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Research paper

Organic nitrogen uptake of Scots pine seedlings is independent of current carbohydrate supply

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In boreal forests, seedling establishment is limited by various factors including soil nitrogen (N) availability. Seedlings may absorb N from soil in a variety of inorganic and organic forms; however, the energy and thus carbohydrate requirements for uptake and assimilation of N vary with N source. We studied the importance of current photoassimilates for the acquisition and allocation of different N sources by Scots pine (*Pinus sylvestris* (L.)) seedlings. Girdling was used as a tool to impair phloem transport of photoassimilates, and hence gradually deprive roots of carbohydrates. Seedlings were cultivated in a greenhouse on equimolar N concentrations of one of the N sources—arginine, ammonium or nitrate—and then girdled prior to a pulse-chase uptake experiment with isotopically labeled N sources. Girdling proved to be efficient in decreasing levels of soluble sugars and starch in the roots. Uptake rate of arginine N was highest, intermediate for ammonium N and lowest for nitrate N. Moreover, the uptake of arginine N was unaffected by girdling, while the uptake of the two inorganic N sources decreased to 45–56% of the ungirdled controls. In arginine-treated seedlings, 95–96% of the acquired arginine N resided in the roots, whereas a significant shift in the N distribution toward the shoot was evident in girdled seedlings treated with inorganic N. This spatial shift was especially pronounced in nitrate-treated seedlings suggesting that the reduction and following incorporation into roots was limited by the availability of current photoassimilates. These results suggest that there are energetic benefits for seedlings to utilize organic N sources, particularly under circumstances where carbohydrate supply is limited. Hence, these putative benefits might be of importance for the survival and growth of seedlings when carbohydrate reserves are depleted in early growing season, or in light-limited environments, such as those sustained by continuous cover forestry systems.

Keywords: amino acid, ammonium, arginine, carbon, conifer, energy, girdling, inorganic nitrogen, nitrate, photoassimilates, *Pinus sylvestris*, uptake rates.

Introduction

A variety of abiotic and biotic factors during cultivation and field establishment restricts the early growth of conifer seedlings in northern boreal forests. Nitrogen (N) is often regarded the most limiting factor for growth of plants in boreal forests (Tamm 1991; Vitousek and Howarth 1991). However, N availability includes both quantitative and qualitative aspects due to its occurrence in different chemical forms in the soil, spanning from simple inorganic ions such as nitrate and ammonium to more complex organic compounds (Miller and Cramer 2004; Näsholm et al. 2009; Inselsbacher and Näsholm 2012; Warren 2012). In boreal forest and tundra ecosystems, plant N demands exceed those supplied by mineralization. Therefore, organic N forms such as amino acids may be of importance in sustaining growth of species inhabiting these areas (Näsholm et al. 1998; Nordin et al. 2001; Persson and Näsholm 2001a;

McFarland et al. 2002; Kielland et al. 2006; Miller et al. 2007; Näsholm et al. 2009; Jones and Kielland 2012). Early growth of seedlings is also dependent on the availability of carbon (C) compounds and their partitioning among various plant organs. Carbon and N cycling within the plant are highly interconnected since energy from the former is required for the uptake and assimilation of the latter, and C skeletons are used for N-containing building blocks of new tissues. Better understanding of these interconnections may help improve the accuracy of the element budget estimates in these ecosystems and also inform silvicultural practices to enhance regeneration success.

Owing to a renewed interest in silvicultural alternatives to clearfellings, such as continuous cover forestry systems, there is an emerging need to understand the complexity and interactions among biotic and abiotic factors affecting regeneration (Pommerening and Murphy 2004; Pommerening 2006). Overstory trees may provide a favorable microenvironment for the regenerating seedlings (Langvall and Ottosson-Löfvenius 2002); however, they also increase competition for light (Mitchell 2001) and nutrients, specifically nitrogen (Strand et al. 2006). Successful regeneration of conifer seedlings, particularly in uneven-aged stands, is enhanced with increasing incoming radiation (de Chantal et al. 2003; Erefur et al. 2008, 2011), emphasizing the importance of adequate carbohydrate supply for early growth and establishment. A tight linkage between new root production and the availability of current photoassimilates has been shown for various conifer species (Burdett 1990). Short days and low sun angles during autumn limit photosynthetic C uptake, and carbohydrate reserves may also be depleted after a long winter as the stored C is consumed in cold hardening processes and cryoprotection (Ögren 1997; Ögren et al. 1997; Tinus et al. 2000). The size of these carbohydrate reserves may impact the survival and growth of seedlings in the following growing season.

Low carbohydrate status of seedlings may also restrict seedling N uptake and assimilation further aggravating growth conditions. This is because of the energetic requirements for reduction and assimilation of N as well as for the demand for C skeletons in synthesis of various organic N molecules. While the most important C source for new root growth is indeed recent photoassimilates fixed by the needles (van den Driessche 1987), seedlings that use amino acids take up C and N concurrently. In contrast to N from nitrate and C from CO_2, C and N from amino acids are already in their respective reduced form, and utilization of the amino acid N leads to lower energetic costs and amino acid C in new structures to decreased construction costs (Zerihun et al. 1998). Consequently, root N uptake and assimilation of seedlings may be less dependent on current photoassimilates in species that rely on organic N in comparison to situations where inorganic N is the main source of N.

