Effect of Ammonium on Nitrate Utilization by Roots of Dwarf Bean

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ABSTRACT

The effect of exogenous NH₄⁺ on NO₃⁻ uptake and in vivo NO₃⁻ reductase activity (NRA) in roots of Phaseolus vulgaris L. cv Witte Krombek was studied before, during, and after the apparent induction of root NRA and NO₃⁻ uptake. Pretreatment with NH₄Cl (0.15–50 millimolar) affected neither the time pattern nor the steady state rate of NO₃⁻ uptake.

When NH₄⁺ was given at the start of NO₃⁻ nutrition, the time pattern of NO₃⁻ uptake was the same as in plants receiving no NH₄⁺. After 6 hours, however, the NO₃⁻ uptake rate (NUR) and root NRA were inhibited by NH₄⁺ to a maximum of 45% and 60%, respectively.

The response of the NUR of NO₃⁻-induced plants depended on the NH₄Cl concentration. Below 1 millimolar NH₄⁺, the NUR declined immediately and some restoration occurred in the second hour. In the third hour, the NUR became constant. In contrast, NH₄⁺ at 2 millimolar and above caused a rapid and transient stimulation of NO₃⁻ uptake, followed again by a decrease in the first, a recovery in the second, and a steady state in the third hour. Maximal inhibition of steady state NUR was 50%. With NO₃⁻-induced plants, root NRA responded less and more slowly to NH₄⁺ than did NUR.

Methionine sulfoximine and azaserine, inhibitors of glutamine synthetase and glutamate synthase, respectively, relieved the NH₄⁺ inhibition of the NUR of NO₃⁻-induced plants. We conclude that repression of the NUR by NH₄⁺ depends on NH₄⁺ assimilation. The repression by NH₄⁺ was least at the lowest and highest NH₄⁺ levels tested (0.04 and 25 millimolar).

NO₃⁻ is the main source of N for crop plants, including legumes (23). At present there is much concern about the ecological and nutritional impact of NO₃⁻ fertilizers. A better understanding of the physiological processes by which the plant responds to the presence of NO₃⁻ in its environment may permit a more intelligent use of NO₃⁻. Despite its biological and agricultural significance, detailed studies on the process of NO₃⁻ acquisition by plants are fairly recent and insight into the regulation of NO₃⁻ uptake is still in its infancy (12).

The process of NO₃⁻ uptake by higher plants differs from that of other inorganic nutrients. For example, the fate of NO₃⁻ in the plant may be accumulation as well as conversion and both uptake and reduction have characteristics of induction. Against this background, we started an investigation into the initial events of NO₃⁻ utilization in dwarf bean, a species which is very flexible in the choice of its N source. Our previous work has identified NO₃⁻ uptake and reduction of NO₃⁻ to NO₂⁻ in the root system as two key steps in NO₃⁻ utilization by N-depleted dwarf bean (3, 6).

Utilization of NO₃⁻ encompasses all processes that deliver exogenous NO₃⁻-N to its final destination which may range from deposition in the root to biosynthesis in the shoot. Assimilation of NO₃⁻ has a number of obligatory chemical intermediates such as NO₂⁻, NH₄⁺, amides, and amino acids. Each intermediate may affect the utilization of NO₃⁻ and thus serve as a metabolic regulator (6).

Numerous reports deal with the effect of NH₄⁺ on NO₃⁻ uptake in higher plants. The effect varies from no effect in detached barley roots (25) to a partial inhibition in maize (13), tomato (14), and sweet potato (16). Recent findings indicate that NH₄⁺ may affect the influx (13) as well as the efflux component (9) of net NO₃⁻ uptake.

It has been proposed that the repression of NO₃⁻ uptake by NH₄⁺ in lower plants is not caused by NH₄⁺ as such. In algae and bacteria, the action of NH₄⁺ on NO₃⁻ uptake seems to be regulated either by an assimilatory enzyme (GS², 18) or a product of NH₄⁺ assimilation (27), e.g. glutamine (1). For maize, Rufty et al. (22) suggested that inhibitory factors generated during NH₄⁺ assimilation may inhibit NO₃⁻ uptake.

