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Compartmentation and flux characteristics of nitrate in spruce

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Abstract. The radiotracer ^{13}N was used to undertake compartmental analyses for NO_3^- in intact non-mycorrhizal roots of *Picea glauca* (Moench) Voss. seedlings. Three compartments were defined, with half-lives of exchange of 2.5 s, 20 s, and 7 min. These were identified as representing surface adsorption, apparent free space, and cytoplasm, respectively. Influx, efflux, and net flux as well as cytoplasmic and apparent-free-space nitrate concentrations were estimated for three different concentration regimes of external nitrate. After exposure to external NO_3^- for 3 d, influx was calculated to be 0.09 $\mu mol \cdot g^{-1} \cdot h^{-1}$ (at 10 μM $[NO_3^-]_o$), 0.5 $\mu mol \cdot g^{-1} \cdot h^{-1}$ (at 100 μM $[NO_3^-]_o$), and 1.2 $\mu mol \cdot g^{-1} \cdot h^{-1}$ (at 1.5 mM $[NO_3^-]_o$). Efflux increased with increasing $[NO_3^-]_o$, constituting 4% of influx at 10 μM, 6% at 100 μM, and 21% at 1.5 mM. Cytoplasmic $[NO_3^-]$ was estimated to be 0.3 mM at 10 μM $[NO_3^-]_o$, 2 mM at 100 μM $[NO_3^-]_o$, and 4 mM at 1.5 mM $[NO_3^-]_o$, while free-space $[NO_3^-]$ was 16 μM, 173 μM, and 2.2 mM, respectively. A series of experiments was carried out to confirm the identity of the compartments resolved by efflux analysis. Pretreatment at high temperature or application of 2-chloro-ethanol, sodium dodecyl sulphate or hydrogen peroxide made it possible to distinguish the metabolic (cytoplasmic) phase from the remaining two (physical) phases. Likewise, varying [Pi] of the medium altered efflux and thereby $[NO_3^-]_{cyt}$, but did not affect $[NO_3^-]_{free\ space}$.

Key words: Compartmental analysis – Nitrate (compartmentation, flux) – Nitrogen-13 – *Picea*

Abbreviations and symbols: $[NO_3^-]_{cyt}$ = cytoplasmic NO_3^- concentration; $[NO_3^-]_{free\ space}$ = apparent-free-space NO_3^- concentration; $[NO_3^-]_o$ = concentration of NO_3^- in the external solution; $\phi = NO_3^-$ flux; ϕ_{co} = efflux from the cytoplasm; ϕ_{oc} = influx to the cytoplasm; ϕ_{net} = net flux; ϕ_{xylem} = flux to the xylem; $\phi_{red/vac}$ = combined flux to reduction and the vacuole

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Introduction

Most conifer species grow naturally on soils poor in nitrate and rich in ammonium (Vogt and Edmonds 1982; Lavoie et al. 1992, and references therein) and seem to preferentially utilize ammonium as their inorganic nitrogen source (Smirnoff and Stewart 1985; Chapin et al. 1986; Kamminga-van Wijk and Prins 1993; Knoepp et al. 1993; and references therein). However, after forest disturbance, such as fire, windthrow, insect calamities or clear-cutting, soil pH generally rises and a new microbial environment appears, which tends to convert the soil nitrogen pool from its predominantly reduced form to mostly nitrate (Likens et al. 1969; Rice and Pancholy 1972; Lodhi 1978). A limited capacity to utilize nitrate by conifer species may therefore be crucial in the success of reforestation trials.

However, detailed studies of nitrate transport have been carried out almost exclusively in angiosperm species and microalgae (see reviews by: Clarkson 1986; Glass 1988), while the physiology of nitrate uptake in conifers has been virtually ignored. Only limited information on nitrate fluxes and no data for cytoplasmic nitrate is available for conifers. A number of studies have been undertaken using depletion methods and experiments with the stable isotopic tracer ^{15}N (Rygiewicz et al. 1984a,b; Marschner et al. 1991; Flaig and Mohr 1992; Lavoie et al. 1992; Kamminga-van Wijk and Prins 1993; Knoepp et al. 1993; Plassard et al. 1993; and references therein). Both these approaches, however, have been limited to long-term estimations of uptake only. They do not take into account differences in influx and efflux, biological rhythms, and longer-term adaptation processes. They further do not provide insight into exchange kinetics across root cell plasmalemmata, the intracellular compartmentation of nitrate, or the magnitude of cytoplasmic nitrate accumulation.

We here report the first application of efflux analysis and of the short-lived radiotracer ^{13}N to a conifer species, and present detailed compartmental analyses for nitrate at three different ecologically relevant concentrations (Vogt and Edmonds 1982). We provide estimates for nitrate influx, efflux, net flux and flux partitioning within the

plant as well as for cytoplasmic and cell wall nitrate concentrations under these conditions. We furthermore present several experimental protocols designed to corroborate the validity of compartment identification in the type of efflux analysis employed in the study.

While it is recognized that most plant species, including spruce, are mycorrhizal in nature, we have employed non-mycorrhizal plants in these initial studies, which were designed to determine component NO_3^- fluxes and to characterize subcellular compartments for NO_3^- in the plant part of the association. Nevertheless, in reported studies which have specifically investigated the influence of ectomycorrhizal fungi on NO_3^- uptake in spruce, the presence of the fungal partner failed to enhance NO_3^- uptake rates above values recorded for the non-mycorrhizal counterparts (Littke et al. 1984; Rygiewicz et al. 1984a,b). Hence, the data reported in the present paper for non-mycorrhizal spruce roots may directly reflect the situation which applies to mycorrhizal plants in the field.

