometer* (IRMS) concentrates on on the ratio between only a few masses, *e.g.* between ^{15}N and ^{14}N, leading to a higher sensitivity and lower detection limits.

Although this approach offers limited sensitivity in comparison to radiotracer techniques, it also offers a range of advantages. First, the possibility of labeling compounds at both N- and C-atoms offers the capability to trace *intact* compounds in plant tissues after uptake, by using separation techniques (in our case gas

chromatography) in combination with mass spectrometry. Second, the use of GC-MS allows for discrimination of different labeled compounds in a sample. Thus, it is possible to simultaneously follow uptake of several compounds. Third, this approach allows for the monitoring of the metabolic fate of different compounds after uptake. In addition, the use of GC-MS to quantitate isotopic enrichment of individual amino acids yields concurrent information about the content of the non-labeled species.

In paper **I**, a simple method for extraction, purification and analysis of amino acids by GC-MS was developed, offering a rapid routine for measurements of isotopic enrichments within single amino acids. Although being in the range of 2 to 3 orders of magnitude less sensitive in measurements of isotopic abundance, the purification and separation steps were found to largely compensate the lower precision, offering detection limits of individual universally labeled amino acids in plant extracts below 1 μM.

A problem in the use of labeled compounds to assess uptake, is metabolic conversions and subsequent losses of isotopic label due to respiration and excretion. Again, this problem concerns the use of ^{13}C to a higher extent than the use of ^{15}N. Large amounts of C is respired in roots (Desrochers, Landhausser & Lieffers, 2002; Högberg, Nordgren & Ågren, 2002), indicating that the analysis of ^{13}C in plant tissues after long incubation times may be misleading.

While IRMS offers a high precision analytical tool for the determination of isotopic ratios, the use of this technique in enrichment studies calls for some precautions in order to avoid misleading results. This is especially true in the use of ^{13}C, for which isotopic dilution is large due to the large background of endogenous ^{13}C, originating largely from structural C, in biological material (*c.f.* Näsholm & Persson, 2001). Unawareness of this problem may be one reason to the varying results regarding plant-mycorrhizal uptake of organic N which have been seen in studies using IRMS to detect uptake (Hodge *et al.*, 1998; Hodge *et al.*, 1999a; Näsholm, Huss-Danell & Högberg, 2000, 2001). Thus, whenever possible, such as in short-term uptake studies, it is advisable to reduce this background by extracting the metabolically active pool of carbon.

In several of the studies presented here, a mix of universally labeled amino acids, obtained from algal extractions, have been used to estimate uptake by different plants (Papers **II**, **III** and **IV**). This has been done in order to mimic natural situation where plant roots can be expected to face a wide array of different amino acids concurrently. The use of such a mix does, however, present limitations to what information that can be obtained. Most importantly, it hinders from observing the kinetic properties of uptake and, hence, prevents from drawing any conclusions concerning the mechanisms behind uptake.

Another source of error becomes obvious in field experiments where labeled compounds are added to the soil in order to follow uptake of isotopic label, as in paper **V**. As the soil already contain a (potentially large) pool of the unlabeled species of the same compound, the isotopic species is diluted and, as a

consequence, total uptake of the compound is underestimated. This source of error may, at least partly, be corrected for by measuring the native pool of the compound in soil before additions and correct uptake data accordingly.

In the physiological studies of uptake regulation, we have used enzyme inhibitors in order to stop metabolic conversions of certain compounds. While this is a common technique in biochemical and physiological studies, the results from such studies should be treated with caution, as the disturbance of central metabolic pathways may induce unpredictable consequences, unrelated to the desired effects. For example, the use of the glutamine synthetase (GS) inhibitor methionine sulfoximine (MSX) leads to elevated levels of ammonium in cells. While this may be a desired effect, high ammonium concentrations can be toxic and may thus lead to unwanted side effects. Moreover, it should be noted that some of the inhibitors used, *e.g.* MSX, aminooxyacetic acid (AOA) and azaserine (AZA) are amino acid analogues and may thus be transported by the same carriers as other amino acids, thereby potentially affecting uptake rates.

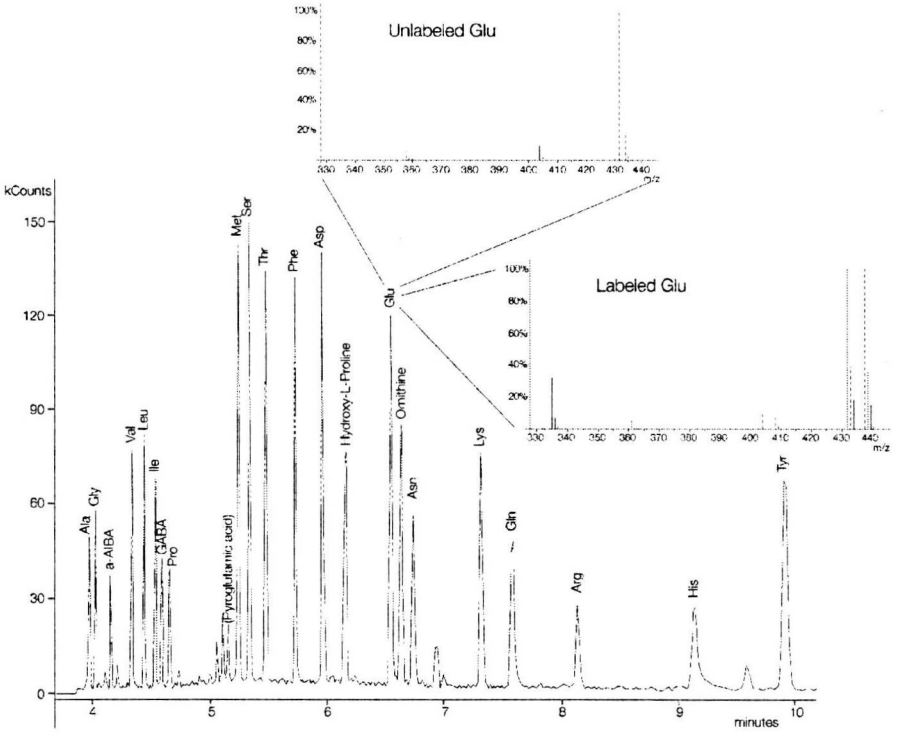

Figure 7. Representative chromatogram and mass spectra from GC-MS analysis of amino acids. The two distinct spectra show ion intensities from 100% unlabeled and 50% universally (U-^{13}C, ^{15}N) labeled glutamic acid, respectively. Y-axes in spectra indicate relative relative intensity (%) of base peak. Spectra are obtained using single ion source, isolating and detecting ions between m/z 325 and m/z 450.

Major results

As mentioned, a wide range of different plants have been shown to take up organic N, and transporters have been identified in several distinct plant species. And although no transporters have yet been identified in boreal forest plants, the large homologies between the fungal, animal and plant transporters identified so far indicate that the presence of these transporters is ubiquitous among eucaryotes (Fischer *et al.*, 1998; Wipf *et al.*, 2002b). As an accumulating amount of research show that different plant species are capable of taking up various types of organic N, several questions come to light. First of all, what is the function of such an uptake? Organic N uptake may function primarily as a way of the plant to acquire a precious resource from a wide variety of N sources in soil. Alternatively, uptake systems may function first and foremost in the retrieval of organic N lost from the root by leakage or exudation (Jones & Darrah, 1993, 1994) or have some other, unknown function.

The function of organic N uptake

Several lines of circumstantial evidence points to the function of organic N as an important nitrogen source for plants. First, the multiphasic uptake seen in several studies (*e.g.* Soldal & Nissen, 1978; Kinraide & Etherton, 1980; Borstlap *et al.*, 1986; Schobert & Komor, 1987) suggests that plants are adapted to adjust its amino acid uptake to the heterogenous situations of the soil environment. Second, the capacity of regulating uptake of some organic N compounds in response to the N- and C-status of the plant, as well as to substrate access suggests that these compounds do indeed serve as N sources for the plant (Desimone *et al.*, 2002, Papers II and III). Moreover, the high relative uptake of organic N as compared to inorganic N sources suggested in paper **V** as well as in other studies (Chapin III, Moilainen & Kielland, 1993; Kielland, 1994; Raab, Lipson & Monson, 1999; Schmidt & Stewart, 1999), implies that organic N may serve as an important source of N in several types of ecosystems.

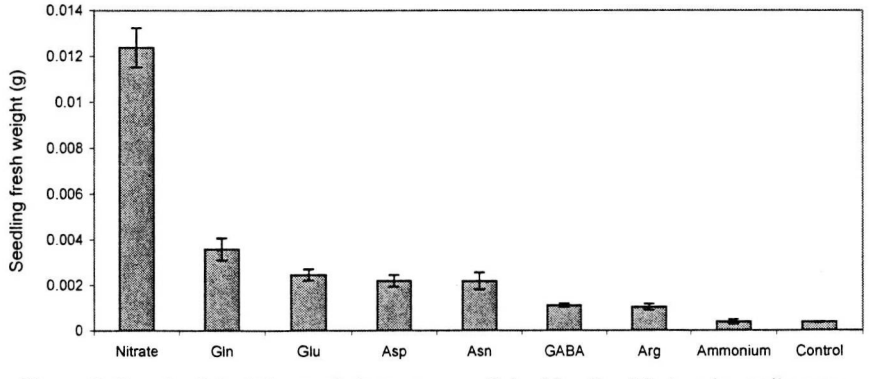

Figure 8. Growth of *Arabidopsis thaliana* ecotype Columbia after 20 days in sterile agar culture containing different nitrogen (N) sources. N was supplied at 3 mM in otherwise N-free MS medium (in 0.8% w/w agar without sucrose). Control was grown in N-free MS medium. Values presented are means. Error bars represent SE. $n = 4$. Data kindly supplied by Oskar Erikson (unpublished).

However, some results also challenge the function of organic N as a primary nitrogen source for plants. Most importantly, some studies have indicated relatively poor growth of non-mycorrhizal plants when supplied with organic N as the single source of nitrogen (*e.g.* Stribley & Read, 1980; Finlay, Frostegård & Sonnerfeldt, 1992; Turnbull, Goodall & Stewart, 1995; Desimone *et al.*, 2002; Erikson and Näsholm, unpublished; Figure 8). The low growth capacity of non-mycorrhizal plants on complex N-sources, such as the protein BSA (Finlay, Frostegård & Sonnerfeldt, 1992), is most likely related to an inability of degrading these compounds.

The slow growth observed on small organic N compounds appears to be unrelated to the capacity of taking up these compounds (Andersson, Ek & Söderström, 1997; Plassard, Bonafos & Touraine, 2000). This suggests that the growth bottleneck in these plants lies in a downstream event from uptake, such as transamination and/or further metabolism. This bottleneck may indeed be an effect of such experiments using a single amino acid as a source of N, rather than the mix of organic N sources that is more likely to occur in natural situations. The relatively good growth performance by non-mycorrhizal plants on glutamine, as compared to other organic N sources, seen in some of these studies may lend support to such an explanation (Turnbull, Goodall & Stewart, 1995, Erikson and Näsholm, unpublished, Figure 8). There may, however, be other reasons for slow growth despite an observed uptake, such as lack of hormonal signaling (*e.g.* Singh *et al.*, 1992, Marschner 1995 p. 565; Samuelson & Larsson, 1993; Wagner & Beck, 1993; Mercier *et al.*, 1997; Tanigushi *et al.*, 1998; Takei *et al.*, 2001) or, in some cases, poisonous effects of high concentrations of specific amino acids (T. Näsholm, pers. comm.). In addition to the varying growth success of non-mycorrhizal plants on organic N sources, the lack of relation between soil organic N content and uptake capacity found in one study (Paper **IV**) may also suggest that amino acid uptake is irrelevant for the N nutrition of plants.

Shaping ecosystems?

The second question that comes to mind when considering organic N uptake in boreal forests regards the extent of this capacity in these ecosystems and its effects on species composition. Or, to put it simply: Is the capacity to take up organic N common, and how does it affect competition between plant species in boreal forests? And while the answer to the first part of this question appears to be rather simple – yes, the second part is more complex.

In paper **IV**, a screening was performed in order to test how common the phenomenon of amino acid uptake is in boreal forest habitats. 31 specific species, exhibiting differing lifestyles and mycorrhizal associations, living in different habitats with varying amounts of amino acids in soil was tested with respect to amino acid uptake. In order to mimic natural conditions, a mix of 15 different amino acids was used for the uptake studies. The results show that all 31 species tested do, in fact, take up amino acids, irrespective of their associated mycorrhiza,

their nitrogen dependence (as measured by Ellenberg's nitrogen indicator values) or soil amino acid concentrations at the growth site. While uptake rates varied widely between species, no correlation could be found to either of the variables. It should, however, be noted that this study constituted a mere snap-shot of plant amino acid uptake, and that the uptake capacity of plants may vary considerably over time and varying conditions (*e.g.* Lipson & Monson, 1998), making it hazardous to draw too far-reaching conclusions from a single sampling occasion. Furthermore, the results from this study stand in contrast to other results suggesting a strong adaption of species growing in different habitats towards the most prevalent nitrogen source (Nordin, Högberg & Näsholm, 2001; McKane *et al.*, 2002).

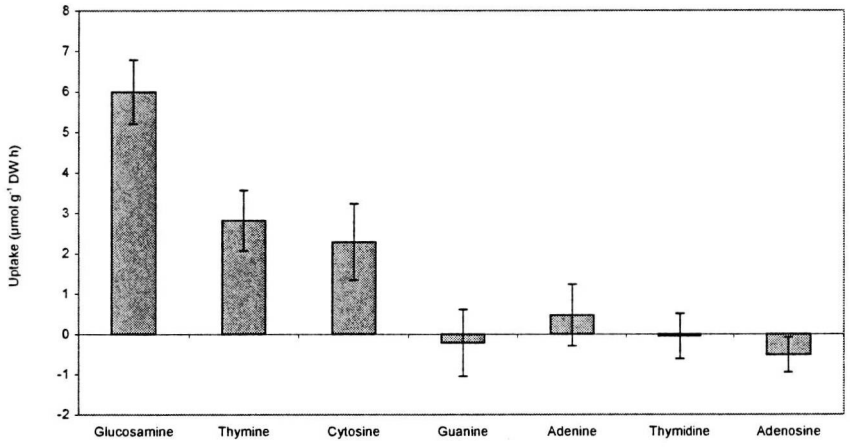

Figure 9. Uptake of a variety of organic N compounds by *Pinus sylvestris* seedlings. Uptake was measured by solution depletion and analyzed using HPLC (glucosamine, guanine, adenine thymidine and adenosine) or GC-MS (thymine, cytosine). Values presented are means ± SE (n = 3). Data presented are unpublished.

The results from paper **IV** confirm the large amount of other studies indicating that amino acid uptake is a common feature of plants (*e.g.* Chapin III, Moilainen & Kielland, 1993; Kielland, 1994, 1997; Näsholm *et al.*, 1998; Raab, Lipson & Monson, 1999; Schmidt & Stewart, 1999; Näsholm, Huss-Danell & Högberg, 2000). Moreover, these results suggest that uptake of amino acids is unrelated to the type of mycorrhizal associations the plant exhibits. This does not mean that mycorrhiza is unimportant for plant amino acid acquisition in the field, as the capacity of ecto- and ericoid mycorrhiza to release hydrolytic enzymes may be very important for the release of amino acids into soil solution. Moreover, the surface increase yielded by mycorrhizal connections (especially EM and AM) may be an important factor for amino acid retrieval, especially concerning basic amino acids, which are relatively immobile in soil (*c.f.* Lipson & Näsholm, 2001; Näsholm & Persson, 2001). However, these results do suggest that mycorrhizal infection is not, in itself, needed to take up amino acids.

The important function of ecto- and ericoid mycorrhiza for N-scavenging from complex or immobile sources was further illustrated in paper **V**, where plants associated with these types of mycorrhiza apparently had larger access to N from ammonium, glycine and a peptide mixture injected into the soil in a long-term (64 days) experiment. Similar to the results in paper **IV** and an earlier study by Näsholm *et al.* (1998), short-term uptake (after 6 h of incubation) in the same field experiment was similar for all 3 species tested (associated with AM, EM and ErM, respectively) disregarding of N source (nitrate, ammonium, glycine, arginine or the peptide mix).

In short, the results above suggest that the answer to the second question might be that the apparently ubiquitous capacity to *take up* amino acids may be of rather low importance in shaping species composition, while the ability of ecto- and ericoid mycorrhiza to release inaccessible N into soil solution may constitute a competitive advantage in N limited systems.

Assorted organic N sources

Another question concerns the ability of plants to take up organic N compounds from other sources than amino acids. The results from papers **II**, **III** and **IV** indicate that plants apparently are capable of taking up a wide range (up to 15) of different amino acids. And although the results from paper **V** concerns the ability to acquire N *from* a variety of sources of varying complexity, some results do

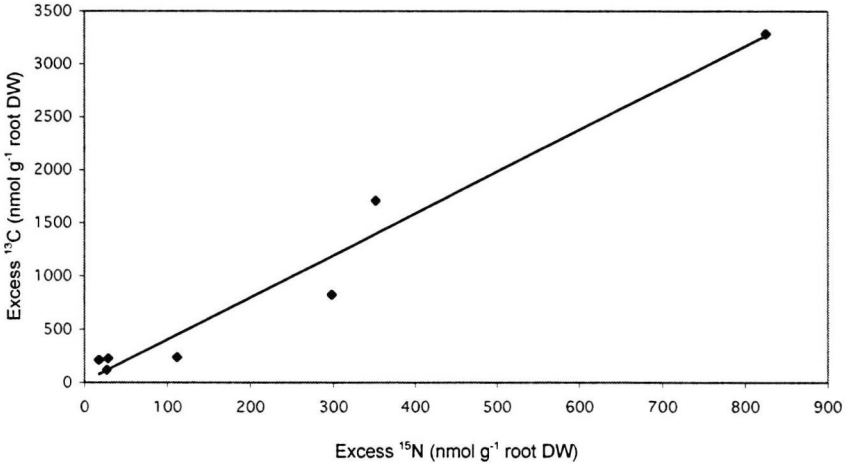

Figure 10. Excess ^{13}C plotted against excess^{15}N in roots of *Vaccinium myrtillus* after injections of universally (U-^{13}C, ^{15}N) labeled peptides into the rhizosphere. Samples were taken after 6 h. $y = 3.949x + 6.969$. $R^2 = 0.964$. The slope of the regression indicate the C:N ratio of the compounds taken up (not accounting for C losses due to metabolism). C:N ratio of the injected peptides was 4.68. Thus, these data suggest that at least 84% of the N taken up from the peptides was in the form of organic compounds (*c.f.* Näsholm *et al.* 1998).

suggest that at least one of the species from that study, *Vaccinium myrtillus* (Bilberry), may have the capacity to take up intact peptides injected into its soil environment (unpublished, Figure 10).

