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Nitrate induction in spruce: an approach using compartmental analysis

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Abstract. Using $^{13}NO_3^-$-efflux analysis, the induction of nitrate uptake by externally supplied nitrate was monitored in roots of intact *Picea glauca* (Moench) Voss. seedlings over a 5-d period. In agreement with our earlier studies, efflux analysis revealed three compartments, which have been identified as surface adsorption, apparent free space, and cytoplasm. While induction of nitrate uptake was pronounced, NO_3^- fluxes in induced plants were decidedly lower and the induction response was slower than in other species. Influx rose from 0.1 $\mu mol \cdot g^{-1} \cdot h^{-1}$ (measured at 100 μM $[NO_3^-]_o$) in uninduced plants to a maximum of 0.5 $\mu mol \cdot g^{-1} \cdot h^{-1}$ after 3 d of exposure to 100 μM $[NO_3^-]_o$ and declined to 0.3–0.4 $\mu mol \cdot g^{-1} \cdot h^{-1}$ at the end of the 5-d period. Efflux remained relatively constant around 0.02–0.04 $\mu mol \cdot g^{-1} \cdot h^{-1}$, but its percentage with respect to influx declined from initially high values (around 30%) to steady-state values of 4–7%. Cytoplasmic $[NO_3^-]$ ranged from the low micromolar in uninduced plants to a maximum of 2 mM in plants fully induced at 100 μM $[NO_3^-]_o$. In-vivo root nitrate reductase activity (NRA) was measured over the same time period, and was found to follow a similar pattern of induction as influx. The maximum response in NRA slightly preceded that of influx. It increased from 25 $nmol \cdot g^{-1} \cdot h^{-1}$ without prior exposure to NO_3^- to peak values around 150 $nmol \cdot g^{-1} \cdot h^{-1}$ after 2 d of exposure to 100 μM $[NO_3^-]_o$. Subsequently, NRA declined by about 50%. The dynamics of flux partitioning to reduction, to the vacuole, the xylem, and to efflux during the induction process are discussed.

Key words: Compartmental analysis – Nitrate (induction of uptake) – Nitrate reductase – Nitrogen-13 – *Picea*

Abbreviations and symbol: $[NO_3^-]_{cyt}$ = cytoplasmic NO_3^- concentration; $[NO_3^-]_{free\ space}$ = NO_3^- concentration in the cell wall free space; $[NO_3^-]_o$ = NO_3^- concentration in the external solution; NRA = nitrate reductase activity; ϕ = NO_3^- flux (for subscripts, see *Materials and methods*)

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Introduction

The uptake of nitrate by plants is unique in that it is considerably enhanced ('induced') by prior exposure to external nitrate (Minotti et al. 1969; Goyal and Huffaker 1986; Lee and Drew 1986; Aslam et al. 1993). Following a lag phase of 1–3 h after first exposure to exogenous nitrate, nitrate uptake rates gradually increase from low 'constitutive' levels (Clarkson 1986) to values as high as 30 times that of the constitutive level (Heimer and Filner 1971; Jackson et al. 1973; Goldstein and Hunziker 1985; Dhugga et al. 1988). A similar induction response to external nitrate provision has also been documented for the activity of assimilatory nitrate reductase (Solomonson and Barber 1990).

Rather than serving simply as an N source during induction, it now seems clear that nitrate functions as a signal, triggering both the inductive enhancement of nitrate uptake as well as that of cytoplasmic nitrate reduction (MacKown and McClure 1988; Tischner et al. 1993). This signal response appears to manifest itself in the specific appearance of several polypeptides, some of which are plasma-membrane-bound and implicated in plasma-membrane nitrate transport (Dhugga et al. 1988; Ni and Beevers 1994). However, direct participation of these proteins in nitrate transport has yet to be demonstrated.

At this point, the most convincing evidence for the inducibility of nitrate transport remains kinetic (Glass and Siddiqi 1994). In addition, studies of the induction of nitrate transport have been limited to only a select group of 'model organisms', while certain taxonomic groups have been completely neglected. Despite their enormous ecological as well as economic importance, coniferous species belong to the latter. While it is known that conifers normally grow in soils poor in nitrate (Lavoie et al. 1992, and references therein), it has also been demonstrated that nitrate can become the predominant nitrogen source available to conifers under a variety of ecological conditions. A concentration-dependent physiological response to external nitrate in terms of NO_3^- fluxes and subcellular concentrations has been shown in our previous studies with white spruce (Kronzucker et al. 1995).

In a previous study we characterized the dependence of $^{13}NO_3^-$ influx and of cytoplasmic $[NO_3^-]$ on the external concentration of nitrate in white spruce (Kronzucker et al. 1995). In this paper we present the first detailed investigation of the process of nitrate induction in a conifer species. We applied the highly sensitive radiotracer ^{13}N to conduct a time series of compartmental analyses (efflux analyses) for nitrate, the aim of which was to monitor simultaneously unidirectional flux processes as well as to estimate cytoplasmic and apoplasmic nitrate concentrations within the time-frame of transporter induction. Together with data on changes in root nitrate reductase activity (NRA) over that time period, this study attempts to further define the limits of nitrate utilization in spruce and to provide, at the same time, a detailed analysis of the dynamics of nitrate flux partitioning and the relative contributions of efflux and of the fluxes to reduction, vacuole and shoot during induction.