Materials and methods

Plant culture. White spruce (*Picea glauca* (Moench) Voss., provenance 29170, from the Prince George region, British Columbia, Canada) seedlings were grown in a peat/perlite (3:1) mixture in styrofoam boxes in an outdoor nursery for a minimum of $3\frac{1}{2}$ months. Seedlings were then transferred indoors and their roots gently washed under running tap water. They were then placed in 24-dm^3 Plexiglas hydroponic tanks containing well-mixed and aerated 1/10-strength modified N-free Johnson's solution, the composition of which was (in mM): KH_2PO_4 0.2, K_2SO_4 0.2, $MgSO_4$ 0.1, $CaSO_4$ 0.05, micronutrients and Fe-EDTA (in μM): Cl 5, B 2.5, Mn 0.2, Zn 0.2, Cu 0.05, Fe 2; NO_3^- was supplied as $Ca(NO_3)_2$ at the desired concentration, i.e. 0.01, 0.1, or 1.5 mM, 3 d prior to experiments. To adapt seedlings to hydroponic culture, minimize post-transfer shock effects and allow for steady-state growth to be established, while keeping the likelihood of microbial and algal contamination small, three weeks preconditioning in hydroponics was found ideal. As confirmed by microscopic examination, seedling roots were non-mycorrhizal. Solutions were monitored daily for $[K^+]$ (using an Instrumentation Laboratory 'model 443' flame photometer), for $[NO_3^-]$ (spectrophotometrically, using the method by Cawse 1967), and for pH (using pH paper). Solutions were buffered using powdered $CaCO_3$ and were exchanged completely every 4 d. Seedlings were maintained in a controlled growth room in a 16 h/8 h photoperiod, 70% relative humidity, and at 20 ± 2°C. Light was provided at approximately 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ 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 at the TRIUMF cyclotron on the University of British Columbia UBC campus in Vancouver, Canada, according to the nuclear reaction:

$$^{16}O + {}^1H \rightarrow {}^{13}N + {}^4He \text{ (Meeks 1993);}$$

A 15-cm^3 target volume was loaded remotely and a pressure of 3 atm was applied during irradiation. Irradiation was for 10–15 min with a 20-MeV proton beam and a beam current of 10 μA. The activity achieved was commonly around 700–750 MBq and the radiochemical purity for $^{13}NO_3^- \geq 90\%$. The target solution was transferred into a 20-cm^3 vial and transported in an underground pipeline with transit times of 2–3 min from the particle-acceleration facility to the UBC hospital. The time taken from pick-up at the hospital to beginning of the chemical purification processes in the laboratory at the UBC Botany Department was commonly 5–10 min. To remove contaminants, two procedures were used. Both procedures were carried out in a lead-shielded fumehood. Procedure I was as described by Siddiqi et al. (1990). Procedure II was as follows: The irradiated solution was taken up by a 20-cm^3 syringe and passed twice through a SEP-PAC Alumina-N cartridge (Waters Associates, Milford, Mass., USA) to remove the main radiocontaminant ^{18}F. The cartridge was rinsed with 5 cm^3 of 2.5 μM $Ca(NO_3)_2$ to remove residual radioactivity. Then the solution was transferred to a 50-cm^3 beaker, made strongly alkaline by addition of 100 mm^3 of 0.2 M KOH and boiled for 2–3 min to volatilize $^{13}NH_3$. Then 200 mm^3 of 1.2 M H_2SO_4 and 1 cm^3 of 10% (v/v) H_2O_2 were added to create an oxidizing and strongly acidic environment, and the solution was boiled for another 2–3 min. This was to oxidize and decompose any residual ^{13}N-nitrite and other nitrous compounds and drive them off as NO_x-gases. The solution was then cooled in an ice bath to room temperature, the pH adjusted to 6.5–7 with approximately 340 mm^3 of 0.2 M KOH (checked with pH paper), and 2 cm^3 catalase (2 $mg \cdot cm^{-3}$) were added to decompose remaining H_2O_2. While about 2 min were given for this reaction to complete, the radioactive solution was transported into a growth room where the experiment was to be carried out, and mixed into a prepared solution of the desired composition on a stirplate behind lead. Thus, approximately 20 min after irradiation of the target, labelled solution was ready to be administered. To obtain high specific activities, usually no more than 400 cm^3 of loading solution was prepared per experimental run.

Efflux analysis. Roots of intact seedlings were equilibrated in non-labelled preloading solution for 5 min, transferred to the $^{13}NO_3^-$-labelled loading solution for 35 min, then seedlings were held upright to drain excess surface label, and the roots eluted successively with 60- to 100-cm^3 aliquots (volume depending on root mass) for varying time periods. With t = 0 as the time of transfer from loading to 'washing' solution and t_{final} = 22 min for the last elution, the time periods for the 25 successive 'washes' were: 5 s (2×), 10 s (2×), 15 s (6×), 30 s (4×), 1 min (4×), 2 min (7×). During elution, seedlings were affixed to 1-dm^3 plastic funnels, the spouts of which had 5-cm lengths of silicone tubing attached to them; metal spring clips on the tubing served as manually operated drainage valves. Aeration and mixing was provided by glass pipettes taped to the insides of the funnels and connected to valve-controlled air lines. The 60- to 100-cm^3 elution aliquots were poured into the funnels at the prescribed times, collected in 100-cm^3 plastic beakers, from which 20-cm^3 subsamples were pipetted into 20-cm^3 scintillation vials. These were then counted in a Packard gamma-counter (Minaxi δ, Auto-γ 5000 Series). Roots were excised from the shoots immediately after the final elution, and the roots spun for 45 s to remove excess surface-bound solution. Both plant organs were weighed, introduced into scintillation vials, and counted for γ-activity.

In experiments designed to check the charge of the ^{13}N-label eluted from plants, 100-cm^3 eluates were collected at 10, 20, and 30 min after plant transfer into non-labelled solution. Then, 20-cm^3 subsamples of these eluates were passed through anion- and cation-exchange resins (analytical-grade AG 1-X 8 anion-exchange resin, 200–400 mesh, acetate form, and analytical-grade AG 50 W-X 8 cation exchange resin, 200–400 mesh, Na^+ form; BIO-RAD Laboratories, Richmond, Calif., USA), and the proportion of ^{13}N going into each fraction was determined by counting the radioactivity of the resins as well as that of the resin filtrates.

