Rapid effects of nitrogen form on leaf morphogenesis in tobacco

Pia Walch-Liu¹, Günter Neumann¹, Fritz Bangerth² and Christof Engels³

¹ Institut für Pflanzenernährung, Universität Hohenheim, D-70593 Stuttgart, Germany
² Institut für Obst-, Gemüse- und Weinbau, Universität Hohenheim, D-70593 Stuttgart, Germany
³ Abteilung Agrarökologie, Fachgruppe Geowissenschaften der Universität Bayreuth, D-95440 Bayreuth, Germany

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Abstract

Ammonium (NH₄⁺) instead of nitrates (NO₃⁻) as the nitrogen (N) source for tobacco (Nicotiana tabacum L.) cultivated in a pH-buffered nutrient solution resulted in decreased shoot and root biomass. Reduction of shoot fresh weight was mainly related to inhibition of leaf growth, which was already detectable after short-term NH₄⁺ treatments of 24 h, and even at a moderate concentration level of 2 mM. Microscopic analysis of the epidermis of fully expanded leaves revealed a decrease in cell number (50%) and in cell size (30%) indicating that both cell division and cell elongation were affected by NH₄⁺ application. Changes in various physiological parameters known to be associated with NH₄⁺-induced growth depression were examined both in long-term and short-term experiments: the concentrations of total N, soluble sugars and starch as well as the osmotic potential, the apparent hydraulic conductivity and the rate of water uptake were not reduced by NH₄⁺ treatments (duration 1–12 d), suggesting that leaf growth was neither limited by the availability of N and carbohydrates, nor by a lack of osmotica or water supply. Although the concentration of K⁺ in leaf press sap declined in expanding leaves by approximately 15% in response to NH₄⁺ nutrition, limitation of mineral nutrients seems to be unlikely in view of the fast response of leaf growth at 24 h after the start of the NH₄⁺ treatment. No inhibitory effects were observed when NH₄⁺ and NO₃⁻ were applied simultaneously (each 1 mM) resulting in a NO₃⁻/NH₄⁺ net uptake ratio of 6:4. These findings suggest that the rapid inhibition of leaf growth was not primarily related to NH₄⁺ toxicity, but to the lack of NO₃⁻-supply. Growth inhibition of plants fed solely with NH₄⁺ was associated with a 60% reduction of the zeatine + zeatine riboside (Z + ZR) cytokinin fraction in the xylem sap after 24 h. Furthermore Z + ZR levels declined to almost zero within the next 4 d after start of the NH₄⁺ treatment. In contrast, the concentrations of the putative Z + ZR precursors isopentenyl-adenine and isopentenyl-adenosine (i-Ade + i-Ado) were not affected by NH₄⁺ application. Since cytokinins are involved in the regulation of both cell division and cell elongation, it seems likely that the presence of NO₃⁻ is required to maintain biosynthesis and/or root to shoot transfer of cytokinins at a level that is sufficient to mediate normal leaf morphogenesis.

Key words: Ammonium, nitrate, leaf growth, cytokinin.
and Barker, 1967; Barker et al., 1966), deficiency of mineral nutrients, such as K⁺ (Barker et al., 1967), Ca²⁺ (Wilcox et al., 1973) and Mg²⁺ (Jungk, 1977), and to carbohydrate limitation (Cramer and Lewis, 1993; Breteler, 1973) due to excessive consumption of soluble sugars for NH₄⁺ assimilation (detoxification). The lack of NO₃⁻ as an important osmoticum (Raab and Terry, 1995; Salsac et al., 1987; Chaillou et al., 1986) and water deficit in response to a reduction in water uptake (Adler et al., 1996; Quebedeaux and Ozbun, 1973) have also been reported as putative growth-limiting factors in plants gently removed. Leaf squares of about 2.5 cm diameter were pressed on to acetone-soaked cellulose acetate sheets supported on a glass slide. After several minutes, when the cellulose acetate sheet had dried, the leaf discs were gently removed. Leaf squares of about 2.5 × 4 cm were pressed on to a nail varnish-soaked glass slide (Hampe, 1979). Photographs were taken from distinct areas and the average cell size was calculated based on cell counts per unit leaf area. Cell number per leaf was determined by extrapolation of cell number per unit leaf area to the total leaf area.