Materials and methods

Plant culture. Several-months-old seedlings of white spruce (*Picea glauca* (Moench) Voss., provenance 29170 from the Prince George region in British Columbia, Canada) were used in all studies. Seedlings were grown for a minimum of $3\frac{1}{2}$ months in a 3:1 peat/perlite mixture in styrofoam boxes in an outdoor nursery located on the University of British Columbia campus. Seedlings were then transferred indoors and, after gentle removal of the rooting medium, adapted to hydroponic culture in 24-dm^3 Plexiglas tanks. Seedling roots were non-mycorrhizal as confirmed by microscopic examination. Tanks contained 1/10-strength N-free Johnson's solution prepared using analytical-grade chemicals in distilled-deionized water (for a detailed description of growth conditions and for exact solution composition see Kronzucker et al. 1995). Plants were adapted under these conditions in the tanks for a period of three weeks prior to the experiments. For the induction experiments, NO_3^- was added at 100 μM as $Ca(NO_3)_2$. Seedlings were maintained in a 16 h/8 h photoperiod, 70% relative humidity, and at 20 ± 2°C. Light was provided at approximately 250 μmol·m^{-2}·s^{-1} at plant level by fluorescent tubes with spectral composition similar to sunlight.

Production of $^{13}NO_3^-$. Nitrogen-13 ($t_{1/2} = 9.96$ min) was produced by proton irradiation of H_2O (Meeks 1993) at the TRIUMF cyclotron on the University of British Columbia (UBC) campus in Vancouver, Canada. The protocol for removal of radiocontaminants followed "Procedure II" described in detail in Kronzucker et al. (1995).

Efflux analysis. Efflux experiments were carried out essentially as described before (Kronzucker et al. 1995). In brief, roots of intact spruce seedlings were equilibrated in non-labelled preloading solution for a minimum of 5 min and then transferred to $^{13}NO_3^-$-labelled loading solution for 35 min. Then, roots of intact seedlings contained in 'efflux funnels' were eluted with 60- to 100-cm^3 aliquots of non-radioactive solution (of a chemical composition otherwise identical to that of the loading solution) for intervals of time ranging from 5 s to 2 min, over an experimental duration of 22 min. Eluates from a total of 25 time intervals were collected separately, and 20-cm^3 subsamples from each eluate were counted for radioactivity in a gamma-counter (Minaxi δ, Auto-γ 5000 Series; Packard, Downers Grove, Ill., USA). After the final elution, roots and shoots were excised, introduced into scintillation vials and also counted for γ-activity.

Plants were maintained under steady-state conditions with regard to all environmental conditions, including nutrient concentrations throughout experiments (i.e. throughout growth, pretreatment, prelabelling, labelling, and elution), except in the case of NO_3^-, which was added as $Ca(NO_3)_2$, either only during labelling and elution (for uninduced plants; added as 10 or 100 μM), or else 1, 2, 3, 4, or 5 d prior to (and then also during) the efflux experiment (for induced plants; added as 100 μM).

Treatment of data. Based on our previously reported tests on compartment identity (Kronzucker et al. 1995), the three phases seen in efflux plots were interpreted as representing surface adsorption, apparent free space, and cytoplasm, respectively. Treatment of data was as outlined by Siddiqi et al. (1991), and was based on the theoretical considerations of Lee and Clarkson (1986). All experiments were performed using two replicates and were repeated at least three times. Representative experiments were chosen for semi-logarithmic plots of the rate of release of ^{13}N versus time of elution. All other data displayed in Figures and Tables represent the means of several experiments ± standard errors (SE). Calculations of fluxes and compartmental NO_3^- concentrations were as described by Siddiqi et al. (1991). All fluxes are expressed in μmol (or nmol) NO_3^-·g^{-1} (root FW)·h^{-1}. Symbols used for fluxes are as follows:

ϕ_{co} = efflux from the cytoplasm, obtained from the rate of ^{13}N release from the cytoplasm at time zero divided by the specific activity of the loading solution;
ϕ_{net} = net flux, obtained directly from the accumulation of ^{13}N in the plants at the end of the elution period;
ϕ_{oc} = unidirectional influx, calculated from $\phi_{net} + \phi_{co}$;
ϕ_{xylem} = flux of ^{13}N to the shoot, obtained directly from count accumulation in the shoot at the end of the elution period;
$\phi_{red/vac}$ = combined flux to reduction and to the vacuole, resulting from $\phi_{net} - \phi_{xylem}$;
ϕ_{vac} = unidirectional flux to the vacuole, obtained from the difference between $\phi_{red/vac}$ and the rate of nitrate reductase, measured in separate experiments, but under identical conditions (see below).

