

Nitrate-Ammonium Synergism in Rice. A Subcellular Flux Analysis¹

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Many reports have shown that plant growth and yield is superior on mixtures of NO_3^- and NH_4^+ compared with provision of either N source alone. Despite its clear practical importance, the nature of this N-source synergism at the cellular level is poorly understood. In the present study we have used the technique of compartmental analysis by efflux and the radiotracer ^{13}N to measure cellular turnover kinetics, patterns of flux partitioning, and cytosolic pool sizes of both NO_3^- and NH_4^+ in seedling roots of rice (*Oryza sativa* L. cv IR72), supplied simultaneously with the two N sources. We show that plasma membrane fluxes for NH_4^+ , cytosolic NH_4^+ accumulation, and NH_4^+ metabolism are enhanced by the presence of NO_3^- , whereas NO_3^- fluxes, accumulation, and metabolism are strongly repressed by NH_4^+ . However, net N acquisition and N translocation to the shoot with dual N-source provision are substantially larger than when NO_3^- or NH_4^+ is provided alone at identical N concentrations.

Although higher plants have the capacity to utilize organic N (Näsholm et al., 1998), the major sources for N acquisition by roots are considered to be NO_3^- and NH_4^+ (Haynes and Goh, 1978). Plants vary substantially in their relative adaptations to these two sources of N (Kronzucker et al., 1997). Although NH_4^+ should be the preferred N source, since its metabolism requires less energy than that of NO_3^- (Bloom et al., 1992), only a few species actually perform well when NH_4^+ is provided as the only N source. Among the latter are boreal conifers (Kronzucker et al., 1997), ericaceous species (Pearson and Stewart, 1993), some vegetable crops (Santamaria and Elia, 1997), and rice (Wang et al., 1993; Kronzucker et al., 1998). Most agricultural species develop at times severe toxicity symptoms on NH_4^+ (Cox and Reisenauer, 1973; Findenegg, 1987); thus, superior growth in these species is seen on NO_3^- (Rideout et al., 1994). However, when both N sources are provided simultaneously, growth and yield are often enhanced significantly compared with growth on either NH_4^+ or NO_3^- alone. The effect is particularly well documented in corn (Below and Gentry, 1987; Smiciklas and Below, 1992; Adriaanse and Human, 1993) and wheat (Cox and Reisenauer,

1973; Heberer and Below, 1989; Chen et al., 1998), but it has also been reported in several other species (Hagin et al., 1990; Cao and Tibbits, 1993; Gill and Reisenauer, 1993), including rice (Ta and Ohira, 1981; Ta et al., 1981). Yield increases of 40% to 70% have been observed in solution culture (Weissman, 1964; Cox and Reisenauer, 1973; Heberer and Below, 1989), although, commonly, somewhat smaller enhancements are obtained in soil culture and under field conditions (Hoeft, 1984; Hagin et al., 1990). Several hypotheses pertaining to the enhanced growth and yield response on mixed N medium have been advanced (Lewis et al., 1982; Findenegg, 1987; Gill and Reisenauer, 1993), but mechanistic examinations of these effects have been lacking. In the present study we have used compartmental analysis with the short-lived radiotracer ^{13}N to examine the reciprocal effects of NH_4^+ and NO_3^- on each other in root tissue of intact rice plants with respect to N-flux partitioning and storage capacity at the subcellular level.

MATERIALS AND METHODS

Plant Growth Conditions

Rice (*Oryza sativa* L. cv IR72) seeds were surface-sterilized in 5% NaOCl for 10 min, rinsed with deionized water, and left to imbibe in aerated deionized water at 30°C in a water bath for 48 h. The partially germinated seeds were then placed onto plastic mesh mounted on Plexiglas discs (Atohaas Americas Inc., Philadelphia, PA) and the discs were transferred to 40-L hydroponic Plexiglas tanks located in walk-in, controlled-environment growth chambers. Growth chambers were maintained at 30°C ± 2°C, 70% RH, and set to a 12-h/12-h photoperiod. A photon flux of approximately 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, measured at plant level (with a light meter [LI-189, Li-Cor, Lincoln, NE] and quantum sensor [LI-190SA, Li-Cor]), was provided by fluorescent lamps (1500, F96T12/CW/VHO, 215 W, Philips, Eindhoven, The Netherlands).