Moreover, some preliminary data (unpublished) from our lab also suggest that Scots pine (*Pinus sylvestris*) may have the capacity to take up the amino sugar glucosamine and the pyrimidines thymine and cytosine, while being unable to acquire purines and nucleosides (Figure 9). The ability of pyrimidine uptake may be of relatively low importance to plant N nutrition due to their low mobility and high tendency to form complexes in soil (Mengel, 1996). The importance of amino sugar and peptide uptake may however be large, as these compounds appears to constitute a relatively large part of the soluble nitrogen in soils (Mengel, 1996; Michalzik & Matzner, 1999; Yu *et al.*, 2002).

Not now – but now!

As nitrogen uptake and assimilation are central processes for plant metabolism, these processes are tightly regulated (Glass *et al.*, 2002; Stitt *et al.*, 2002). With this in mind, the regulation of amino acid uptake may contribute with important information about the role of such an uptake for the nitrogen nutrition of the plant. Hence, an important question in this context is how uptake is regulated in response to plant nitrogen and carbon status, as well as in response to substrate availability.

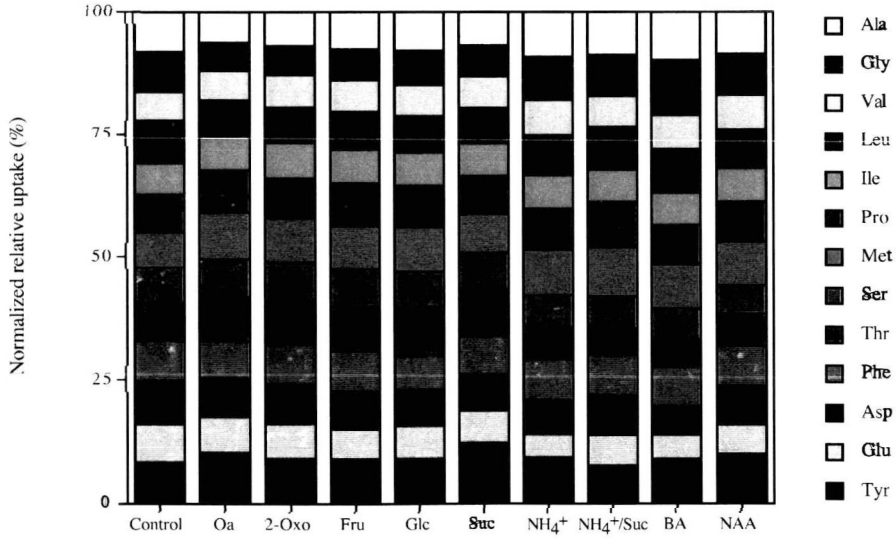

Figure 11. Relative uptake of individual amino acids by *P. sylvestris* after different pre-treatments. An entire bar indicate total uptake, while the coloured fields within the bar indicate the relative contribution to total uptake of each individual amino acid. Total uptake varied widely as an effect of pre-treatments (Paper **III**). Oa: oxaloacetic acid, 2-oxo: 2-oxoglutaric acid, Fru: fructose, Glc: glucose, Suc: sucrose, BA: 6-benzylaminopurine, NAA: 1-naphtaleneacetic acid.

In papers **II** and **III**, the regulation of amino acid uptake was investigated in non-mycorrhizal *Pinus sylvestris* plants. These studies suggest that uptake may be down-regulated by an improved plant nitrogen status and by endogenous ammonium levels, while treatments with sucrose, glucose and amino acids may lead to augmented uptake rates. The iHATS nitrate transporter system has been observed to be regulated by cellular ammonium, most likely by a post-transcriptional mechanism (Kronzucker, Glass & Siddiqi, 1999; Fraisier *et al.*, 2000). Moreover, it has been suggested that sucrose serves as a signal up-regulating nitrate uptake and transporter transcript abundance in several species (Lejay *et al.*, 1999; Ono, Frommer & von Wiren, 2000; Matt *et al.*, 2001).

Similarly, a study of *Chlorella* amino acid uptake indicated that glucose additions increased uptake rates of amino acids (Cho *et al.*, 1981) and in a study of *Lolium* glycine uptake, sucrose and glucose had favourable effects on uptake (Thornton, 2001). In paper **II**, pre-treatments with amino acids prior to the uptake assays also resulted in increased uptake rates. However, although these pre-treatments brought about dramatic increases in endogenous amino acid concentration, uptake did not correlate to the levels of any specific amino acid. This suggests that regulation may be an effect of sensing of outside amino acid concentrations, similar to the sensing of outside hexose or amino acid levels in some systems (Özcan, Dover & Johnston, 1998; Kim *et al.*, 2001).

While sucrose and glucose both increased amino acid uptake in paper **III**, fructose and the 2 central Krebs cycle organic acids oxaloacetate and 2-oxoglutarate had no effect on uptake, indicating that the regulation was not due to the addition of easily metabolizable substrates. As in the case of amino acid induction of uptake, the regulatory effect was also unrelated to concentrations of any of the two sugars. Thus, again, the regulation may be due to the flux of sucrose and glucose (or possibly only glucose, resulting from breakdown of sucrose) through some kind of sensor, such as a membrane transporter or a cytosolic protein, such as hexokinase, which has shown strong indications of having signaling functions (*e.g.* Jang *et al.*, 1997; Smeekens, 2000).

In papers **II** and **III**, the simultaneous uptake of at least 12 amino acids was recorded and total uptake was downregulated by almost 50% in one treatment (ammonium). The relative uptake of the specific amino acids, however, did not show any major variations, but exhibited a relatively strong co-variation (Figure 11). This suggests that the uptake system contains at least one transporter with broad substrate specificitiy, providing a "buffer" of uptake of the individual amino acids, concealing drastic variations in uptake by any presumed more specific transporters.

The regulation of amino acid transport in relation to plant C- and N-status suggests that the uptake of the compounds does, in fact, contribute significantly to plant N uptake and illustrate the potential importance of soil organic N to plant nitrogen nutrition. It is not impossible, however, that the regulatory patterns of amino acid uptake by *P. sylvestris* could by drastically altered following mycorrhization, which constitutes the naturally occuring state for these plants.

Is organic N feeding boreal forest plants?

As the potential of organic N as a nitrogen source for plants is becoming well accepted, the question of its importance in real-life situations inevitably arises. As is implied from the preceding text, actual field uptake of N is influenced by a wide variety of factors. Uptake capacity, soil fluxes and competitive capabilities, which are all in themselves affected by an array of variables, are important parts in deciding actual plant uptake of any compound.

Amino acid uptake appears to be a common feature among boreal forest plants (Papers **IV** and **V**). Thus, this basic requisite may be fulfilled in many cases. In order to utilize a resource effectively however, the capacity to regulate uptake in response to sudden changes in substrate availability and plant needs may be of large importance. Our studies indicate that at least one species in boreal forests, the ecologically important species *Pinus sylvestris*, appears to have the capacity to regulate its uptake of amino acids (Papers **II** and **III**).

Several studies have indicated that soil content of amino acids are, indeed, large in boreal systems, in many cases larger than the content of inorganic N (*e.g.* Näsholm *et al.*, 1998, Figure 12; Nordin, Högberg & Näsholm, 2001). Soil fluxes towards root (and mycorrhizal) surfaces have been suggested to be the most important factor controlling plant root uptake in N limited systems, such as the boreal forest (Leadley, Reynolds & Chapin III, 1997).

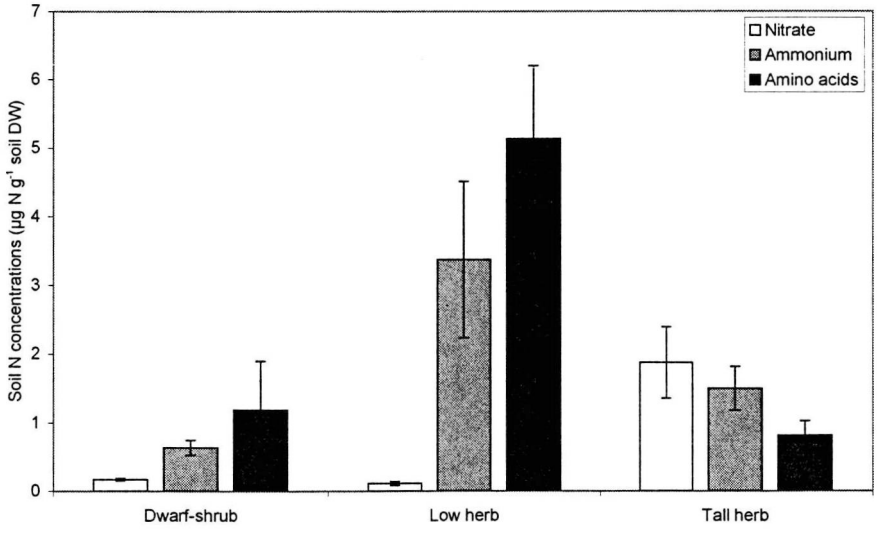

Figure 12. Soil content of nitrate, ammonium and amino acids in 3 different habitats of a boreal forest in north Sweden. Values presented are means ± SE. Figure re-drawn from Nordin *et al.* 2001.

The competitive capability of plants regarding organic N uptake has been lively discussed, and it has been suggested that plants are to be regarded as inferior competitors for organic N (*e.g.* Hodge *et al.*, 1999a; Hodge, Robinson & Fitter, 2000b; Owen & Jones, 2001). Some of these studies do, however, suffer from methodological shortcomings (see Some methodological reflections above). Moreover, other studies have, indicated that plants do indeed compete well for organic N, especially in the long-term perspective (*e.g.* Schimel & Chapin III, 1996; Näsholm *et al.*, 1998; Lipson *et al.*, 1999; Raab, Lipson & Monson, 1999; Paper **V**). The difficulty of separating mycorrhizal fungi from other microorganisms probably also lead to underestimation of plant N uptake in short-term field studies (*c.f.* Lipson & Näsholm, 2001; Näsholm & Persson, 2001).

Apparently, the major criteria for organic N to contribute significantly to plant N nutrition seems to be fulfilled. Taken together, available data – among that the results presented in this thesis - suggests that organic N, in particular amino acids, may be of large importance to plant nitrogen nutrition in many systems, among them boreal forests.

Conclusions and future objectives

The "traditional" view concludes that only inorganic N is available for plant uptake. An accumulating amount of results now indicate that current conceptual models of nitrogen uptake and cycling may have to be revided, as nitrogen fluxes appear to be more complex than earlier presumed. The consequences of plant organic N uptake as a possible major part of N fluxes in natural systems are far-reaching, not only from a theoretical perspective. Practical consequences may concern the way we consider important occurrences, such as N deposition and N fertilization as well as global warming and the role of N in carbon sequestration.

Although the recent decade have seen an increasing amount of research concerning the role of organic N for plant nutrition, there are large gaps of knowledge to fill, both in the field of plant physiology and in ecology. Among plant physiological aspects to be illuminated, the mechanism behind uptake is clearly in need of further research, as well as the regulation and variation in uptake capacities of different species. The fate of different organic N sources after uptake, and the effects of these on metabolism and plant performance also needs to be elucidated. Moreover, the interactions between plants and various types of mycorrhiza need to be further studied, primarily concerning both qualitative and quantitative aspects of N flow between symbionts.

Concerning the ecological consequences of organic N uptake, the overlying question naturally concerns the role of organic N in shaping plant communities. Within this question, short- and long-term competition in between plants as well as between plant and soil microorganisms needs to be studied. As does the

relative contribution of different N sources to the N nutrition of distinct plant species.

In conclusion, the potential importance of organic N in plant nutrition clearly merits further research from several angles of approach in order to elucidate its physiological, ecological and economical roles. Much remains to be done!

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Appendix list

The present thesis is based on the following papers.

Paper I. Persson, J. & Näsholm, T. 2001. A GC-MS method for determination of amino acid uptake by plants. *Physiologia Plantarum 113,* 352-358.

Paper II. Persson, J. & Näsholm, T. 2002. Regulation of amino acid uptake in conifers by exogenous and endogenous nitrogen. *Planta 215,* 639-644.

Paper III. Persson, J. & Näsholm, T. 2003. Regulation of amino acid uptake by carbon and nitrogen in *Pinus sylvestris. Planta, Accepted.*

Paper IV. Persson, J. & Näsholm, T. 2001. Amino acid uptake: a widespread ability among boreal forest plants. *Ecology letters 4,* 434-438.

Paper V. Persson, J., Högberg, P., Ekblad, A., Högberg, M.N., Nordgren, A. & Näsholm, T. XXXX. Nitrogen acquisition from inorganic and organic sources by boreal forest plants in the field. *Submitted.*

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A GC-MS method for determination of amino acid uptake by plants

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In this study, we present a rapid, robust and sensitive method for quantification of plant amino acid uptake using universally (U) (^{13}C, ^{15}N)-labelled amino acids and gas chromatography-mass spectrometry (GC-MS). Amino acids were analysed as their *tert*-butyldimethylsilyl (tBDMS) derivatives and displayed detection limits in the range 10–100 fmol on column, depending on the amino acid. The technique allows for simultaneous detection and quantification of both unlabelled and isotopically labelled species of amino acids. This makes simple quantification of plant amino acid uptake from an isotopically labelled source possible. The analytical variation was low, concerning total amino acid concentrations (relative standard deviation, RSD, less than 5.3%) as well as enrichment of U-^{13}C, ^{15}N-labelled glycine (Gly), arginine (Arg) and glutamic acid (Glu) (RSD < 2.1%). An application of the GC-MS method was conducted on non-mycorrhizal *Pinus sylvestris* roots supplied with U-^{13}C, ^{15}N-labelled amino acids. Intact, labelled amino acids were traced in root extracts. This provided conclusive evidence of plant root uptake of intact amino acids. Uptake rates of the three amino acids Gly, Glu and Arg in the range 0.5–37.9 μmol g^{-1} dry weight h^{-1} were recorded. These rates are comparable with those recorded in earlier studies of amino acid uptake, using other methods, as well as uptake rates measured for nitrate and ammonium.

Introduction

It has been known for a long time that plants are able to use amino acids as sources of nitrogen for growth and development (e.g. Virtanen and Linkola 1946, Melin and Nilsson 1953). A general problem when trying to estimate amino acid uptake is the difficulty of verifying the uptake of intact compounds. Amino acids are subject to biological as well as physical breakdown processes. Thus, the use of indirect methods or methods based on the tracing of single constituents of the amino acids may be inaccurate, since such methods may actually report on uptake of products resulting from the breakdown of the amino acid compounds. In this context, the use of dual-labelled, e.g. (^{13}C, ^{15}N), amino acids is essential. Recent research has, by the use of dual-labelled (^{13}C, ^{15}N) amino acid tracers and isotope ratio mass spectrometry (IRMS), studied amino acid nitrogen uptake by plant roots in both field and laboratory settings (e.g. Näsholm et al. 1998, Hodge et al. 2000, Streeter et al. 2000). These studies have, however, come to different conclusions as to whether plants can actually access intact amino acids, highlighting the need for alternative methods to study such uptake. In this context, IRMS techniques have some major shortcomings that limit the possibility of achieving unambiguous results. The most obvious problem is that of detecting ^{13}C from absorbed tracers. Thus, the large amount of endogenous ^{13}C in plants (ca 450 μmol g^{-1} root dry weight) leads to a very high dilution of absorbed C-tracer. Compared with ^{15}N, ^{13}C is diluted some 100–150 times more (Näsholm and Persson 2001). Moreover, although IRMS can give support for plant uptake by simultaneous measurements of ^{13}C and ^{15}N, detection of intact amino acids is not possible and thus it cannot provide conclusive evidence that uptake of intact compounds has occurred.

In order to overcome these problems we have developed a method for the simultaneous quantification of amino acid content and isotopic enrichment using gas chromatography-mass spectrometry (GC-MS). The applicability of this method was tested in a minor study of amino acid uptake by non-mycorrhizal *Pinus sylvestris* seedlings.

Materials and methods

Amino acid derivatization

Amino acid solutions were evaporated to dryness and then re-dissolved in 40 μl of N,N-dimethylformamide (DMF). The dissolved amino acids were derivatized to their *tert*-butyldimethylsilyl (tBDMS) derivatives using 10 μl N-

methyl-N-*tert*-butyldimethylsilyl-trifluoroacetamide (MTBSTFA) as the derivatization agent (Mawhinney et al. 1986, Woo and Lee 1995). Vials were then heated in 70°C for 25 min and allowed to cool to room temperature prior to analysis. All derivatization steps were conducted under a stream of dry argon gas. The resulting amino acid derivatives were subsequently analysed by GC-MS. Internal standards (α-aminoisobutyric acid and hydroxy-L-proline) were added to the samples prior to evaporation.

Amino acids, amino acid standards and the derivatization agent were purchased from Sigma-Aldrich, St Louis, MO, USA.

GC-MS analysis and quantification of amino acids

GC-MS was performed using a Varian 3800 gas chromatograph connected to a Varian Saturn 2000 ion trap mass spectrometer. GC separation of amino acids was carried out on a 30-m Chrompack CP-Sil 5 capillary column.

Samples were introduced by split injection using a Varian 1079 injector set at 270°C. Split settings generally varied between 30:1 and 50:1, depending on amino acid concentrations. GC oven temperature gradient was programmed at an initial 130°C, holding for 2 min followed by a ramping at 30°C min^{-1} up to 290°C, holding for another 6 min. The GC-MS interface was held at a temperature of 270°C and the ion trap was kept at 250°C.

Mass spectra were collected using electron impact (EI) ionization at 70 eV. The selected ion source (SIS) mode was used, trapping an individual range of ions for each amino acid analysed. Thus, mass spectra collected also exhibited a mass range specific for each amino acid. All spectra acquired were within the m/z range 200–510.

Quantifications were performed by the use of two internal standards (α-aminoisobutyric acid and hydroxy-L-proline) to which a 10-point calibration curve between 10 and 1 000 μ*M* had been established for each of the analysed amino acids. For compounds present in two isotopic species, quantifications were made from the mass fragment peak areas of both species, being m/z 218 and 220 (glycine, Gly), m/z 432 and 438 (glutamic acid, Glu) and m/z 442 and 451 (arginine, Arg). All other amino acids were quantified using the mass fragment from the unlabelled species. For Gly, where the mass peak from the labelled amino acid coincided with a peak resulting from natural abundance of the isotopes, the natural abundance was subtracted prior to quantification.

During analyses, standards were run in an interval of known concentrations in order to verify the reproducibility of the calibration curves.

Quantification of U-^{13}C, ^{15}N amino acid enrichment

Amino acid standards enriched with different proportions (0, 20, 40, 60, 80 and 100%) of universally (U) ^{13}C, ^{15}N-labelled Gly, Arg or Glu (i.e. amino acids containing > 98% ^{13}C and ^{15}N at all carbon and nitrogen positions), were used to calibrate the method for isotopic enrichment of the individual amino acids.