Nitrate reductase. For analysis of root NRA the in-vivo assay as described by King et al. (1992) was used. It was modified as follows: 0.1–0.4 g (FW) of root segments were excised and submersed in KPi buffer (100 μM at pH 7.7) in 10-cm^3 test tubes, to each of which was added 0.2 cm^3 *iso*propanol (99% v/v). In some control experiments, test tubes were closed off with air-tight caps, and N_2-purging was performed for 10 min. The test tubes were then covered with aluminum foil to keep out light, and the desired nitrate concentration (0.1–50 mM) was added from a 1-M KNO_3 stock solution, so that a final volume of 4 cm^3 was achieved in the test tubes. The tubes were then incubated for 30–90 min at 25°C in a waterbath. After incubation, the tubes were transferred to a second waterbath preheated to 95–100°C and were boiled for 10 min to quantitatively extract NO_2^- from the root tissue. After removal of the tubes from the waterbath, 1.5-cm^3 samples were withdrawn and transferred into 5-cm^3 test tubes, to which were added 0.25 cm^3 N-(1-naphthyl)-ethylene-diamine-hydrochloride and 0.5 cm^3 sulfanilamide. The detection reaction was started by adding approximately 60 mm^3 1% (v/v) HCl to each test tube (colour reaction was completed in ≈ 2 min). The tubes were left to stand for 30 min to allow for plant debris to settle. The absorbance was then read at 540 nm in a spectrophotometer (PU 8820 UV/VIS, Philips, Eindhoven, The Netherlands). As a standard, 100 nmol NO_2^-/1.5 cm^3 in an otherwise identical solution was used. Activities of root nitrate reductase (NRA) are expressed in μmol (or nmol) NO_2^- produced·g^{-1} (root FW)·h^{-1}.

Results

Compartment regression. In agreement with an earlier study (Kronzucker et al. 1995), three distinct phases were identified by linear regression of semi-logarithmic plots of ^{13}N efflux versus elution time. Figure 1 shows a representative plot for spruce seedlings, which were induced for 3 d

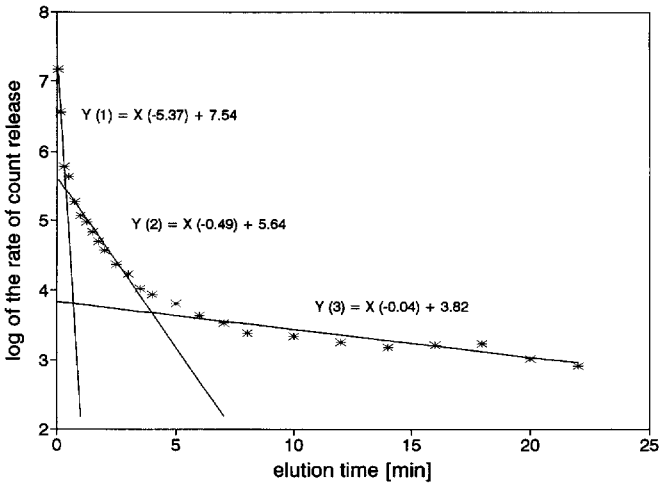

Fig. 1. Representative semi-logarithmic plot of the rate of release of ^{13}N [log (cpm released)·g^{-1}·min^{-1}] versus time of elution for intact roots of white spruce. Plants were induced at 100 μM $[NO_3^-]_o$ for 3 d, and efflux analysis was conducted at the same concentration. The plot includes linear regression lines as well as the respective linear equations for the three 'compartments' I(*1*), II(*2*), and III(*3*)

at 100 μM NO_3^- in the external solution ($[NO_3^-]_o$). The plot includes linear regression equations for each phase (r^2 = 0.89–0.99). The three phases exhibited half-lives of exchange of 2–3 s ('compartment I'), 20–30 s ('compartment II'), and ≈ 7 min ('compartment III'). Using multiple-range testing according to Newman-Keuls, no significant differences in these half-lives of exchange could be found between uninduced seedlings and seedlings which had been induced for varying periods of time (see Table 1).

Nitrate fluxes. Fluxes and compartmental NO_3^- concentrations were obtained by analyzing the semi-logarithmic efflux plots. As illustrated by Table 2, a clear increase in NO_3^- influx was evident with time of exposure to 100 μM NO_3^- in the external solution, from 0.1 μmol·g^{-1}·h^{-1} in seedlings which had not been previously exposed to NO_3^- (fluxes in these 'uninduced' plants were measured in transition from 0 to 100 μM $[NO_3^-]_o$), to a maximum of 0.5 μmol·g^{-1}·h^{-1} after 3 d of exposure to 100 μM $[NO_3^-]_o$. Thereafter, a decline in influx could be observed, to values around 0.3–0.4 μmol·g^{-1}·h^{-1}. Most of the ^{13}N taken up went to reduction or was sequestered in the vacuole (Table 2), while only negligible translocation to the shoot was measured over the duration of the experiment (47 min). Since efflux remained relatively constant at 0.02–0.04 μmol·g^{-1}·h^{-1}, net flux exhibited essentially the same pattern as influx over the 5-d time period. Only initially did efflux constitute a high percentage with regard to influx (around 30% in uninduced, 25% in 1-d-induced plants). After only 2 d of exposure to NO_3^-, apparent steady-state values around 4–7% were achieved.

Compartmental nitrate concentrations. Cytoplasmic NO_3^--concentration ($[NO_3^-]_{cyt}$) followed a pattern of rise, maximization and decline, comparable to that of influx and net flux. Differences as a function of induction state were substantial. Values of $[NO_3^-]_{cyt}$ increased from virtually undetectable, in the low-micromolar range, in uninduced plants to maximum values around 2 mM at 100 μM $[NO_3^-]_o$, and declined thereafter to around 1.2 mM (Table 3). Since $[NO_3^-]_{cyt}$ is calculated from the quotient of the rate of ^{13}N release (in a time period of 5 × the half-life for cytoplasmic exchange) and of the ratio of efflux to all fluxes removing $^{13}NO_3^-$ from the cytoplasm (Lee and Clarkson 1986; Siddiqi et al. 1991), the intercept of the presumed cytoplasmic regression line with the Y-axis in an efflux plot (corrected for specific activity in the labelling solution) is an indication of the magnitude of cytoplasmic NO_3^- accumulation, unless differences in NO_3^- accumulation are overcompensated for by high efflux percentages (see Kronzucker et al. 1995). Despite the fact that significant differences in efflux percentage did exist in our experiments between plants uninduced for NO_3^- and plants induced to a maximum, the differences in $[NO_3^-]_{cyt}$ were large enough to manifest themselves in significantly different Y-intercepts. To illustrate this effect,