Nutrient Solutions

Seedlings were cultivated for 3 weeks in hydroponic medium contained in 40-L Plexiglas tanks. Deionized, distilled water and reagent-grade chemicals were used in the preparation of all nutrient solutions. N was provided either as 100 μM NH_4^+ (in the form of $(\text{NH}_4)_2\text{SO}_4$), as 100 μM

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NO_3^- (in the form of $\text{Ca}(\text{NO}_3)_2$), or as $100 \mu\text{M}$ NH_4NO_3 . Other nutrient salts added were as follows: 1 mM K_2SO_4 , 2 mM MgSO_4 , 1 mM CaCl_2 , $300 \mu\text{M}$ NaH_2PO_4 , $100 \mu\text{M}$ Fe-EDTA, $9 \mu\text{M}$ MnCl_2 , $25 \mu\text{M}$ $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, $20 \mu\text{M}$ H_3BO_3 , $1.5 \mu\text{M}$ ZnSO_4 , and $1.5 \mu\text{M}$ CuSO_4 . Nutrient solutions in tanks were continuously mixed via electric circulating pumps (model IC-2, Brinkmann). Continuous infusion of nutrient stock solution via peristaltic pumps (Technicon Proportioning Pump II, Technicon Instrument, Tarrytown, NY) allowed steady-state control of nutrient concentrations in the tanks. Solutions were checked daily for $[\text{K}^+]$ using a spectrophotometer (model 443; Instrumentation Laboratory, Lexington, MA). The solution pH was maintained at 6.5 ± 0.3 by addition of powdered $\text{Ca}(\text{CO}_3)_2$. pH was monitored daily using a microprocessor-based, pocket-size pH meter (pH Testr2 model 59000-20, Cole Parmer, Chicago, IL). $[\text{NH}_4^+]_o$ was measured (using a Philips PU 8820 UV/visible spectrophotometer) according to the method described by Solorzano (1969). $[\text{NO}_3^-]_o$ was measured spectrophotometrically by the method of Cawse (1967).

Compartmental Analysis

The radiotracer ^{13}N (half-life = 9.98 min) was produced by the cyclotron facility (Tri-University Meson Facility) at the University of British Columbia. Proton irradiation of a water target was used to generate ^{13}N , a procedure that provides chiefly $^{13}\text{NO}_3^-$ with high radiochemical purity (Kronzucker et al., 1995b). The irradiated solutions were supplied in sealed 20-mL glass vials, with a starting activity of 700 to 740 MBq. At this activity sufficient counts were present in both eluates and plant samples following loading periods of up to 60 min and a total elution period of 22 min (see below). Procedures for the removal of radiocontaminants and conversion of $^{13}\text{NO}_3^-$ to $^{13}\text{NH}_4^+$ were as described in detail elsewhere (Kronzucker et al., 1995a, 1995b, 1995c). A volume of 20 to 100 mL of ^{13}N -containing "stock" solution was prepared in a fume hood and was transferred into the controlled-environment chambers where experiments were carried out. All uptake solutions were premixed and kept behind lead shielding. The chemical composition of the labeling solutions was identical to that of the growth solutions in the hydroponic tanks (see above). The protocol for efflux experiments was essentially as described elsewhere (Kronzucker et al., 1995b, 1995d, 1995e). Roots of intact rice seedlings were immersed for 60 min in 120-mL darkened plastic beakers containing the $^{13}\text{NO}_3^-$ - or $^{13}\text{NH}_4^+$ -labeled solution. Steady-state conditions with respect to all nutrients were maintained throughout growth, loading, and elution. The duration of the loading period was chosen on the basis of the half-lives of exchange for the cytoplasmic compartment, i.e. approximately 14 min for NH_4^+ and 16 min for NO_3^- . Therefore, 60 min of exposure to tracer should ensure that cytoplasmic specific activity approximate 95% of that in the loading solution (Kronzucker et al., 1995e). Following loading with ^{13}N , seedlings were transferred to efflux funnels (Wang et al., 1993), and the roots were eluted with 20-mL aliquots of nonradioactive solution after varying time intervals. These time intervals ranged from 5 s to 2 min over an experimen-