The effect of NH₄⁺ on NRA during and after induction has been reported to be none, positive, or negative, depending on species, genotype, tissue, and nutrient conditions (26). It is unclear whether the effect on root NO₃⁻ reduction is related to the effect of NH₄⁺ on uptake.

The aim of the present study was to elucidate the role of NH₄⁺ in the initial utilization of NO₃⁻ by roots of dwarf bean. Short reports of our results have appeared (4, 8).

MATERIALS AND METHODS

Plant Cultivation and Experiments. Phaseolus vulgaris L. cv Witte Krombek was germinated in perlite and grown unnodulated for 7 ± 1 d on an N-free basal medium (BM) at pH 5.0 ± 0.2 under a 16-h photoperiod (30 W m⁻²) at 65% RH and 20°C. NO₃⁻ nutrition started by the addition of Ca(NO₃)₂, with or without NH₄Cl, to plants grown in BM. During uptake experiments, the concentration of NO₃⁻ was 0.15 or 0.4 mM which rendered the NUR independent of NO₃⁻ concentration (7). Plant to solution ratios were chosen such that the ambient pH fluctuations were kept within the range 4.9 to 5.4. For further details on plants and experiments, we refer to earlier papers (3, 5–7).

All experiments were repeated at least twice and results are expressed per unit of root dry mass.

Analytical Procedures. Uptake of NO₃⁻ and NH₄⁺ was meas-

1 M. S. was a student from the Department of Soil Science and Plant Nutrition of the Agricultural University at Wageningen.

1 Abbreviations: GS(A), glutamine synthetase (activity); BM, basal medium; NUR, NO₃⁻ uptake rate; GDH(A), glutamate dehydrogenase (activity); LN₂, liquid nitrogen; NR(A), NO₃⁻ reductase (activity); 2-OG, 2-oxoglutarate; MSO, methionine sulfoximine; AS, asaservine; GOGAT, glutamine-oxoglutarate aminotransferase (glutamate synthase).
ured by ambient depletion. NO₃⁻ was determined continuously or discontinuously by UV spectrophotometry, or colorimetrically with Szechrome reagent or reduction with hydrazine as indicated previously (6, 7). Data obtained by UV analyses were checked with data from other assays and only used when the difference in concentration obtained by independent methods was less than 6%. The Berthelot reaction was used to assay ambient NH₄⁺ (2), and plant NH₄⁺ was measured in a cold water extract of LN₂-powdered tissue material with an Ultrarax pistol blender. The extracts were steam-distilled at pH 10.5 into boric acid and the trapped NH₃ was determined acidimetrically (2). Under these conditions, amide breakdown was <5% and NH₄⁺ recovery was >98%.

NO₃⁻ reductase activity was measured by an anaerobic in vivo test (5) that gave results in agreement with the rate of in situ reduction (3). The activity of GS and GDH was measured in vitro in an extract prepared with a mortar and pestle from fresh LN₂-powdered roots mixed with sand. The extraction buffer (pH 7.2, 4°C) contained imidazole, DTT and Na₂EDTA at concentrations of 50, 1, and 0.5 mM, respectively. Crude extracts were centrifuged (2°C, 5 min, 5000g) and kept on ice until the enzyme assays. The synthetase reaction of GS was measured by the formation of L-glutamate-5-monohydroxamate after 30 min of incubation at 30°C in a pH 7.2 buffer containing K-glutamate, imidazole, MgSO₄, hydroxylamine, and ATP at concentrations of 80, 50, 20, and 8 mM, respectively. The reaction was stopped by addition of a mixture of TCA and FeCl₃ in HCl (20). After centrifugation (5 min, 5000g), the absorbance was measured at 546 nm. The absorbance of extracts incubated without ATP was subtracted and the absorbance difference was measured after standard series of L-glutamate-5-monohydroxamate subjected to the same procedure as the plant extracts.