Table 1. Half-lives of exchange ($t_{1/2}$) for NO_3^- of compartments I, II, and III (believed to represent surface film, apparent free space, and cytoplasm, respectively). Plants of white spruce were either grown without NO_3^- (experiments a and b; see also Table 2) or induced at 100 μM NO_3^- for the indicated time periods (c–g). Data ± SE (n = 2–3)

(Pre)treatment	Compartment I $t_{1/2}$ (s)	Compartment II $t_{1/2}$ (s)	Compartment III $t_{1/2}$ (min)
(a) uninduced ('0–10')	2.14 ± 0.27	16.95 ± 4.87	7.31 ± 2.35
(b) uninduced ('0–100')	2.27 ± 0.08	27.96 ± 5.71	6.12 ± 0.39
(c) 1-d ind.	2.41 ± 0.1	28.98 ± 2.37	6.38 ± 0.79
(d) 2-d ind.	2.38 ± 0.19	21.82 ± 3.89	8.44 ± 0.28
(e) 3-d ind.	2.78 ± 0.001	28.07 ± 3.26	7.49 ± 0.7
(f) 4-d ind.	2.45 ± 0.13	21.16 ± 2.09	7.51 ± 0.71
(g) 5-d ind.	2.64 ± 0.2	22.65 ± 3.22	6.64 ± 0.48

Table 2. Nitrate fluxes as estimated from compartmental analysis (for symbols see text). Plants of white spruce were either grown without NO_3^- (experiments a and b) or induced at 100 μM NO_3^- for the indicated periods of time. Efflux analyses were undertaken in perturbation (experiments a and b; measurement was at 10 or 100 μM $[NO_3^-]_o$, indicated as '0–10' and '0–100') and under steady-state conditions at 100 μM $[NO_3^-]_o$ (experiments c–g). Data ± SE (n = 2–3).

(Pre)treatment	NO_3^- fluxes ($nmol \cdot g^{-1} \cdot h^{-1}$)				
	ϕ_{oc}	ϕ_{co}	ϕ_{net}	$\phi_{red/vac}$	ϕ_{xylem}
(a) uninduced ('0–10'):	12.99 ± 1.44	4.51 ± 0.55	8.49 ± 0.89	8.48 ± 0.89	0.0089 ± 0.0039
(b) uninduced ('0–100'):	104.86 ± 15.09	28.6 ± 6.54	76.26 ± 8.56	74.48 ± 8.99	1.43 ± 0.44
(c) 1-d ind.:	165.06 ± 6.28	39.76 ± 3.78	125.3 ± 10.05	124.84 ± 10.21	0.45 ± 0.16
(d) 2-d ind.:	241.36 ± 10.92	17.16 ± 0.06	224.2 ± 10.98	223.45 ± 10.63	0.75 ± 0.42
(e) 3-d ind.:	482.81 ± 41.37	17.45 ± 4.02	465.36 ± 37.89	461.47 ± 36.7	3.89 ± 2.02
(f) 4-d ind.:	360.45 ± 23.57	26.67 ± 2.49	333.78 ± 20.63	333.19 ± 20.09	0.59 ± 0.23
(g) 5-d ind.:	394.77 ± 30.25	24.26 ± 3.14	370.51 ± 27.11	369.02 ± 26.38	1.49 ± 0.73

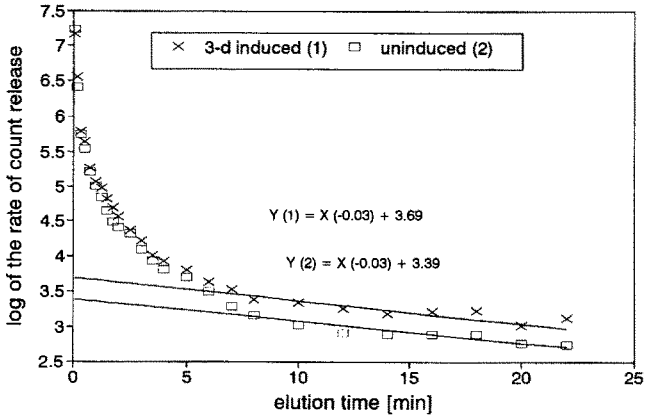

Fig. 2. Superimposed semi-logarithmic plots of the rates of release of ^{13}N [log (cpm released)$\cdot g^{-1} \cdot min^{-1}$] versus time of elution for intact roots of white spruce. Plants were either uninduced (□) or induced for 3 d (×) at 100 μM $[NO_3^-]_o$. Plots include linear regression lines and equations for the presumed cytoplasmic phases ('compartment III'). The significantly different Y-intercepts in the plants at different stages of induction indicate differences in cytoplasmic accumulation of NO_3^-

Table 3. Compartment concentrations of NO_3^- as a function of NO_3^-, estimated from compartmental analysis. Tissue volume was assumed to be 5% for the cytoplasm and 10% for the free space. Plants of white spruce were either grown without NO_3^- and then measured at 10 or 100 μM $[NO_3^-]_o$ (experiments a and b; see also Table 2), or they were exposed to 100 μM $[NO_3^-]_o$ for the indicated time periods (c–g). Data ± SE (n = 2–3).