Quantification of isotopic enrichment was calculated using the formula:

$$C_l = (A_l - A_{na})/(A_l - A_{na} + A_u) \times C_{tot}$$

where C_l represents the concentration of the U-^{13}C, ^{15}N amino acid (M); A_l equals the area of the U-^{13}C, ^{15}N peak (counts × min); A_{na} denotes the area of natural abundance from unlabelled compound (counts × min); A_u equals the area of unlabelled (^{12}C, ^{14}N) peak (counts × min); and C_{tot} represents the total concentration of the amino acid (M).

To provide method evaluation, GC-MS analysis of an amino acid standard enriched with an unknown amount of U-^{13}C, ^{15}N Glu was compared to results from continuous flow-isotope ratio mass spectrometry (CF-IRMS) analyses of the same standard sample. IRMS results were obtained in atom% and recalculated to concentrations. CF-IRMS on ^{13}C and ^{15}N was performed on an Europa Scientific, Europa 20-20 stable isotope analyser.

Plant material

Pinus sylvestris (L. Karst.) seeds were surface sterilized for 20 min using 15% H_2O_2 in sterile water. Sterilized seeds were washed in sterile water and transferred to 1-l pots containing sand, sterilized at 130°C for 96 h. The pots were placed in a greenhouse under 24 h light, 20/18°C (day/night), being watered every 24 h until germination.

From 2 weeks after germination, the seedlings were supplied with either of two fertilizer treatments, adding nitrogen at 0.2 or 10 m*M*. Nitrogen forms added were equal parts of NO_3^-, NH_4^+, Gly, Arg and Glu. Other nutrients were supplied at supplemental concentrations to the nitrogen treatments in accordance with the industrial fertilizer Superba S™ (Hydro Agri AB, Landskrona, Sweden).

The sand was kept constantly moist and fertilization was conducted twice a week until harvesting the seedlings 17 weeks after germination. The relatively high nutrient additions were made in order to achieve rapid growth. Plants were harvested by gentle removal of the sand surrounding the root, followed by mild rinsing with water. Caution was taken to keep the entire root system of the seedlings intact and unharmed.

Amino acid uptake experiment

Immediately following harvest, the entire root systems of the seedlings were submerged in 7 ml of 1 m*M* amino acid solution, containing either of the U-^{13}C, ^{15}N-labelled amino acids Gly, Arg or Glu at pH 4.5. Controls were submerged in 7 ml of sterile water at pH 4.5. The relatively high concentration of the incubation solution was chosen in order to reveal differences in uptake rates between roots from differing fertilizer pretreatments in an environment where nitrogen availability was not limited.

The uptake experiment lasted for 20 min, after which seedlings were removed from the nitrogen solutions. Shoots were cut off and roots thoroughly washed three times for 4 min in 20 ml of 0.5 m*M* $CaCl_2$. After washing, roots were

immediately frozen in liquid nitrogen and subsequently stored at −22°C.

The efficiency of the $CaCl_2$ washing was tested by incubating dead, but not dry, roots in labelled compounds (Gly, Glu or Arg) as in the uptake study. Roots were then rinsed and samples taken from the rinsing solution after each of the three washing steps. These samples, as well as extracts from the roots, were then analysed for content of the labelled compound.

Amino acid extraction and purification

Root samples were milled in liquid nitrogen using a ball-mill. Soluble amino acids were extracted by shaking the pellet (the entire root material from each plant) in 0.01 *M* HCl for 45 min. The amount of root material used for extraction varied between 30 and 200 mg dry weight. The extracted pellet was dried and weighed. The resulting extract was purified for amino acids by solid phase extraction (SPE) using Alltech strong cation exchange cartridges, containing 200 mg ion-exchange resin. Subsequent to conditioning the cartridges with 10 ml of sterile water, 0.2–1.5 ml of amino acid extract was added and drawn through the cartridges at 0.5 ml min^{-1}. SPE columns were then washed with 2 ml of a methanol/water mixture (8:1). Amino acids were eluted using 2 ml of 4 *M* NH_4OH. Internal standard was added to the SPE-purified samples. The mixes were evaporated to dryness and subsequently derivatized as above.

The efficiency of the extraction and purification steps was tested by spiking milled samples with known amounts of U-^{13}C, ^{15}N amino acids (Gly, Glu or Arg). Subsequent to HCl extraction and SPE purification, the spiked samples were analysed for the labelled compounds. The efficiency of the extraction and purification steps was calculated as the percentage remaining compound of that added.

Chitin analysis

As a measure of root fungal content, and thus an indicator of mycorrhizal infection, chitin content in plant roots from all treatments was analysed using HPLC (Ekblad and Näsholm 1996).

Statistical analyses

Calibration data were made subject to linear regressions yielding calibration curves for the individual amino acids. Root amino acid content and uptake data as well as amino acid quantification and determination of isotopic ratios were made subject to analysis of variance (ANOVA) followed by Tukey's post hoc test.

Results

Method development

Gas chromatography gave rise to well-resolved single peaks for all 20 amino acids analysed as well as the internal standards α-aminoisobutyric acid and hydroxy-L-proline (Fig. 1). Due to temperature sensitivity, small fractions of glutamate and glutamine (Gln) were converted to pyroglutamic acid during sample preparation (cf. Woo and Lee 1995).

Mass spectroscopy gave rise to characteristic spectra for all analysed compounds (Gly, Glu and Arg are shown in Fig. 2). Fragmentation patterns were stable and quantification could be performed on single m/z fragments. Detection limits for amino acid standards, as well as isotopic enrichment of standard samples, were, by analysis, found to be between 10 and 100 fmol on column depending on the amino acid, equivalent to sample concentrations of 1 and 10 n*M*, respectively.

Calibration yielded linear responses between all amino acids and internal standards in the analysed interval 10–1 000 μ*M*. Multiple injections of single samples at different concentrations displayed only minor variation (RSD < 5.3%) due to MS quantification (Table 1). This was also true for analyses of differing isotopic enrichments (RSD < 2.2%; Table 2).

A comparative analysis between IRMS and GC-MS of an amino acid standard containing an unknown fraction of U-^{13}C, ^{15}N-Glu unveiled a good match between the two techniques, GC-MS quantification yielding a result of 0.95 m*M* ± 4.0% (mean ± RSD; n = 3), while CF-IRMS reported a result of 1.03 m*M* ± 3.8% (mean ± RSD; n = 6).

Results from the evaluation of the root-washing technique showed that the $CaCl_2$ rinsing was efficient in removing the labelled compounds from root surfaces. The amount of labelled compounds in the rinsing solution from the third washing was found to be 0–2.72 nmol, corresponding to a theoretical maximum of 0.54 nmol g^{-1} root dry weight. No labelled amino acids could be detected in analyses of extracts from the rinsed roots.

Recovery of amino acids after extraction and purification was 90.2 ± 5.4 (Gly), 90.7 ± 6.2 (Glu) and 71 ± 9.4 (Arg) (percentage of added amount amino acid ± RSD).

The effect of fertilizer pretreatment on amino acid content in *P. sylvestris* roots was large, giving rise to distinct differences in amino acid concentrations and composition (Fig. 3). Roots from the 10 m*M* pretreatment exhibited higher concentrations of all amino acids than roots from the 0.2 m*M* pretreatment. This effect was particularly evident concerning Arg, for which root concentrations were 1.8 and 38.1 μmol g^{-1} dry weight in the 0.2 and 10 m*M* pretreatments, respectively, asparagine (Asn) (0.4 and 15.0 μmol g^{-1} dry weight), serine (Ser) (0.9 and 6.8 μmol g^{-1} dry weight) and Gln (0.3 and 4.6 μmol g^{-1} dry weight, respectively) (Fig. 3).

Mass analysis of root extracts from *P. sylvestris* roots incubated in U-^{13}C, ^{15}N-labelled Gly, Glu or Arg solutions exhibited fractions of the labelled species of between 1.2 and 89.5% of the total root content of these compounds (data not shown).

Depending on the total root concentrations of Gly, Glu and Arg, these fractions corresponded to uptake rates between 0.5 and 37.9 μmol g^{-1} root dry weight h^{-1} (Fig. 4). Differences in uptake rates depending on amino acid as well as pretreatment were significant, showing higher uptake rates by roots from the 10 m*M* pretreatment than roots

from the 0.2 m*M* pretreatment. Roots from both pretreatments exhibited the same pattern of the highest uptake rates for Arg, followed by Gly and Glu, for which uptake was lowest during the 20-min incubation.

Chitin analyses of root samples showed a very low root chitin content (0.02–0.07% root dry weight), indicating negligible fungal infection (Ekblad and Näsholm 1996).

Discussion

Our results show that GC-MS of amino acid tBDMS-derivatives is a robust method for separation, identification and quantification of amino acids. This has also been established in earlier studies using this technique (e.g. Calder et al. 1999).

The relatively low detection levels found in this study (10–100 fmol on column), as compared with similar studies showing detection levels of some 30 pmol on column (Rhodes et al. 1981), are partly due to the ion trap technique used for mass analysis. This technique allows for a large analyte enrichment by consequently ejecting unwanted ions from the ion trap, trapping only ions wanted for analysis. For each compound eluted from the GC, different ions may be selectively trapped and analysed, as shown in this study. By doing this, signal-to-noise ratios can be drastically improved (Wells and Huston 1995).

Detection levels are, however, highly dependent on sample purity, since impure samples may provide a very large background. The root extracts used in this study exhibited practical detection levels of 0.1–0.5 μ*M* after purification by SPE. Due to the relatively high concentrations of amino acids in root extracts, a further lowering of detection levels was not considered critical. Clearly, detection levels are good in comparison to other quantitative techniques, e.g. HPLC in combination with fluorescence detection (Näsholm et al. 1987).

By analysing the different m/z peaks resulting from different molecular masses, identical compounds, differing only in isotopic composition, can be simultaneously quantified. This methodology allows for determination of the total amount of uptake of the intact molecule as well as quantification of the total pool of the compound, in this case different amino acids. This is illustrated by the results in this study as well as preliminary results from our laboratory, where uptake of 16 different U-^{13}C, ^{15}N-labelled amino acids has been quantified simultaneously.

Although the method for uptake studies presented here is applicable using singly labelled compounds, the use of dual labelled compounds offers special advantages. First, studies using singly labelled organic compounds cannot separate uptake of intact molecules from uptake of catabolites. Second, assessing the fraction of a labelled compound in a large pool of the non-labelled species is somewhat problematic

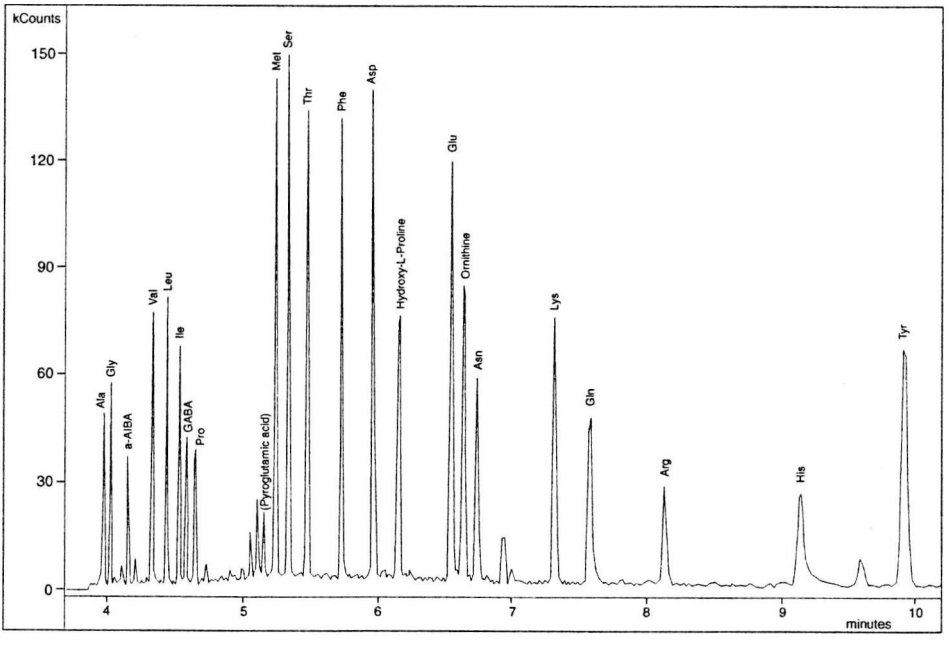

Fig. 1. Representative chromatogram from GC-MS analysis of amino acids. Chromatogram shown is a reconstructed ion chromatogram from MS analysis. a-AIBA denotes DL-α-aminoisobutyric acid, which, together with hydroxy-L-proline, was used as an internal standard.

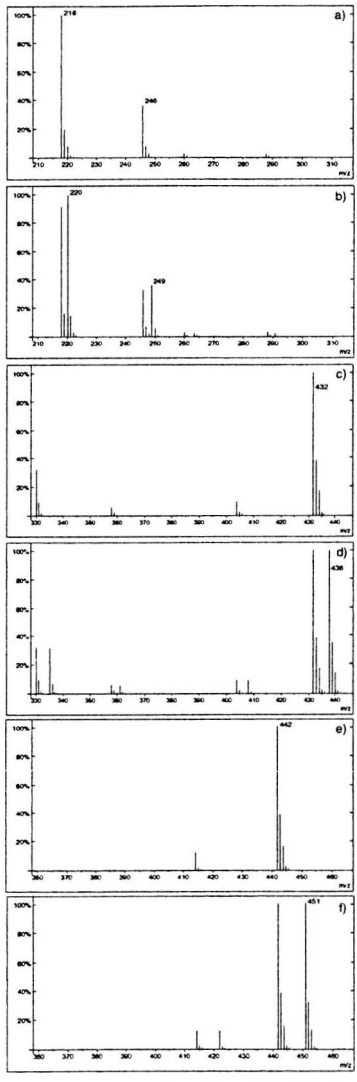

Fig. 2. Representative spectra from (a) unlabelled Gly, (b) 50% U-^{13}C, ^{15}N Gly, (c) unlabelled Glu, (d) 50% U-^{13}C, ^{15}N Glu, (e) unlabelled Arg, (f) 50% U-^{13}C, ^{15}N Arg. m/z denotes mass/charge. Scale is % relative abundance. Note that the m/z fragment from the U-^{13}C, ^{15}N Gly coincides with a m/z fragment resulting from natural isotopic abundance (m/z 220).

when using singly labelled substances. This is due to the natural background of especially ^{13}C and, in this case, ^{29}Si and ^{30}Si from the derivatization agent, which produces clusters of ions around (behind) each non-labelled ion (cf. Fig. 2). Thus, measurements of the isotopic label of singly labelled compounds are always hampered by a background, especially when trying to measure small amounts of labelled compounds in a large pool of the unlabelled species. When using dual labelled compounds, the isotopic shift is larger, placing the peak from the labelled compound outside the cluster of peaks resulting from natural background. Therefore, the precision of the isotopic analysis becomes higher and detection limits lower. Using this method, the detection limit for the labelled compound is equal to the detection limit for the amino acid itself, i.e. 10–100 fmol on column.

Table 1. GC-MS quantification of amino acid standards of known concentrations. Values presented are means in μM, numbers in brackets are RSD (%), n = 5.

Added concentration (μM)	Measured concentration (μM)		
	Gly	Glu	Arg
10	9.6 (2.2)	9.8 (2.3)	9.7 (1.0)
50	49.5 (4.8)	48.9 (2.5)	48.4 (5.3)
100	98.2 (3.1)	98.6 (2.7)	100.7 (3.3)
150	146.6 (1.5)	148.9 (1.3)	153.6 (1.7)
500	497.8 (0.9)	494.2 (1.9)	508.0 (0.4)

The major advantage with GC-MS is the ability to analyse the isotopic enrichment within individual molecules. This ability more than compensates for the relatively low precision in analysis of isotopic abundance, as compared to IRMS, for which the precision is two to three orders of magnitude higher than for GC-MS. In short-term uptake studies with isotopically labelled compounds, the label is present in only one or a few substances. Hence, high isotopic enrichment of a few compounds can be predicted, while the effect of uptake of dual labelled tracers on total levels (i.e. atom% or δ-values) of ^{13}C and ^{15}N may be relatively small. The GC-MS technique takes advantage of this condition, while IRMS techniques suffer from the limitation of a large ^{13}C and ^{15}N background.

Caution should however be exercised, since metabolic conversion and shoot translocation of the acquired compounds may give rise to a deception of apparently lower uptake rates than real. Hence, if long incubation times (many hours to days) are used, GC-MS is not an appropriate method for uptake quantification, especially if compounds exhibiting only partial isotopic labelling are used. The problem of metabolic conversion of acquired compounds is, however, not specific for GC-MS quantification

Table 2. GC-MS measurements of amino acid standards with known fractions of added U-^{13}C, ^{15}N Gly, Glu or Arg. Values presented are means, numbers in brackets are RSD (%), n = 5.

Added U-^{13}C, ^{15}N-amino acid (%)	Measured labelling (%)		
	Gly	Glu	Arg
0	0 (0)	0 (0)	0 (0)
20	18.4 (2.1)	17.7 (1.3)	19.0 (1.6)
40	38.8 (1.2)	38.9 (0.7)	39.0 (0.6)
60	56.6 (0.6)	58.6 (0.5)	57.3 (0.4)
80	79.6 (0.5)	77.7 (0.5)	81.6 (0.5)
100	99.0 (0.3)	99.4 (0.3)	99.7 (0.4)

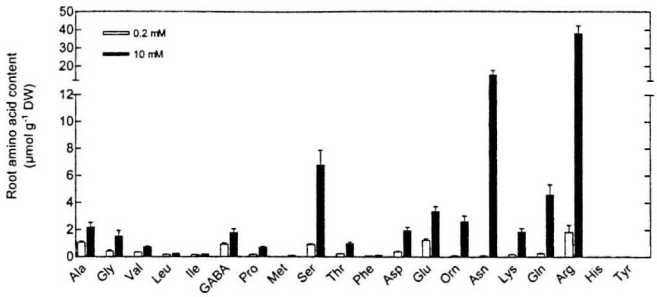

Fig. 3. Amino acid concentrations in SPE-purified 0.01 *M* HCl extracts of *P. sylvestris* roots. Values are means ± SE (n = 20). 0.2 and 10 m*M* are nitrogen concentrations in fertilizer pretreatments. Please note the broken y-axis.

of amino acid uptake. Any method dealing with measurements of carbon label is facing the problem of losses of labelled carbon as CO_2 with time, inducing underestimations of carbon label content (cf. Näsholm and Persson 2001). Metabolic conversion that does not remove labelled carbon from tissues, however, only affects GC-MS measurements, while IRMS results are not affected.

In this study, metabolic activities in the roots were stopped ca 35 min after the incubation started. No label was detected in either Ser, Gln, or ornithine as would have been expected if metabolic conversion of the added amino acids had occurred (cf. Miflin 1980). Schobert and Komor (1990) found that amino acids fed to roots of *Ricinus communis* appeared in the xylem sap after less than 20 min. Thus, shoot translocation of acquired amino acids is a possible source of uptake underestimation in our study.