The NADH-dependent activity of GDH was estimated spectrophotometrically at 340 nm by measuring the oxidation of NADH by a crude enzyme extract mixed with a pH 7.2 buffer which contained Tricine, (NH₄)₂SO₄, 2-OG, CaCl₂, and NADH in final concentrations of 50, 50, 1, and 0.2 mM, respectively. NADH consumption by other enzyme systems was corrected for by measuring GDHA against a reference cuvette with identical composition, except that K₂SO₄ replaced (NH₄)₂SO₄ and 2-OG was omitted. The activity in the sample cuvette was 5 to 6 times higher than the reference cuvette.

RESULTS

Pretreatment with NH₄⁺. An 18-h pretreatment with NH₄Cl (0.15–50 mM) did not affect the duration of the apparent induction phase of NO₃⁻ uptake (data not shown). Induction took about 6 h in all treatments, and the shape of the NUR-time curve (cf. Fig. 1) was not significantly altered by the NH₄⁺ history. After induction of NO₃⁻ uptake, the NUR did not differ significantly between NH₄⁺-pretreated and control plants (data not shown).

As compared with control plants, initial potential NRA (5) was unaffected in primary leaves, but 50% lower in roots of NH₄⁺-pretreated (18 h, 0.15 and 10 mM) plants. Two and 6 h after NO₃⁻ supply, however, actual NRA equaled potential NRA in roots and leaves, and no pretreatment effect of NH₄⁺ was observed (data not shown).

Concomitant Supply of NH₄⁺ and NO₃⁻. When N nutrition was initiated with 0.15 mM NH₄NO₃, the time pattern of NO₃⁻ uptake was the same as with Ca(NO₃)₂ (Fig. 1), but steady state NUR was about 30% lower with NH₄NO₃. The inhibition was aggravated at higher exogenous NH₄⁺ levels (Fig. 2) and became maximal (40–50%) between 1 and 10 mM. NH₄⁺ levels between 1 and 10 mM had no effect on the time pattern of NO₃⁻ uptake during concomitant supply of the two N sources (data not shown). NH₄⁺ uptake was not affected by NO₃⁻ (Fig. 1).

![FIG. 1. Uptake of NO₃⁻ and NH₄⁺ by dwarf bean in the absence and presence of each other. N sources added at t = 0.15 mM. The average coefficients of variation for the data are 9% (NO₃⁻) and 4% (NH₄⁺).](image)

![FIG. 2. Effect of NH₄⁺ concentration on NO₃⁻ uptake rate (NUR) and NO₃⁻ reductase activity (NRA) of dwarf bean roots after 6 h of NO₃⁻ (0.15 mM) supply in the absence or presence of NHCl. Data are given ± SD, n = 3 (NUR) or n = 5 (NRA). Control values: NUR, 34.8 μmol NO₃⁻ g⁻¹ h⁻¹; NRA, 11.2 μmol NO₃⁻ g⁻¹ h⁻¹. Values of NaCl controls are given for 25 and 50 mM. Controls with KCl at 2 and 10 mM yielded similar values.](image)
FIG. 3. Effect of NH₄⁺ on the NO₃⁻ uptake rate (NUR) of dwarf bean. NO₃⁻-induced plants (18 h, 0.4 mM) were supplied with NH₄Cl (concentration indicated in figure) at t = 0 (arrows). The NUR was computed from continuous recordings of the disappearance of NO₃⁻ versus time and expressed as per cent of the initial rate (t = 0) for each NH₄⁺ concentration. Control values varied between experiments from 37 to 48 μmol NO₃⁻ g⁻¹ h⁻¹.