(Pre)treatment	$[NO_3^-]_{cyt}$ (μM)	$[NO_3^-]_{free\ space}$ (μM)
(a) uninduced ('0–10'):	43.66 ± 9.3	16.06 ± 0.44
(b) uninduced ('0–100'):	253.25 ± 18.21	166.39 ± 16.21
(c) 1-d ind.:	427.17 ± 76.9	226.4 ± 7.6
(d) 2-d ind.:	978.41 ± 38.58	203.29 ± 90.07
(e) 3-d ind.:	1837.29 ± 381.37	203.67 ± 39.5
(f) 4-d ind.:	1281.24 ± 225.24	206.62 ± 19.77
(g) 5-d ind.:	1240 ± 10.01	184.39 ± 42.9

we overlaid two efflux plots, one from an experiment conducted on uninduced seedlings, the other on seedlings fully induced for 3 d at 100 μM $[NO_3^-]_o$ (Fig. 2), and included regression lines for the presumed cytoplasmic phases.

By contrast, NO_3^- in the cell wall free space ($[NO_3^-]_{free\ space}$) did not change with time of exposure to external NO_3^-. It was dependent only on the magnitude of $[NO_3^-]_o$, and increased from 16 μM at 10 μM $[NO_3^-]_o$ to 160–230 μM at 100 μM, with no significant differences at the different stages of induction (Table 3).

Root nitrate reductase and unidirectional flux to the vacuole. The NRA was inducible: in vivo NRA, as measured by the in vivo assay at 100 μM $[NO_3^-]_o$, increased from low 'constitutive' levels in uninduced plants (25 nmol $NO_2^- \cdot g^{-1} \cdot h^{-1}$) to maximal values (150 nmol $NO_2^- \cdot g^{-1} \cdot h^{-1}$) after a period of 2 d of exposure to 100 μM $[NO_3^-]_o$. After this, NRA declined to a steady value of 140 nmol $NO_2^- \cdot g^{-1} \cdot h^{-1}$ at the end of the 5-d period (Fig. 3, lower graph). Subtracting these values for NRA at 100 μM $[NO_3^-]_o$ from the values for the combined fluxes to vacuole and reduction as obtained from efflux analyses conducted at the same external NO_3^- concentration (Fig. 3, upper graph) allowed for estimates of the unidirectional flux to the vacuole (see dashed line connections of the two graphs in Fig. 3). This unidirectional flux to the vacuole stayed virtually unchanged at around 50 $nmol \cdot g^{-1} \cdot h^{-1}$ until after 24 h of exposure to external NO_3^-, when it slowly started to rise, reaching a maximum

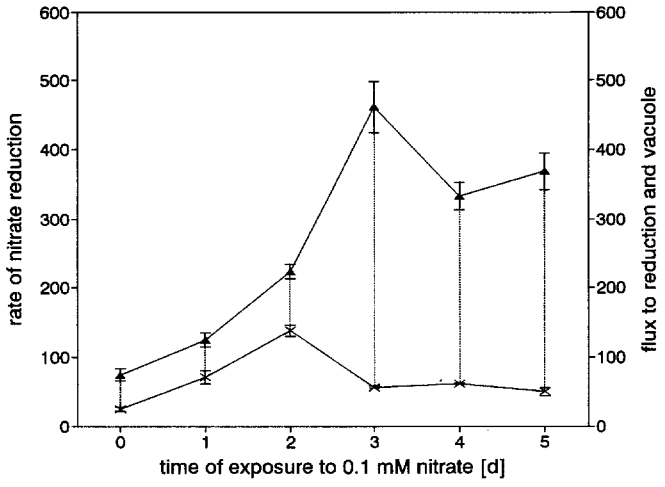

Fig. 3. In-vivo rate of root nitrate reductase (nmol·g^{-1}·h^{-1}) as measured at 100 μM $[NO_3^-]_o$ (×) and the combined flux to NO_3^- reduction and to the vacuole [nmol·g^{-1}·h^{-1}] as estimated from compartmental analysis (▲). The difference between the two rates represents the unidirectional flux of NO_3^- to the vacuole (nmol·g^{-1}·h^{-1}) (indicated as *dashed line* segments connecting the two graphs)

of 400 nmol·g^{-1}·h^{-1} at day 3 of induction. Thereafter, a 20–30% decline was observed.

Discussion

The steady-state assumption in compartmental analysis – is it violated in the present study? This study represents the first application of compartmental analysis in a time-dependent context to the problem of nitrate induction. As such, this application must be evaluated carefully. It may be argued that the approach of monitoring a time-dependent change in physiological plant activity violates a basic assumption underlying the theory of efflux analysis, which is the requirement of steady state, particularly with respect to compartment sizes and fluxes to and from compartments (Cram 1968; Walker and Pitman 1976). However, growing plants rarely, if ever, achieve a condition of true steady state. Nevertheless, provided that the duration of the efflux analysis is relatively short by reference to the time scale of developmental change and that environmental conditions are held constant during analysis, it can be assumed that the rate of change of compartmental parameters approximates zero during the course of such an analysis. In the present study, nitrate is slowly accumulated in the plants over time by continued exposure to 100 μM NO_3^-, and hence, strictly speaking, tissue $[NO_3^-]$ is not at steady state. However, during a 57-min experiment, it can be assumed that compartmental parameters remain essentially constant with respect to NO_3^- status of the tissue. Moreover, spruce, even in the seedling stage, is a genus characterized by an extremely low inherent growth rate (Chapin et al. 1986), making it a model organism for compartmental analysis. Likewise, the process of nitrate induction investigated in our study, compared to other plant systems, proceeds quite slowly on a time scale of several days. By contrast, the actual efflux experiment is completed in less than 1 h (35 min isotopic loading + 22 min elution). Therefore, we consider the physiological changes occurring during the experimental probing negligible on kinetic grounds, and believe the thermodynamic prerequisite of steady state to be closely approximated in our plant system. The validity of these assumptions/approximations regarding steady state is confirmed by the close agreement between influx values calculated on the basis of these assumptions and independent (direct) measurements of influx (see following section).