Uptake and assimilation of the inorganic N forms—nitrate and ammonium—have been subject to extensive research. The uptake across the cell plasma membrane is an active process mediated by carrier proteins and both high- and low-affinity transport systems have been characterized (Miller and Cramer 2004). Following uptake, nitrate is reduced in two steps to ammonium via nitrite before assimilation into amino acids occurs. The transporter responsible for the uptake of arginine, the amino acid investigated in the present study, has been identified in Arabidopsis (*Arabidopsis thaliana* (L.) Heynh.) (Svennerstam et al. 2008). In order for uptake of arginine to occur, a highly efficient uptake system must be present. High affinity of roots for arginine uptake has indeed been shown in *Arabidopsis* (Svennerstam et al. 2011), barley (*Hordeum vulgare* (L.); Jämtgård et al. 2008) as well as in Scots pine (*Pinus sylvestris* (L.)) and Norway spruce (*Picea abies* (L.) Karst.; Öhlund and Näsholm 2001). Owing to the aforementioned differences in uptake and assimilation between N sources, the energetic costs associated with their respective usage also differ; the cost for using ammonium and nitrate as N sources has been estimated to comprise 14 and 23% of the total root C catabolism, respectively (Bloom et al. 1992).

Reduction and subsequent assimilation of reduced nitrate N is primarily taking place in roots when external nitrate concentrations are low and is almost exclusively localized in roots of gymnosperms under natural conditions (Smirnoff et al. 1984; Andrews 1986). It has also been suggested that, from an energetic point of view, the reduction of nitrate in photosynthetic tissues (through photoreduction) should be more beneficial than the respiratory-driven reduction in roots (Andrews 1986). Although different among species, nitrate assimilation in shoots becomes increasingly important at higher external nitrate concentrations (Andrews 1986). In contrast, earlier studies indicate that organic N may preferentially be assimilated (Persson et al. 2006), and also to a large extent incorporated, into roots (Cambui et al. 2011). Results from a split-root experiment on *Arabidopsis* suggested that a significant share of the absorbed organic N resided at the site of primary assimilation where it promoted root growth (Cambui et al. 2011). Schmidt and Stewart (1999) showed that the amino acid glycine was to a high degree metabolized and retained in roots of Hakea (*Hakea* sp.) Based on these findings, we suggest that, compared with ammonium and nitrate, organic N may to a larger extent be incorporated in roots. At least in the short run, and particularly under circumstances where plant growth is limited by the supply of carbohydrates, the use of organic N would lead to decreased energetic expenditures in roots (Zerihun et al. 1998).

We studied the importance of current photoassimilates transported from the shoot to the roots for uptake and allocation of different N sources (arginine, ammonium and nitrate) in Scots pine seedlings. The choice of arginine was based on the relatively high presence of this amino acid in the soil of boreal

forests (Nordin et al. 2001) but also on the fact that this amino acid is used as N fertilizer in conifer nurseries (cf. Gruffman et al. 2012). We used girdling as a tool to deplete seedling roots of carbohydrates and measured seedling N uptake and allocation by pulse-chase labeling the N source with the stable isotope ^{15}N (and ^{13}C in the case of the organic N source). We hypothesized that girdled seedlings would show a decreased uptake of N, and that this would be more pronounced for the uptake of inorganic N as compared to organic N. Based on earlier findings in *Arabidopsis* (Cambui et al. 2011), it was also hypothesized that seedlings would, to a larger extent as compared to that of ammonium and nitrate, incorporate acquired organic N in the roots.

Materials and methods

Plant material and growth conditions

Scots pine seeds originating from seed orchard Pålberget T5 (65°15′N), Sweden, were sown in 0.2 dm^3 pots containing a mixture of unfertilized peat and vermiculite in the proportions 3 : 1. Seedlings were cultivated in greenhouse conditions in an 18/6 h light/dark cycle with a photon flux density of 200–250 µmol m^{-2} s^{-1}. The relative humidity was 70–80%. Seedlings were rotated three times a week and from day 15, fertilizers were applied to each individual seedling with a pump (Midi-Digital, IsmaTec, Glattbrugg-Zürich, Switzerland, www.ismatec.com) twice a week. Three different fertilizer treatments were given throughout the growing period differing only with respect to the N sources. The target compositions of the fertilizers were (g l^{-1}): N, 78 (originating from any of the N sources arginine, ammonium or nitrate); K, 56; P, 12; Mg, 7.2; S, 7.2; Fe, 0.84; Mn, 0.42; B, 0.18; Zn, 0.13; Mo, 0.03; Cu, 0.02. The fertilizer was given at an N concentration of 7 mM (in the case of arginine this corresponds to a concentration of 1.75 mM since each mole of arginine contains 4 moles of N) and each seedling received 20 ml of the solution each time, corresponding to an amount of 1.96 mg N. The nutrient solutions were kept frozen in order to guarantee the N content and diluted fresh for every fertilization event. pH of the solutions was set to 5.8. After 11 weeks, the fertilizer concentration was raised to 10 mM so that seedlings received 2.80 mg N per fertilization event.

Girdling pretreatment

In order to stop phloem transport of photoassimilates from shoot to roots, a one-centimeter-wide strip of the bark was carefully removed with a scalpel just beneath the first needle pairs of the stem. The plant material was divided into three groups (24 seedlings per group) of which one-third of the plant material was girdled 7 days before the pulse-chase study and one-third of the plant material 3 days prior to the pulse-chase study, while the rest of the seedlings were used as ungirdled control plants (Figure 1).

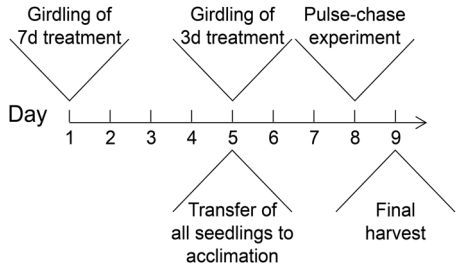

Figure 1. Time course of girdling pre-treatments, transfer to acclimation solutions, pulse-chase experiment and final harvest.