FIG. 4. Effect of NH₄⁺ on NO₃⁻ reductase activity (NRA) of roots of NO₃⁻-induced (18 h, 2 mM) dwarf bean. NH₄Cl (2 mM) was added to the NO₃⁻ medium at t = 0. Data are given as per cent of the control (t = 0; 20.8 μmol NO₃⁻ g⁻¹ h⁻¹) ± SD (n = 5).

rate were consistently highest at 25 mM NH₄⁺. At 0.04 mM NH₄⁺, the NUR after 3 h was also consistently higher than at 2 mM NH₄⁺. Maximum inhibition of the NUR over the whole NH₄⁺ concentration range tested was about 50%. Parallel experiments with NaCl or KCl proved that the effect of NH₄Cl was due to the NH₄⁺ ion (data not shown). Removal of exogenous NH₄⁺ (2 mM) after 3 h resulted in a further decrease of the NUR. Uptake recovered, however, within 3 h to the NH₄⁺-inhibited rate (data not shown). Washing of roots during NO₃⁻ uptake in the absence of NH₄⁺ did not alter the NUR. Similarly, a second wash, 3 h after NH₄⁺ removal, did not affect the NUR. Prolonged NH₄⁺ supply (3–10 h at 0.04–25 mM) did not reduce the NUR by more than 50% (data not shown).

Root NRA, in contrast to NUR, was unaffected by 5 min of NH₄⁺ supply (2 mM; data not shown). Similarly, when NH₄⁺ (5 mM) was added to the incubation buffer in an in vivo NRA assay of NO₃⁻-induced roots, the rate of NO₃⁻ release was not influenced for at least the subsequent 45 min. At 2 mM of nutrient NH₄⁺, root NRA was only significantly affected after 2 h, and the inhibition was about 35% after 6 h of mixed NH₄⁺ + NO₃⁻ nutrition (Fig. 4).

NH₄⁺ Content in Roots. Plants kept on BM contained very little NH₄⁺, typically 1 μmol/g dry root, corresponding to a mean cellular concentration of about 0.1 mM. After 6 h of N supply (0.4 mM) NH₄⁺ in roots increased 3-fold and 50-fold with NO₃⁻ and NH₄⁺, respectively (Fig. 5). Accumulation of NH₄⁺ was similar in roots of induced and uninduced plants for at least 3 h.

Effect of Methionine Sulfoximine and Azaserine. MSO and AS, inhibitors of GS and GOGAT, respectively, were used to assess whether the effect of exogenous NH₄⁺ is exerted by NH₄⁺ per se or by an event or compound related to its primary assimilation in the root. When the NUR of induced plants was about 50% inhibited by 2 mM NH₄⁺ (cf. Fig. 3), the addition of MSO rapidly accelerated NO₃⁻ uptake (Fig. 6). The NUR gradually declined upon removal of the inhibitor, but the GSA was irreversibly inhibited by MSO (Fig. 7). Without removal of MSO, the NUR did not decrease for the subsequent 1 to 2 h (data not shown). When added to NH₄⁺-free media, MSO did not alter the NUR of induced plants for at least 0.5 h, but caused a 30% lower NUR than in control plants after 3 h (data not shown). In mixed NO₃⁻ + NH₄⁺ nutrition, glutamate, of which MSO is an analogue, did not stimulate the NUR (data not shown). AS also rapidly stimulated the NUR of NH₄⁺-affected plants (Fig. 6).

Pretreatments (1 h, 1 mM) with MSO and AS caused a transiently decreasing initial NO₃⁻ uptake. After 6 h of NO₃⁻ supply, the NUR was 45% (MSO) and 25% (AS) of that of nonpretreated
GOGAT exceeded GDH. equimolar estimate the NH4+, the with NH4+ was measured of induced plants, plants, which showed an induction pattern similar to that in Figure 1 (data not shown).

Six h after the start of NO3− supply in the presence of MSO, root NRA was only 10% of the control. A short revival of root NRA occurred consistently after 4 h (data not shown). Root NRA of induced plants was decreased more by MSO than by NH4+ (Table 1; cf. Fig. 4), and the effects of MSO and NH4+ did not appear to be additive.