tal duration of 22 min. Eluates from a total of 25 time intervals were collected separately, and the radioactivities of each eluate were determined in a gamma-counter (Minaxi δ , Auto- γ 5000 series, Hewlett-Packard), measuring the 511-keV positron-electron annihilation radiation generated by recombination of ambient electrons and β^+ particles emitted from ^{13}N . After the final elution seedling roots were excised from the shoots, the roots were spun in a low-speed centrifuge for 30 s to remove surface liquid, and the fresh weights of roots and shoots were determined. The plant organs were then introduced into 20-mL scintillation vials, and the radioactivities of roots and shoots were determined.

Data Analysis

All experiments were repeated five to eight times, with two replicates per experiment. Data from several experiments were pooled ($n \geq 10$) for calculations of means and SE. Symbols and calculation of fluxes were as follows: ϕ_{cor} , efflux from the cytoplasmic compartment at time 0 divided by the specific activity of ^{13}N in the loading solution; ϕ_{net} , net flux, obtained from the accumulation of ^{13}N in the plants at the end of the loading period (60 min); ϕ_{cor} , unidirectional influx, calculated from $\phi_{net} + \phi_{co}$; ϕ_{xylem} , flux of ^{13}N to the shoot at the end of the elution period; and $\phi_{vac./ass.}$, combined flux to N assimilation and the vacuole, resulting in $\phi_{net} - \phi_{xylem}$. Half-lives of exchange and pool sizes were determined as described in detail elsewhere (Siddiqi et al., 1991; Kronzucker et al., 1995a, 1995b, 1995c, 1995e).

RESULTS AND DISCUSSION

For both NO_3^- and NH_4^+ , compartmental analyses by efflux revealed exchange with three subcellular compartments (Fig. 1), identified as a surface film (I), a binding component in the cell wall (II), and the cytoplasm (III), in keeping with previous studies in which detailed compartment identity tests were carried out using membrane perturbation, ion-exchange series, and metabolic modifiers (Siddiqi et al., 1991; Kronzucker et al., 1995e). The short isotopic half-life of ^{13}N (9.98 min) made it impossible to trace vacuolar exchange in our study. Half-lives of exchange for the three compartments identified in our study were approximately 2 s, 30 s, and 16 min, respectively, for NO_3^- , and 2 s, 40 s, and 14 min, respectively, for NH_4^+ (data not shown). These half-lives were very similar to those reported for N exchange in other studies (Wang et al., 1993; Kronzucker et al., 1995e, 1997), with no significant differences in the presence of the other ion. Cytoplasmic NO_3^- exchange, however, exhibited half-lives that were about two to three times as long as those observed for other species (compare Devienne et al., 1994; Kronzucker et al., 1995a). The relatively long half-life for cytosolic exchange of NO_3^- in rice may be seen as an indication of a relatively small negative feedback upon NO_3^- influx by cytoplasmic NO_3^- , in keeping with a high cytosolic accumulation capacity and efficiency of uptake for NO_3^- in this species (H.J. Kronzucker, A.D.M. Glass, M.Y. Siddiqi, and G.J.D.