Acclimation and pulse-chase experiment

After 16 weeks, the seedlings were transferred to solutions in order to acclimate to hydroponic conditions that were to be used for the following uptake experiments. Seedlings were gently removed from the growth substrate and cleansed thoroughly in tap water. Extra care was taken not to damage the root systems. The seedlings were washed in three aliquots of 0.5 mM $CaCl_2$ in order to remove any adsorbed compounds before they were transferred to the respective acclimation-solution (Persson and Näsholm 2001b). These solutions were identical to the fertilizer solutions, differing only with respect to the N sources and provided at an N concentration of 1 mM. Seedlings were placed in black plastic containers (9.5 × 9.5 × 14 cm) filled with one of the three different solutions. The solutions were aerated from an aquarium pump with an outlet to each container. Seedlings were maintained afloat at the surface by the support of a piece of black foam plastic with a slot on each side of the piece. Neither growth conditions nor the subsequent pulse-chase experiments were axenic and mycorrhizal infection was not controlled. Acclimation solutions were mixed fresh daily and seedlings were allowed to acclimate for 3 days before the uptake experiment.

On the third day in hydroponics, an uptake study with ^{15}N- and ^{13}C-labeled N sources was carried out. To follow the fate of the acquired ^{15}N (and in the case of arginine also ^{13}C) from the respective N source, the uptake experiment was set up as a pulse-chase experiment, where seedlings were allowed to take up N from N sources containing 10 atom% excess of ^{15}N during 2 h followed by 24 h in identical, but unlabeled solutions. The plants were removed from the acclimation solution, patted dry with tissue paper, washed three times in 0.5 mM $CaCl_2$, patted with tissue paper again and thereafter immersed in a new plastic container containing one of the respective labeled N sources. The solutions were aerated throughout the uptake experiment. Seedlings were thereafter patted dry, washed in $CaCl_2$, patted dry and moved to freshly prepared unlabeled solutions. The seedlings were harvested 24 h after the uptake experiment; roots were washed three times in $CaCl_2$ and patted dry with tissue paper. Roots and shoots were separated at the uppermost lateral root and the two parts

immediately immersed in liquid N_2, and kept frozen until further analyses. For clarification of the time course of these events, see Figure 1.

Stable isotope analysis

Frozen samples were transferred to a freeze drier for 5 days. The dried root and shoot samples were weighed and milled in a bead mill to a fine powder. The total N, ^{15}N and ^{13}C content in the plant material was analyzed with an isotope ratio mass spectrometer (DeltaV, Thermo Fisher Scientific, Bremen, GmbH) interfaced to an element analyzer (Flash EA 2000) modified from Ohlsson and Wallmark (1999).

Soluble sugars and starch

In order to quantify the effect of the girdling pretreatments on carbohydrate levels in roots, soluble sugar and starch contents were analyzed by ion chromatography (Metrohm, IC Net 2.3 with the column Metrosep Carb 1–250 run isocratically with the eluent 0.1 M NaOH at 1 ml min^{-1}). For the extraction of soluble sugars, 50 mg of freeze-dried and milled root samples were extracted twice in 0.5 ml 80% ethanol containing 4 mM Hepes (pH 7.5 with KOH). Samples were thereafter shaken, heated at 80 °C for 30 min and centrifuged 15 min at 14,000 rpm. The supernatant was removed and kept cold while the pellet was extracted once more. The procedure was thereafter repeated twice; however, with the ethanol concentration decreased to 50%. Supernatants were collected, mixed, evaporated and thereafter analyzed. The pellet from the soluble sugar extraction was mixed with 500 µl milli-Q water of which an aliquot of 50 µl was added to 450 µl 50 mM NaAc buffer containing 6.3 units of amyloglucosidase (pH 4.8). The samples were incubated at 40 °C for 16 h and centrifuged 15 min at 14,000 rpm. The samples were thereafter diluted and glucose resulting from starch hydrolysis was analyzed by ion chromatography.

Ergosterol and chitin analyses

Ergosterol and chitin contents of roots were used as indicators for mycorrhizal infection. The ergosterol analysis was carried out according to Sundberg et al. (1999) with a few modifications. Fifty milligrams of dried, milled root material was dissolved in 1000 µl 99.5% ethanol. Samples were shaken for 30 min and centrifuged in 4 °C, 14,000 rpm for 15 min and 100 µl of the supernatant was analyzed by high-performance liquid chromatography (HPLC; Waters Corp., Milford, MA, USA). Ergosterol was separated on a reversed phase column (Merck LiChrospher RP-18, 250 × 4.6 mm; ID 5 µm; Merck, Darmstadt, Germany). Chitin measurements were modified from Ekblad and Näsholm (1996). Briefly, the pellet from the above-described ergosterol extraction was diluted and treated with 0.2 M NaOH for 1.5 h in room temperature and then at 100 °C for 17.5 h in order to eliminate interference of other organic compounds in the analysis. Glucosamine residues were released by acid hydrolysis with 6 M HCl and subsequently converted to fluorescent derivatives by treatment with 9-fluorentylmethylchloroformate (FMOC-Cl). Twenty microliters of the acid hydrolysate was dissolved in 200 µl, 10 µM homocysteic acid. The fluorescent derivatives were thereafter analyzed by HPLC. Glucosamine separates as three peaks of which the third was used for quantification (Ekblad and Näsholm 1996; Ekblad et al. 1998).

pH measurements of growth substrate

Growth substrate was sampled at harvest and stored at 5 °C. The $pH_{(H_2O)}$ was measured on three replicates from each treatment. The peat substrate was thoroughly homogenized using a mortar and pestle and thereafter 1.5 g (FW) was shaken for 2 h with 20 ml distilled water. The samples were centrifuged for 5 min (3000 rpm) and the pH of the supernatant was measured after 30 min.

Treatment of data and statistical analyses

Atom% excess of the ^{15}N content in the seedlings was calculated by subtracting the natural abundances of the heavier isotopes from the atom% from each labeled sample and N source. This theoretical natural abundance for each N source was calculated from a former study on seedlings grown on the same sources. The values used for natural abundance were 0.36640, 0.36715 and 0.37078 atom%, respectively, for arginine, ammonium and nitrate. The atom% excess was thereafter converted to µmol acquired N root DW^{-1} h^{-1}.