Plants received steady-state treatment with regard to nutrient concentrations throughout all experiments (3 d pretreatment + preloading-loading-elution; see earlier description of growth and pretreatment solutions for exact composition). In experiments conducted to test the identity of the compartments, perturbation designs were used for technical reasons. In sodium-dodecyl-sulphate (SDS)-experiments, plants were pretreated for 30 min, loaded and eluted in the presence of 1% SDS. In high-temperature experiments, plants were pretreated for 20 min at 75°C. In H_2O_2 treatments, H_2O_2 ($\leq$ 0.07% v/v) was present only in the loading solution (from incomplete catalase reactions – see above). In 2-chloro-ethanol experiments, gas exposure (0.075% v/v) was confined to a 4-d pretreatment in a 4-dm^3 gas chamber (enclosing the shoots) prior to the experiment.

Treatment of data was as outlined by Siddiqi et al. (1991), and essentially followed 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 ^{13}N release versus elution time, while all other data shown in tables and graphs represent means of several experiments ± standard error ($n = 2$–4).

Flux and pool-size calculations. Since NO_3^- is a metabolized ion, the commonly employed procedure in efflux analyses of non-metabolized ions, where the logarithm of radioactivity remaining in the plant tissue is plotted versus time of elution (Walker and Pitman 1976), is not applicable. However, assuming that efflux was constant during the steady-state conditions of the experiment and that specific activity in the plant compartments during elution was declining exponentially, the logarithm of the rate of release of radioactivity from the plant tissue can be plotted versus elution time (Lee and Clarkson 1986).

Linear regression on the semi-logarithmic plots was then used to resolve separate phases. The intercept with the ordinate of the regression line for the presumed cytoplasmic phase (i.e. the rate of ^{13}N release from the cytoplasm at time zero, R_o) divided by S_o, the specific activity of the loading solution, yielded the rate of efflux from the cytoplasm ϕ_{co} (divided, as all other fluxes, by root fresh weight), while net flux ϕ_{net} was obtained directly from the accumulation of ^{13}N in the plants. Influx ϕ_{oc} was calculated as $\phi_{oc} = \phi_{net} + \phi_{co}$. Flux to the xylem was obtained from count accumulation in the shoot, and the combined flux to reduction and to the vacuole $\phi_{red/vac}$ results from $\phi_{net} - \phi_{xylem}$. Because the identity of the chemical species (NO_3^-, NH_4^+, amino acids?) translocated to the xylem is unknown in our study, it is not possible to calculate the chemical flux for the shoot translocation term. Therefore, ϕ_{xylem} (as shown in Table 2) represents isotopic flux only; it was, however, calculated using $[NO_3^-]_o$ as the specific term. Thus, its value is for the derivative calculation of other flux terms (see above) and as an indicator of the relative proportion of ^{13}N translocated to the shoot. All fluxes were expressed in nmol (or μmol)·g^{-1} (FW)·h^{-1}.

Cytoplasmic NO_3^- concentration ($[NO_3^-]_{cyt}$) was calculated from the quotient of the rate of ^{13}N release during 5 × the half-life of cytoplasmic exchange and the ratio of efflux to all fluxes removing $^{13}NO_3^-$, and assuming 5% for cell volume occupied by the cytoplasm. Similarly, apparent-free-space NO_3^- concentration ($[NO_3^-]_{free\ space}$) was obtained by assuming 10% cell volume for the free space (see Siddiqi et al. 1991, for more details on calculation methods).

Results

Phase regression. Figure 1 shows a representative efflux plot for spruce seedlings at 100 μM external NO_3^- ($[NO_3^-]_o$). Three different exponential phases, presumably corresponding to physiological NO_3^- compartments, were identified. Fig. 1 includes linear regression lines for these kinetically distinct 'compartments' in a semi-logarithmic plot. High r^2 values (0.82–0.99) were usually found in the linear-regression approach. Half-lives of exchange for the 'compartments' were ≈ 2.5 s, ≈ 20 s, and ≈ 7 min (see Table 1), respectively. Multiple range testing according to Newman-Keuls indicated that there was no significant change in these half-lives with variation in pretreatment (e.g. $[NO_3^-]_o$, SDS, etc.). Compartments tentatively assigned to the exponential phases were the film of surface-adsorbed label carried over from the loading solution (the fastest exchanging phase), the apparent free space (the intermediate phase), and the cytoplasm (the slowest phase) (see Fig. 1).

Nitrate fluxes. As illustrated by Table 2a, a clear increase in NO_3^- influx was evident with increasing $[NO_3^-]_o$, from 0.095 μmol·g^{-1}·h^{-1} at 10 μM to 0.5 μmol·g^{-1}·h^{-1} at 100 μM and 1.2 μmol·g^{-1}·h^{-1} at 1.5 mM. Efflux rose from 0.004 μmol·g^{-1}·h^{-1}, to 0.03 μmol·g^{-1}·h^{-1}, and 0.3 μmol·g^{-1}·h^{-1}, constituting 4.3% of influx at 10 μM, 5.6% at 100 μM, and 20.9% at 1.5 mM $[NO_3^-]_o$. Net flux therefore was 0.091 μmol·g^{-1}·h^{-1} at 10 μM, 0.48 μmol·g^{-1}·h^{-1} at 100 μM, and 0.98 μmol·g^{-1}·h^{-1} at 1.5 mM (see Fig. 2). Most of the ^{13}N taken up went to reduction or was sequestered in the vacuole (Table 2a), while only negligible translocation to the shoot (< 1%) was noted over the duration of the experiments, which, including loading and elution, was 47 min. Unfortunately, due to low uptake rates for NO_3^- in spruce combined with high specific activities in the loading solutions, cross-contamination of shoots during plant handling unavoidably resulted in high errors for the shoot translocation term.

