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Comparative kinetic analysis of ammonium and nitrate acquisition by tropical lowland rice: implications for rice cultivation and yield potential

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SUMMARY

Nitrogen limitation compromises the realization of yield potential in cereals more than any other single factor. In rice, the world's most important crop species, the assumption has long been that only ammonium-N is efficiently utilized. Consequently, nitrate utilization has been largely ignored, although fragmentary data have suggested that growth could be substantial on nitrate. Using the short-lived radiotracer ^{13}N, we here provide direct comparisons of root transmembrane fluxes and cytoplasmic pool sizes for nitrate- and ammonium-N in a major variety of *Indica* rice (*Oryza sativa*), and show that nitrate acquisition is not only of high capacity and efficiency but is superior to that of ammonium. We believe our results have implications for rice breeding and molecular genetics as well as the design of water-management and fertilization regimes. Potential strategies to harness this hitherto unexplored N-utilization potential are proposed.

Key words: ammonium, compartmental analysis, nitrate, rice, yield.

INTRODUCTION

More than 70% of global rice production occurs in intensely managed irrigated systems in the lowlands of Asia (IRRI, 1998). Yield maxima in current genotypes of lowland rice are approx. 10 t per ha. It is now recognized that nitrogen supply during vegetative growth poses the most critical limitation to the realization of yield potential in the field (Cassman *et al.*, 1993, 1998; Kropff *et al.*, 1993; Sheehy *et al.*, 1998). Significant yield increases are usually observed following the application of N fertilizer, with as much as 7×10^6 t of elemental N presently applied each year to irrigated rice fields (Kronzucker *et al.*, 1998; Sheehy *et al.*, 1998). However, fertilizer-use efficiency is poor in tropical lowlands, with large amounts of N being lost to the atmosphere through NH_3 volatilization and denitrification; typically, less than a third of fertilizer N is recovered in the rice plant (Vlek & Byrnes, 1986; Cassman *et al.*, 1993, 1998). Based on current predictions of world population growth, rice production must increase by at least 70% in the next 35 yr if the demand for food is to be met. At present levels of fertilizer-use efficiency, this will entail a threefold increase of N fertilizer application (IRRI, 1998). Clearly, N-use efficiency in rice is of key concern. In sharp contrast to most agricultural soils, where nitrate (NO_3^-) is the predominant N species

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(Kronzucker *et al.*, 1998), hypoxic conditions in the paddy environment largely preclude the microbial formation of NO_3^- through nitrification (Bouldin, 1986; Arth *et al.*, 1998) and, consequently, ammonium (NH_4^+) is the main form of N available to rice in the field (Shen, 1969; Wang *et al.*, 1993; Arth *et al.*, 1998; Kronzucker *et al.*, 1998). It is therefore not surprising that NH_4^+ nutrition, as opposed to NO_3^- nutrition, has received almost exclusive attention in rice (Bonner, 1946; Fried *et al.*, 1965; Shen, 1969; Wang *et al.*, 1993). However, some reports have indicated that rice does possess some capacity for root NO_3^- absorption (Ismunadji & Dijkshoorn, 1971; Sasakawa & Yamamoto, 1978; Youngdahl *et al.*, 1982; Raman *et al.*, 1995) and for the reduction of NO_3^- in leaves (Tang & Wu, 1957). Isolated nursery trials have shown that NO_3^- pretreatment of rice seedlings can result in enhanced transplanting success (Yamasaki & Seino, 1965), and, perhaps most strikingly, several varieties of *Indica* rice have been shown to exhibit superior growth on NO_3^- under certain conditions (Ta & Ohira, 1981; Ta *et al.*, 1981).

In the present paper we report results from influx analyses and compartmental (efflux) analyses conducted in controlled-environment conditions, with the positron emitter ^{13}N. Our analyses permitted the noninvasive determination of cellular turnover kinetics and cytoplasmic pool sizes for NO_3^- and NH_4^+ as well as the high-precision measurement of subcellular fluxes for the two N sources (component fluxes: influx, efflux, flux to assimilation and the vacuole, and N flux to the shoot) in root tissue of intact rice seedlings. The goal of our study was to afford a direct comparison of both the relative capacities and efficiencies of NO_3^- and NH_4^+ acquisition in *Indica* rice during vegetative growth and in conditions representative of irrigated rice fields in tropical lowlands. The results are discussed with respect to their relevance to rice-breeding strategies and to rice cultivation in tropical lowlands.