Intact uptake of arginine was evaluated by regression analysis (detected ^{13}C vs. theoretical ^{13}C calculated as product of the ratio 1.5, corresponding to 100% of arginine N absorbed as intact arginine, and the detected ^{15}N) to test for significant deviation of slopes from the theoretical maximal slope (i.e., 1.5).

The effects of pretreatment and N source on N acquisition, the distribution of ^{15}N to roots as well as differences in soluble sugars and starch levels were evaluated by separate one-way analysis of variance (ANOVA). The following statistical model was used:

$$Y_{ij} = \mu + \alpha_i + e_{ij}$$

where Y is the response, μ is the overall mean, α_i is the effect of the ith pretreatment (i.e., girdling) or N source and e is the error. The factors were pretreatment ($i = 1,2,3$) or N source ($i = 1,2,3$). One-way ANOVAs were also used to test for differences in N concentration, biomass data, pH of growth substrate as well as chitin and ergosterol contents between N sources. All analyses were followed by Tukey's post hoc test. Data were transformed prior to analyses when required to meet the assumptions of ANOVA. Minitab 16 Statistical Software (Minitab Inc., State College, PA, USA) was used for all

statistical analyses and P values ≤ 0.05 were considered to indicate a significant difference among means.

Results

Biomass and internal N concentrations

Seedlings grown on different N sources, arginine, ammonium or nitrate, displayed similar total biomasses and similar biomass distribution between roots and shoots (Table 1). Average total biomasses of seedlings were 1.92, 1.87, 1.78 g DW for the arginine, ammonium and nitrate treatment, respectively ($P = 0.51$), while root : shoot ratios were 0.31, 0.27 and 0.31, respectively ($P = 0.11$). The N concentrations of roots from the arginine and ammonium treatments were similar: 2.22 and 2.19%, respectively, while nitrate-treated seedlings displayed lower N concentration: 1.92% ($P < 0.001$). However, no differences among treatments could be found in the shoot N with corresponding average N concentrations of 1.67, 1.78 and 1.71% for the arginine, ammonium and nitrate treatments, respectively ($P = 0.38$).

Ergosterol and chitin

Neither growth conditions nor the following uptake conditions were axenic, hence mycorrhizal association was not controlled in this study. Ectomycorrhizal associations may affect N uptake; however, ergosterol and chitin analyses of control roots from the different N treatments indicated low mycorrhizal infection with similar ergosterol contents ranging between 48.40 and 53.52 μg g^{-1} ($P = 0.54$). Yet, the chitin content of nitrate-treated seedlings was significantly higher (1.36 mg g^{-1}) than that of both arginine (0.51 mg g^{-1}) and ammonium (0.52 mg g^{-1}) ($P < 0.001$). Concentrations of both ergosterol and chitin in the root systems were generally very low, <5% of those reported from mycorrhizal root tips of Scots pine (Ekblad et al. 1998), indicating a low degree of mycorrhizal association (Ekblad and Näsholm 1996). It should, however, be noted that in contrast to Ekblad et al. (1998), the whole root systems were analyzed in the present study inevitably resulting in a dilution of the mycorrhizal content.

Table 1. Biomass (g DW), root : shoot ratio and N concentration (%) in roots and shoots at harvest of Scots pine seedlings grown in a greenhouse and fertilized with N in the form of arginine, ammonium or nitrate.

Nitrogen source	Biomass (g DW)	R : S ratio	*N* (%) root	*N* (%) shoot
Arginine	1.92 ± 0.07	0.31 ± 0.02	2.22 ± 0.07a	1.67 ± 0.06
Ammonium	1.87 ± 0.10	0.27 ± 0.02	2.19 ± 0.07a	1.78 ± 0.06
Nitrate	1.78 ± 0.10	0.31 ± 0.02	1.92 ± 0.04b	1.71 ± 0.05
P values	0.51	0.11	<0.001	0.38

Different letters indicate significant difference between N sources at $P \leq 0.05$ (average values across girdling pretreatments ± SE, $n = 24$).

pH of growth substrate

The pH of the separate fertilizer solutions was set to 5.8 before every fertilizing event. Measurements of $pH_{(H_2O)}$ of the growth substrates from the three N sources at harvest showed that growth substrate pH differed significantly ($P < 0.001$) being 4.7 and 4.4 for the arginine and ammonium treatments, respectively, and 5.2 for the nitrate treatment.

Soluble sugars and starch

Girdling was effective in decreasing the nonstructural carbohydrate content of roots in all fertilizer treatments (Figure 2;