The differing root amino acid content and composition between different pretreatments (0.2 or 10 m*M* N) confirm earlier studies, showing increasing relative levels of Arg and Gln in plants experiencing high nitrogen availability (Kim et al. 1987, Näsholm and McDonald 1990, Schneider et al. 1996). In addition, the current study displayed notably higher relative abundance of Asn in the high nitrogen treatment. This may be an effect of light (carbon) limitation (Durzan and Steward 1967, Gezelius 1986, Lam et al. 1996) increasing the levels of asparagine synthase, and thus the production of Asn from Gln (Lam et al. 1996).

The amino acid uptake rates demonstrated in this study are quite comparable to uptake rates observed for ammonium or nitrate (e.g. Gessler et al. 1998, Min et al. 2000). In addition, uptake rates perceived in this study are in good agreement with measurements made in earlier studies using other techniques (Schobert and Komor 1987, Jones and Darrah 1993, 1994, Wallenda and Read 1999).

The observed difference in uptake rates by roots from different fertilizer pretreatments could indicate the expression of transport systems with differing affinity and transport rates (cf. Fischer et al. 1998). The roots from the 0.2 m*M* treatment, being acclimated to relatively low levels of amino acids in soil solution, would be expressing high-affinity transporters with corresponding low maximum transport rates. Accordingly, roots from the 10 m*M* treatment would express high-rate, low-affinity transporter proteins.

We found significant differences in uptake rates between the three tested amino acids in this study. Arg was subject to the highest uptake rates, followed by Gly and Glu, for which uptake was the lowest. These differences may be an effect of differing affinities for different amino acids of the active amino acid transporters and/or a higher relative expression of basic/neutral transporters as compared to acid amino acid transporters. Extraction and SPE purification of spiked samples displayed a recovery of 71–91%, being lower for the basic amino acid Arg than for Gly and Glu. During calculation of root uptake, no compensation for losses has been made. Therefore, uptake rates, especially for Arg, should be considered low estimations.

The results presented here give evidence of non-mycorrhizal plant uptake of intact Gly, Glu and Arg. Uptake rates detected are in good agreement with earlier studies on other plant species, as well as being well within the range of uptake rates measured for ammonium and nitrate.

In conclusion, GC-MS provides a simple and powerful method for amino acid quantification. The method presented here is most suitable for short-term studies of amino acid uptake and metabolism, using isotopic tracers. Although IRMS is a more appropriate method than GC-MS for long-term experiments using labelled compounds, GC-

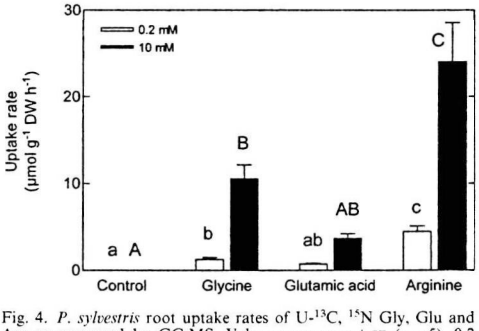

Fig. 4. *P. sylvestris* root uptake rates of U-^{13}C, ^{15}N Gly, Glu and Arg as measured by GC-MS. Values are means ± SE (n = 5). 0.2 and 10 m*M* are nitrogen concentrations in fertilizer pretreatments. All treatments differed significantly between fertilizer pretreatments (0.2 and 10 m*M* N). Different letters denote statistically different root uptake rates between amino acids (Gly, Glu or Arg; Tukey's post hoc test). Capitals are used for the 10 m*M* fertilizer treatment, lowercase letters for the 0.2 m*M* fertilizer treatment.

MS has the capacity to give structural information about the compounds of interest, thereby allowing for detection of intact labelled compounds and thorough studies of metabolic conversions and pathways.

Acknowledgements – Margareta Zetterström, Jonas Öhlund and Lena Dahlman made a huge contribution to this work with great assistance and good fun. We thank Dr Tomas Moritz for providing some very helpful suggestions on the manuscript. We would also like to thank two anonymous reviewers who have given well-needed opinions on the manuscript. This work was financially supported by grants to T.N. from the National Council for Forestry and Agricultural Research (SJFR) and the program of Abatement Strategies for Transboundary Air Pollution (ASTA) under the Foundation for Strategic Environmental Research (MISTRA).

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ORIGINAL ARTICLE

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Regulation of amino acid uptake in conifers by exogenous and endogenous nitrogen

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Abstract Although an accumulating amount of research clearly indicates that plants are capable of taking up exogenous amino acids, the actual importance of such organic N sources for plant N nutrition is under debate. In this study, we show that amino acid uptake by Scots pine (*Pinus sylvestris* L.) is significantly decreased by elevated internal NH_4^+ levels, while it increases following exposure to exogenous amino acids. Furthermore, amino acid uptake is larger in N-deficient plants than in plants grown with a large access of N. The regulatory pattern of amino acid uptake shows important similarities to the regulation of NO_3^- and NH_4^+ transport as well as to the regulation of yeast amino acid transporters. In addition, our data suggest that uptake may be regulated by factors not originating from N metabolism. The up-regulation of uptake in response to N deficiency suggests that amino acid uptake may be a significant contributor to the N economy of *P. sylvestris*.

Keywords Amino acid uptake · Nitrogen nutrition · *Pinus* (N nutrition) · Regulation (amino acid uptake)

Abbreviations CCCP: carbonyl cyanide *m*-chlorophenylhydrazone · Glu: glutamate · Gln: glutamine · NR: nitrate reductase

Introduction

Amino acids constitute a potentially important source of nitrogen (N) for plants, especially in areas where low temperature and/or pH makes mineralization processes slow, such as arctic and alpine areas, or the boreal forest ecosystems (Chapin et al. 1993; Näsholm et al. 1998; Raab et al. 1999). Accordingly, various studies add to the picture of a large array of plant species being capable of taking up exogenous amino acids at relatively high rates, both without, and in association with, mycorrhizal fungi of differing types (e.g. Kielland 1994; Lipson and Monson 1998; Schmidt and Stewart 1999; Persson and Näsholm 2001a). Moreover, a wide array of amino acid transporters has been identified in several different plants species, thereby providing the actual mechanisms behind amino acid uptake (e.g. Frommer et al. 1993; Montamat et al. 1999; Neelam et al. 1999; Zhou et al. 1999). The actual importance of amino acids and other sources of organic N for plant nutrition in the field is however under debate, since plants are generally considered to be inferior competitors for organic nitrogen (Lipson et al. 1999; Hodge et al. 2000; Owen and Jones 2001).

In order to optimize N uptake, plant uptake systems for NO_3^- and NH_4^+ are tightly regulated in complex interactions between external levels of the substrates and endogenous concentrations of differing N compounds (Glass et al. 2001). Although a large number of amino acid transporters have been identified and characterized, data on regulation of amino acid transport and uptake in plants are scarce. Since the regulation of uptake systems in response to plant N status and resource access may give important information on the actual role of amino acid uptake in plant N nutrition, we conducted a study following amino acid uptake by plants exposed to differing growth conditions and pre-treatments. As a model plant we used Scots pine, an ecologically and economically important plant of the boreal forest ecosystem.

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Materials and methods

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 and rinsed in sterile water. Seeds were sown in 3-l pots containing an autoclaved peat/sand mixture (50/50, v/v) and

allowed to grow in a greenhouse for 90 days under 24 h light at 20–25 °C. The substrate was kept continuously moist and from day 14, plants were fertilized twice a week using 100 ml Superba S fertilizer (Hydro Agri, Landskrona, Sweden) containing (% w/w): N 6.5 (of which NO_3^--N 4.0, NH_4^+-N 2.5); P 1.0; K 4.7; Mg 0.6; S 0.5; B 0.01; Cu 0.003; Fe 0.07; Mn 0.04; Mo 0.001; Zn 0.01. Fertilizer was added at an N concentration of 10 mM. All plants grew well and looked healthy. From day 91 up to harvest, half of the plants were deprived of N, but given all other nutrients in proportion to 10 mM N. The remaining plants were kept on the Superba S treatment. These treatments resulted in plants of differing N status. Plants were harvested at day 116–117 by gently removing the roots from the substrate, taking great care to keep the root system intact and unharmed. Roots were then cleaned and rinsed in 0.5 mM $CaCl_2$ in order to remove soil particles and desorb charged compounds from root surfaces.

All roots except controls (C) were immediately transferred to a solution containing 10 mM of NO_3^-, NH_4^+, glutamate (Glu), glutamine (Gln), an amino acid mixture (aa-mix) composed as the mix used in the uptake experiment (see below) or a nutrient solution without N (–N). In order to stop further metabolism of the added N sources, the pre-treatment solution contained combinations of inhibitors for key N-metabolism enzyme: tungstate (WO_4^{2-}), methionine sulfoximine, aminooxyacetic acid and azaserine (Brunk and Rhodes 1988; Garcia-Fernandez et al. 1995; Barthes et al. 1996; Oaks et al. 1998; Schmidt and Stewart 1999) (Table 1). Pre-treatments lasted for 6 h, after which roots were rinsed in 0.5 mM $CaCl_2$. Control roots were immediately transferred to the uptake experiment.

Incubation solutions contained a mixture of 12 universally (U-^{13}C, ^{15}N, >98%) labelled amino acids, composed as: alanine (Ala, 14.2%), glycine (Gly, 7.9%), valine (Val, 5.6%), leucine (Leu, 8.7%), isoleucine (Ile, 4.2%), proline (Pro, 4.4%), serine (Ser, 7.5%), threonine (Thr, 8.2%), phenylalanine (Phe, 4.5%), aspartic acid (Asp, 14.6%), Glu (15.3%) and lysine (Lys, 4.9%) (proportions of total amino acid concentration). The amino acid mixture was obtained from Cambridge Isotope Laboratories, Mass., USA. The incubation medium also contained supplemental amounts of all other plant nutrients. Roots from each plant were excised and split into five parts of which one was immediately frozen in liquid N and stored at –22 °C, while the remaining four parts were submerged in 50, 100, 200 or 400 µM amino acid mixtures held at pH 5.0 and 20 °C. Great care was taken to keep the root cuttings above the incubation solution surface. The uptake experiment lasted for 30 min, after which roots were rinsed three times in $CaCl_2$, frozen in liquid N and stored at –22 °C. After removal of roots, the incubation solutions were immediately frozen at –22 °C.

Passive amino acid uptake was controlled using the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Roots were pre-treated in a solution containing 50 µM CCCP and, subsequently, tested for amino acid uptake at 400 µM amino acid concentration as above.

Root amino acid uptake was recorded as solution depletion, and cross-checked by analyzing root extracts for labelled amino acids. Amino acid concentrations were analyzed by gas chromatography-mass spectrometry (GC-MS) as in Persson and Näsholm (2001b). Root concentrations of NO_3^- and NH_4^+ were determined on root extracts by flow injection analysis (FIA) using a Tecator 5012 (Foss Tecator, Sollentuna, Sweden). Root chitin content, as a measurement of root mycorrhizal status, was determined using HPLC as in Ekblad and Näsholm (1996).

Uptake data were subjected to linear regressions. Data were normalized with respect to amino acid concentration in the incubation solution and pooled within pre-treatments and N status for further analyses. Normalized uptake data, as well as endogenous levels of N compounds were subjected to ANOVA. All statistical analyses were performed using Statview 5.0 statistical software.

Table 1. N sources and combinations of inhibitors used in pre-treatments. N sources were added at 10 mM. All inhibitors were added at 1 mM. *n.a.* Not applicable, *–N* minus nitrogen, *aa-mix* amino acid mixture as specified in *Materials and methods*

Pre-treatment	Inhibitor	Enzyme inhibited
Control	n.a.	n.a.
–N	n.a.	n.a.
NO_3^-	Tungstate	Nitrate reductase
NH_4^+	Methionine sulfoximine	Glutamine synthetase
Glu	Aminooxyacetic acid	Aminotransferases
	Methionine sulfoximine	Glutamine synthetase
Gln	Azaserine	Glutamate synthase
aa-mix	n.a.	n.a.

Results

All roots generally took up all amino acids, at a rate depending on amino acid, external concentration, N status of the plant and pre-treatments. Total uptake rates (uptake of all amino acids added) varied between 0.63 and 13.5 µmol (g DW)$^{-1}$ h^{-1}. Uptake of all amino acids increased linearly with increasing external concentrations in the analyzed concentration range (50–400 µM; Fig. 1). CCCP treatment confirmed that amino acid uptake was mediated by active transport, and that passive uptake (background) was negligible in this context (45.9 ± 3.5 nmol (g DW)$^{-1}$; mean ± SE, $n = 3$). GC-MS analyses of root extracts quantitatively confirmed uptake data from depletion measurements (Fig. 2). Endogenous root amino acid concentrations were significantly ($P < 0.05$) higher in N-fertilized plants than in N-starved plants: 10.72 ± 0.61 and 3.77 ± 0.44 µmol (g DW)$^{-1}$, respectively (mean ± SE, $n = 2$; Table 2). All pre-treatments gave rise to substantially increased levels of the specific N compound added (Table 2). NO_3^--fed plants, however, also exhibited increased NH_4^+ concentrations, suggesting that the tungstate treatment did not lead to complete inhibition of nitrate reductase (NR) activity.

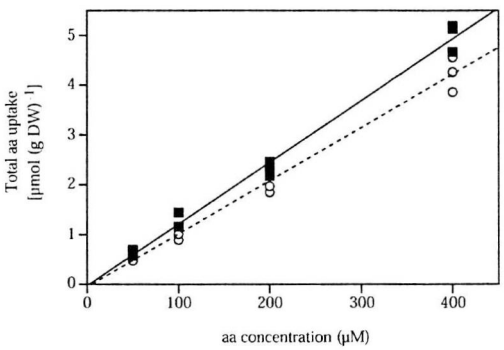

Fig. 1. Total amino acid (*aa*) uptake by roots of Scots pine (*Pinus sylvestris*) plotted against aa concentrations in the initial incubation solution. *Filled squares* and *unbroken regression line* represent uptake by N-starved plants ($y = 0.012x + 0.28$, $R^2 = 0.988$, $n = 3$). *Circles* and *broken regression line* represent uptake by N-fertilized plants ($y = 0.01x - 0.09$, $R^2 = 0.982$, $n = 3$)

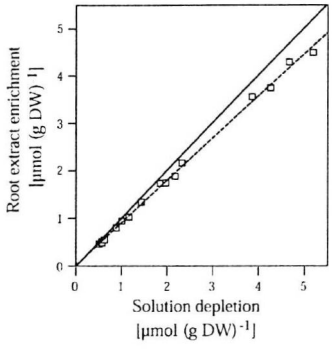

Fig. 2. Amino acid uptake by Scots pine as measured by analysis of root extracts plotted against uptake as measured by solution depletion. $y = 0.89x + 0.013$, $R^2 = 0.998$, $n = 3$. The *unbroken line* shows a 1:1 relation

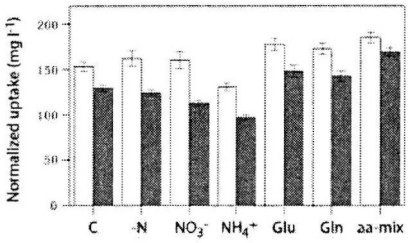

Fig. 3. Normalized amino acid uptake by roots of Scots pine following pre-treatments. *White bars* N-starved plants, *grey bars* N-fertilized plants. Values presented are means ± SE ($n = 12$). *C* Control, *–N* minus nitrogen, *aa-mix* amino acid mixture as specified in *Materials and methods*

N-starved control plants exhibited 18.6% higher total uptake than their N-fertilized counterparts ($P < 0.001$; Fig. 3). For N-starved plants, pre-treatment with NH_4^+ significantly ($P < 0.05$) decreased total amino acid uptake by 14.7%, while pre-treatments with Glu, Gln or the amino acid mixture increased uptake by 12.8% ($P < 0.05$), 16.1% ($P < 0.05$) and 20.9% ($P < 0.01$), respectively. Pre-treatments with N-free medium or NO_3^- did not affect uptake significantly as compared to controls (Fig. 3). Amino acid uptake by N-fertilized plants was decreased by pre-treating with NO_3^- and NH_4^+, by 12.8 ($P < 0.05$) and 25.1% ($P < 0.0001$), respectively. Total uptake was not affected by the N-free pre-treatment, while exposure to Glu, Gln and the amino acid mixture increased uptake by 14.4% ($P < 0.01$), 10.2% ($P < 0.05$) and 30.8% ($P < 0.0001$), respectively (Fig. 3). The variation in uptake of the specific amino acids between the different treatments generally followed the pattern of total amino acid uptake (Fig. 4).

Chitin analyses confirmed that roots were non-mycorrhizal, showing very low levels of chitin (0.02–0.05% of root dry weight), indicating no or negligible amounts of fungi in roots from all treatments (data not shown).

Discussion

Recent studies have reported significant amino acid uptake by plants in the field (Schimel and Chapin 1996; Näsholm et al. 1998), thereby showing that plants are well capable of competing for organic N sources under field conditions. The actual importance of amino acid uptake for plant N nutrition is, however, not well understood. In this context, information on the regulatory pattern of amino acid uptake as a function of resource access and N status of the plant constitutes an important clue to the actual significance of amino acid uptake for plant N nutrition. Thus, an uptake system that is regulated in response to resource access and, foremost, to plant N status may putatively function as an N-scavenging system for the plant.