Agreement between flux values estimated from compartmental analysis and those obtained by independent methods. In our efflux analyses in white spruce, NO_3^- fluxes were found to be very low, even after full induction. This is in agreement with our earlier studies (Kronzucker et al. 1995). We employed, however, three independent approaches to confirm the magnitude of these fluxes. Firstly, we undertook measurements of unidirectional NO_3^- influx based on the accumulation of ^{13}N after a 10-min exposure of roots to labelled solution. Secondly, we determined the 'quasi steady' flux to the vacuole based on the accumulation of ^{13}N over 30- to 60 min periods (cf. Cram 1968), and, thirdly, we measured net flux, based on the depletion of $^{14}NO_3^-$ over 4- to 6-h periods. Since it was particularly critical to ascertain the validity of the use of compartmental analysis in a time-dependent study of nitrate induction (see previous paragraph), all of the above approaches were applied to seedlings at varying stages of induction (uninduced, 1-d-induced, and 3-d-induced seedlings). Flux values obtained from these independent assessments were in close agreement with values derived from compartmental analysis (see Table 4), which provides confirmation of the methodology of compartmental analysis and, in particular, its use in the present study. In addition, while unidirectional NO_3^- fluxes in conifers have remained undetermined by other workers, the 'steady state' values for net flux at around 0.3 μmol·g^{-1}·h^{-1} as seen in our experiments after 4–5 d of seedling exposure to external NO_3^- (see Table 2) agree well with values reported from longer-term net-uptake studies in other conifer species (see Kronzucker et al. 1995 for references). Similar agreement with work published by other groups was found for values of root NRA (Peuke and Tischner 1991; Schmidt et al. 1991; and references therein).

Magnitude and time profile of nitrate-induced nitrate uptake in spruce. Both in terms of magnitude and time profile, the induction response in spruce differs markedly from what is seen in other species. Constitutive levels of NO_3^- influx as low as 0.1 μmol·g^{-1}·h^{-1} (very close to our observed values in spruce) have been previously recorded in wheat (Goyal and Huffaker 1986) and in the barley variety 'Klondike' (Siddiqi et al. 1989). Nevertheless, constitutive levels of NO_3^- influx in particular varieties may be more than 10 times higher than this (Lee and Drew 1986; King et al. 1993). Estimates by other workers commonly fall between these extremes (Clarkson and Lüttge 1991; Aslam et al. 1992; Aslam et al. 1993). It is generally assumed that constitutive uptake is mediated by

Table 4. Nitrate influx and net flux at 100 μM $[NO_3^-]_o$ as determined by methods independent of compartmental analysis. Influx was measured concomitantly by the accumulation of ^{13}N in intact roots of white-spruce seedlings after immersion in isotopic solution for 10 min (a) and by the depletion of ^{13}N from solution during this time (b). Net flux was determined from the depletion of $^{14}NO_3^-$ over a period of 4–6 h (c). An additional estimate of influx (d) was obtained from the sum of ^{14}N-depletion values and ^{13}N-efflux estimates (see Table 2). Seedlings were either uninduced, or induced at 100 μM $[NO_3^-]_o$ for 1 or 3 d, as indicated. Data ± SE (n = 3–4).

Method of measurement	Induction state of seedlings		
	Uninduced	1-d induced	3-d induced
(a) ^{13}N accumulation (ϕ_{oc})	109.53 ± 10.59	295.01 ± 20.41	403.17 ± 67.49
(b) ^{13}N depletion (ϕ_{oc})	134.58 ± 11.3	326.02 ± 9.6	594.68 ± 27.15
(c) ^{14}N depletion (ϕ_{net})	94.01 ± 31.69	309.01 ± 29.59	497.02 ± 48.81
(d) ^{14}N depl. + ^{13}N effl. (ϕ_{oc})	122.61 ± 19.12	348.77 ± 16.69	514.47 ± 26.42

a low-capacity uptake system ('CHATS') and serves the function of 'NO_3^--sensing' (King et al. 1993). It is conceivable that the considerable differences reported between species and varieties for the activity of 'CHATS' in the uninduced state simply indicates genetic variability, but it is equally possible that the 'true' values for 'CHATS' are indeed quite similar in different plant systems, and that the differences observed are instead attributable to varying degrees of internal NO_3^- formation. This might be achieved via oxidative processes in the plant tissue (see Clarkson and Lüttge 1991, and Aslam et al. 1993 for references), to endogenous seed NO_3^- reserves, varying with the nature of the seed and the age of the plant material, or to trace NO_3^- contamination in chemicals or growth media (Mäck and Tischner 1986). Given that the spruce seedlings used in our study were several months old, that they were starved of NO_3^- for a period of three weeks prior to experiments, and that both analytical-grade chemicals and high-purity water were used in growth media and in induction experiments, the constitutive level of NO_3^- uptake determined is probably a realistic value for this species, and not different from values for certain varieties of barley (see above).