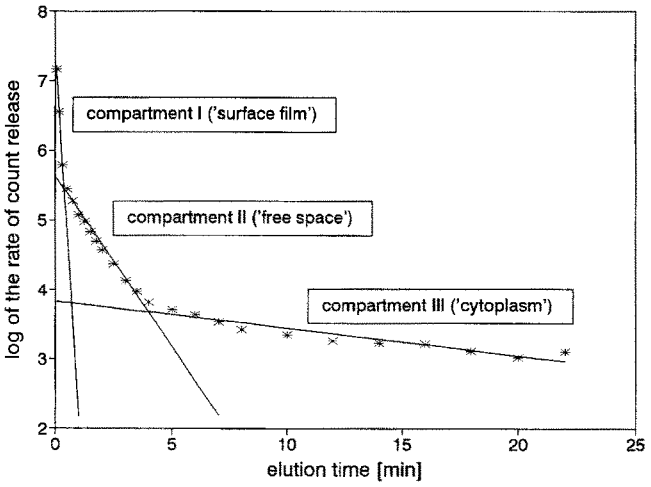

Fig. 1. Representative semi-logarithmic plot of the rate of release of ^{13}N [log (cpm released)·g^{-1}·min^{-1}] versus time of elution for intact roots of white spruce at 100 μM $[NO_3^-]_o$. The plot includes linear regression lines and equations for the three phases resolved in efflux analysis.

Table 1. Half-lives of exchange ($t_{1/2}$) for NO_3^- of compartments I, II, and III (identified as surface film, apparent free space, and cytoplasm, respectively) at three different external concentrations as estimated from compartmental analysis. Data ± SE ($n = 2$–3)

(Pre)treatment	Compartment I $t_{1/2}$ (s)	Compartment II $t_{1/2}$ (min)	Compartment III $t_{1/2}$ (min)
10 μM NO_3^-	2.11 ± 0.13	30.4 ± 1.11	7.5 ± 0.47
100 μM NO_3^-	2.62 ± 0.16	26.67 ± 2.35	8.45 ± 1.04
1.5 mM NO_3^-	2.4 ± 0.12	17.45 ± 2.04	6.9 ± 0.73

Table 2a. Nitrate fluxes as estimated from compartmental analysis (for symbols see text). Plants were induced for 3 d at the indicated external NO_3^- concentrations. Data ± SE ($n = 2$–3)

(Pre)treatment	NO_3^- fluxes (nmol·g^{-1}·h^{-1})				
	ϕ_{oc}	ϕ_{co}	ϕ_{net}	$\phi_{red/vac}$	ϕ_{xylem}[a]
10 μM NO_3^-	94.75 ± 2.64	4.06 ± 0.87	90.7 ± 3.51	90.05 ± 3.45	0.64 ± 0.06
100 μM NO_3^-	508.39 ± 84.48	28.22 ± 7.13	480.17 ± 81.64	473.43 ± 77.66	6.74 ± 4.67
1.5 mM NO_3^-	1242.39 ± 61.46	259.2 ± 25.8	983.2 ± 87.12	942.02 ± 80.34	41.17 ± 11.05

Table 2b. Nitrate fluxes as affected by high temperature of H_2O_2. Plants were induced at 100 μM $[NO_3^-]_o$ for 3 d (see also Table 2a)

(Pre)treatment	NO_3^- fluxes [nmol g^{-1}h^{-1}]:				
	ϕ_{oc}	ϕ_{co}	ϕ_{net}	$\phi_{red/vac}$	ϕ_{xylem}[a]
100 μM NO_3^- (control)	448.3 ± 64.11	38.56 ± 8.44	409.74 ± 56.83	406.49 ± 55.96	3.25 ± 2.03
100 μM NO_3^-/ 75 °C	21.09 ± 2.21	18.07 ± 2.49	3.03 ± 0.39	2.84 ± 0.35	0.19 ± 0.09
100 μM NO_3^-/ H_2O_2	45.71 ± 3.75	16.96 ± 2.78	28.75 ± 1.02	28.22 ± 1.23	0.53 ± 0.26

Table 2c. Nitrate fluxes at 1.5 mM $[NO_3^-]_o$ as affected by 4 d exposure to 0.075% (v/v) of the gaseous metabolic poison 2-chloro-ethanol (see also Table 2a)

(Pre)treatment	NO_3^- fluxes (nmol·g^{-1}·h^{-1})				
	ϕ_{co}	ϕ_{co}	ϕ_{net}	$\phi_{red/vac}$	ϕ_{xylem}[a]
1.5 mM NO_3^- (control):	1347.56 ± 105.24	218.67 ± 29.45	1128.89 ± 134.34	1084.85 ± 117.71	44.04 ± 15.41
1.5 mM NO_3^-/ 2-ChEth:	279.71 ± 16.72	217.94 ± 15.95	61.78 ± 4.39	60.09 ± 0.42	1.69 ± 0.55

Table 2d. Nitrate fluxes at 100 μM $[NO_3^-]_o$ as affected by varying $[Pi]_o$. Plants were induced at 100 μM $[NO_3^-]_o$ for 3 d (see also Table 2a)

(Pre)treatment	NO_3^- fluxes (nmol·g^{-1}·h^{-1})				
	ϕ_{oc}	ϕ_{co}	ϕ_{net}	$\phi_{red/vac}$	ϕ_{xylem}[a]
100 μM NO_3^-/ 20 μM Pi	469.87 ± 44.36	21.76 ± 2.79	448.1 ± 46.33	446.6 ± 44.57	1.48 ± 0.58
100 μM NO_3^-/ 200 μM Pi	514.99 ± 76.98	18.61 ± 4.33	496.38 ± 76.03	490.81 ± 73.69	5.57 ± 3.58
100 μM NO_3^-/ 2 mM Pi	524.17 ± 32.55	52.74 ± 4.43	471.43 ± 37.62	470.48 ± 35.71	0.95 ± 0.4