The uptake rates detected in the present study correspond well to uptake rates seen in an earlier study of Gly, Glu and arginine uptake by Scots pine (Persson and Näsholm 2001b). Complementary root extract analyses also confirmed that amino acids were, in fact, absorbed intact (Persson and Näsholm 2001b; Fig. 2). The linearly

Table 2. Endogenous concentrations of N compounds in Scots pine roots following pre-treatments. Values presented are means ± SE ($n = 2$). *Boldface* denotes significant differences from controls ($P < 0.05$). Units are µmol (g root DW)$^{-1}$. *–N* Minus nitrogen, *aa* amino acids, *aa-mix* amino acid mixture as specified in *Materials and methods*

N status	Pre-treatment	NO_3^-	NH_4^+	Glu	Gln	Total aa
N-starved	Control	0.004 ± 0.003	2.15 ± 0.39	0.29 ± 0.07	0.55 (0.25	3.77 (0.44
	–N	0.002 ± 0.002	2.55 ± 0.28	0.28 ± 0.01	0.42 ± 0.04	4.29 ± 0.13
	NO_3^-	**0.680 ± 0.066**	4.97 ± 0.75	0.20 ± 0.00	0.22 ± 0.02	3.89 ± 0.25
	NH_4^+	0.035 ± 0.001	**28.73 ± 2.67**	0.22 ± 0.03	0.62 ± 0.10	4.32 ± 0.40
	Glu	0.000 ± 0.001	3.16 ± 0.35	**1.19 ± 0.21**	0.64 ± 0.12	**7.47 ± 0.32**
	Gln	0.000 ± 0.001	2.63 ± 0.03	0.38 ± 0.02	**2.79 ± 0.17**	**7.19 ± 0.17**
	aa-mix	0.000 ± 0.001	2.95 ± 0.32	**0.65 ± 0.03**	**1.66 ± 0.13**	**13.48 ± 1.24**
N-fertilized	Control	0.073 ± 0.052	4.46 ± 1.43	0.69 ± 0.05	1.76 ± 0.32	10.72 ± 0.61
	–N	0.000 ± 0.002	4.27 ± 0.49	0.46 ± 0.07	1.76 ± 0.18	11.56 ± 0.41
	NO_3^-	**1.198 ± 0.190**	**15.76 ± 3.38**	0.37 ± 0.03	2.39 ± 0.21	12.23 ± 1.17
	NH_4^+	0.018 ± 0.020	**33.39 ± 2.19**	0.60 ± 0.28	2.96 ± 0.92	13.15 ± 2.59
	Glu	0.001 ± 0.001	4.58 ± 0.55	1.02 ± 0.05	1.27 ± 0.14	**17.82 ± 0.61**
	Gln	0.000 ± 0.000	3.92 ± 0.35	0.42 ± 0.02	**3.40 ± 0.71**	**20.37 ± 2.35**
	aa-mix	0.000 ± 0.000	2.15 ± 0.52	0.52 ± 0.57	1.48 ± 0.46	**21.37 ± 0.86**

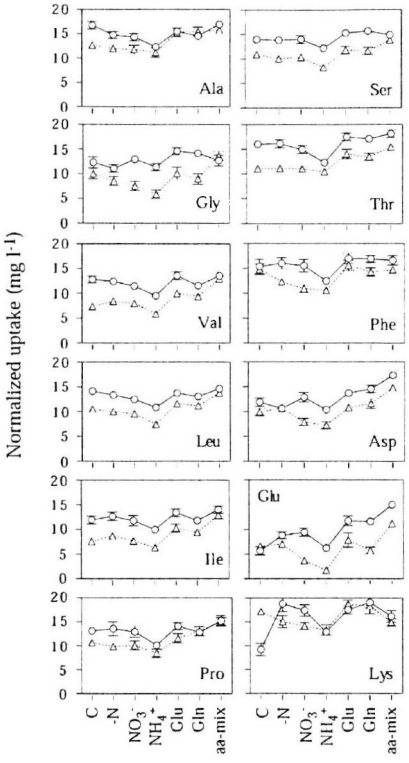

Fig. 4. Normalized uptake by Scots pine roots of the specified amino acids plotted against pre-treatments. *Circles* N-starved plants, *triangles* N-fertilized plants. Values presented are means ± SE ($n = 12$); error bars may be hidden by symbol size. (Points of different treatments are joined by a line to reveal the pattern of co-regulation.) *C* Control, *–N* minus nitrogen, *aa-mix* amino acid mixture as specified in *Materials and methods*

increasing uptake with increasing substrate concentrations (Fig. 1) indicates that the accountable uptake system was not saturated or was in the lower end of its uptake capacity during this study. Endogenous amino acid concentrations in control roots from the N-starved plants were substantially lower than in N-fertilized plants, indicating that their N statuses were thoroughly different. Plants pre-treated with NO_3^- and tungstate displayed augmented NH_4^+ concentrations, indicating that the tungstate treatment did not completely inhibit NR activity (Table 2). This was most likely an effect of an insufficient amount of tungstate in the pre-treatment. All other enzyme inhibitors appeared to have inhibited their respective target enzymes.

Recent research has disclosed a large number of amino acid transporters from a number of different plant species (Montamat et al. 1999; Neelam et al. 1999; Zhou et al. 1999), although not all of these transporters have been found to be expressed in roots (Fischer et al. 1998). Albeit such transporters are yet to be described in *P. sylvestris*, the large sequence homology between the so-far-identified transporters in different plants, as well as in, for example, yeast and mammals suggests that the presence of specific amino acid transporters is ubiquitous among organisms (Fischer et al. 1998). Although a number of transporters have been identified and characterized, data on the regulation of amino acid transport and uptake are scarce.

In our study, N-starved plants exhibited significantly higher uptake rates than their N-fertilized counterparts. A similar pattern has been observed for *Zea mays* amino acid uptake (Jones and Darrah 1994). The actual mechanism behind this regulation cannot be pinpointed using our data. The data do, however, provide an indication that the regulation may not be mediated by Glu or Gln concentrations, which have been suggested as strong candidates for regulating transcription of the N-uptake transporters for NO_3^- and NH_4^+ in plants (Rawat et al. 1999; Vidmar et al. 2000). It is possible that the regulatory signal may not be an N compound at all, but a compound derived from, for example, carbon metabolism. Indeed, both glucose and non-metabolizable glucose analogues have been reported to induce amino acid transporters in algae, illustrating the tight interdependence of carbon and nitrogen metabolism (Cho et al. 1981). The ability to up-regulate amino acid uptake in response to N deficiency suggests that amino acid uptake plays a significant role in the N scavenging of the plant.

All roots pre-treated with NH_4^+, as well as N-fertilized roots pre-treated with NO_3^- exhibited decreased amino acid uptake (Fig. 3). The NO_3^--fed roots did, however, exhibit increased endogenous NH_4^+ concentrations, probably due to incomplete NR inhibition (Table 2). This suggests that increased endogenous levels of NH_4^+ have a negative effect on amino acid uptake. Similar effects of NH_4^+ have been seen on high-affinity NO_3^--uptake systems as well as on NH_4^+ uptake in earlier studies (Rawat et al. 1999; Vidmar et al. 2000), where increased NH_4^+ concentrations were believed to have post-transcriptional effects on the respective transporters. It is, however, possible that the damping effect on amino acid uptake may have other causes, such as NH_4^+ working as a signal down-regulating transcription of the gene encoding the transporter.

Although the increased uptake seen in all plants pre-treated with amino acids coincides with increased root amino acid content (Table 2, Fig. 3), it does not correlate with endogenous concentrations of any specific amino acid (Glu and Gln shown in Table 2). Conversely, previous studies of NO_3^- and NH_4^+ transport indicate that Gln may be a powerful agent for down-regulating expression of NO_3^- and NH_4^+ transporter genes (Rawat et al. 1999; Glass et al. 2001). It does, however, seem likely that the increased uptake is an effect of the high external levels of amino acids in the pre-treatment medium. This would indicate some sort of external sensing,

similar to the NO_3^- sensing which increases expression of the mRNA for the high-affinity NO_3^- transporter and increases NO_3^- uptake (Glass et al. 2001). The regulation of amino acid uptake seen in the present study also displays similarities to the regulation of several amino acid permeases in yeast, such as GAP1. The transcription of these genes is up-regulated by low external N concentrations or by the presence of their substrate, while it is down-regulated by high levels of NH_4^+, asparagine or Gln. Furthermore, NH_4^+ causes a rapid inactivation of GAP1 (Grenson 1992).

The amino acid transporters that to date have been identified and characterized generally, but not ubiquitously, exhibit wide substrate specificity concerning individual amino acids (e.g. Fischer et al. 1995; Montamat et al. 1999). As mentioned, however, there is evidence for the expression of only a fraction of the so-far-identified transporters in root tissue (Fischer et al. 1998). Our data indicate that all individual amino acids tested exhibit a rough, but general co-variation in uptake regulation (Fig. 4). Thus, uptake may be mediated by a single amino acid transporter with a very broad substrate specificity, being capable of transporting a wide range of amino acids, from basic to acidic and from polar to non-polar, with similar rates. Alternatively, the uptake system is comprised of several transporters exhibiting a more-or-less general substrate specificity. According to our data, these separate transporters are in that case tightly co-regulated, forming a combined system for uptake of exogenous amino acids. Our data are, however, not sufficient to elucidate which of these possibilities is correct.

As a whole, our data indicate that amino acid uptake in Scots pine is down-regulated by NH_4^+, while it is up-regulated by the presence of amino acids in the external solution and by plant N deficiency. The fact that N deficiency induces amino acid uptake suggests that it is, in fact, a significant contributor to the N economy of *P. sylvestris*. Furthermore, it could be argued that the decreased amino acid uptake following increased endogenous NH_4^+ concentrations is an effect of the high energy cost associated with NH_4^+ assimilation and a subsequent lack of energy for amino acid uptake. Consequently, since there is no such high energy cost associated with amino acid assimilation or nitrate storage, increased endogenous levels of amino acids or nitrate do not affect amino acid uptake. The actual up-regulation of amino acid uptake in response to substrate access even in the N-fertilized plants suggests that amino acid uptake is regulated, not by N status, but rather by the C status of the plant.

Thus, while the uptake of NO_3^- and NH_4^+ is down-regulated by improved plant N status, amino acid uptake is rather regulated by the energy status of the plant. This suggests that amino acids, from a plant perspective, may be a preferred source of N even under situations of high access to NO_3^- or NH_4^+, although the low relative concentrations of amino acids in systems exhibiting high inorganic N may limit the actual contribution of these compounds to plant N nutrition. The practical consequences of amino acid uptake not being down-regulated by improved plant N status are far-reaching and may have implications on the way we consider, for example, industrial fertilizers and N deposition.

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III

Regulation of amino acid uptake by carbon and nitrogen in *Pinus sylvestris*

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Abstract

The simultaneous uptake of 13 different amino acids by Scots pine (*Pinus sylvestris* L.) was characterized and its regulation investigated after pre-treatments with a range of C and N metabolites. The uptake system exhibited a broad substrate specificity, acquiring all tested amino acids at similar uptake rates. Uptake of all tested amino acids by excised roots was linear over a time period of 150 minutes and exhibited pH-dependency, showing a peak at pH 4.0-5.0. Uptake was increased following pre-treatments with glucose and sucrose, while ammonium pre-treatments had a negative effect on amino acid acquisition. Pre-treatments with the important Krebs cycle intermediates oxaloacetate or 2-oxoglutarate did not result in altered amino acid uptake. It is speculated that the up-regulation of uptake may be due to an increased flow of glucose through a sensor mechanism, such as hexokinase. The regulation of transport by N and C suggests a function of amino acid uptake for the N nutrition of the plant, thus further highlighting the importance of organic nitrogen for plant N nutrition suggested by an increasing amount of research.

Introduction

Amino acids constitute a major proportion of the soluble nitrogen in many soils, especially in areas exhibiting low mineralization rates due to low temperature and/or pH, such as tundra and boreal forest soils (e.g. Kielland 1995; Jones and Kielland 2002). Concurrently, plants in these ecosystems generally exhibit a profound nitrogen limitation with respect to growth and development, inevitably leading to the notion of amino acids as nitrogen sources for plants in these systems. Consequently, an increasing amount of research is accumulating, showing uptake and utilization of amino acids by a large array of differing plant species from a range of differing habitats (e.g. Kielland 1994; Schmidt and Stewart 1999; Persson and Näsholm 2001a).

Due to the pivotal role of nitrogen for plant growth, N uptake and assimilation are strictly regulated processes. Uptake systems for the inorganic nitrogen compounds nitrate and ammonium appear to be transcriptionally as well as post-transcriptionally regulated by their substrates and by downstream metabolites, predominantly glutamine (Rawat et al. 1999; Vidmar et al. 2000). Moreover, the activity of nitrate reductase, glutamine synthetase and glutamate synthase, enzymes essential for the assimilation of nitrate and ammonium, appears to be regulated by substrate access as well as by glutamate and/or glutamine, but also respond to changes in carbon metabolism, such as changes in the levels of sucrose and the organic acids 2-oxoglutarate and malate (e.g. Morcuende et al. 1998; Lejay et al. 1999).

During recent years, a large number of amino acid transporters have been identified and characterized from several different plant species, and from homology studies it has been suggested that as many as 53 different amino acid transporters may be present in *Arabidopsis* (Wipf et al. 2002). Notwithstanding the large number of identified and characterized amino acid transporters (e.g. Montamat et al. 1999; Chen et al. 2001; Fischer et al. 2002), data on the regulation of these transporters are hard to come by. Such data would, however, be highly valuable for the understanding of the role of amino acid uptake for plant N nutrition and metabolism.

Correspondingly, in a recent study we demonstrated that amino acid uptake by *Pinus sylvestris* may be regulated by several factors, including plant N status, substrate access and endogenous levels of ammonium. Moreover, the study gave indications that C compounds may be of importance to the regulation of amino acid uptake (Persson and Näsholm 2002). In addition to indicating that amino acids are important for, and may constitute a significant proportion of *P. sylvestris* N nutrition, these findings further highlight the firm connection between C and N metabolism

Table 1. Pre-treatments and concentrations of compounds in the pre-treatment solutions.

Pre-treatment	Concentrations
Oxaloacetate	3 mM
2-oxoglutarate	3 mM
Fructose	3 mM
Glucose	3 mM
Sucrose	3 mM
NH_4^+/methionine sulfoximine (MSX)	3 mM/1 mM
NH_4^+/Sucrose/methionine sulfoximine (MSX)	3 mM/3 mM/1 mM
6-benzylaminopurine (BA)	1 µM
1-naphthaleneacetic acid (NAA)	1 µM

and signaling (e.g. Lejay et al. 1999; Coruzzi and Zhou 2001).

In the present study, we characterized the simultaneous uptake by *P. sylvestris* roots of 13 different amino acids as a function of substrate concentration, time and pH. Moreover, in order to learn more of the regulation of uptake, the effect of a range of important C and N metabolites on amino acid uptake was studied. The results are discussed in relation to possible mechanisms behind uptake regulation, and the potential importance of amino acid uptake for plant N nutrition.

Materials and methods

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 minutes, rinsed in sterile water and planted in 0.5 dm^3 pots containing autoclaved quartz sand. Plants were grown in a greenhouse for 60 days under a 16/8 h light/dark period at 20-25°C. The substrate was kept constantly moist and plants were, from day 14 to day 53, fertilized twice a week using Superba S™ (Hydro Agri, Landskrona, Sweden) at an N concentration of 5 mM (for fertilizer composition see Persson and Näsholm 2002). From day 53, plants were fertilized using an N-free fertilizer, in all other aspects similar to the Superba S™. At day 59, all plants were fertilized with a mixture of amino acids with the same composition as the experiment incubation solution (although unlabeled). Plants were harvested at day 60. At harvest, plants were carefully cleaned from sand, rinsed in 0.5 mM $CaCl_2$ followed by sterile water and immediately transferred to pre-treatment solutions. Control roots were immediately excised and transferred to experiment incubations.

Pre-treatments are shown in table 1. In addition to the specific compounds, all pre-treatment and experimental solutions contained the N-free fertilizer, complementary to 10 mM N. During pre-treatments as well as experimental incubations, roots were intensely bubbled with air. Pre-treatments were performed at pH 5.0, 20°C and lasted for 2 h, after which roots were rinsed in 0.5 mM $CaCl_2$, excised and immediately transferred to experimental incubations. Small parts of the roots were however immediately frozen in N_2 (liq.) for subsequent root analyses.

Incubation solutions contained a universally (U-^{13}C, ^{15}N, >98%) labeled amino acid mixture composed as: Ala (15.5%), Gly (7.5%), Val (6.6%), Leu (10.4%), Ile (4.7%), Pro (6.1%), Met (0.7%), Ser (7.7%), Thr (7.9%), Phe (4.4%), Asp (13.7%), Glu (12.1%), Tyr (2.7%) (percentages refer to proportion of total amino acid concentration in incubation solutions). Lys, Arg and His were also present in the amino acid mixture, but not analyzed. Labeled amino acids were obtained from Cambridge Isotope Laboratories, Inc., MA, USA. Incubations lasted for 1 h, except time series which lasted for 150 minutes and were sampled by removing a 100 µl aliquot every 30 minutes. Other incubations were sampled by removal of 1 ml aliquots after incubations. All samples were immediately frozen at -22°C for later analysis. Root cuttings were carefully kept above the incubation solution surface during incubations. Incubations were performed at pH 5.0, 20°C, except pH-gradient studies which were performed at 3.0, 4.0, 5.0, 6.0 or 7.0. Uptake kinetics were determined at 74, 266, 648, 830 and 1553 µM, while pH-dependence, time-dependence and regulation were tested at 435 µM. All incubations were performed in triplicates (n = 3) using a single plant for each replicate.

Passive amino acid uptake was tested in a prior study using the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) and found negligible (Persson and Näsholm 2002). In the same study, the accuracy of solution depletion as a measurement of uptake was tested and found high. Amino acid, organic acid and sugar analyses were

Table 2. Root content of pre-treatment compounds. Values presented are means ± SE (n = 1-3). No SE indicates only 1 sample (n = 1). N.d. denotes not determined. Lack of values indicate that concentrations were below detection limits. Oa, oxaloacetate; 2-oxo, 2-oxoglutarate; BA, 6-benzylaminopurine; NAA, 1-naphthaleneacetic acid; MSX, methionine sulfoximine.

Pre-treatment	Endogenous concentration (μmol g^{-1} root DW)					
	Oa	2-oxo	Fru	Glc	Suc	NH_4^+
Control	-	0.20±0.02	23.7±0.5	13.4±2.7	27.6±1.3	1.8±0.3
Oa	-	0.22±0.02	15.0±1.1	12.7±1.3	5.9±1.9	0.8±0
2-oxo	-	0.44±0.03	56.8±3.4	49.8±3.8	3.2±1.4	2.8±0
Fructose	n.d.	n.d.	33.4±1.1	20.7±0.1	33.2±1.5	2.3±0.2
Glucose	n.d.	n.d.	38.1±4.3	24.3±2.7	41.2±6.0	2.8±0
Sucrose	n.d.	n.d.	65.4±7.2	39.1±8.4	33.0±0.6	2.4±0.7
NH_4^+/MSX	n.d.	n.d.	24.6±1.1	18.1±1.8	16.5±2.8	33.0±1.5
NH_4^+/Suc/MSX	n.d.	n.d.	36.6±2.6	24.8±3.0	45.5±2.8	29.0±2.6
BA	n.d.	n.d.	23.8±2.4	17.0±1.6	28.3±1.3	1.5±0.2
NAA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

performed using GC-MS (Persson and Näsholm 2001b). Organic acids and sugars were, however, analyzed after trimethylsilylation using N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA)(Pierce Biotechnologies, Rockford, IL, USA) at 70°C for 30 minutes. Sugars were also methoximated using methoxyamine in pyridine (40 μg/ml) at 30°C for 1 h prior to silylation. NH_4^+ was analyzed on root extracts by Flow Injection Analysis (FIA) using a Tecator 5012, Foss Tecator, Sollentuna, Sweden. All chemicals used were supplied by Sigma-Aldrich, St. Louis, MO, USA, unless specified.

Uptake and root content data were made subject to ANOVA. Regression was performed on uptake data from the time study. Individual amino acid uptake data were normalized with respect to incubation solution concentration. All statistical analyses were performed using Statview 5.0 statistical software.