Glutamine Synthetase and Glutamate Dehydrogenase. GSA was measured at various times during NH4+ inhibition of the NUR of induced plants (Fig. 7). The initial drop in GSA coincided with a transient stimulation of NO3− uptake by NH4+ and the plateau of GSA in the 3rd h coincided with a constant (about 50% inhibited) NUR and with a constant level of endogenous NH4+ (Fig. 5). The in vitro activities of GS and GDH in induced roots were measured after 3 h of NH4+ (0–25 mm) supply to estimate the partition of NH4+ assimilation in roots over the GS-GOGAT route and the GDH route (Fig. 8). At low ambient NH4+, the activity of GS was about 2 times higher than that of GDH. At 25 mm NH4+, GDHA and GSA were equal.

Table 1. Effect of MSO and NH4+ on the NRA of Dwarf Bean Roots

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NRA (%)</th>
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<tbody>
<tr>
<td>NO3−</td>
<td>100 ± 1*</td>
</tr>
<tr>
<td>NO3− + NH4+</td>
<td>71 ± 8</td>
</tr>
<tr>
<td>NO3− + MSO</td>
<td>54 ± 6</td>
</tr>
<tr>
<td>NO3− + MSO + NH4+</td>
<td>56 ± 5</td>
</tr>
</tbody>
</table>

* Per cent of control (NO3− only; 6.3 μmol NO3− g−1 h−1) and given ± SD (n = 5).

DISCUSSION

Uptake and Accumulation of NH4+. Uptake of NH4+ proceeded at a constant rate after 0.5 h and was unaffected by an equimolar concentration of NO3− (Fig. 1). The initial phase probably reflects the filling of a free space (2). NH4+ was taken up faster than NO3− in the absence and in the presence of NO3−. After 6 h of NO3− supply, the concentration of NH4+ in the roots of about 3 μmol g−1 (Fig. 5) was much higher than that of NO3− (<0.5 μmol g−1 [6]) and much lower than that of NO3− (about 40 μmol g−1 [3]) under similar conditions. In induced and uninhibited plants, NH4+ were not only limited to a plateau level of about 50 μmol g−1 after a few hours of NH4+ supply (Fig. 5).

NO3− Uptake. The apparent induction of NO3− uptake proceeded identically in the absence and presence of NH4+ (Fig. 1), but the NUR was rapidly and severely affected by NH4+ (Figs. 2 and 3). The lack of a reverse effect (Fig. 1) shows that NH4+ and NO3− are not competing for a common uptake site. Inhibition of NH4+ assimilation by blocking the GS-GOGAT cycle prevented the normal induction of NO3− uptake. The increase of the NUR from an initial low to a high constant value thus seems to require normal NH4+ assimilation or its products, e.g., newly made amino acids and proteins. An altered N status due to NH4+ pretreatment did not affect the induction of NO3− uptake. For wheat (28) and cultured tobacco cells (11), the timing of NO3− uptake is also independent of exogenous NH4+.

NH4+ could not repress the NUR after 6 h NO3− supply by more than 40% to 50% (Fig. 2). Similarly, the steady state NUR of NH4+ affected induced plants was at least 50% of the uninhibited rate (Fig. 3). These findings suggest that only part of the NO3− uptake system can be influenced by NH4+, and that NH4+ in the millimolar range causes maximal inhibition. In induced plants, NH4+ at 2 mm and above caused a rapid but transient increase of the NUR, and the inhibition was lower at 25 than at 0.2 to 10 mm. Rao and Rains (19) found a similar concentration-dependent effect of NH4+ on the NUR of barley plants. At 0.1 mm, they reported an inhibition of the NUR of about 35% whereas the NUR was higher below and above that concentration. It was recently reported for tomato that inhibition of the NUR decreases when the NH4+ level exceeds 50 μM (14).