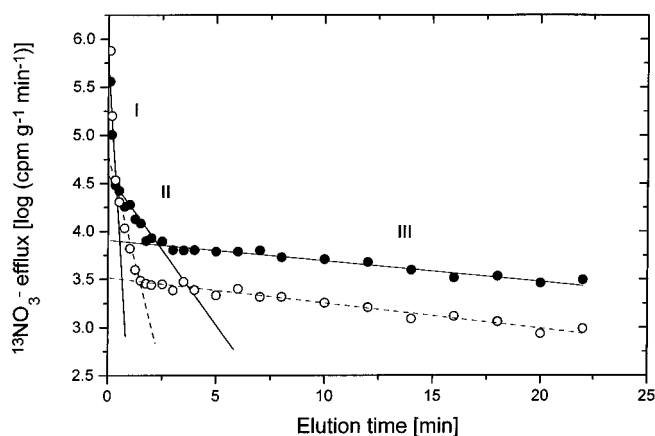


Figure 1. Representative semilogarithmic plots for the rate of release of $^{13}\text{NO}_3^-$ [$\log(\text{cpm released}) \text{ g}^{-1} \text{ h}^{-1}$] versus time of elution for roots of intact cv IR72 rice seedlings maintained at $100 \mu\text{M} [\text{NO}_3^-]_o$ with NH_4^+ (○) or without NH_4^+ (●). Plots include linear regression lines for the three phases of efflux (I, surface film; II, cell wall; III, cytoplasm). Regression lines are dashed for the $+\text{NH}_4^+$ treatment and solid for the control (phase I overlapped). See text for derivation of compartmental parameters.

Kirk, unpublished results). It is surprising to find such high capacity and efficiency for NO_3^- capture in rice, which traditionally has been assumed to prefer NH_4^+ -N (compare Wang et al., 1993; Kronzucker et al., 1998). Notwithstanding the substantial rates of both NO_3^- influx and net flux, we found a strong inhibitory effect of NH_4^+ on the latter (Table I). Such repression of NO_3^- uptake by NH_4^+ has been documented in many species (Jackson et al., 1976; MacKown et al., 1982; Lee and Drew, 1989; Aslam et al., 1997; Colmer and Bloom, 1998), although there has been an ongoing debate about whether the effect is primarily upon influx or efflux (Kronzucker et al., 1999).

Our present study shows that, under steady-state coprovision of the two N sources, NO_3^- influx and efflux are both significantly repressed by NH_4^+ , compared with plants fed only with NO_3^- (Table I). Influx was repressed by approximately 50% (Colmer and Bloom, 1998) and efflux by almost 40%, so that NO_3^- -net acquisition in the presence of NH_4^+ was 2.2 times less than with NO_3^- -only provision. Thus, it is clear that, under steady-state conditions, the principal effect of NH_4^+ on net NO_3^- uptake is through its repressive action on influx, not through en-

hancement of efflux, which supports the conclusions by Lee and Drew (1989) and our own group (Kronzucker et al., 1999; compare Aslam et al., 1997). Also, since NO_3^- efflux constituted only 8.7% (with NO_3^-) to 11.4% (with NO_3^- plus NH_4^+) of NO_3^- influx, any effect on efflux could make only a negligible contribution to net NO_3^- acquisition. The same trend as for NO_3^- fluxes was observed for cytosolic NO_3^- accumulation capacity. Figure 1 shows overlaid efflux plots for NO_3^- in the presence and absence of NH_4^+ , with a significant downward y -axis shift being evident for NO_3^- efflux from the cytoplasmic compartment in the presence of NH_4^+ . By contrast, half-life for cytoplasmic exchange, as seen in the slope of the regression line for compartment III, was not changed. Given this half-life constancy, the y -axis intercepts for ^{13}N efflux from compartment III in Figure 1 reflect directly the relative sizes of the cytoplasmic NO_3^- pools. As shown in Figure 2, cytoplasmic $[\text{NO}_3^-]$ was depressed from $36 \pm 4.5 \text{ mM}$ with NO_3^- -only provision to $17.8 \pm 3.6 \text{ mM}$ in the presence of NH_4^+ .