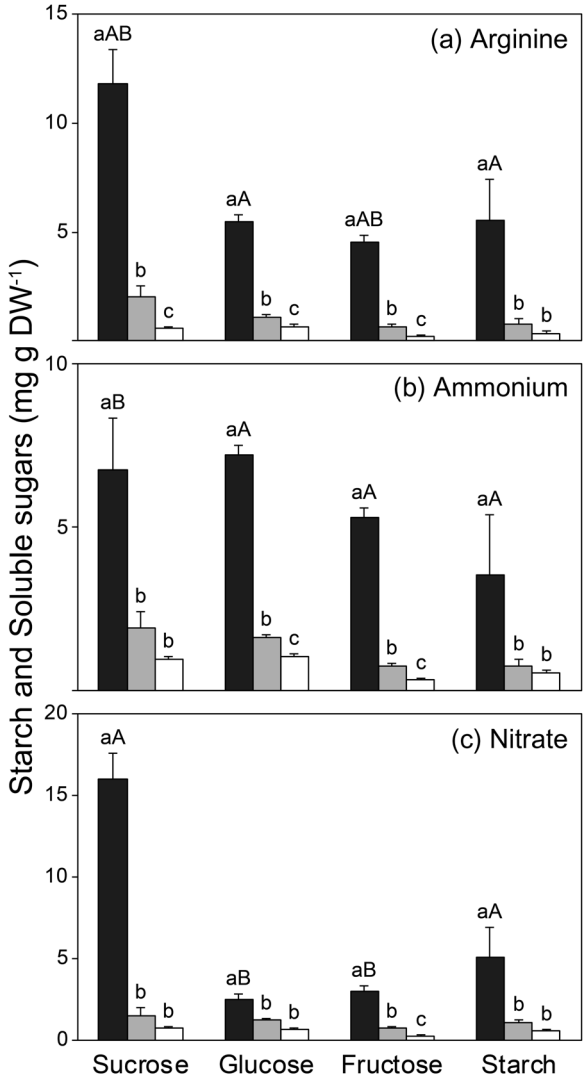

Figure 2. Soluble sugars and starch contents (mg g DW^{-1}) of roots from Scots pine seedlings that have been grown on the N sources (a) arginine, (b) ammonium or (c) nitrate and thereafter girdled for 3 or 7 days. Black, gray and white bars indicate ungirdled control, 3- or 7-day girdling treatments. Note the different scales of the *y*-axis. Different lower case letters indicate significant differences in carbohydrate levels within each N source caused by the girdling treatments and different capital letters indicate significant differences in carbohydrate levels between ungirdled control seedlings at $P \leq 0.05$ (average values ± SE, $n = 8$).

$P < 0.001$). Total non-structural carbohydrate concentrations in ungirdled control seedlings were 27.4, 22.8 and 26.7 mg g DW^{-1}, for the arginine, ammonium and nitrate treatment, respectively, while the corresponding carbohydrate contents for seedlings that had been girdled for seven days were 1.87, 2.85 and 2.31 mg g DW^{-1}, respectively (i.e., a decrease by c. 90%). Starch content was similar for ungirdled controls across treatments ($P = 0.49$), while the initial concentration of soluble sugars differed among treatments. Sucrose concentration of roots was higher in the nitrate-fertilized seedlings compared with ammonium-fertilized seedlings ($P < 0.001$), while the concentration in roots of the arginine-fertilized seedlings was similar to those found in seedlings treated with the two inorganic N sources. Glucose and fructose concentrations were the highest in the ammonium, intermediate in the arginine, and lowest in the nitrate treatment; however, fructose concentration in arginine-fertilized seedlings was not different from concentration in the other two treatments (Figure 2; $P < 0.001$–0.01).

Nitrogen acquisition

Uptake rates of arginine N were higher than those of inorganic N acquired from either ammonium or nitrate. Nitrogen uptake rates of ungirdled control seedlings were 15.8 for arginine N, and 5.0 and 2.2 μmol N root DW^{-1} h^{-1}, for ammonium and nitrate N, respectively (Figure 3; $P < 0.001$). Uptake rates of arginine N were also very high regardless of girdling pretreatment (Figure 3a). Seedlings that were girdled 3 or 7 days prior to the uptake experiment displayed similar uptake rates to control seedlings ($P = 0.72$). In contrast, as a result of girdling, the uptake of ammonium N declined to 3.4 and 2.8, and of nitrate N to 1.6 and 1.0 μmol N root DW^{-1} h^{-1} 3 and 7 days after girdling, respectively (Figure 3b and c; $P < 0.001$).

Nitrogen distribution between roots and shoot

In order to follow the fate of the acquired ^{15}N within the seedlings, the uptake study was conducted as a pulse-chase experiment. Twenty-four hours after the pulse of ^{15}N-labeled sources was applied, the distribution of acquired N between roots differed among N sources ($P < 0.001$) such that, on average, 95% of the arginine N, 73% of the ammonium N and 72% of the nitrate N resided in the roots. Almost all, i.e., 95–96%, of the acquired arginine N resided in the roots irrespective of girdling pre-treatment (Figure 4; $P = 0.51$). However, in the ammonium treatment, N remaining in the roots tended to decline over time (Figure 4; $P = 0.06$). Tukey's post hoc test showed that relatively more N remained in the control seedlings (73%) compared with those girdled 7 days prior to the pulse-chase experiment (63%), whereas seedlings girdled three days before (68%) could not be separated from those in the other two girdling treatments. In the control seedlings, 72% of the acquired nitrate N resided in the roots. However, there was a shift in the distribution of N from the roots to the shoots between the control and girdled seedlings (Figure 4; $P < 0.001$). Seedlings that had been girdled for 3 or 7 days allocated relatively more N to the shoots and only 40 and 29%, respectively, resided in the roots. Hence, girdling shifted allocation of N acquired as ammonium and nitrate, but not of N acquired as arginine.