NH4+ assimilation in higher plants usually involves the high affinity GS-GOGAT route, the low affinity GDH route only being significant at high endogenous NH4+ concentrations (21). Our results from in vitro enzyme activities in roots, and data from duck weed (21) and maize leaves (24), are consistent with the prevalence of the GS-GOGAT route at low and an increasing contribution of the GDH route at high NH4+ concentrations (Fig. 8). Inhibition of NO3− uptake may be related to the assimilation route of the absorbed NH4+. Apparently, a high GDHA or a low GSA is associated with less inhibition of NO3− uptake.
by NH₄⁺. Recently, labeling experiments with ¹⁵NH₄⁺ have produced results that are inconsistent with any significant role of the GDH route (10, 15). Although ¹⁵N data are by far more conclusive than data from in vitro enzyme activities, it should be noted that no ¹⁵N labeling data are available for the highest NH₄⁺ levels used in our experiments.

The time pattern of NH₄⁺ inhibition of NUR in induced plants is consistent with an inhibitory role of the GS-GOGAT route (Fig. 7). It is also possible that an unknown critical factor, e.g. an amino acid, is responsible for the time pattern and concentration dependence of the inhibition by NH₄⁺, but this explanation would require a lower concentration of this factor at high NH₄⁺ levels.

Other evidence for the involvement of the GS-GOGAT route in the inhibition of NO₃⁻ uptake comes from experiments with the specific enzyme inhibitors MOSO and AS (Fig. 6). MOSO rapidly and irreversibly inactivated GS (Eq. 7) and caused a swift increase of the NUR of NH₄⁺-inhibited plants. AS, although to a lesser extent, also relieved the NH₄⁺ inhibition of NO₃⁻ uptake (Fig. 6). Removal of MOSO gradually decreased the NUR, despite the further decrease in GSA. This effect of MOSO may be due to its function as glutamate analog, since Breitler and Arnon (in preparation) found that inhibition of NUR by glutamate was aggravated rather than relieved upon removal of the inhibiting compound. Moreover, removal of NO₃⁻ (6) and NH₄⁺ also drastically lowered NO₃⁻ uptake. Continuation of an NH₄⁺ effect for several hours in the absence of exogenous NO₃⁻ is another indication of the involvement of NH₄⁺ assimilation in the suppression of the NUR. Since MOSO and AS have at least qualitatively the same effect on NO₃⁻ uptake, it is unlikely that the accumulation of NH₄⁺, glutamine, or glutamate is responsible for the repression of the NUR. Aspects of the GS-GOGAT route other than the primary intermediates seem to be involved. A strong candidate is the ATP-requiring reaction catalyzed by GS, which may divert ATP from sustaining active NO₃⁻ uptake (3) to NH₄⁺ assimilation, as suggested by Ohmori et al. for an alga (17).

NO₃⁻ Reductase. A lower amount of entering substrate for NO₃⁻ reduction was not the sole effect of NH₄⁺ on root NUR. During initial NO₃⁻ supply, root NUR was more affected by NH₄⁺ than was the NUR, and maximal inhibition of NUR was already exerted by 0.1 mm NH₄⁺, the lowest concentration tested (Fig. 2). In induced plants, NUR was more affected by NH₄⁺ than was NUR. NUR was affected immediately (Fig. 3), whereas root NUR started to decrease between 1 and 2 h, and to a lesser extent than the NUR, under similar conditions (Fig. 4). NUR and root NUR thus seem to be independently regulated during and after the apparent induction of uptake and reduction of NO₃⁻.

Independent of the presence of NH₄⁺, root NUR of induced plants was lowered by MOSO (Table 1). Events or compounds associated with assimilation of NH₄⁺ via the GS-GOGAT route thus were required for both induction and high activity of root NUR.

NO₃⁻ Uptake and N Assimilation. Uptake of NO₃⁻ appeared more related to NH₄⁺ assimilation than to NO₃⁻ assimilation (Figs. 3, 4, and 6). The NO₃⁻ history of the plants determined whether NH₄⁺ affects NUR more or less than NRA, and also determined the timing and magnitude of the response of NUR to NH₄⁺ (Figs. 1-3). We therefore conclude that NH₄⁺ does not exclusively affect NO₃⁻ utilization by the generation of a common regulator of uptake and reduction. Rao and Rains (19) similarly reached the conclusion that in barley the effect of NH₄⁺ on NO₃⁻ uptake is independent of its effects on root NRA.

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