Results

Uptake system characterization

Total amino acid uptake exhibited a peak at pH 4.0-5.0, while being significantly lower at pH 3.0, as well as at pH 6.0 and 7.0 (Fig. 1). Uptake of all individual amino acids followed this pattern, with the exception of Ala and Gly, which did not exhibit lower uptake at pH 3.0 (data not shown). Total amino acid uptake exhibited a linear pattern over time (Fig. 2). Likewise, uptake of all specific amino acids displayed linear uptake, although uptake of glycine demonstrated a slight decrease after 90 minutes (data not shown). The linearly increasing uptake suggest that the observed solution depletion is, in fact, the result of active uptake and not of cell wall binding.

Total amino acid uptake rates at pH 5.0 varied between 1.56 ± 0.09 and 6.98 ± 0.42 μmol g^{-1} DW h^{-1} (mean ± SE), depending on concentration in the incubation solution. Total uptake exhibited saturation kinetics in the analyzed concentration range 74-1553 μM (Fig. 3). Uptake of all individual amino acids displayed similar kinetics, with the exception of Thr, which was taken up in a linear manner in the analyzed concentration range. Uptake of Ser, Asp and Glu declined dramatically at their lowest concentrations (3.4, 5.9 and 5.3 μM, respectively).

The relative uptake of the specific amino acids varied over the differing concentrations (data not shown). Uptake of Val, Leu, Ile, Pro, Met and Phe showed a trend of contributing relatively more to total uptake at 74 μM (P < 0.05, except Pro P = 0.092). Ser, Asp and Glu contributed significantly less at 74 μM than at

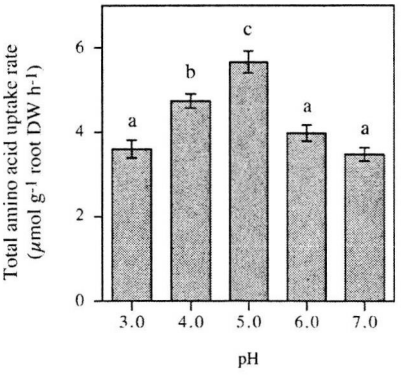

Fig. 1. Total amino acid uptake rates at different pH. Bars indicate mean uptake rates (μmol g^{-1} root DW h^{-1}), while error bars display SE (n = 3). Different letters indicate significantly different uptake rates.

the higher concentrations ($P < 0.05$). Ala, Gly and Tyr contributed equally to total uptake at all concentrations, while Thr exhibited a trend of increased relative uptake at higher concentrations ($P < 0.05$ at 1553 μM vs. 74, 266 and 648 μM). Pro contributed significantly less at 1553 μM ($P < 0.01$). Relative uptake was found not to be related to availability (i.e. concentration in incubation solution).

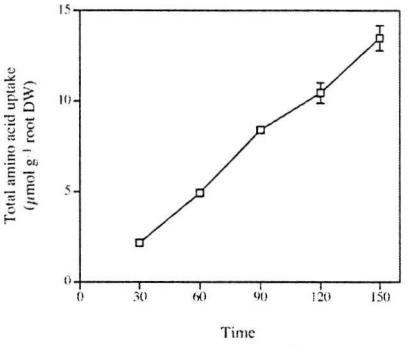

Fig. 2. Amino acid uptake (μmol g^{-1} root DW) plotted against time. Values presented are means ± SE (n = 3). Error bars may be hidden by symbol size. y = 0.094x – 0.568; R^2 = 0.995.

Regulation of amino acid uptake

Pre-treatments with glucose and sucrose resulted in 27.7% ($P < 0.001$) and 21.0% ($P < 0.01$) increased amino acid uptake rates respectively as compared to controls, while pre-treatments with fructose, 2-oxoglutarate or oxaloacetate had no significant effect on uptake rates (Fig. 4). Pre-treatment with NH_4^+ decreased uptake rates with 44.5% ($P < 0.0001$), while NH_4^+ in combination with sucrose caused a 21.9% ($P < 0.01$) reduced uptake rate.

Although several of the pre-treatments brought about significant changes in the endogenous pools of several of the measured metabolites (table 2, some data not shown), none of the changes in amino acid uptake was directly connected to endogenous concentrations of either sugars, amino acids, ammonium or organic acids as measured in this study. Likewise, no correlation between uptake and ratios between any of the measured endogenous compounds could be found. Pre-treatments with the synthetic plant hormones 6-benzylaminopurine (BA) or 1-naphthaleneacetic acid (NAA) did not result in altered amino acid uptake rates. Uptake of the specific amino acids generally followed the pattern of total amino acid uptake (Fig. 5).

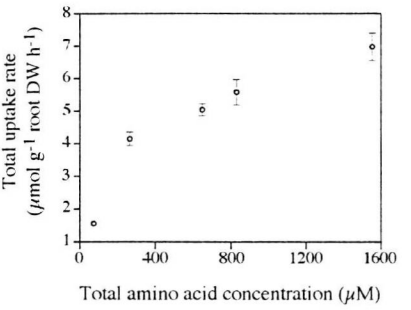

Fig. 3. Total amino acid uptake rates (μmol g^{-1} root DW h^{-1}) plotted against substrate concentrations (μM). Values presented are means ± SE (n = 3). Error bars may be hidden by symbol size.

Discussion

Uptake system characterization

The observed peak of uptake at pH 4.0-5.0 (Fig. 1) conforms well to earlier studies of amino acid transport (Borstlap et al. 1986), as well as to the stochiometrical properties of amino acid transporters, being proton symporters (Fischer et al. 1998). Since the pK_a of the amino acid carboxyl group is below pH 3.0 for all amino acids, the lower uptake at pH 3.0 is more likely a result of physiological processes affected by the low pH, rather than a transport system dependence of substrate charge. Alternatively, transporter function is inhibited at pH 3.0, as compared to higher pH.

The use of excised roots excludes possible shoot-to-root signaling which cannot be evaluated using our data. The linear uptake of amino acids over the observed time period (Fig. 2) 150 minutes does, however, indicate that uptake is not regulated by the actual transport of, or by the exposure to exogenous amino acids, at least at the concentration and time period used in this study (435 μM for 150 min). These results contrasts to results from an earlier study, where a 6 h pre-treatment with exogenous amino acids at 10 mM resulted in an increased root uptake (Persson and Näsholm 2002). These contrasting results are likely to be a consequence of the highly different concentrations used in the pre-treatment solution. In support of amino acid sensing are the recent findings of an amino acid sensor in *Saccharomyces cerevisiae* (Bernard and André 2001) and glutamate receptor genes in *Arabidopsis thaliana* (Lam et al. 1998).

The transport system exhibited a very broad substrate specificity, transporting all examined amino acids as well as arginine and lysine

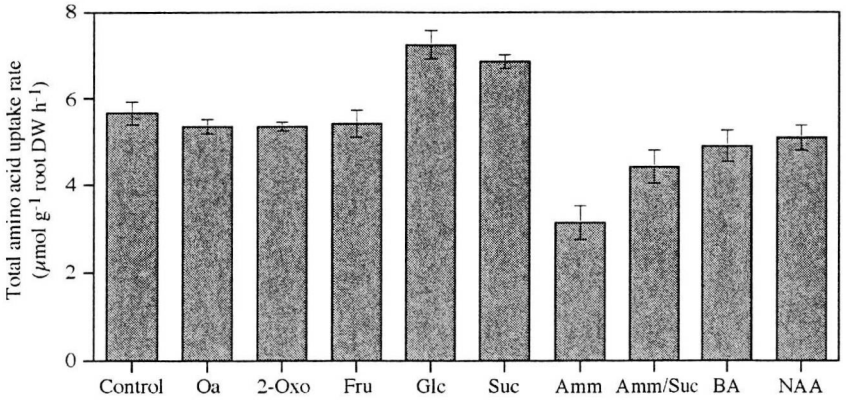

Fig. 4. Total amino acid uptake rates (μmol g^{-1} root DW h^{-1}) following pre-treatments. Values presented are means ± SE (n = 3). Oa, oxaloacetate; 2-oxo, 2-oxoglutarate; Fru, fructose; Glc, glucose; Suc, sucrose; Amm, ammonium; BA, 6-benzylaminopurine; NAA, 1-naphthaleneacetic acid.

(data not shown), although all amino acids exhibited differing uptake rates. Uptake of all amino acids except Thr, which displayed linear uptake, exhibited saturation kinetics. The dramatically lower uptake of Asp, Ser and Glu at the lowest concentration indicate that the uptake system have a lower affinity for the 3 latter amino acids, while uptake of Thr appears to be efficient over a wider concentration range than uptake of all other amino acids.

Although these results do not contrast to results from an earlier study of amino acid uptake by *P. sylvestris*, showing linearly increasing uptake in the concentration range 50-400 μM, the actual uptake rates differ markedly, being only about 50% at similar incubation solution concentrations in the present study. While the observed discrepancy is not a result of differing N status as measured by root amino acid content, any other factor may affect uptake rates, particularly as the plants from the 2 studies were harvested at differing ages (116 days in the prior study vs. 60 days in the present study).

The presented kinetic data should be treated with caution, since the evaluation of individual amino acid uptake in the presence of a range of other amino acids, inhibiting uptake of each other, is precarious. Thus, our data should be seen upon, not as absolute uptake values for single amino acids comparable to data presented in earlier amino acid transporter characterizations (Fischer et al. 2002), but as a general estimate of uptake when presented to a wide range of substrates. As such, it is a valuable description of the transport system functioning in a normal situation, where uptake systems are likely to encounter a wide range of amino acids and other substrates simultaneously. In addition, the amino acid mixture is derived from algae protein, thus representing a biological composition of amino acids likely to be roughly similar to what plant roots actually encounter in natural soils. Consequently, the total uptake kinetic parameters may be considered as reasonably representative for field situations.

Uptake regulation

In order to optimize nitrogen uptake in relation to carbon and nitrogen status, N uptake and assimilation are processes that, in a complex manner, are tightly regulated transcriptionally as well as post-transcriptionally by a range of differing carbon and nitrogen metabolites (e.g. Morcuende et al. 1998; Glass et al. 2002). Due to the potentially large importance of amino acids for the N nutrition of many plants, it is of large importance to learn more of the regulation of amino acid uptake and assimilation.

Data from this, as well as a previous study (Persson and Näsholm 2002), suggest that the amino acid uptake system in *P. sylvestris* roots is regulated by several factors. Moreover, regulation of individual amino acid uptake is strikingly similar, suggesting a tight co-regulation of contributing transporters (Fig. 5).

In previous studies, exposure to nitrate and ammonium have been seen to have profound impact on the flux of carbon into organic acids for assimilation of the acquired N into amino acids (e.g. Scheible et al. 1997). The organic acids oxaloacetate and 2-oxoglutarate are important intermediates in N- and C-metabolism, imperative for the transamination of amino acids, thus being important

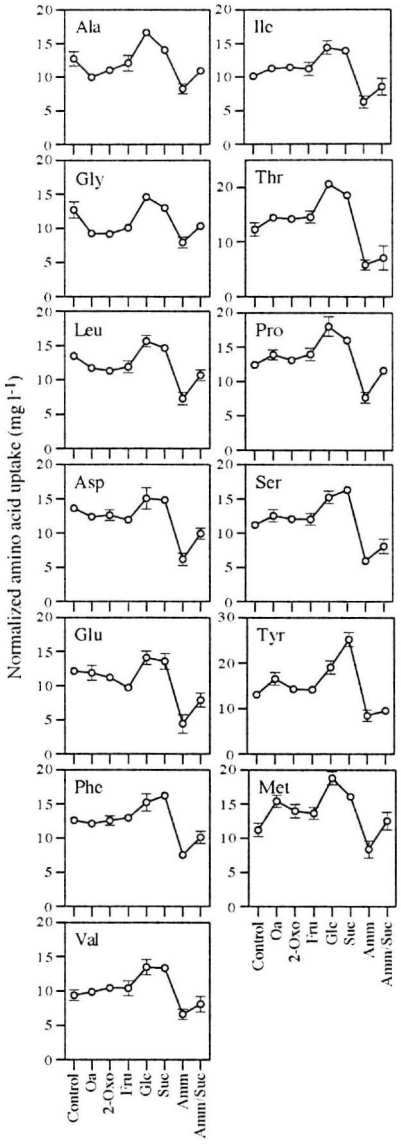

Fig. 5. Normalized uptake of individual amino acids (mg l^{-1}) plotted against pre-treatments. Values presented are means ± SE (n = 3). Error bars may be hidden by symbol size. Points are connected by a line to reveal patterns of co-regulation. Oa; oxaloacetate, 2-oxo; 2-oxoglutarate, Fru; fructose, Glc; glucose, Suc; sucrose, Amm; ammonium. Please observe differing scale for Thr and Tyr.

regulatory factors of the relative levels of differing amino acids. Pre-treatments with these organic acids did, however, not result in changes of amino acid uptake (Fig. 4). Root content of oxaloacetate was below detection limits in all roots, including those pre-treated with this metabolite, while incubation with 2-oxoglutarate resulted in significant increases of root content (Table 2). The oxaloacetate pre-treatment did however, similarly to 2-oxoglutarate incubation, lead to significant changes in other carbon metabolites (Table 2), indicating that the compound was, in fact, taken up.

While fructose failed to affect amino acid uptake, glucose as well as sucrose had a stimulatory effect on uptake (Fig. 4). As fructose as well as the important Krebs cycle intermediates oxaloacetate and 2-oxoglutarate failed to increase uptake, it seems unlikely that the observed effect of glucose and sucrose was a result of increased carbon, and thus energy, flows. Rather, the observed changes in uptake may be a result of regulation of the uptake system. Such up-regulation of amino acid uptake by glucose has previously been observed in *Chlorella* cells (Cho et al. 1981), while in another study, glucose and sucrose failed to affect alanine transport in *Beta vulgaris* leaf cells (Chiou and Bush 1998). Such a failure may, however, be attributed to the wide array of plant amino acid transporters with differing functions and, presumably, regulations (Fischer et al. 1998; Wipf et al. 2002). Sucrose and light have also been shown to initiate increased activity and transcript abundance of ammonium as well as nitrate transporters in plants (e.g. Lejay et al. 1999; von Wirén et al. 2000).

The increased uptake in the present study was not related to any specific changes in absolute or relative levels of any of the measured endogenous metabolites, indicating that uptake regulation may be an effect of flux, rather than actual pools of metabolites. Hexose sensing is generally thought to be conveyed by either of 2 systems, a hexokinase dependent or a hexokinase independent system, the former being dependent upon flux of primarily glucose, but not fructose, through hexokinase, while the latter is thought to sense actual hexose levels (Smeekens 2000). In a previous study of glycine uptake by *Lolium perenne*, uptake was increased by glucose and sucrose, but not by the glucose analogue 3-O-methyl-D-pyranose (Thornton 2001). While the author concluded that the sugar effect was due to the addition of energy, this study actually lends support to the presence of a hexokinase dependent regulation of uptake, as 3-O-

methyl-D-pyranose is not metabolized by hexokinase (Gibson 2000).

Although sucrose has been observed to be sensed by itself, leading to sucrose-specific regulation of several genes (Gibson 2000; Smeekens 2000), the apparent rapid hydrolyzation of sucrose after uptake suggest that the effect of sucrose may, in fact, be an effect of glucose in the present study. The effect of sucrose was also replicated using intact (i.e. roots of non-excised) plants incubated with sucrose vs. intact control plants (data not shown), indicating that the sugar effect was most likely not an effect of the removal of shoots before the uptake experiments.

Pre-treatment with ammonium in combination with the glutamine synthetase inhibitor methionine sulfoximine (MSX) resulted in significantly decreased amino acid uptake rates (44.5%; Fig. 4). The same pre-treatment, but with the addition of sucrose led to a smaller damping of uptake (21.9%; Fig. 4). At least 2 alternative explanations can be presented in order to explain these results. The increased endogenous ammonium levels may lead to major energy costs for NH_4^+-efflux (Britto et al. 2001), energy costs that are alleviated by exogenously applied sucrose. Such an explanation is, however, somewhat unlikely due to the relatively unchanged sugar levels in the ammonium treatment as compared to controls (Table 2). Alternatively, ammonium and sucrose (or glucose resulting from hydrolyzed sucrose) may regulate uptake in opposite directions, leading to a smaller decrease in uptake when sucrose is supplied in combination with ammonium. Such a regulation may be performed on a transcriptional as well as post-transcriptional level with regard to both compounds. Due to the apparently efficient blocking of glutamine synthetase, and the consequently lowered endogenous concentrations of Gln and Glu, a down-regulation of uptake by these compounds, as indicated in the case of ammonium and nitrate uptake (Rawat et al. 1999; Glass et al. 2001), is unlikely.

In order to test whether amino acid uptake was regulated in a short-time perspective by plant growth factors, uptake was tested following pre-treatments with either of the plant hormone homologues BA or NAA. A previous study has reported on regulation of the *Arabidopsis AtNRT1.1* dual affinity nitrate transporter by auxin (Guo et al. 2002), thereby targeting expression to active uptake sites such as root tips. Auxin and nitrate also have key roles in the regulation of root branching and development (Zhang et al. 1999). In addition, auxins are structurally related to amino acids, and both compound groups are mutually transported by common permeases (Young et al. 1999).

Cytokinins have been reported to influence root growth processes as well as regulation of N metabolism enzymes such as nitrate reductase (Schmülling et al. 1997; Silverman et al. 1998). Moreover, the production and flux of cytokinins are strongly influenced by nitrogen status and uptake (e.g. Takei et al. 2001).

Pre-treatments with BA or NAA failed to affect amino acid uptake in the subsequent uptake experiment. This does, however, not exclude that plant hormones may have an effect on amino acid uptake in a longer time-perspective, or that naturally occurring auxin or cytokinins (in contrast to the synthetic species used in this study) have a direct or indirect effect on amino acid transporter expression and activity.

Conclusions

Data on regulation of amino acid transporters are scarce and transporters are to be expected to display widely varying regulatory patterns, due to the large number of transporters (Wipf et al. 2002), their differing expression patterns and putative functions (Fischer et al. 1998). As an example, proline transporter expression and activity have been shown to increase strongly as a response to osmotic stress, while other amino acid permeases were simultaneously down-regulated (e.g. Ueda et al. 2001). The pattern of regulation of *P. sylvestris* amino acid uptake seen in our studies is quite different and rather indicates the importance of amino acid uptake for the N nutrition of the plant, as it is displaying variations in concordance with varying N- and C-status of the plant. Thus, in addition to an increasing amount of research, our results further point out the importance of organic N for plant nutrition.

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IV

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LETTER

Amino acid uptake: a widespread ability among boreal forest plants

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Abstract

Amino acids constitute a potentially important source of nitrogen for plants in boreal forest ecosystems. Accordingly, it may be suggested that distinct plant species differing abilities to take up amino acids constitutes an important factor in determining plant ecosystem composition. Using GC-MS and isotopically labelled amino acids, we measured the simultaneous uptake of 15 different amino acids by 31 common boreal forest plant species. The results from this study show that all plant species tested, representing a wide variety of plant types, have the ability to take up amino acids from an incubation solution. Furthermore, uptake rates were unrelated to mycorrhizal associations as well as habitat soil amino acid concentrations and plant nitrogen availability dependence as measured by Ellenberg nitrogen indicator values. These results suggest that mycorrhiza is of minor importance for discrete plant amino acid uptake rates and further points out the potential importance of amino acids to plant nitrogen nutrition in boreal forest ecosystems.