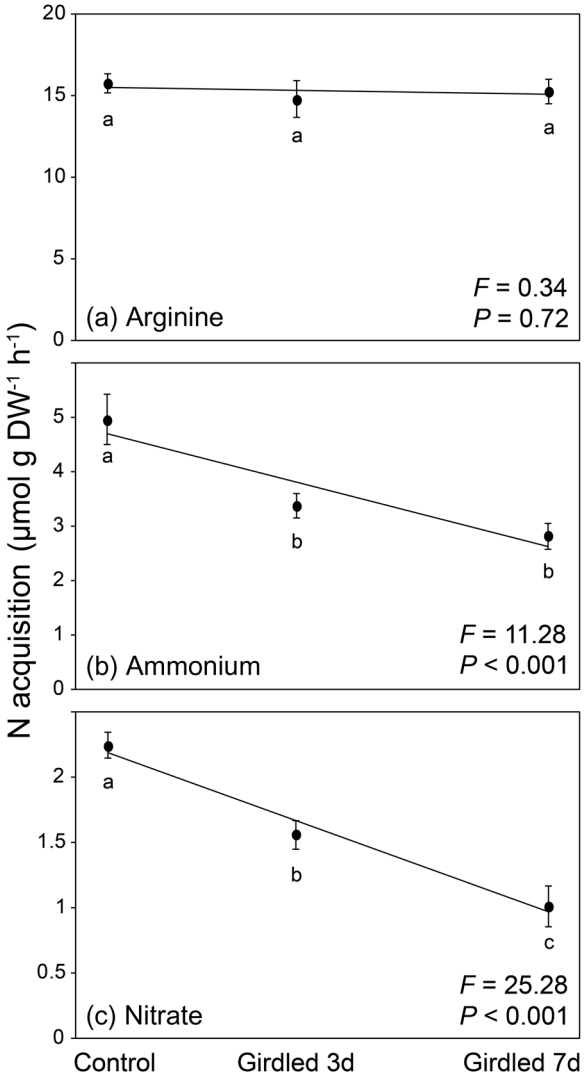

Figure 3. Nitrogen acquisition of girdled Scots pine seedlings from separate uptake solutions with (a) arginine, (b) ammonium or (c) nitrate provided at equimolar N concentrations. Note the different scales of the *y*-axes. Different letters indicate significant differences in acquisition between girdling time points at $P \leq 0.05$ (average values ± SE, $n = 8$).

Intact uptake of arginine

Intact uptake of arginine was verified in several ways. Firstly, roots of arginine-treated seedlings displayed higher δ ^{13}C values (–15.27‰) compared with roots of the ammonium- and nitrate-treated seedlings (–29.51‰ and –29.78‰), respectively ($P < 0.001$). The corresponding shoot values were –30.70, –30.78 and –31.14 ‰, respectively. Hence, shoots

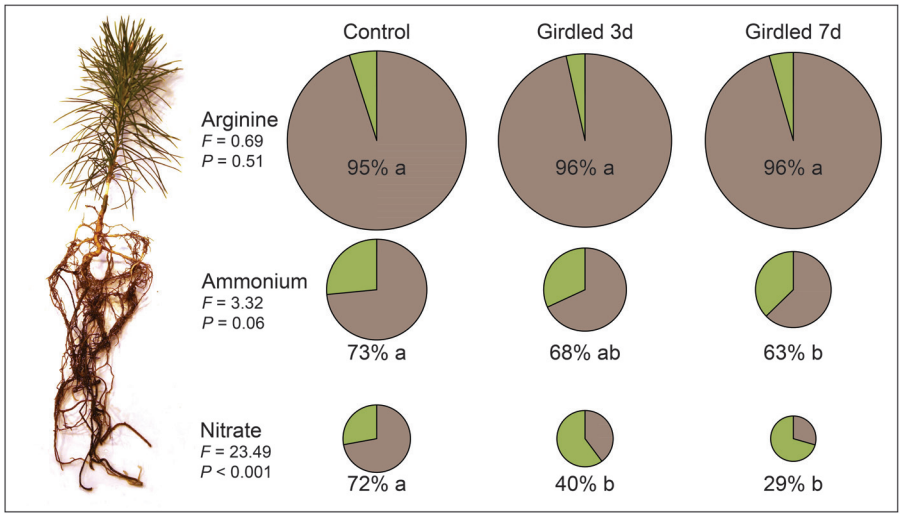

Figure 4. Nitrogen distribution between roots and shoots of N acquired from the different N sources arginine, ammonium and nitrate when phloem transport had been disrupted by girdling 3 or 7 days prior to uptake. Brown tone indicates the fraction of N residing in roots after 24 h, while green tone indicates N transported to shoots. The size of separate charts is representative of the relative amount of N that seedlings have acquired. Different letters indicate significant differences in N residing in roots between girdling time points at $P \leq 0.05$ (average values ± SE, $n = 8$).

displayed similar δ ^{13}C values across treatments ($P = 0.06$). Secondly, in roots, excess ^{13}C content was related to excess ^{15}N content for seedlings labeled with $U^{13}C_6$, $U^{15}N_4$ arginine (Figure 5), but not for seedlings labeled with either $^{15}NH_4^+$ or $^{15}NO_3^-$ (data not shown). The slope of the regression for ungirdled, arginine-labeled seedlings was not different from that of the theoretical maximum indicating that all N acquired from arginine was through intact amino acid uptake (i.e., slope = 1.5; $P = 0.13$). However, for seedlings girdled 3 or 7 days before labeling, slopes >1.5 were found (Figure 5; $P = 0.02$). Moreover, the differences in internal distribution of ^{15}N acquired from arginine or ammonium suggest that arginine was taken up intact rather than in mineralized form (Figure 4).

Discussion

We investigated the importance of current photoassimilates for N acquisition using girdling as a tool to gradually deplete roots of carbohydrates. Girdling proved to be effective in decreasing root C status of the seedlings as concentrations of both soluble carbohydrates and starch decreased in girdled seedlings (Figure 2). Hence, girdling enabled us to study the effect of root carbohydrate status on the uptake rates of N and whether this impact differed among different chemical forms of N. We hypothesized that girdled seedlings would show decreased N uptake rates due to the restricted availability of photoassimilates needed for the uptake and further assimilation into nitrogenous compounds. Specifically, we hypothesized that the uptake of arginine would be less affected by girdling compared with the inorganic N forms—ammonium and nitrate, the rationale for this assumption being the smaller energetic costs associated with this N form compared with the inorganic N sources (Zerihun et al. 1998). Because biomass allocation has been shown to depend on N concentration as well as the developmental stage of seedlings (Ericsson 1995; Ingestad and Ågren 1991), to test the hypotheses, we aimed at using seedlings with as similar traits as possible across the treatments. At harvest, total biomass, root : shoot ratio and N concentration of shoots were indeed similar between treatments; however, the N concentration of the roots of nitrate-treated seedlings was lower than that of arginine- and ammonium-treated seedlings (Table 1). Lower root N status may affect uptake rates of N positively (Persson et al. 2006). This was not the case, however, since nitrate uptake was generally low (Figure 3c).