Unlike 'CHATS' activity, however, the magnitude of induced NO_3^- fluxes (commonly attributed to an 'IHATS', i.e. an induced high-affinity transport system; see King et al. 1993) is decidedly lower than in any other plant system so far investigated. Upon exposure to external NO_3^-, increases in the amplitude of NO_3^- influx of at least 5–10 times are normally observed in other species (Siddiqi et al. 1989; Warner and Huffaker 1989; King et al. 1993; Glass and Siddiqi 1994). In 'Klondike' barley, with its constitutive fluxes around 0.1 $\mu mol \cdot g^{-1} \cdot h^{-1}$, the increase of 'IHATS' was as high as 30-fold (Siddiqi et al. 1989). With its maximum influx around 0.5 $\mu mol \cdot g^{-1} \cdot h^{-1}$ at full induction, spruce sets a 'record low' in terms of inductive flux enhancement. White spruce seedlings do not normally encounter appreciable quantities of NO_3^- in their environment, and NH_4^+ serves as the main inorganic source of nitrogen (see Kronzucker et al. 1995, for a detailed discussion). This inherently low rate of inducible NO_3^- uptake may therefore represent an evolutionary adaptation to these particular environmental conditions. Since it is now believed that constitutive and inducible transporters (i.e. 'CHATS' and IHATS') are separate protein entities and appear to be regulated and coded for by different genes (Clarkson and Lüttge 1991; Aslam et al. 1992), an adaptive modification toward reduced expression (atrophy?) of the inducible but not of the constitutive transport component in spruce appears plausible from an evolutionary perspective.

The limited capacity of white–spruce seedlings to use NO_3^- as a nitrogen source is also seen in the time profile of induction. It is known that the time of exposure necessary for maximum fluxes to be achieved varies between species. In intact roots of higher plants, it ranges from 4–6 h in corn (Jackson et al. 1973) to 6–8 h (Warner and Huffaker 1989) and 24 h in different varieties of barley (Siddiqi et al. 1989; King et al. 1993). By comparison, the inductive response in spruce is much slower. The use of NO_3^- pulses has provided strong evidence for a 'signal' function of exogenous NO_3^- (MacKown and McClure 1988; Tischner et al. 1993). It has been postulated that quite low concentrations of NO_3^- can be effective in inducing NO_3^- uptake, and that once the induction process has been initiated, the presence of exogenous NO_3^- is no longer necessary (Tischner et al. 1993). However, Siddiqi et al. (1989) demonstrated that the time to peak induction varied inversely with $[NO_3^-]_o$ in barley, when exogenous NO_3^- was supplied continuously. In our studies with white spruce, there appeared to be no significant differences, with the time resolution used, in the kinetics of the induction response at different $[NO_3^-]_o$. Three days of exposure to exogenous NO_3^- were necessary at 10 μM, at 100 μM and at 1.5 mM $[NO_3^-]_o$ (Kronzucker et al. 1995).

Clearly, with its requirement for a 3-d exposure to external NO_3^- to maximize NO_3^- fluxes, white spruce is the slowest responding amongst the species investigated. With a view to the typical transient appearance of NO_3^- in forest soils, as in the form of seasonal NO_3^- flushes (Vitousek and Melillo 1979), such a slow induction response would appear to put spruce seedlings at a disadvantage in a plant competition scenario on soil habitats poor in other N sources such as NH_4^+ or organic N. Thus, combined with the extremely low inductive enhancement of NO_3^- influx, we see additional evidence in the slow kinetics of the induction response to external nitrate for the physiological classification advanced in a previous communication (Kronzucker et al. 1995) of white spruce as an 'NH_4^+ species' rather than an 'NO_3^- species'. This basic conclusion is also shared by other workers (Lavoie et al. 1992). The practical implications of this inability to utilize NO_3^- efficiently may be considerable with a view to large-scale reforestation of such species on disturbed (i.e. NO_3^--rich) forest soils. Such practices are common after clear-cut forest harvesting in large parts of North America (Kronzucker et al. 1995).

The role of nitrate flux partitioning and negative feedback regulation during induction. The fact that, kinetically, the

induction of NO_3^- uptake by external NO_3^- is so slow in spruce seedlings offers the interesting possibility of exploring the dynamics in flux partitioning and of monitoring the development of positive and negative feedback on NO_3^- influx during the process of induction. The mechanisms of negative feedback of NO_3^- uptake have been much discussed in the literature but few details have been resolved (Breteler and Siegerist 1984; Lee and Rudge 1986; Cooper and Clarkson 1989; Siddiqi et al. 1989; Lee et al. 1992; King et al. 1993). The discussion has particularly centered around the agent responsible for the 'negative feedback' effect on NO_3^- uptake (Siddiqi et al. 1989), seen in the decline of flux values following inductive rise to a maximum. Based on work with the glutamine synthetase inhibitor MSO (methionine sulphoximine), which appeared capable of overcoming this negative-feedback effect, Breteler and Siegerist (1984) and Lee et al. (1992) concluded that the effect arose from a product of ammonium assimilation rather than from nitrate or possibly nitrite (see also Lee and Rudge 1986). However, King et al. (1993) pointed out some of the difficulties in interpreting results from experiments with MSO, and presented data of their own, which showed that a 24-h exposure of Steptoe barley to 250 μM MSO failed to prevent negative feedback. Thus, either nitrate or nitrite would have to initiate negative feedback rather than reduced N products emanating from ammonium assimilation. This notion was further supported by the fact that barley mutants deficient in both the NADH-specific and the NAD(P)H-bispecific nitrate reductases displayed essentially the same pattern of induction and subsequent negative feedback as the respective wild type (Warner and Huffaker 1989; King et al. 1993). Similar evidence comes from studies with *Lemna gibba* (Ingemarsson et al. 1987), where inactivation of NRA was achieved by adding WO_4^{2-}, yet negative feedback was unaffected (Mattson et al. 1991).