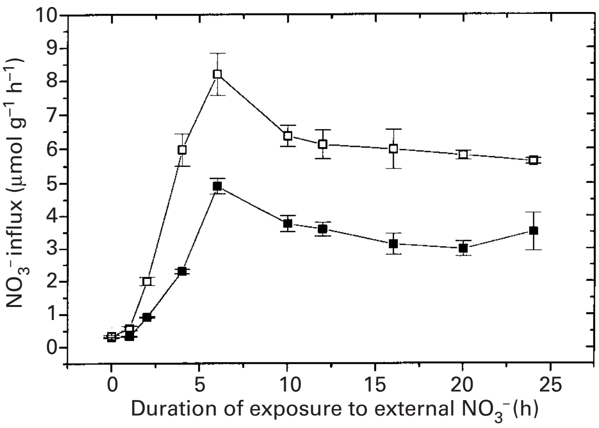

Fig. 1. Induction profile of NO_3^- influx into roots of intact 3-week-old (closed symbols) and 4-week-old (open symbols) 'IR72' rice seedlings. Plants were maintained in N-free solution for 24 h before NO_3^- resupply. Re-exposure and flux determination was at 100 μM $[NO_3^-]_o$. Error bars indicate SE ($n \geqslant 16$).

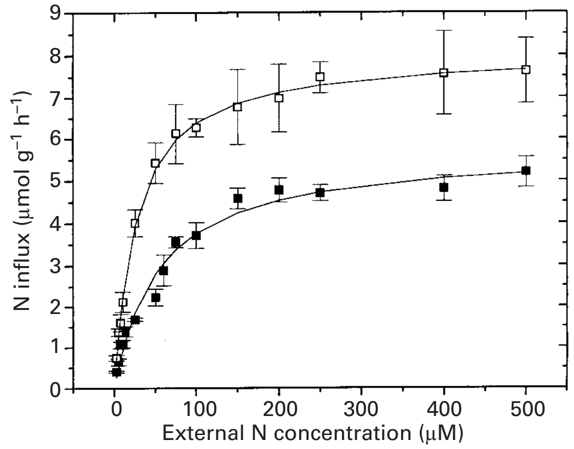

Fig. 2. Comparative concentration dependence of steady-state NO_3^- influx (open symbols) and NH_4^+ influx (closed symbols) in roots of intact 4-wk-old 'IR72' rice in the range of environmentally relevant N concentrations. All plants were grown at 100 μM $[NO_3^-]_o$ or $[NH_4^+]_o$, respectively. Error bars indicate SE ($n \geqslant 16$).

MATERIALS AND METHODS

Influx experiments

Rice seedlings (*Oryza sativa* L., cv. IR72) were cultivated hydroponically for 3–4 wk in controlled-environment chambers (Kronzucker *et al.*, 1998). The hydroponic tanks contained aerated one-tenth-strength modified N-free Johnson's solution (Kronzucker *et al.*, 1995a, 1998). NO_3^- was supplied as $Ca(NO_3)_2$, and NH_4^+ as $(NH_4)_2SO_4$. The concentration of N during growth was 100 μM, except in NO_3^--induction experiments, in which seedlings were maintained in N-free solution for 24 h, then re-exposed to 100 μM NO_3^- for the periods of time indicated (Fig. 1). During ^{13}N-labelling experiments, N was also provided at 100 μM, except in kinetic experiments, in which concentrations ranged from 2.5–500 μM (Fig. 2). The radiotracer ^{13}N was provided by the Tri-University Meson Facility (TRIUMF) at the University of British Columbia, Canada, and $^{13}NH_4^+$ and $^{13}NO_3^-$ were prepared according to previously described procedures (Siddiqi *et al.*, 1989; Kronzucker *et al.*, 1998). Roots of intact seedlings were equilibrated for 5 min in nonlabelled solutions chemically identical to the uptake solutions, transferred to uptake vessels containing $^{13}NH_4^+$- or $^{13}NO_3^-$-labelled solution for 10 min, and desorbed in nonlabelled solution for 2 min (NO_3^-) or 3 min (NH_4^+) to desorb tracer contained in the free space (Kronzucker *et al.*, 1996). Seedling roots were then excised from shoots, the roots spun in a low-speed centrifuge for 30 s to remove surface liquid, and the fresh weights of roots and shoots determined. The radioactivities of roots