[a]Note that ϕ_{xylem}, unlike the remaining flux terms, represents the flux of ^{13}N rather than NO_3^- (see text for explanation)

Compartmental nitrate concentrations. The concentration of NO_3^- in the cytoplasm increased with $[NO_3^-]_o$, almost sevenfold from 0.3 mM at 10 μM $[NO_3^-]_o$ to 2 mM at 100 μM $[NO_3^-]_o$, and a further twofold to 4 mM with $[NO_3^-]_o$ at 1.5 mM, while $[NO_3^-]_{free\ space}$ was estimated to be 0.016, 0.173, and 2.2 mM at the respective same $[NO_3^-]_o$ regimes (Table 3a), and was therefore about 50% higher for all concentrations used than would be expected on the presumption that zero Donnan binding of NO_3^- occurs in the free space.

Compartment identification. Since the validity of our calculations of the above parameters depends upon the correct assignment of the compartments identified in efflux plots, several approaches were used (see *Materials and methods* for details of protocols) to confirm the assignment of the phases found to the specific subcellular and extracellular compartments.

As shown in Tables 2b–c, all treatments used to perturb the cytoplasm, i.e. H_2O_2, 2-chloro-ethanol, and high temperature, led to substantial reduction in count accumulation in the roots, both at 100 μM and at 1.5 mM $[NO_3^-]_o$. Treatment with the detergent SDS gave essentially similar results (data not shown). The reduction in count accumulation was evident in reduced estimates of $[NO_3^-]_{cyt}$ obtained from regression of compartment III

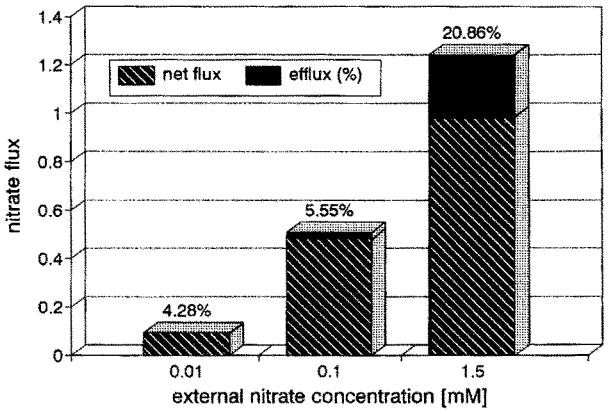

Fig. 2. Concentration dependence of net flux, influx and efflux ($\mu mol \cdot g^{-1} \cdot h^{-1}$) for NO_3^- in white spruce as determined by efflux analysis. *Dashed line segments* in stacked bar graph represent net flux, *dark filled segments* represent efflux (percentage of influx indicated above bar). Influx results from the sum of the former (see Table 1a)

Table 3a. Compartment concentrations of NO_3^- as a function of $[NO_3^-]_o$ as estimated from compartmental analysis. Tissue volume was assumed to be 5% for cytoplasm and 10% for free space. Plants were exposed to the indicated concentrations of external NO_3^- for 3 d. Data ± SE (n = 2–3)

(Pre)treatment	$[NO_3^-]_{cyt}$ (μM)	$[NO_3^-]_{free\ space}$ (μM)
10 μM NO_3^-	328.69 ± 27.07	15.79 ± 3.25
100 μM NO_3^-	2002 ± 332.74	173.47 ± 30.36
1.5 mM NO_3^-	4037 ± 251.44	2232.33 ± 109.8

Table 3b. Compartment concentrations of NO_3^- after disruptive treatment at 75°C or with H_2O_2. Plants were induced at 100 μM $[NO_3^-]_o$ for 3 d (see also Table 3a)

(Pre)treatment	$[NO_3^-]_{cyt}$ (μM)	$[NO_3^-]_{free\ space}$ (μM)
100 μM NO_3^- (control)	1449.87 ± 381.9	164.41 ± 35.18
100 μM NO_3^-/ 75°C	60.22 ± 25.78	256 ± 11.03
100 μM NO_3^-/ H_2O_2	148.46 ± 17.51	212.04 ± 14.78

Table 3c. Compartmental concentrations of NO_3^- as affected by 4 d exposure to the gaseous metabolic poison 2-chloro-ethanol at 0.075% v/v. Plants were induced at 1.5 mM $[NO_3^-]_o$ for 3 d (see also Table 3a)

(Pre)treatment	$[NO_3^-]_{cyt}$ (μM)	$[NO_3^-]_{free\ space}$ (μM)
1.5 mM NO_3^- (control)	3757 ± 458.32	2020 ± 189.27
1.5 mM NO_3^-/ 2-ChEth:	685.46 ± 64.71	1250 ± 73.63

Table 3d. Compartmental NO_3^- concentrations as a function of Pi concentration in the medium. Plants were induced for 3 d at 100 μM NO_3^-. $[NO_3^-]_o$ and $[Pi]_o$ during the experiment were as indicated (see also Table 3a)

(Pre)treatment	$[NO_3^-]_{cyt}$ (μM)	$[NO_3^-]_{free\ space}$ (μM)
100 μM NO_3^-/ 20 μM Pi	1737 ± 319.96	260 ± 36.32
100 μM NO_3^-/ 200 μM Pi	1462 ± 220.76	230 ± 45.93
100 μM NO_3^-/ 2 mM Pi	1396.1 ± 192.24	255 ± 36.41

(Table 3b–c) and of net flux values and was both attributable to depressed influx and to an increase in efflux percentage from the presumed cytoplasmic phase (Table 2b–c). On the other hand, $[NO_3^-]_{free\ space}$ was significantly affected only in 2-chloro-ethanol pretreatment (Table 3c), not by any of the other treatments (Table 3b).