In the reverse experimental design, compartmental analysis revealed unexpected effects of NO_3^- on NH_4^+ fluxes. Cytosolic $[\text{NH}_4^+]$ was not affected significantly by the presence of NO_3^- (Fig. 2). Due to this, efflux plots for NH_4^+ with or without NO_3^- virtually overlapped (data not shown). However, NH_4^+ influx was increased by almost 25% when NO_3^- was provided at the same time (Table I). Concurrently, NH_4^+ efflux was decreased by NO_3^- almost 2-fold. As a result, net NH_4^+ acquisition was improved by as much as 50% compared with the NH_4^+ -only control. Under perturbational conditions, since N-deprived plants were resupplied with N, a stimulatory effect of NO_3^- on NH_4^+ uptake has been recorded previously for soybean (Rideout et al., 1994; Saravitz et al., 1994). Here we show that NH_4^+ uptake is stimulated substantially as well under steady-state conditions.

Perhaps even more important, however, is the finding that N-flux partitioning patterns changed significantly when both N sources were provided compared with either NH_4^+ or NO_3^- alone. For both NH_4^+ and NO_3^- , if supplied alone, approximately 50% of incoming N remained in roots, either channeled to assimilation or to the vacuole, whereas a relatively smaller proportion was translocated to the shoot, approximately 38% of incoming ^{13}N in the case of NO_3^- and 26% in the case of NH_4^+ . With coprovision of

Table I. Component fluxes for NH_4^+ and NO_3^- as determined by compartmental analysis

Rice plants were grown on $100 \mu\text{M} \text{NO}_3^-$, $100 \mu\text{M} \text{NH}_4^+$, or $100 \mu\text{M} \text{NH}_4\text{NO}_3$. The bottom row indicates combined N fluxes in the NH_4NO_3 treatment. For flux symbols, see "Materials and Methods." Data are means \pm SE ($n \geq 10$).

N Source	N Fluxes				
	ϕ_{oc}	ϕ_{co}	ϕ_{net}	$\phi_{vac./ass.}$	ϕ_{xylem}
	$\mu\text{mol g}^{-1} \text{ h}^{-1}$				
NO_3^-	5.98 ± 0.44	0.52 ± 0.04	5.46 ± 0.49	3.18 ± 0.27	2.28 ± 0.32
$\text{NO}_3^- (+\text{NH}_4^+)$	2.81 ± 0.23	0.32 ± 0.09	2.49 ± 0.24	1.12 ± 0.08	1.37 ± 0.19
NH_4^+	4.08 ± 0.31	0.99 ± 0.12	3.09 ± 0.33	2.04 ± 0.09	1.05 ± 0.24
$\text{NH}_4^+ (+\text{NO}_3^-)$	5.07 ± 0.38	0.51 ± 0.07	4.56 ± 0.41	1.74 ± 0.11	2.82 ± 0.17
$\Sigma (\text{NH}_4^+ + \text{NO}_3^-)$	7.88	0.83	7.05	2.86	4.19

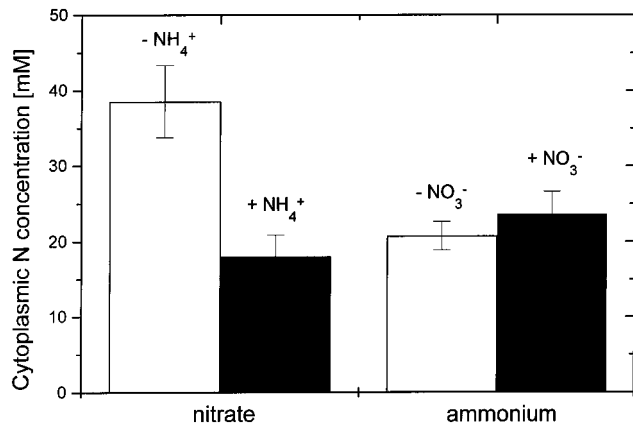


Figure 2. Cytoplasmic pool sizes (in mM) of NO₃⁻ and NH₄⁺ in roots of intact cv IR72 rice seedlings in the presence (black bars) or absence (white bars) of the other N source. Plants were under steady-state conditions with respect to N treatments. Error bars indicate SE ($n \geq 10$).

the other N source, xylem-N translocation increased substantially, to approximately 49% on NO₃⁻ (in the presence of NH₄⁺) and to approximately 56% on NH₄⁺ (in the presence of NO₃⁻). Our compartmental analyses do not allow us to determine the biochemical profiles of N-translocation compounds, nor can the specific activities of the respective xylem-loading pools of these compounds be known. Hence, the xylem-translocation data presented here include not only the NO₃⁻ and NH₄⁺ species, respectively, but also N metabolites and, thus, a fraction of the assimilatory flux.