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INTRODUCTION

During the last decade, a number of studies have confirmed earlier reports showing uptake of amino acids by plants (e.g. Miller & Schmidt 1965; Newton 1974; Chapin *et al.* 1993). Although the ability of plants to take up and use amino acids as a source of nitrogen is becoming widely accepted, the mechanisms behind this uptake as well as its actual importance in the field are not well understood.

Nitrogen is generally a limited resource in the boreal forest, and nitrogen availability is one of the factors regulating growth in these ecosystems. Since amino acids constitute a relatively large part of plant available nitrogen in boreal forest soils (Ivarson & Sowden 1969; Näsholm *et al.* 1998), it may be argued that the ability to take up these compounds may be an important factor in determining plant ecosystem composition in the boreal forest.

In order to test the importance of the type of mycorrhizal connections, habitat and Ellenberg's nitrogen indicator values for amino acid uptake rates by different plant species, we conducted a study testing uptake of 15 different universally (U-^{13}C, ^{15}N) labelled amino acids simultaneously by 31 different plant species. The different species were taken from six different habitats of the boreal forest exhibiting differing soil amino acid content. The species were chosen to represent a wide range of plant types and to include natively nonmycorrhizal, as well as arbuscular mycorrhizal, ericoid- and ectomycorrhizal species. The total amino acid uptake rates of the different species were subsequently related to mycorrhizal connections, habitat soil amino acid concentrations and Ellenberg's nitrogen indicator values as a measure of plant dependence on nitrogen availability.

The results show that amino acid uptake is a common feature of plants growing in boreal forest habitats of greatly varying types. Furthermore, uptake of amino acids was not correlated to either mycorrhizal type, soil amino acid concentrations or Ellenberg's nitrogen indicator values.

MATERIALS AND METHODS

Fine roots from 31 differing plant species from six different locations were carefully removed from the soil and kept moist during transport to the laboratory. Roots were cleaned and carefully rinsed under tap water and thereafter incubated in a solution containing 15 universally (U-^{13}C, ^{15}N, >98%) labelled amino acids at a total concentration of 941 μM for 20 min. The amino acid concentration used is within range of K_m found for amino acid uptake of a range of differing plant species (e.g. Kielland 1994; Boorer & Fischer 1997).

Individual amino acid concentrations in the incubation solution were; Ala 98 μM, Gly 99 μM, Val 52 μM, Leu 97 μM, Ile 37 μM, Pro 68 μM, Met 8 μM, Ser 47 μM, Thr 46 μM, Phe 33 μM, Asp 86 μM, Glu 85 μM, Lys 112 μM, Arg 47 μM and Tyr 26 μM.

In order to keep root functioning intact for all of the widely differing plant species used in this study, pH of the incubation solutions was set to 6.8 (Schubert *et al.* 1986). Each species was replicated 4–5 times ($n = 4$ or 5).

Root cleaning, extraction, SPE-purification and GC-MS analysis was performed according to Persson and Näsholm (in press). In short, root amino acids were extracted using 10 mM HCl and the resulting extracts were purified using strong cation exchange (SCX) SPE-cartridges. The resulting purified amino acid extracts were derivatized to their *tert.*-butyltrimethylsilyl (tBDMS) derivatives. The derivatized amino acids were separated on a 30-m Chrompack CP-Sil 5 MS capillary column and subsequently mass analysed on a Varian Saturn 2000 ion trap mass spectrometer. Amino acid uptake was quantified by measuring the mass peak resulting from the added universally labelled amino acids in the mass spektra from each individual amino acid.

Soil samples ($n = 4$) from each sampling location were collected and extracted by 10 mL of sterilized water. Soil extracts were analysed for amino acid content by HPLC according to Näsholm *et al.* (1987). Labeled amino acids were obtained from Cambridge Isotope Laboratories (Cambridge, MA). Mycorrhizal connections were deduced after Harley & Harley (1987). Ellenberg nitrogen indicator values were taken from Ellenberg *et al.* (1992).

Plant amino acid uptake rates were grouped and made subject to ANOVA. Uptake rates of the individual amino acids from all species were pooled and made subject to correlation analysis. All statistics were performed using Statview 5.0 statistical software.

RESULTS

Roots from all species acquired amino acids from the solution at total rates varying between 0.25 and 9.2 μmol g^{-1} DW h (2.71 ± 0.47 μmol g^{-1} DW h; mean ± SE) (Table 1). All plant species did not take up all of the different amino acids (data not shown). Total uptake of amino acids was not related to plant-mycorrhizal connections (nonmycorrhizal, arbuscular-, ericoid- or ectomycorrhizal) (Fig. 1a). Nor could we find any relation between total amino acid uptake and habitat soil amino acid concentrations or Ellenberg nitrogen indicator values (Fig. 1b, c).

Uptake rates of the individual amino acids were not correlated to abundance in the uptake solution. The uptake of Asp, Glu and Tyr was very low (0.033 ± 0.007, 0.004 ± 0.004 and 0.004 ± 0.002 μmol g^{-1} DW h, mean ± SE, respectively) by all species. Apart from the low uptake of the acid amino acids, no correlation between charge or polarity and uptake of the individual amino acids was found (data not shown).

Normalized relative uptake rates (corrected for relative abundance in the incubation solution) of all amino acids are shown in Fig. 2. Normalized uptake rates of Gly, Val, Leu, Ile, Pro, Ser, Thr, Phe and Lys exhibited strong positive correlation to each others ($r = 0.783$–0.973) as well as to total uptake rates ($r = 0.777$–0.931) (data not shown).

DISCUSSION

The results presented in this study indicate that the ability to acquire exogenous amino acids is a widespread character among boreal forest plants. This supports earlier studies suggesting amino acid uptake to be a common feature of plants from widely differing habitats (e.g. Kaye & Hart 1997; Schmidt & Stewart 1999). Although varying greatly between individual species, mean amino acid uptake rates found in this study (2.71 ± 0.47 μmol g^{-1} DW h) are in the range of uninduced nitrate and ammonium uptake rates at similar concentrations recorded in several studies of a number of differing plants (e.g. Aslam *et al.* 1992; Kielland 1994; Høgh-Jensen *et al.* 1997; Min *et al.* 2000). The present study does not account, however, for soil–amino acid interactions or plant–plant and plant–microorganism competition. Therefore the uptake rates recorded here cannot be used as a measurement of the importance of amino acids for plant nitrogen nutrition in the field.

Amino acid uptake rates varied greatly between plant species, but were not significantly related to the type of mycorrhizal connections of the plant. Plant maintaining arbuscular-, ericoid or ectomycorrhiza as well as nonmycorrhizal plants apparently took up amino acids at similar rates. Thus, this study implies that the type of plant–mycorrhizal connections have an inferior role in determining the ability of plant roots to take up amino acids.

These results, however, do not rule out an important role of mycorrhiza for long-term plant uptake of amino acids in the field, since the ability of ericoid- and ectomycorrhiza to release exogenous proteolytic enzymes (Chalot & Brun 1998) may be of great importance for the release of amino acids from complex soil compounds. This ability, combined with the surface-extending role of mycorrhiza for plant roots, may be of great importance in the exploitation of amino acids for plant nitrogen nutrition.

Although the varying uptake rates recorded between species could be an effect of the expression of amino acid transport systems with differing affinities (Fischer *et al.* 1998) due to growth in habitats with differing soil amino acid concentrations, we could not find any relation between habitat soil amino acid concentrations and measured uptake rates (Fig. 1b).

By using GC-MS and isotopically labelled amino acids, we were able to follow the uptake of 15 different amino acids simultaneously. This allows for a better simulation of field conditions where plant roots encounter a wide range of

Table 1 Root uptake rates, mycorrhizal connections, habitat soil amino acid concentrations and individual species Ellenberg nitrogen indicator values of the 31 species tested

Species	Uptake rate	Mycorrhiza (nmol g^{-1} soil DW)	Soil aa concentration	Ellenberg NIV (μmol g^{-1} root DW h)
Carex canescens	0.32 ± 0.17	non	5 ± 0.3	2
Carex magellanica	0.72 ± 0.05	non	5 ± 0.3	n.a.
Cornus suecia	4.07 ± 0.76	non	68 ± 4	2
Melampyrum pratense	0.74 ± 0.15	non	29 ± 2	2
Potentilla palustris	5.69 ± 0.81	non	92 ± 19	2
Rumex acetosella	1.90 ± 0.34	non	29 ± 2	2
Silene dioica	0.56 ± 0.04	non	27 ± 4	8
Achillea millefolium	2.22 ± 0.11	AM	5 ± 0.3	5
Agrostis capillaris	5.81 ± 0.77	AM	5 ± 0.3	4
Calamagrostis canescens	1.24 ± 0.17	AM	27 ± 4	5
Dechampsia caespitosa	4.32 ± 0.61	AM	27 ± 4	3
Juniperus communis	4.70 ± 0.48	AM/EM	29 ± 2	n.a.
Maianthemum bifolium	0.25 ± 0.06	AM	330 ± 124	3
Molinia caerulea	9.22 ± 1.32	AM	27 ± 4	2
Paris quadrifolia	1.00 ± 0.09	AM	27 ± 4	7
Ranunculus acris	2.52 ± 0.39	AM	5 ± 0.3	n.a.
Rubus idaeus	0.59 ± 0.12	AM	29 ± 2	6
Sorbus aucuparia	0.63 ± 0.02	AM	29 ± 2	n.a.
Trientalis europaea	2.17 ± 0.27	AM	330 ± 124	2
Viola epipsila	6.89 ± 0.28	AM	27 ± 4	2
Andromeda polifolia	8.13 ± 1.07	ErM	68 ± 4	1
Calluna vulgaris	1.28 ± 0.28	ErM	68 ± 4	1
Ledum palustre	2.76 ± 0.26	ErM	68 ± 4	2
Vaccinium myrtillus	3.76 ± 0.21	ErM	330 ± 124	3
Vaccinium oxycoccus	0.35 ± 0.11	ErM	92 ± 19	1
Vaccinium vitis-idaea	0.45 ± 0.04	ErM	29 ± 2	1
Betula pendula	7.57 ± 0.71	EM	330 ± 124	n.a.
Picea abies	2.29 ± 0.15	EM	330 ± 124	n.a.
Pinus sylvestris	0.65 ± 0.16	EM	29 ± 2	n.a.
Populus tremula	0.67 ± 0.07	EM	330 ± 124	n.a.
Salix repens	0.63 ± 0.02	EM	92 ± 19	n.a.

Aa denotes amino acid. Uptake rates are quoted in μmol g^{-1} root DW h. Non denotes nonmycorrhizal; AM, ErM and EM denotes arbuscular mycorrhizal, ericoid and ectomycorrhizal, respectively. Amino acid soil concentrations are quoted in nmol g^{-1} soil DW. N.a. in the Ellenberg NIV column denotes not applicable (plant species having no Ellenberg NIV). Values presented are means ± SE.

compounds, a large advantage over studies utilizing only one compound.

The uptake rate correlations found between differing amino acids when pooling all species suggest that similar, but not necessarily equal, uptake mechanisms or transport systems were responsible for uptake in most species. The very low uptake rates found for Asp and Glu can most likely be explained by the high pH (6.8) of the incubation solution (e.g. Borstlap *et al.* 1986). We cannot, however, see any similar simple explanation to the low general uptake rates of Tyr.

No significant correlation between Ellenberg's plant nitrogen indicator values and uptake rates could be found, indicating that plant amino acid uptake is a general ability found in many different types of plants from widely differing habitats. These results are further supported by several other studies showing amino acid uptake by a wide array of different plant species and types (e.g. Soldal & Nissen 1978; Schobert & Komor 1987; Schimel & Chapin 1996; Raab *et al.* 1999; Näsholm *et al.* 2000; Streeter *et al.* 2000).

Although amino acid uptake rates displayed significant interspecies variation in this study, this variation does not appear to be systematic in any lucid way. Since all species tested exhibited the ability to take up amino acids, this ability in itself does not appear to be an important determinant of plant ecosystem composition. Rather, availability of amino acids and the capacity to compete for these compounds appear to be more important factors regulating plant uptake of amino acids. Similar results have

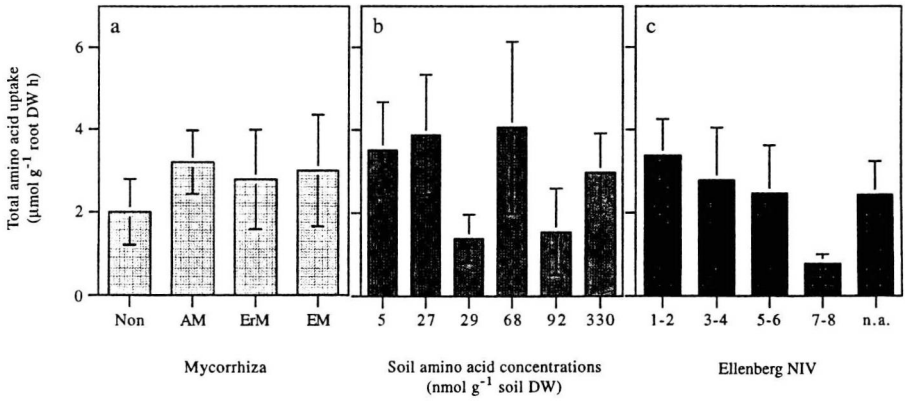

Figure 1 Amino acid uptake rates (μmol g^{-1} root DW h) related to: (a) plant-associated mycorrhizal type; Non denotes nonmycorrhizal; AM, ErM and EM denote arbuscular mycorrhizal, ericoid and ectomycorrhizal, respectively. (b) Habitat soil amino acid concentrations in nmol g^{-1} soil DW. (c) Ellenberg nitrogen indicator values, n.a. denotes not applicable (plant species having no Ellenberg NIV). Grouping of species can be deduced from Table 1. Values presented are means ± SE.

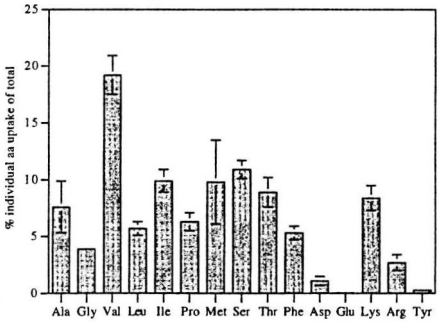

Figure 2 Relative uptake of individual amino acids. Values presented are means ± SE of all species. Uptake rates have been corrected for presence in the incubation solution and are expressed as percentage uptake of individual amino acid of total amino acid uptake.

also been deduced from studies of plant nitrogen availability, uptake and competition (Leadley *et al.* 1997; Hodge *et al.* 2000; Näsholm & Persson 2001; Owen & Jones 2001).

Although our study did not take into account seasonal variation in soil amino acid concentrations and plant uptake, the uptake rates give an indication of the potential role of amino acids for plant nitrogen nutrition. The present study also clearly points out that the need for long-term studies of plant acquisition of amino acids in the field is compelling in order to learn more of the importance of these compounds for plant nitrogen nutrition.

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BIOSKETCH

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Nitrogen acquisition from inorganic and organic sources by boreal forest plants in the field

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Abstract

We investigated short- as well as long-term plant nitrogen (N) uptake by *Deschampsia flexuosa*, *Picea abies* and *Vaccinium myrtillus* from $^{15}NO_3^-$, $^{15}NH_4^+$ and (U-^{13}C, ^{15}N) arginine, glycine or peptides. Root N uptake was analyzed after 6 h and 64 days following injections. Our results show that all species, irrespective of their type of associated mycorrhiza, rapidly acquired similar amounts of N from the entire range of added N sources. After 64 days, *P. abies* and *V. myrtillus* again had acquired similar amounts of N from all N sources, while for *D. flexuosa*, the uptake from all other N sources except ammonium was significantly lower than that from nitrate. Furthermore, soil analyses indicate that glycine was rapidly decarboxylated after injections, while other organic compounds exhibited slower turnover. In all, these results suggest that a wide range of N compounds may be of importance for the N nutrition of these boreal forest plants, and that the type of mycorrhiza may be of large importance for N scavenging, but of less importance to N uptake rates of plants.

Introduction

Nitrogen (N) is a fundamental element regulating plant growth and development (Vitousek et al. 1997). Recent research does, however, give disparate pictures of key elements of the N cycle, particularly concerning the role and importance of organic N for plant N nutrition. Several studies have shown amino acid uptake by a wide range of different plants (Kielland 1994; Raab et al. 1999; Schmidt and Stewart 1999; Persson and Näsholm 2001a; Henry and Jefferies 2002) and plant uptake of small peptides have also been observed (Sopanen et al. 1985; Steiner et al. 1994). Moreover, some studies have shown that plants in symbiosis with ecto- or ericoid mycorrhiza have the ability to grow on complex N sources, such as protein (Abuzinadah and Read 1986; Finlay et al. 1992; Turnbull et al. 1995) and chitin (Kerley and Read 1995, 1997). In addition, a large number of transporters for organic N compounds have been identified in several plant and mycorrhizal species (Chalot et al. 1996; Ortiz-Lopez et al. 2000; Stacey et al. 2002).

In all, these studies suggest that organic N compounds may function as N sources for plant growth, and since the bulk of soil N in many ecosystems is present in organic form (Schulten and Schnitzer 1997; Murphy et al. 2000; Jones and Kielland 2002), the potential use of these compounds for plant N nutrition, bypassing mineralization, may be of large importance and could have profound effects on our understanding of N cycling in these ecosystems. In contrast, however, several studies have implied that plant uptake of organic N sources is small and may be of only limited significance to plant N nutrition (Hodge et al. 2000b; Owen and Jones 2001).

In parallel with the discussion of the importance of organic N for plant N nutrition, recent studies have debated the relative importance of nitrate and ammonium in different ecosystems (Eviner and Chapin III 1997; Grogan et al. 2000; Min et al. 2000). Studies of plant uptake of these inorganic nitrogen compounds show that conifers exhibit a strong preference for ammonium (Kronzucker et al. 1997), while analyses of soil N turnover, on the other hand, have suggested that nitrate may be an important N source even in ecosystems where earlier studies have failed to detect a significant pool of nitrate (Stark and Hart 1997), such as coniferous forests.

Most of the above-mentioned studies have, however, been performed in laboratory settings, which limit their value as they do not

account for important factors such as soil interactions, plant-microbial and plant-plant competition and mycorrhizal associations, factors which may seriously affect plant acquisition of different N sources (Jones and Hodge 1999; Hodge et al. 2000a). Hence, the need for field studies testing the relative importance of different N compounds, inorganic as well as organic, for plant N nutrition is compelling.

The present study addresses two fundamental questions regarding the N ecology of boreal forest plants. First, what importance do different N compounds of increasing complexity have for plant N nutrition in the field? Second, do different plant species, associated with different kinds of mycorrhiza, have differing abilities to acquire organic nitrogen compounds, in a short- as well as a long-term perspective?