Increasing the concentration of orthophosphate in the medium ($[Pi]_o$) led to a more than twofold increase in NO_3^- efflux in the range of 0.2 to 2 mM $[Pi]_o$ (Table 2d). Consequently, $[NO_3^-]_{cyt}$ appeared somewhat lower, albeit not significantly, at 2 mM $[Pi]_o$ than at the other concentrations (Table 3d). However, $[NO_3^-]_{free\ space}$ was unaffected by these variations in $[Pi]_o$ (Table 3d).

Discussion

Validity of efflux calculations. The present study is the first to examine NO_3^- exchange kinetics in conifer roots using the technique of compartmental analysis. Consistent with data on cereal species (Presland and McNaughton 1984; Lee and Clarkson 1986; Siddiqi et al. 1991), we found three distinct phases from which NO_3^- was exchanging over a 22-min experimental duration, with half-lives of exchange of 2.5 s, 20 s, and 7 min. Since the slowest exchanging phase detected and used for calculations in these experiments possessed a 7-min half-life, we employed a 35-min loading period to label seedling roots with the tracer. This represents 5× the half-life for the slowest phase, after which the specific activity of tracer in this compartment theoretically should have reached 96.86% of its value in bulk solution, and, hence, the assumption of steady state with regard to specific activity of the tracer, a prerequisite to using compartmental analysis, can be made with reasonable justification (Walker and Pitman 1976; Lee and Clarkson 1986). In order to ensure validity of flux and pool-size calculations, it was also important to ascertain whether the ^{13}N effluxing from the root cells was in the form of unconverted NO_3^-, and not as NO_2^-, NH_4^+ or possibly assimilation products (Lee and Clarkson 1986; Siddiqi et al. 1991). By passing efflux eluates collected at several different times during the washing period through anion- and cation-exchange resins and by determining both column-retained and column-eluted radioactivities, we found that ≥ 98.8% of the ^{13}N-containing species released from the plant roots were negatively charged. This rules out confounding effects due

to effluxing NH_4^+ or positively charged amino acids. Since the rates of nitrite reductase normally greatly exceed those of nitrate reductase (up to 30-fold higher rates have been reported; Aguera et al. 1990) and NO_2^- therefore does not normally accumulate in plant cells (Solomonson and Barber 1990; Siddiqi et al. 1992), a significant $^{13}NO_2^-$ pool available to efflux from the cytoplasm was unlikely under the conditions of our experiments. While the slight possibility of negatively charged ^{13}N-labelled amino acids contributing to efflux remains untested in the present study, the associated error can probably be taken as minimal (Lee and Clarkson 1986; Siddiqi et al. 1991). Since root cell processing of NH_4^+ derived from reduction of NO_3^-, as opposed to NH_4^+ absorbed directly from the external solution in the reduced form, appears to be compartmentalized in proplastids and carried out by plastidic glutamine synthetase and ferredoxin-dependent glutamate synthase (Redinbaugh and Campbell 1993), a large cytoplasmic pool of negatively charged ^{13}N-labelled amino acids is considered unlikely in the time-frame of our experiments.

It needs to be stressed that the validity of the calculations used to obtain values for compartmental concentrations and fluxes of NO_3^- crucially rests upon the correct assignment of root cell compartments to the phases seen in regression analysis of the semi-logarithmic efflux plots (see Fig. 1). Therefore we have undertaken several experiments designed to test the initial tentative interpretation of the regression lines, which took compartment I, II, and III to represent surface adsorption, apparent free space, and the cytoplasm, respectively. In previous studies, independent assessments of $[NO_3^-]_{cyt}$ (Ferrari et al. 1973) or apparent-free-space volume (Lee and Clarkson 1986) or a phase assignment based on a presumed in-series arrangement of subcellular compartments (Macklon et al. 1990) have been employed to identify the kinetically defined compartments. In the present study we have used special (pre-)treatments of the plants in an effort to confirm the sub-cellular assignment. Such an approach was first used by Siddiqi et al. (1991) and is expanded in the present study. In our experiments, seedlings were subjected to H_2O_2, SDS, high temperature, or the metabolic poison 2-chloro-ethanol. We found a consistent reduction of ^{13}N accumulation in the plants (attributed to both reduced influx and enhanced efflux), which we explain by the destructive effects of the given treatments on membrane integrity and metabolic processes. Compartment III, the presumed cytoplasmic phase, was therefore either partially (H_2O_2) or almost completely (other treatments) eliminated, while the presumed 'physical' phases (surface film and free space) were unaffected by the same treatments. To illustrate this finding, we overlaid two efflux plots in Fig. 3, one from an experiment conducted in the presence of H_2O_2, the other without. The significantly different Y-intercepts of the regression lines demonstrate the specific inhibitory effect on compartment III. The absence of such an effect on the other two compartments is demonstrated by the lack of a significant reduction in calculated $[NO_3^-]_{free\ space}$ (see Tables 3b–c and following section).