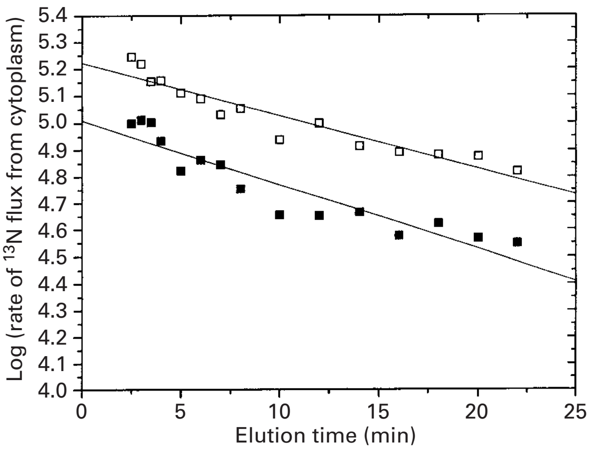

Fig. 3. Representative semi-logarithmic plots of the rates of release of $^{13}NO_3^-$ (open symbols) and $^{13}NH_4^+$ (closed symbols) in [log (cpm released) g^{-1} min^{-1}] vs time of elution for roots of intact 'IR72' rice seedlings at 100 μM $[NO_3^-]_o$ or $[NH_4^+]_o$. Plots include linear regression lines for the cytoplasmic efflux phase for both sets of experiments. Counts eluting from root tissues were corrected for differences in specific activity of the radiotracer, root mass and differences in the ratios of efflux to all N fluxes from the cytoplasm. Y-intercepts indicate directly the relative sizes of the respective cytoplasmic pools.

and shoots were determined in a Packard γ-counter (Minaxi δ, Auto-γ 5000 Series, Canberry-Packard, Mississauga, Ontario, Canada). Using the value for specific activity ($^{13}N/(^{13}N + {}^{14}N)$) of the loading solution and the total fresh weight of the roots of each seedling, NO_3^- or NH_4^+ fluxes were calculated and expressed in μmol g^{-1} (f. wt) h^{-1} (Siddiqi *et al.*, 1989). Experiments were repeated four times, each experimental treatment consisting of four replicates. Data from several experiments were pooled ($n \geqslant 16$) for calculations of means and SE. These values were used for plotting the representative concentration-dependent curves as well as for calculating V_{max} and K_m values according to a method described elsewhere (Kronzucker *et al.*, 1996, 1997).

Compartmental analysis

Roots of intact seedlings (grown at steady-state provision of 100 μM NO_3^- or NH_4^+) were equilibrated in nonlabelled preloading solution for 5 min before transfer to the ^{13}N-loading solution. Roots were then immersed in ^{13}N-labelled loading solution for 1 h to bring the cytoplasmic phase to a specific ^{13}N activity close to that of the loading solution (Siddiqi *et al.*, 1991). Seedlings were then transferred to efflux funnels (Wang *et al.*, 1993) and the roots eluted successively with 20-ml aliquots of nonlabelled solution for different durations of time. With t = 0 as the time of transfer from loading to washing solution and t_{final} = 22 min for the final elution, the time periods and number (in brackets) for the 25 successive washes were: 5 s (2), 10 s (2), 15 s (6), 30 s (4), 1 min (4), 2 min (7). The eluates were then counted in a Packard γ-counter (Minaxi δ, Auto-γ 5000 Series). Roots were excised from the shoots after the final elution, and spun for 30 s, and plant organs were weighed and counted. Treatment of data was as described by Lee & Clarkson (1986) and Siddiqi *et al.* (1991). Experiments were repeated eight or nine times. Standard errors for various derived parameters (half-lives, fluxes, pool sizes) were within 15% of the means ($n \geqslant 8$). Representative experiments were chosen for semi-logarithmic plots of the rate of ^{13}N-release vs elution time (Fig. 3). Since the specific activity in the plant compartments during elution declines exponentially, the logarithm of the rate of release of radioactivity from the plant tissue can be plotted vs elution time (Lee & Clarkson, 1986; Siddiqi *et al.*, 1991). Linear regression on the semi-logarithmic plots was then used to resolve separate phases. The slopes of the regression lines, after conversion to natural logarithm, yielded kinetic (first-order) decay constants (k) for the respective phases, which could be expressed as half-lives of exchange ($t_{0.5} = 0.693/k$). Cytoplasmic concentrations of NO_3^- or NH_4^+ ($[NO_3^-]_{cyt}$ and $[NH_4^+]_{cyt}$) (Fig. 3) were calculated from the quotient of the integrated rate of ^{13}N release during five times the half-life of cytoplasmic exchange and the ratio (R_e) of efflux to all fluxes removing $^{13}NO_3^-$ or $^{13}NH_4^+$ from the cytoplasm, and assuming 5% for cell volume occupied by the cytoplasm (Lee & Clarkson, 1986; Siddiqi *et al.*, 1991). The efflux plot in Fig. 3 shows the overlaid cytoplasmic phases for NO_3^- and NH_4^+ exchange after correction for differences in specific activity and root mass. Moreover, the values of log(R_e) for NO_3^- and NH_4^+ efflux (see above) were subtracted from all respective logarithmically transformed data points so as to correct for differences in flux partitioning; thus, the intercepts with the ordinate of the regression lines for the phases of cytoplasmic exchange directly indicate the relative sizes of the cytoplasmic NO_3^- and NH_4^+ pools (Kronzucker *et al.*, 1997).