Results from the pulse-chase experiment supported in part our hypothesis on the dependency of N uptake on recently assimilated C; girdling significantly decreased the uptake of both ammonium and nitrate N, but not of arginine N (Figure 3). Already 3 days after girdling, the uptake of ammonium decreased to 68% of the control; by 7 days, it had decreased further to 56% of the control (Figure 3b). Nitrate uptake of the girdled seedlings was 73% of the control seedlings three days after girdling and, seven days after girdling, their uptake capacity was further reduced to 45% of the control (Figure 3c) suggesting an even stronger dependency of nitrate uptake on carbohydrate transport from the shoot. These results are consistent with those reported by Bloom et al. (1992) in suggesting that, compared with ammonium, plant growth on nitrate may be more energy limited. Here, the N form of whose uptake was most affected by a sudden girdling-induced decrease in C supply was the one that is most energetically expensive to acquire.

In contrast to seedlings grown on ammonium or nitrate, arginine-fertilized seedlings were able to take up and incorporate

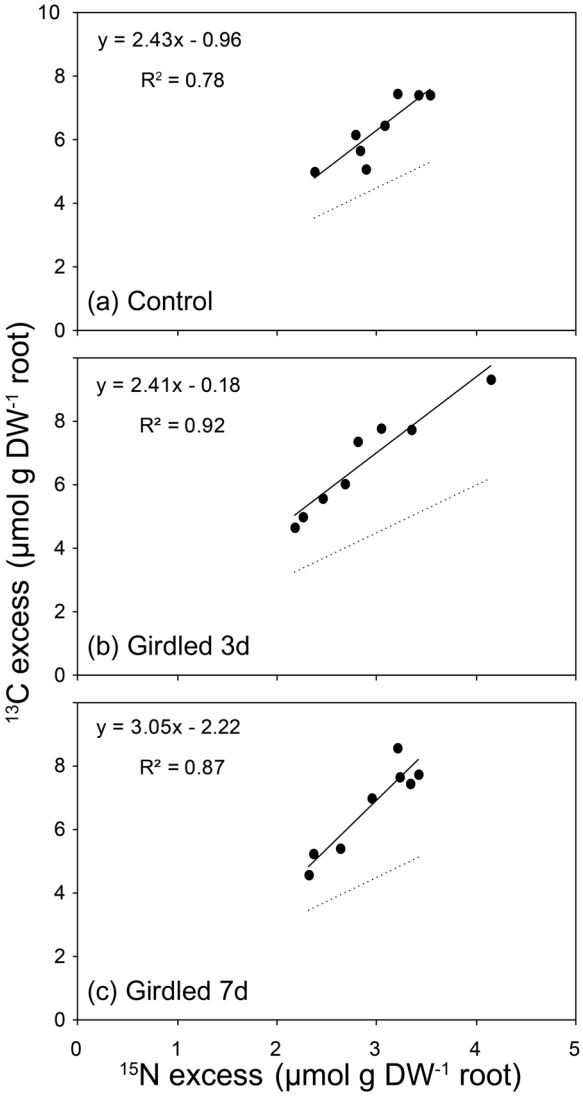

Figure 5. Linear regressions of ^{13}C and ^{15}N excess in roots of Scots pine seedlings 24 h after a pulse-chase experiment with universally labeled ^{13}C, ^{15}N-arginine. (a) Ungirdled control, (b) seedlings girdled for 3 days and (c) seedlings girdled for 7 days prior to uptake. Dotted lines indicate the theoretical relationship between isotopes corresponding to 100% of arginine N being absorbed in the form of intact amino acid.

organic N at an unaffected rate even when the starch and soluble carbohydrate reserves were significantly depleted after girdling. In a field study of beech trees, Dannenmann et al. (2009) showed that girdled trees acquired higher amounts of arginine compared with ungirdled control trees one year subsequent to girdling. The uptake of nitrate in girdled trees was also higher during the first season; however, the following year nitrate uptake decreased to almost zero or even negative values (probably indicating efflux of this N form from roots). This observation was accompanied by a decrease in dissolved organic C and microbial biomass in the soil of girdled plots, and it was suggested that lower C efflux from girdled trees reduced soil microbial competition for N, hence favoring plant N acquisition. In the current study, the uptake of both ammonium and nitrate decreased significantly but arginine uptake was high and remained unaffected by girdling (Figure 3). Hence, our results suggest that in the present experiment, the effect of girdling on uptake rates of the different N forms was mediated by a shift in root internal C-status and not by altered microbial competition for various N forms.

Nitrate uptake is often substantially lower than that of ammonium (Kamminga-van Wijk and Prins 1993; Kronzucker et al. 1997; Miller and Hawkins 2007), and the observed uptake of these N sources in control seedlings were within the range of those reported for conifers (Rygiewicz et al. 1984*a*, 1984*b*; Kamminga-van Wijk and Prins 1993; Öhlund and Näsholm 2001), and much lower than the acquisition of arginine N (Figure 3). Given that the seedlings acquired much more N from arginine in solution than from ammonium or nitrate in solution in the pulse-chase experiment, one may wonder why the arginine-grown seedlings did not reach a much larger size over the study period. Arginine is a strong cation at the relevant pH (4.7) of the growth substrate, and binds strongly to the growth substrate (peat). Thus, plants grown in peat and supplied with arginine as N source would experience a low arginine concentration even though the concentration in the added fertilizer may be high. Our studies of how carbohydrate depletion affects the regulation of the uptake of different N sources were carried out in solutions. Hence, the very high rates of arginine uptake may not occur for seedlings grown in a solid substrate. Moreover, seedlings across all treatments were given equimolar N concentrations, during growth, implying that the actual arginine concentration of the fertilizer was four times lower than those of ammonium and nitrate; while in the uptake experiments, we used equimolar substrate concentrations. Thus, uptake studies were performed using identical concentrations of the different N sources, while cultivation employed identical N concentrations of solutions and thus the concentration of arginine was four times lower than those of either nitrate or ammonium. Although not directly comparable, Öhlund and Näsholm (2001) also reported approximately four times as high uptake of arginine N as compared with ammonium N in an uptake experiment from mixed N sources conducted on containerized Scots pine seedlings.