Nitrate in the apparent free space. Interestingly, estimates for $[NO_3^-]_{free\ space}$ obtained from analysis of compartment

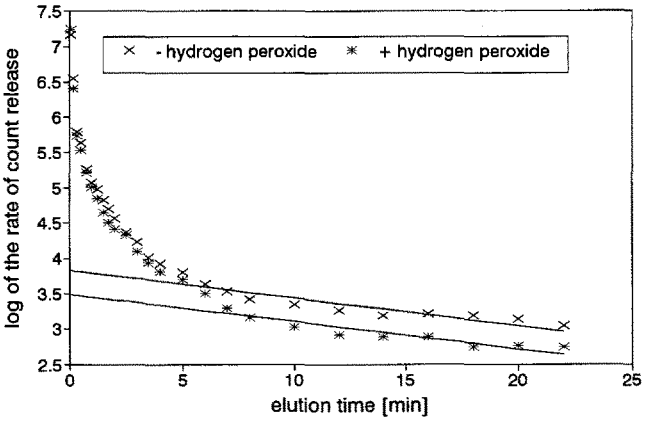

Fig. 3. Combined semi-logarithmic plots of the rate of release of ^{13}N [log (cpm released) $\cdot g^{-1} \cdot min^{-1}$] versus time of eluton for intact roots of white spruce at 100 μM $[NO_3^-]_o$ from experiments with (*bottom line*) and without (*top line*) H_2O_2 present in the loading solution. Plots include linear regression lines for the presumed cytoplastic phase (compartment III)

II in our experiments consistently yielded an overestimate of about 50–150% with regard to $[NO_3^-]_o$ (Tables 3a–d). Were NO_3^- in the free space present only in the water free space (i.e. without Donnan inclusion), the same concentrations should be expected for free-space NO_3^- as for the external solution. There is the possibility that an error has been introduced into the calculation by the assumption of a value of 10% cell volume for the free space. Yet, similar overestimates have previously been found in completely different plant systems (McNaughton and Presland 1983; Siddiqi et al. 1991), which leads us to believe that the effect is real rather than an artifact of calculation. Given the surplus of negative charges present in the Donnan free space, which should lead to an exclusion of anions from the free space rather than an enrichment, such overestimates for $[NO_3^-]_{free\ space}$ are surprising. We believe this effect to be due to the presence of specific NO_3^--binding proteins, in analogy to the anion-binding proteins found in other species, e.g. for phosphate and sulphate (Siddiqi et al. 1991, and references therein; Arthur Grossmann, Department of Plant Biology, Stanford, Calif., USA, personal communication). There are, indeed, indications that the binding is NO_3^--specific. Varying the external concentration of orthophosphate Pi, which, according to the lyotropic series of ion binding strength, should outcompete NO_3^- at non-specific positive binding sites, failed to alter $[NO_3^-]_{free\ space}$ in our studies (Table 3d). The fact that after 4 d exposure to the gaseous metabolic poison 2-chloro-ethanol, our only longer-term disruptive treatment, $[NO_3^-]_{free\ space}$ actually decreased to that of the external solution may indicate involvement of 'plastic' metabolically produced binding components (Table 3c). However, in SDS and high-temperature experiments, which should have led to the denaturation of proteins, the overestimation was not abolished. It may therefore be that the presumed apparent-free-space proteins are especially resistant to denaturing treatments, or else that an as yet unidentified non-proteinaceous positively charged cell wall component is responsible for binding NO_3^-. Siddiqi et al. (1991) advanced a proposal, according to which

binding to the external face of the NO_3^- transporter proteins may be responsible for the enrichment of NO_3^- in the free space. This proposal seemed attractive, since an increase in the NO_3^--concentrating effect was seen with induction of NO_3^- transport (McNaughton and Presland 1983; Siddiqi et al. 1991). Also, in contrast to our studies with spruce, in which we found a similar overestimate at 1.5 mM $[NO_3^-]_o$ as at the other concentrations, these workers did not find the effect to be present at $[NO_3^-]_o \geq 1$ mM, which seemed to argue for the phenomenon to be directly connected to the inducible high-affinity transporter active at $[NO_3^-]_o \leq 1$ mM. However, on the basis of our peroxide/detergent/high-temperature work, this proposal has to be rejected, given the clear disruptive effect of these treatments on membrane proteins as seen in our efflux analyses.

At present, it is difficult to advance an explanation that reconciles all the observed phenomena. Possibly, non-proteinaceous cell wall components are responsible for the concentrating of NO_3^- to a 'baseline' value, while inducible proteins lead to the up to 13-fold increase that has been seen upon induction (McNaughton and Presland 1983). The latter effect may be somewhat obscured in the several-months-old spruce seedlings used in the present study, due to the presence of large proportions of lignified/suberized roots, which may display non-proteinaceous binding but may not express inducible proteins. Clearly, this warrants further investigation.

Nitrate in the cytoplasm. The half-life of exchange (7 min) observed for compartment III, the presumed cytoplasm, is in close agreement with the values obtained by others in cereal species (Presland and McNaughton 1984; Lee and Clarkson 1986; Siddiqi et al. 1991; Devienne et al. 1994, and references therein). In our group, we found similar $t_{1/2}$ values (≤ 10 min) for NO_3^- in control experiments with barley and tomato plants (data not shown). Macklon et al. (1990), on the other hand, reported a significantly longer $t_{1/2}$ value (107 min) for the presumed cytoplasmic compartment in their ^{15}N studies of onion root segments. This may be a system-specific difference, but given the closeness of values in systems as far apart systematically as grasses, tomato, and conifers, such a drastic difference in onion seems surprising. We believe, instead, that this apparent contradiction is attributable to an arbitrary assignment by Macklon and coworkers of the phase displaying the 107 min half-life to the cytoplasm, which was not tested for in their study. The real cytoplasmic compartment may have been incorrectly interpreted as part of the cell wall fraction. In our perturbation experiments (H_2O_2, SDS, 2-chloro-ethanol, high temperature) and those by Siddiqi et al. (1991) we see convincing evidence that our 7-min half-life compartment is not of a primarily physical nature (as expected for cell wall fractions), but is indeed plasmalemma-bound and metabolically dependent, and that therefore estimations of the cytoplasmic pool size of NO_3^- can be undertaken with reasonable confidence on the basis of regressing this compartment.