These issues were studied by injecting a range of isotopically labeled N compounds of increasing complexity into field soil. The added isotopes were subsequently traced in plant roots after two time intervals, 6 hours and 64 days after injections. Moreover, we followed the turnover of the added compounds in the soil in addition to analyzing the decomposition of the organic compounds by ^{13}C-measurements on soil CO_2 efflux.

Materials and Methods

Experimental site and design

The study was performed at the edge of a 15-year-old clear-cut at Renberget (64° 14′N, 19° 46′E), situated 60 km northwest of Umeå in northeast Sweden. The vegetation consisted of small plants of *Picea abies* (L. Karst.) and *Pinus sylvestris* (L.) above a field layer of mainly *Vaccinium myrtillus*, *Vaccinium vitis-idaea* and *Deschampsia flexuosa*. The site was chosen due to its homogeneity and its large abundance of small *P. abies* plants. Experimental plots, containing all three species of which *P. abies* plants were 10-20 cm tall, were circular, covering an area of 0.018 m^2 each. The plots were replicated 8 times (n=8) for each of 6 treatments and for each of two sampling occasions, resulting in 96 plots. Treatments were applied to plots randomly.

Treatments and sampling

The experiment was started at 11.00 a.m. on July 1, 1999 and consisted of 6 different treatments; water, $^{15}NH_4^+$, $^{15}NO_3^-$, (U-$^{13}C_6$, $^{15}N_4$) arginine, (U-$^{13}C_2$, ^{15}N) glycine or (U-^{13}C, ^{15}N) peptides. The latter N source was produced from re-wetted, dry lyophilized algal cells universally labeled with ^{15}N and ^{13}C. All isotopes, including labeled algal cells, were purchased from Cambridge Isotopes Ltd, Cambridge, UK. To achieve a polypeptide fraction from the isotopically labeled algae, cells were fissured and peptides precipitated by shaking cells in acetone. After centrifugation, the supernatant was discarded and the resulting pellet containing peptides was dissolved in 0.1 M NaOH. The resulting solution contained small amounts (less than 3% of total N) of free amino acids and NH_4^+. The solution had a C/N-ratio of 4.68 and consisted to an extent of 90 and 50% of peptide fragments heavier than 0.7 and 4 kDa, respectively. All compounds had an isotopic enrichment of 96-99 atom percent of both ^{13}C and ^{15}N. The N concentration of each added solution was; 1.3 mM NH_4^+, NO_3^-, glycine and arginine while the N concentration of the peptide solution was 2.3 mM. pH of all added solutions was set to 7.5.

Each plot was injected with 100 ml of either of the solutions in five 20 ml doses. The solutions were injected into the mor layer using a 4-sideport needle syringe, one injection in the center of the plot, while the four other injections were made halfway out to the edge of the plot in four perpendicular directions. No attempts were made to hinder lateral or vertical flow of the injected solutions. N additions amounted to ca. 1 per mil of total soil N in the mor layer.

Roots of all species as well as soil samples were harvested at two separate occasions, 6 hours and 64 days after tracer injections. Harvesting plots was conducted by cutting shoots, and thereafter immediately retrieving the entire mor layer, containing roots of the plants, of each plot. Shoots were discarded, while other sampled material was instantly put on carbon-dioxide ice. Root- and soil samples were then retrieved from the soil cores within three hours after harvesting. Only roots that could be connected to shoots were used. After retrieving roots, they were rinsed under tap water and thereafter washed three times in 0.5 mM $CaCl_2$, in order to remove added solutes from root surfaces. Soil samples were sieved to remove roots and litter. Root samples were freeze-dried under low pressure for 72 h, and milled to a fine powder using a ball mill. Samples were then stored at −22°C until analyzed. Soil samples were dried at 70°C for 48 h before subsequent IRMS analyses.

Measurements of soil respiration of added, labeled substances were conducted on separate plots. Four different treatments, (U-$^{13}C_2$, ^{15}N) glycine, (2-^{13}C, $^{15}N_4$) glycine, (U-$^{13}C_6$, $^{15}N_4$)

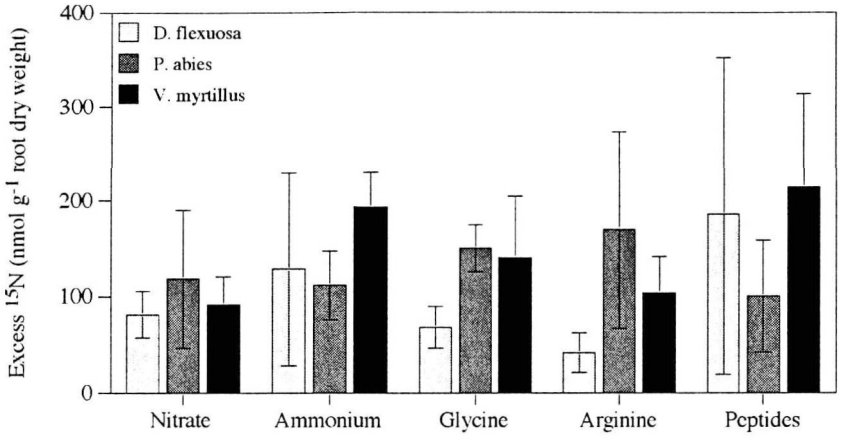

Figure 1. Levels of ^{15}N excess, expressed as nmol g^{-1} root dry weight, in roots of *D. flexuosa*, *P. abies* and *V. myrtillus* harvested 6 h after tracer additions. Values presented are means (n = 3-8). Error bars represent SE.

arginine and (U-^{13}C, ^{15}N) peptides plus a control (water) were applied on 4 plots per treatment. Thus, an additional form of labeled glycine was added to plots used for measurements of soil respiration compared to plots used for plant harvests. The size of the treated plots and the rate of additions were identical as for plots used for plant harvests. Field sampling of soil respiration were performed on 5 occasions; 1.2, 7.5, 27, 118 and 193 h after tracer additions, as described in Högberg & Ekblad (1996).

Analyses

The root powder and soil samples were analyzed for ^{13}C, ^{15}N, total C and total N by Continuous Flow-Isotope Ratio Mass Spectrometry (CF-IRMS), using an Europa Scientific, Europa 20-20 stable isotope analyzer (Ohlsson & Wallmark 1999).

For gas chromatography-mass spectrometry (GC-MS) detection of intact labeled amino acids, 0.01 M HCl extracts of milled root samples were evaporated under reduced pressure, redissolved in N, N-dimethylformamide (DMF) and derivatized using N-methyl-N-*tert.*-butyldimethylsilyl-trifluoroacetamide (MTBSTFA) under a constant flow of argon gas (Persson and Näsholm 2001b). Amino acid and NH_4^+ content of plant roots, the peptide solution and of hydrolyzed (6M HCl, 100°C, 16 h) polypeptide solution were analyzed by HPLC (Näsholm et al. 1987). The composition of polypeptide fragment sizes in the extract was determined by FPLC (gel filtration).

Concentration of CO_2 and ^{13}C abundance of the CO_2 were measured as previously described (Högberg & Ekblad 1996), except that the standard in this study was CO_2 with a $\partial^{13}C$ value of -29.56‰ relative to V-PDB (AGA Specialgas, Stockholm, Sweden). Results are reported in $\partial^{13}C$ (‰):

$\partial^{13}C = 1000((R_{sample} - R_{standard}) / R_{standard})$ (‰), where $R = {}^{13}C/{}^{12}C$.

Statistics

^{15}N and ^{13}C data were analyzed by ANOVA or made subject to regression analyses, using Statview 5.0 statistical software.

Results

Root ^{15}N data from the first harvest, 6 h after injections, indicated that all species rapidly acquired N in similar amounts from all added N sources (Fig. 1). However, large variations of ^{15}N root content (0-850.7 nmol g^{-1} root dry weight) between replicates within treatments were found, indicating that injections did not result in a uniform distribution of labeled compounds in the soil.

Inter-specific comparisons of uptake at the 64 days harvest revealed a similar uptake of N from nitrate and arginine by all species, a significantly higher uptake of ammonium and peptides by *P. abies* and *V. myrtillus* as compared to *D. flexuosa*, while the ericoid species *V. myrtillus* acquired significantly more N from glycine, compared to the two other species (Fig. 2).

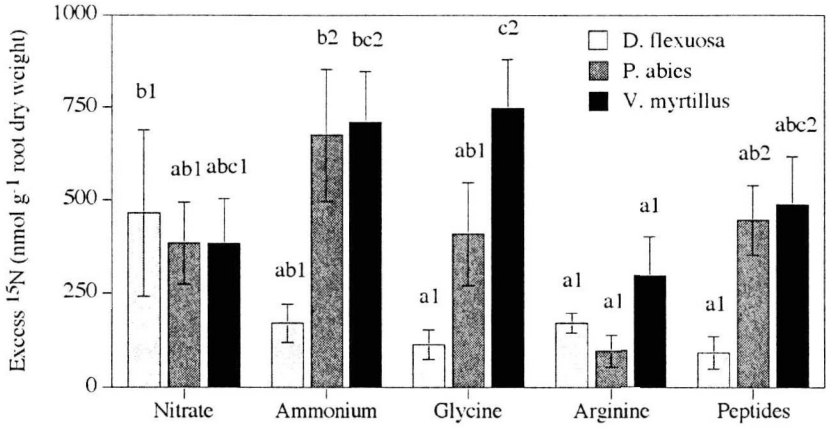

Figure 2. Levels of ^{15}N excess, expressed as nmol g^{-1} root dry weight, in roots of *D. flexuosa*, *P. abies* and *V. myrtillus* harvested 64 days after tracer additions. Values presented are means (n = 4-8). Error bars represent SE. Different letters on top of bars indicate significant differences ($P < 0.05$) between treatments (within species). Different numbers on top of bars indicate significant differences ($P < 0.05$) between species (within treatment).

After 64 days, the 3 species exhibited significant differences in N uptake from the distinct N-sources (Fig. 2). The grass *D. flexuosa* exhibited a four times higher uptake from the nitrate addition than from any of the organic N treatments ($P < 0.05$). Other than a notably lower uptake from the arginine addition as compared to glycine uptake ($P < 0.05$), *P. abies* exhibited relatively high uptake from all N sources. *V. myrtillus* exhibited a consistently high uptake from all N sources, although uptake from the arginine treatment was significantly lower than uptake from the glycine and ammonium treatments (40% and 42% of glycine and ammonium uptake, respectively; $P < 0.05$). In addition, nitrate uptake was significantly lower than glycine uptake (51% of glycine uptake; $P < 0.05$).

^{13}C levels in roots from the organic N treatments were generally not found to significantly differ from controls, at neither of the two harvests. Consequently, plotting excess ^{13}C against excess ^{15}N did not results in significant correlations for any but one treatment, in which *V. myrtillus* roots from the peptide additions yielded a significant regression ($y = 3.95x + 6.97$; $R^2 = 0.96$; $n = 7$) between ^{13}C and ^{15}N. The low enrichment levels of ^{13}C in combination with the large dilution by endogenous C precluded other significant regressions (Näsholm and Persson 2001).

GC-MS analyses of root extracts revealed universally (U-^{13}C, ^{15}N) labeled glycine in *D. flexuosa* and *P. abies* roots at the 6 h harvest (data not shown). Labeled amino acids were not detected in root extracts from any other treatments.

Analyses of ^{13}C and ^{15}N in soil from the 6 h sampling showed a decrease of $^{13}C/^{15}N$-ratios by 42% ($P < 0.05$) in soil samples from the glycine treatments, as compared to start values, while $^{13}C/^{15}N$-ratios in the peptide treatment displayed a non-significant decrease by 10.3% and ratios in the arginine treatment exhibited a non-significant increase. At the second harvest, decreases were 39% ($P = 0.055$) and 33% ($P < 0.05$) for glycine and peptides, respectively, as compared to initial values (Fig. 3), while arginine did not exhibit any significant change. Measurements of $^{13}CO_2$ losses from (U-^{13}C, ^{15}N) glycine, arginine and peptides, and from (2-^{13}C, ^{15}N) glycine additions revealed markedly different respiration rates from the differing compounds (Fig. 4). The (U-^{13}C, ^{15}N) glycine exhibited the relatively highest loss of $^{13}CO_2$, followed by arginine, peptides and (2-^{13}C, ^{15}N) glycine, in falling order.

Discussion

Although within-species variation of uptake was very large due to the injection technique, our results show that all 3 species, irrespective of associated mycorrhiza, have the ability to rapidly (after 6 h) take up N from all compounds tested in this study (Fig. 1). Moreover, GC-MS analysis confirms uptake of intact glycine for *D. flexuosa* and *P. abies*,

while IRMS analysis of *V. myrtillus* indicate that N from peptides was taken up in organic form by this species (Näsholm and Persson 2001). Due to the low tracer levels used in this study, our data can not unequivocally show organic N uptake in all treatments and species or determine to what extent uptake of organic N actually occurred.

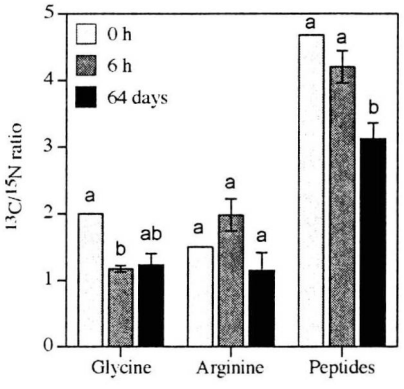

Figure 3. Decomposition of added organic compounds as revealed by $^{13}C/^{15}N$-ratios in soil at addition 0 h, and at 6 h and 64 days after soil injections. Glycine and arginine initial values (0 h) are theoretical, while the initial $^{13}C/^{15}N$-ratio of the peptide solution was determined analytically before injections. Values presented are means ± SE (n = 5-8). Error bars of the initial $^{13}C/^{15}N$-ratio of the peptide solution are too small to be visible. Different letters indicate significant differences between harvests ($P < 0.05$).

At the second harvest, 64 days after injections, the grass species *D. flexuosa* had acquired relatively more N from nitrate than from organic N treatments. Such preferential uptake of nitrate has earlier been interpreted as a key component in the process where grasses out-compete other plants, e.g. after a clear-cutting (Kronzucker et al. 1997). In contrast, *P. abies* and *V. myrtillus* had acquired similar amounts of N from all added N sources after 64 days, suggesting that these species have access to N from a wide array of compounds in field situations. This may be a significant advantage in situations of low N availability. The large ability to acquire N from complex sources may be a consequence of the ecto- or ericoid mycorrhizal connections exhibited by these two species, as these types of mycorrhiza have been shown to have proteolytic abilities (Abuzinadah et al. 1986; Kerley and Read 1995). The relatively low uptake of N from arginine by all species may be due to the very low mobility of this strongly cationic amino acid, and its large potential immobilization.

Interestingly, our data indicate that the capacity of *P. abies* and *V. myrtillus* to acquire N from nitrate does not differ from the capacity to utilize other N sources in a field situation, either in a short- or long-term perspective. This stand in contrast to earlier laboratory studies of conifer roots showing a reduced capacity to utilize nitrate-N as compared to ammonium-N (Kronzucker et al. 1997), and further highlight the need for field studies in order to elucidate the actual importance of different N compounds to plant N nutrition.

Soil ^{13}C and ^{15}N analyses indicate that arginine and peptides were turned over at a relatively slower rate as compared to glycine (Fig. 3). A previous study demonstrated glycine-N to be rapidly immobilized by soil microbial biomass, associated with rapid decarboxylation (Näsholm et al. 1998). Our present data suggest that arginine and peptides were also rapidly immobilized, but that the C of these compounds was retained in the soil. Although one cannot, from our data, determine whether immobilization was biotic or abiotic, soil data in combination with respiration data, showing a comparatively slow respiration of arginine and peptide ^{13}C (Fig. 4), suggest that these compounds were abiotically immobilized.

$^{13}CO_2$ measurements revealed that ^{13}C from (U-^{13}C, ^{15}N) glycine was rapidly respired, while $^{13}CO_2$ emissions from (2-^{13}C, ^{15}N) glycine were 10 times lower. These data indicate that glycine was metabolized by glycine decarboxylase (Ireland and Hilz 1995; Näsholm et al. 2001), and that the serine produced was preferentially used for anabolic, and not catabolic reactions. The fraction of supplied ^{13}C lost as $^{13}CO_2$ during the first week after additions was relatively low (1-14%; Fig. 4) for all treatments, indicating that a significant part of the added C was not turned over. This was also supported by a high ^{13}C concentration in the mor layer of the soil (data not shown).

Together, these data suggest that most of the organic N compounds added were either incorporated in plant and/or microbial biomass or immobilized in soil organic matter, while only a fraction was used in respiratory reactions. ^{15}N concentrations in soil samples from the two harvests (data not shown) suggest that the form of N added was decisive for the recovery of N in the soil organic fraction. Large parts of arginine-^{15}N and peptide-^{15}N were found in soil samples, again suggesting a higher extent of immobilization of these compounds as compared to nitrate, ammonium and glycine, for which a large part of the added

^{15}N could not be recovered in soil, possibly due to plant uptake and/or leaching.

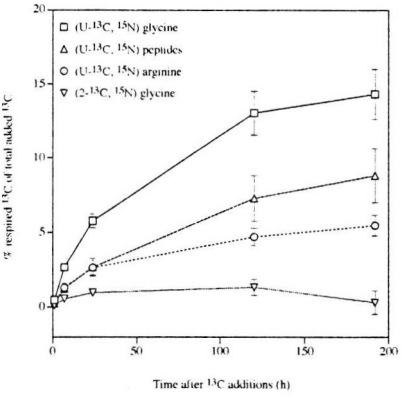

Figure 4. Cumulative soil respiration of added organic compounds, expressed as percent respired ^{13}C of added. Values presented are means from 8 samplings (n = 8). Error bars represent SE.

In conclusion, our data show that all 3 species have the ability to rapidly, within 6 h, acquire N from all sources tested, including N from such complex sources as a mixture of peptides. Moreover, our data suggest that at least some of this N was taken up in organic forms. This suggests that uptake capacity of different N compounds do not differ markedly in field situations, regardless of species and mycorrhizal symbioses.

After 64 days, the ectomycorrhizal *P. abies* and the ericoid mycorrhizal *V. myrtillus* exhibited roughly similar uptake of N from all N sources, while the arbuscular mycorrhizal grass species *D. flexuosa* had taken up relatively more N from the NO_3^- treatment than from the other N sources. These data do suggest that the relative content of nitrate in the plant available N pool is of importance for the growth of grasses, but also that conifers, as well as ericaceous plants, may be better adapted to use this N form in the field than is suggested in laboratory studies. Moreover, data from the 64 days harvest suggest that the type of mycorrhizal symbiosis may be of large importance for the retrieval of immobilized nitrogen, while being of less importance to actual uptake capacity, as suggested by data from the 6 h harvest.

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