We also hypothesized that in the short term, a larger fraction of N absorbed as arginine would reside in roots compared to N absorbed as either ammonium or as nitrate. The acquired arginine N in the present study was indeed almost exclusively found in roots 24 h following the pulse-chase experiment and 95–96% of ^{15}N remained in roots regardless of girdling (Figure 4). This short-term preferential allocation of arginine N to roots parallels the greater biomass distribution to roots noted for arginine-cultivated Scots pine and Norway spruce seedlings compared to seedlings cultivated on conventional ammonium nitrate fertilizers (Gruffman et al. 2012). However, the long-term

distribution of arginine-N will obviously be different from the short-term distribution as evident from growth studies employing arginine as the single N source (Öhlund and Näsholm 2001; Gruffman et al. 2012).

A comparison of δ ^{13}C among treatments indicated the intact uptake of arginine rather than mineralized amino acid N. Arginine-treated seedlings displayed significantly higher δ ^{13}C values in the roots compared with ammonium- and nitrate-treated seedlings, while δ ^{13}C values in the shoots were similar across treatments, further demonstrating that both arginine N and C resided in roots during the 24-h chase period. This short-term distribution of ^{15}N and ^{13}C to roots indicates that seedlings are able to make use of the energy gained from the organic C during carbohydrate limitation, at least in the short run.

Regressions between excess amounts of ^{13}C and excess amounts of ^{15}N have been used to indicate N uptake in the form of intact amino acids (cf. Näsholm et al. 1998; Näsholm and Persson 2001). This method relies on the assumption that ^{13}C is lost during mineralization of amino acids in soil (and potentially during metabolism in the root) resulting in a lower slope of the regression ^{13}C-excess : ^{15}N-excess compared with that of the supplied, labeled compound. In the present study, the population of ungirdled seedlings displayed a ^{13}C-excess : ^{15}N-excess ratio that was higher, but not statistically different ($P = 0.13$) from that of the supplied dual labeled arginine, suggesting that all N taken up by these seedlings was actually in the form of intact amino acid (Figure 5a). However, seedlings girdled three and seven days before labeling displayed ^{13}C-excess : ^{15}N-excess ratios higher than that of the supplied dual labeled arginine ($P = 0.017$ for both; Figure 5b and c). This high ratio of ^{13}C-excess : ^{15}N-excess may, theoretically, have at least two different causes. Firstly, it might result from a high retention of ^{13}C in the root, while ^{15}N is transported to the shoot. Secondly, plants have the capacity to exude NH_4^+ (Kronzucker et al. 1995), and hence, in this case, $^{15}NH_4^+$ resulting from root metabolism of labeled arginine might have been exuded. There was no relationship between slopes of ^{13}C-excess : ^{15}N excess of roots and ^{15}N of the shoots of seedlings supplied labeled arginine and hence the second explanation seems more probable. Also, the gradual increase in ^{13}C-excess : ^{15}N-excess of roots from ungirdled to 3 and 7 days after girdling suggests that at more severe carbohydrate status, seedlings were more inclined to use arginine as a source of C rather than as a source of N.

The reduced N uptake in girdled seedlings grown on ammonium was accompanied by a shift in the distribution of ^{15}N within the seedlings (Figure 4); compared with the control seedlings less of the N taken up by girdled seedlings was retained in roots seven days after girdling. It has been argued that ammonium N must be directly assimilated in roots following uptake in order to avoid risks of negative effects on ATP synthesis and potential disturbance in intracellular pH. However, the assimilation of N requires energy, and may be dependent on the relative availabilities of both ammonium and carbohydrates. For example, Schjoerring et al. (2002) showed that high external concentrations of ammonium repressed glutamine synthetase activity in roots, resulting in a concurrent increase of ammonium in xylem sap and leaves of tomato (*Solanum lycopersicum* (L.) H. Karst.) as well as oilseed rape (*Brassica napus* (L.)), perhaps indicating a shortage of carbohydrates. Our result shows that, although the primary site of ammonium assimilation is in the roots, the increased allocation of ammonium N to shoots was most likely caused by carbohydrate limitation in roots of girdled seedlings which could still assimilate ammonium N in their shoots.

At low external nitrate concentrations, the reduction and assimilation of nitrate N is primarily localized to roots; hence, during natural conditions, root assimilation of nitrate N predominates in gymnosperms (Smirnoff et al. 1984; Andrews 1986). Although variable among species, nitrate assimilation in shoots becomes increasingly important at higher external nitrate concentrations (Andrews 1986). In this study, the ungirdled control seedlings showed opposite ^{15}N distribution patterns compared to seedlings that had been girdled 7 days prior to the uptake experiment. In ungirdled control seedlings, 72% of the nitrate N was retained in the roots, while 71% of the nitrate N was transported to shoots of the girdled seedlings, a shift that was already noticeable following 3 days of girdling. Thus, our results provide further insight on the cause of a spatial shift and suggest that nitrate incorporation in roots is highly dependent on the availability of current photoassimilates.

There were no obvious growth benefits for seedlings supplied with arginine as N source. However, based on the results from this study, we conclude that there may be energetic benefits for plants to utilize organic N sources for their N nutrition, and that these benefits are displayed when carbohydrate supply to roots is reduced. A range of environmental conditions may lead to restrictions in root carbohydrate supply and root energy metabolism of seedlings, including low light, low soil temperatures and prolonged periods of seedling storage. Consequently, organic N would be of particular value for seedlings under these conditions.

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