To our knowledge, no data on $[NO_3^-]_{cyt}$ in conifers have been published. The present study is the first to provide such values. However, in several other systems $[NO_3^-]_{cyt}$ has been determined using a number of different techniques. Based on compartmental analysis with the tracer ^{13}N, as in the present study, $[NO_3^-]_{cyt}$ values of 50–100 mM were reported for maize roots (Presland and McNaughton 1984), and 26 mM (Lee and Clarkson 1986) for barley roots. Siddiqi et al. (1991) examined the dependence of $[NO_3^-]_{cyt}$ on $[NO_3^-]_o$ in intact barley roots and found $[NO_3^-]_{cyt}$ to vary from 0.95 to 36.5 mM as $[NO_3^-]_o$ increased from 0 to 1 mM. Macklon et al. (1990), using the stable tracer ^{15}N in compartmental analyses of onion root segments, reported higher values in the 40- to 50-mM range for the compartment they identified as the cytoplasm. Devienne et al. (1994) undertook $^{15}NO_3^-$-compartmental analyses in intact roots of 30-d-old wheat plants and reported values in the 10- to 20-mM range at $[NO_3^-]_o$ from 0.1 to 5 mM. Miller and Smith (1992), using nitrate-specific electrodes, reported 4.9 mM and 2.7 mM at 10 mM $[NO_3^-]_o$ in barley for epidermal and for cortical root cells, respectively. These values are lower than those published by the previous workers, but they are still in the millimolar range.

Using a method based on an analysis of in-vivo and in-vitro rates of nitrate reductase, however, Robin et al. (1983), Belton et al. (1985) and King et al. (1992) all reported values for $[NO_3^-]_{cyt}$ in the micromolar range. Robin et al. (1983) determined a range of 10–100 μM in leaf tissue of barley, corn, rice, alfalfa, pea and soybean. Belton et al. (1985) obtained values of 65–140 μM from data gathered on wheat plants and rose cells, also confirming these rather low values by ^{14}N-nucler magnetic resonance spectroscopy, although the latter technique provided only low sensitivity for NO_3^- (in contrast to the situation for NH_4^+). King et al. (1992) found slightly higher values, from 0.66 mM at zero $[NO_3^-]_o$ to 3.9 mM at 20 mM $[NO_3^-]_o$, but likewise significantly lower than estimates obtained by compartmental analysis or microelectrodes.

Why these estimates are so widely spread is not entirely clear. It has been advanced that different pools of $[NO_3^-]_{cyt}$ may be measured by the nitrate-reductase method as opposed to compartmental analysis (Siddiqi et al. 1991; King et al. 1992), the latter perhaps measuring a (cortical) storage pool with higher $[NO_3^-]_{cyt}$, while the former would determine a pool rich in nitrate reductase and therefore lower in $[NO_3^-]_{cyt}$, possibly the epidermis.

The $[NO_3^-]_{cyt}$ values obtained in our present study in spruce are in the range of 0.3–4 mM at $[NO_3^-]_o$ from 0.01 to 1.5 mM and are therefore closer in magnitude to some of the values reported using the nitrate-reductase and microelectrode methods than to other compartmental-analysis data. This is, however, not to suggest a methodological reconciliation. It rather needs to be pointed out that a direct comparison of values is only legitimate between data obtained via the same method, because of the unresolved discrepancy of which pools are measured by which method. Given the fact that great care is taken in compartmental analysis to achieve a steady-state labelling condition with the tracer before measurements are undertaken, we can assume to be reporting cross-sectionally (and longitudinally) averaged values for $[NO_3^-]_{cyt}$ in the root cortex. A possible difference in epidermal cells would certainly be masked by the much larger volume of the cortex and the therefore much stronger cortical 'signal'.

It emerges clearly, however, by comparing our compartmental-analysis data to those of other workers (see above), that spruce root cells accumulate significantly less NO_3^- than other species examined by the same method. Interestingly, cytoplasmic concentration estimates for NH_4^+ in spruce (data not shown) are much closer in magnitude to the NO_3^- values found in these other species and to NH_4^+ levels in a plant such as rice which is especially adapted to NH_4^+ (Wang et al. 1993). This apparent discrimination against oxidized N is also seen on the level of NO_3^- fluxes.

Nitrate fluxes. The NO_3^- fluxes in white spruce as determined by our compartmental analyses were low, despite the fact that they represent maximal values in a state of full induction for NO_3^-(Kronzucker et al. 1995a). The fluxes derived by compartmental analysis are in close agreement with values obtained in independent experiments relying on three different methods, firstly with measurements of unidirectional NO_3^- influx based on accumulation of ^{13}N after a 10-min exposure of roots to labelled solution, secondly with determinations of 'quasi steady' flux to the vacuole based on the accumulation of ^{13}N over 30- to 60-min periods (cf. Cram 1973), and thirdly with net flux measurements based on the depletion of $^{14}NO_3^-$ over 4- to 6-h periods (Kronzucker et al. 1995a). This provides good confirmation for the technique employed in this study.

Fluxes for NO_3^- were found to be much lower than is typical in agricultural species (Clarkson 1986; Glass 1988). Interestingly, fluxes of NH_4^+ measured by the same technique in the same plant system are in the order of 4–5 times higher than those for NO_3^- (Kronzucker et al. 1995b). We believe that this, along with the lower values for $[NO_3^-]_{cyt}$, may be a manifestation of a lack of adaptation to NO_3^- as an N source in spruce, and not simply of an inherently lower growth rate (cf. Chapin et al. 1986). Evidence for this apparent lack of adaptation to NO_3^- has also been seen in other conifer species (Marschner et al. 1991; Flaig and Mohr 1992; Lavoie et al. 1992; Kamminga-van Wijk and Prins 1993; Knoepp et al. 1993; Plassard et al. 1994; and references therein). In an evolutionary sense, this N-source discrimination may be explained by the fact that later successional conifers, such as white spruce, do not normally encounter appreciable soil NO_3^- concentrations, while for most agricultural species NO_3^- is the predominant N form available in soils (see *Introduction* for references). Since such a specialized adaptation could have important implications for the replantation success of a species like white spruce on disturbed forest sites, where reduced N is in short supply and NO_3^- is the predominant N source (see *Introduction*), this question warrants further investigation.

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