Whereas in the case of NO₃⁻ long-distance N translocation increased only in percentage terms, an absolute increase was seen in the case of NH₄⁺. It has been suggested that the inhibition of NO₃⁻ uptake might be accompanied by an inhibition of nitrate reductase in roots (Smith and Thompson, 1971; Radin, 1975; MacKown et al., 1982); therefore, the increased proportion of N translocated to the shoot in the case of NO₃⁻ is likely to be accompanied by a decreased rate of N metabolism and hence a lower ratio of N metabolites to free NO₃⁻ in the xylem. Since, under most conditions, NH₄⁺ is not transported as such in the xylem of rice at appreciable concentrations (Wang et al., 1993; Kronzucker et al., 1995e), the translocation increase with NH₄⁺ in the presence of NO₃⁻ must be due to a stimulation of NH₄⁺ assimilation. A similar NO₃⁻-specific stimulation of NH₄⁺ assimilation has been reported elsewhere for radish plants (Goyal et al., 1982; Ota and Yamamoto, 1989). We propose that the specific induction by NO₃⁻ of the proplastidic glutamine synthetase/glutamate synthase pathway (Redinbaugh and Campbell, 1993), in addition to the one localized in the cytoplasm, opens up an assimilatory flux potential that is not available to plants grown on pure NH₄⁺. It is possible that significant portions of N derived from both incoming NO₃⁻ and NH₄⁺ could be channeled through this pathway. The increased shoot translocation of N is likely to have important agronomic consequences. In the case of rice, in excess of 70% of N in the grain at harvesting and more than 50% of N in photo-

synthetically active leaves during grain filling are drawn from N that accumulated in shoot tissue during vegetative growth (Mae et al., 1985, and refs. therein); on the other hand, the rice root system during grain filling is subject to senescence.

In summary, our analyses document distinct changes in the pattern of N-flux partitioning when NO₃⁻ and NH₄⁺ are supplied together, compared with provision of either NO₃⁻ or NH₄⁺ alone. At least in part, the frequently observed growth and yield maximization on a combined N-source diet (see the introduction) can be attributed to an up-regulation of NH₄⁺ uptake and metabolism by NO₃⁻. Although uptake, metabolism, and cytosolic accumulation of NO₃⁻ are depressed by as much as 50% by the simultaneous presence of NH₄⁺, when contributions to the N budget from both NO₃⁻ and NH₄⁺ are taken into account (see Table I), a substantially larger N-acquisition rate is achieved than would be possible with either NH₄⁺ or NO₃⁻ alone at an identical external N concentration (i.e. 200 μM in our experiments). The Michaelis-Menten saturation kinetics of the individual influx components allow for an increase in influx of approximately only 12% with an increase in external N from 100 to 200 μM (compare Siddiqi et al., 1990; Kronzucker et al., 1998); by contrast, the combined N intake from the NO₃⁻/NH₄⁺ mixture is approximately 20% and 75% larger than individual fluxes at 200 μM in the case of NO₃⁻ and NH₄⁺, respectively. It is clear that this benefit from combined N-source provision must be most pronounced at higher concentrations of external N, as influx isotherms are near or at saturation (Siddiqi et al., 1990; Kronzucker et al., 1995d, 1996). In our study with rice, the additive N-budget advantage due to the combined influx components was further enhanced by a reduction in N loss through efflux. In addition, a significant shift in N partitioning was observed in favor of N allocation to the shoot, with agronomic consequences that are likely not trivial.

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