RESULTS AND DISCUSSION

Our first goal was to characterize the profile of NO_3^- influx over time when NO_3^- was resupplied to rice seedlings previously grown without N. This was necessary since NO_3^- influx in many species is significantly enhanced (induced) by external NO_3^- provision (Siddiqi *et al.*, 1989, 1991; Kronzucker *et al.*, 1995a,b, 1997), whereas no such inductive response is seen for NH_4^+ uptake (Glass & Siddiqi, 1995; Kronzucker *et al.*, 1996, 1998). Our results show that rice is indeed highly responsive to external NO_3^- provision. Following a brief lag period of ~ 2 h, influx increased rapidly from low constitutive values (~ 0.3 μmol g^{-1} h^{-1}) to peak values (~ 8.5 μmol g^{-1} h^{-1}) after 5 h of NO_3^- resupply (Fig. 1). This shows a strong physiological plasticity of the NO_3^- influx apparatus in rice. In species whose adaptation to the NO_3^- source is poor (Kronzucker *et al.*, 1997), an inductive peak is seen only after days of exposure to NO_3^- (Kronzucker *et al.*, 1995a). Even in the barley variety *Klondike*, one of the most efficient of the higher plants at NO_3^- utilization (Siddiqi *et al.*, 1989, 1991), full induction is not achieved until 24 h after exposure to NO_3^- (Siddiqi *et al.*, 1989; Kronzucker *et al.*, 1995a). Interestingly, induction in rice was considerably greater in 4-wk-old than in 3-wk-old seedlings (27-fold vs 15-fold above the constitutive influx). The extent of this response rivalled the largest recorded inductive amplitude in barley (Siddiqi *et al.*, 1989), and was significantly larger than that observed in any other species (Kronzucker *et al.*, 1995a). By 10 h of NO_3^- exposure, NO_3^- influx declined in rice, and, by 15–20 h, steady-state values of influx were achieved (Fig. 1). Although below the inductive peak, these remained substantial ($\sim$ 3.5–6 μmol g^{-1} h^{-1}) and in 3-wk-old plants were similar to NH_4^+ influx (Kronzucker *et al.*, 1998). In 4-wk-old seedlings, NO_3^- uptake rates exceeded those of NH_4^+. A kinetic study for unidirectional influx of both N species in the 2.5–500 μM concentration range revealed Michaelis–Menten saturation patterns (Fig. 2), typical of high-affinity transport systems (Glass & Siddiqi, 1995; Kronzucker *et al.*, 1995b, 1997, 1998). For these transport systems, K_m values were 26 ± 5.6 μM for NO_3^- and 51 ± 18.4 μM for NH_4^+, indicating a higher substrate affinity in NO_3^-. More importantly, NO_3^- influx exceeded NH_4^+ influx by $\sim$ 50 % at all concentrations examined (V_{max} values were $\sim$ 8.1 and 5.7 μmol g^{-1} h^{-1}, respectively).