We believe that some of the data presented in this paper may contribute to our understanding of the nature of induction and negative feedback. Firstly, the relatively constant absolute value of efflux (Table 2) during the entire course of induction in our study unequivocally identifies unidirectional influx as the target of negative feedback, not efflux, which would be a theoretical possibility for both up- and downregulation of net flux values. Only the percentage of efflux with respect to influx changes significantly, from high initial values to rather low steady-state values (Table 2). This high initial efflux percentage presumably signifies an initial imbalance of NO_3^- fluxes into the different compartments, and therefore a low efficiency in NO_3^- utilization. Interestingly, the unidirectional flux to the vacuole remained constant at around 50 $nmol \cdot g^{-1} \cdot h^{-1}$ during this time of poor NO_3^- utilization efficiency (i.e. up to day 2 of the induction treatment), while both nitrate reduction and cytoplasmic accumulation of nitrate were positively correlated with influx (see Table 3 and Fig. 3). Apparently, a low flux to the vacuole accounted for the relatively high amounts of NO_3^- lost to the ambient solution through efflux. Only after day 2, when ϕ_{vac} clearly increased, did the efficiency of NO_3^- utilization improve and efflux percentage assume a lower value. Thus, the enhancement of influx during induction appeared to be partitioned mainly to nitrate reduction initially (see the parallel rise of NRA and $\phi_{red/vac}$ in Fig. 3). During this time, the efficiency of NO_3^- utilization, as judged from the percentage of efflux, seemed to be low (see Table 2). Corresponding to the development of ϕ_{vac} and the downregulation of NRA, ϕ_{co} as a proportion of influx was much reduced.

In spruce, ϕ_{vac} also appeared to be the factor determining the onset of negative feedback. While influx continued to increase until day 3, the rate of nitrate reduction had achieved its maximum at day 2 and was immediately followed by a decline. It seems unlikely, therefore, that either nitrite or products of ammonium assimilation were responsible for the downregulation of influx. Were this to have been the case, influx should have been at a minimum as the production of nitrite and the flux to glutamine synthetase/glutamate synthase were at their maxima, i.e. at day 2. When negative feedback began to take effect, NRA had already been down-regulated to a steady-state level (Figure 3). This agrees with the conclusion arrived at by Mattson et al. (1991) and King et al. (1993), who considered nitrate itself to be an important candidate for the feedback effector. These workers were, however, unable to determine whether the cytoplasmic or the vacuolar nitrate pool was ultimately responsible (King et al. 1993). Data in the present study suggest that cytoplasmic nitrate concentration may be an unlikely candidate as the negative-feedback agent, because the peaks of influx and of cytoplasmic accumulation coincided at day 3 (Tables 2 and 3). In the down-regulated state, $[NO_3^-]_{cyt}$ was also lowered compared to the value at maximal induction. It seems more plausible that the signal originates from the vacuole, which also represents the largest sink for NO_3^-. This is, however, not intended to suggest that under different conditions other agents may not play a role in negative feedback upon NO_3^- influx. For instance, the cycling of amino-N through the whole plant (Cooper and Clarkson 1989; Muller and Touraine 1992) will almost certainly exert a regulatory effect on NO_3^- uptake. Our concern here is that before definitive evidence for a role of amino-N in the down-regulation of N uptake has been established, many workers appear to have rejected the potential role of NO_3^- itself in this process.

Conclusion

The present study has established that white spruce is poorly adapted to employ NO_3^- as a primary N source. Both the extent of the inductive enhancement of NO_3^- influx by exogenously supplied nitrate, and the time required to achieve maximum influx support this conclusion. The inherently low capacity for NO_3^- uptake, even after full induction, reflects the natural distribution of white spruce on soils generally deficient in nitrate. It seems that this feature alone renders white spruce an unsuitable species for the reforestation of clear-cut or otherwise disturbed sites, which are rich in NO_3^- rather than NH_4^+ or organic N. It is interesting to note that in studies by other workers (see Kronzucker et al. 1995 for references) no enhancement of NO_3^- uptake rates was observed in spruce

after inoculation of seedling roots with strains of their native ectomycorrhizal fungi. Thus, although the present studies were conducted with non-mycorrhizal plants, we believe that they also have relevance from an ecological point of view.

In addition, the important role of nitrate reduction in the inductive upregulation and that of NO_3^- flux to the vacuole and of vacuolar filling state in the subsequent downregulation of NO_3^- influx has been identified. Based on this work, we believe we are able to rule out several potential candidates for negative feedback upon NO_3^- uptake under the conditions of induction. These include nitrite, ammonium, ammonium assimilation products, as well as the cytoplasmic nitrate pool.

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