The technique of compartmental analysis (Lee & Clarkson, 1986; Siddiqi *et al.*, 1991) allowed us to quantify cytosolic pool sizes of NO_3^- and NH_4^+ in root cells, half-lives of cytosolic exchange, and N-flux partitioning at the subcellular level. We measured substantial levels of free cytosolic NO_3^- and NH_4^+ in seedling roots; $[NH_4^+]_{cyt}$ was 20 ± 3.21 mM, very similar to values previously reported for rice (Wang *et al.*, 1993; Kronzucker *et al.*, 1998) and spruce (Kronzucker *et al.*, 1995a,b, 1997). Both rice and spruce are considered especially well adapted to utilizing NH_4^+ as an N source (Kronzucker *et al.*, 1997). Cytoplasmic levels of NO_3^-, however, were even larger than those of NH_4^+ (Fig. 3; $[NO_3^-]_{cyt} = 37 \pm 4.18$ mM), surpassing peak $[NO_3^-]_{cyt}$ levels reported for barley (Siddiqi *et al.*, 1991). Interestingly, imposition of hypoxia ($\sim$ 50 % O_2 saturation), which more closely resembles conditions in rice fields (Arth *et al.*, 1998; Kronzucker *et al.*, 1998), further increased (approx. 1.6-fold) both NO_3^- influx and cytoplasmic accumulation capacity (data not shown), a result similar to that shown in some detail for NH_4^+ influx in an earlier communication (Kronzucker *et al.*, 1998). This indicates that rice has a unique ability to respond to an increased N requirement under conditions of oxygen limitation. Notwithstanding the differences in cytosolic concentrations, the kinetics of cellular N turnover, as evidenced by half-lives of cellular exchange (slopes of regression lines in Fig. 3), were remarkably similar for the two N species (14 ± 0.9 min for NH_4^+ and 16 ± 2.3 min for NO_3^-). Half-lives of exchange give an indication of the magnitude of influx that can be sustained at a given cytosolic concentration (Kronzucker *et al.*, 1995a,b, 1997). It is especially noteworthy that rice displays $t_{0.5}$ values for NO_3^- exchange that are substantially larger than those observed in other species, including highly efficient users of NO_3^- (Siddiqi *et al.*, 1989, 1991; Kronzucker *et al.*, 1995a), whereas its $t_{0.5}$ values for NH_4^+ are more typical (Kronzucker *et al.*, 1997, 1998). The larger $t_{0.5}$ values for NO_3^- provide additional evidence for unusually high capacity for N capture and retention when NO_3^- is the N source.

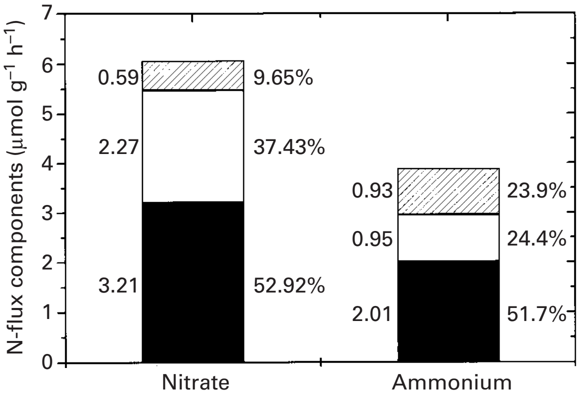

Fig. 4. N flux components for NO_3^- and NH_4^+ estimated by compartmental analysis. 'IR72' rice seedlings were grown and maintained for 4 wk at 100 μM $[NO_3^-]_o$ or $[NH_4^+]_o$, respectively. Total bar graph heights indicate influx (ϕ_{oc}). Component fluxes are efflux from the cytoplasm (ϕ_{co}, hatched bar segments), combined flux to assimilation and the vacuole ($\phi_{assimilation/vacuole}$, black bar segments) and flux to the shoot (ϕ_{xylem}, white bar segments) (Siddiqi *et al.*, 1991; Kronzucker *et al.*, 1995a,b). Absolute flux contributions are indicated to the left of respective bar segments, percentages of influx are indicated to the right. Data are the means of eight or nine experiments ($n \geqslant 8$). SE < 15 %.

Perhaps most importantly, however, compartmental analysis revealed pronounced differences in subcellular N-flux partitioning patterns between seedlings provided with NH_4^+ and those provided with NO_3^- (Fig. 4). Although almost identical proportions (52–53 %) of incoming N were channelled into assimilation and to the vacuolar compartment under the two N regimes, the proportions of N translocated to the shoot and lost through efflux were quite different. Translocation of N to the shoot comprised 37.43 % of incoming $^{13}NO_3^-$ compared with only 24.4 % of incoming $^{13}NH_4^+$. In absolute terms, more than twice as much N was made available to the shoot with NO_3^- provision. Given that $> 70\%$ of N entering the rice caryopsis and $> 50\%$ of N in growing leaves during the grain filling stage is derived from remobilization of N-storage compounds accumulated in the shoot during the vegetative stage (Mae *et al.*, 1985; Sheehy *et al.*, 1998), this difference is potentially of great significance. Moreover, loss of N from roots through efflux was minimized when NO_3^- was provided. Efflux was $< 10\%$ for NO_3^- and $\sim 24\%$ for NH_4^+; in absolute terms, 1.8 times more N was lost with NH_4^+. This difference is striking and shows a superior efficiency of N utilization by NO_3^-.

CONCLUSIONS

Our data show that both capacity for and efficiency of NO_3^- utilization in *Indica* rice are greater than for NH_4^+, indicating a highly specialized adaptation to the NO_3^- source which has not hitherto been recognized. Current growing regimes for lowland rice fail to harness this potential fully, as flooded paddy conditions are neither conducive to the formation of NO_3^- nor to its stability. However, NO_3^- is produced in the O_2-rich surface layer of irrigated paddy soils as well as in well drained upland rice fields and in rain-fed environments during the dry season (Bouldin, 1986; Arth *et al.*, 1998). Given the potential importance of NO_3^- acquisition to the enhancement of yield (see below), we propose the following strategies to capitalize on the high capacity of rice for NO_3^- acquisition:

(1) In breeding, direct efforts towards maximizing the area of the surface root system exposed to NO_3^- (Bouldin, 1986). The area of the surface root system of some new high-yielding hybrid rice varieties is substantially larger than that of traditional inbred varieties, accounting for $\sim 40\%$ of root fresh weight (Yang & Sun, 1987). These varieties also show high nitrate reductase and tissue NO_3^- accumulation, coupled with overall improved N-use efficiency. A direct connection between enhanced NO_3^- acquisition and increased yield seems possible. Field trials at the International Rice Research Institute (IRRI) confirm that the correlation between NO_3^- acquisition capacity and yield might indeed be stronger than previously assumed (X. E. Yang *et al.* unpublished).

(2) Breeding for enhanced formation of NO_3^- in the bulk rhizosphere by root-released O_2. It is established that rice roots can release O_2 at rates sufficient to support nonspecific aerobic microbial processes (Armstrong *et al.*, 1990; Bedford *et al.*, 1991; Begg *et al.*, 1994; Kirk & Du, 1997) and to oxidize substantial quantities of iron and sulphate at the rhizosphere (Kirk & Bajita, 1995; Wind & Conrad, 1995, 1997; Arth *et al.*, 1998). The oxidation of NH_4^+ to NO_3^- appears equally possible (Reddy & Patrick, 1986; Mosier *et al.*, 1990; Buresh *et al.*, 1991; Arth *et al.*, 1998) and must be tested.

(3) Customization of water management practice to include intermittent drainage of rice paddies during vegetative stages of rice growth, allowing nitrification to occur (Arth *et al.*, 1998). Such a regimen has also been shown to favour the development of the surface root system in rice and is already practised by some farmers in southern China and Japan (Greenland, 1997; Guerra *et al.*, 1998).

It is important to note that, regardless of which strategies are employed, neither N source is likely to become the sole constituent in the rice rhizosphere under field conditions. Rather, at best, N mixtures of varying proportions must be expected in soil solution. Although the uptake of NO_3^- is inhibited by the presence of NH_4^+ in many species by as much as 50% (Glass & Siddiqi, 1995; Kronzucker *et al.*, 1999b), with a similar extent of inhibition observed in rice (Fried *et al.*, 1965; Colmer & Bloom, 1998; Kronzucker *et al.*, 1999a), co-provision of NO_3^- and NH_4^+ has been shown to facilitate a significant enhancement of growth and yield (Cox & Reisenauer, 1973; Ta & Ohira, 1981; Ta *et al.*, 1981; Ancheng *et al.*, 1993; Kronzucker *et al.*, 1999b) compared with provision of either N source alone. Increases as high as 40–70% in controlled-culture conditions have been reported, and somewhat lower, but still appreciable, enhancements are seen in field conditions (Gill & Reisenauer, 1993). The nature of this N-source synergism is poorly understood, but, importantly, the enhancement effect is most pronounced when the relative proportions of NO_3^- within the N mixture are higher, and is therefore directly related to the acquisition potential of NO_3^- in a given species (Cox & Reisenauer, 1973). This emphasizes the importance in the field of the high potential for NO_3^- acquisition we have identified in rice.

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