Materials and methods

Plant cultivation

Tobacco (Nicotiana tabacum L. cv. Samsun and cv. Gatersleben) was grown in a climate chamber under controlled environmental conditions with a 14 h light period (light intensity of 450 μE m⁻² s⁻¹), a 25/20 °C light/dark temperature regime, and 60% relative humidity. Tobacco seeds were germinated in a mixture consisting of 90% (w/w) peat culture substrate (Euflor GmbH, München, Germany), 7% (w/w) perlite and 3% (w/w) sand. About 2 weeks after sowing, plants were transferred to an aerated, hydroponic culture system, and supplied with saturated CaSO₄ solution during the first 24 h, and thereafter with a full-strength nutrient solution consisting of H₂BO₃, 10 μM, MnSO₄, 0.5 μM, ZnSO₄, 0.5 μM, CuSO₄, 0.1 μM, (NH₄)₂MoO₄, 0.01 μM, Fe-EDTA 15 μM, KH₂PO₄, 0.5 mM, MgSO₄, 1.2 mM, CaCl₂ 2.0 mM. Nitrogen was applied either as KNO₃ or (NH₄)₂SO₄ at a concentration of 2 mM N or as mentioned in the text. For NH₄NO₃ treatments, K₂SO₄ was added to compensate for potassium applied in the KNO₃ variants. Complete N depletion in the nutrient solution was avoided by checking the N concentration in the solution at least once a day using a ROFlex reflectometer (Merck, Darmstadt, Germany) and by adding appropriate N amounts. The nutrient solution was renewed completely every 2 d and pH was held between 6.8 and 7.2 by addition of CaCO₃.

Leaf area and expansion

Leaf length and width were determined daily at the same time using a ruler and leaf area (A) was calculated assuming an ellipsoidal leaf shape using the formula:

\[ A = \pi ab \]

where \( a \) is the leaf length × 0.5, \( b \) is the leaf width × 0.5. Leaf expansion rate was expressed as the increase in leaf area h⁻¹.

Cell size and cell number

Size of epidermal cells was estimated by microscopic analysis of cellulose acetate or nail varnish imprints. Leaf discs of about 1 cm diameter were pressed on to acetone-soaked cellulose acetate sheets supported on a glass slide. After several minutes, when the cellulose acetate sheet had dried, the leaf discs were gently removed. Leaf squares of about 2.5 × 4 cm were pressed on to a nail varnish-soaked glass slide (Hampe, 1979). Photographs were taken from distinct areas and the average cell size was calculated based on cell counts per unit leaf area. Cell number per leaf was determined by extrapolation of cell number per unit leaf area to the total leaf area.

Carbohydrate analysis

Reducing sugars and sucrose were determined according to a modified method of Blakeney and Mutton (Blakeney and Mutton, 1980). About 20 mg of freeze-dried plant material was extracted in 5 ml of 70% (v/v) ethanol by swirling in a tube and centrifuged at 2500 g (Minifuge, Hereaus GmbH, Stuttgart, Germany). Chlorophyll in leaf extracts was removed by addition of activated charcoal (about 10 mg ml⁻¹), swirling and centrifuging at 18000 g (Mikro Centrifuge, Hettich, Tuttingen, Germany). For determination of reducing sugars, 0.2 ml of the clear supernatant was mixed with 0.8 ml of 0.1 M sodium acetate (pH 4.5). Sucrose was determined by mixing 0.1 ml of supernatant with 0.1 ml invertase solution (50 units ml⁻¹ buffered in 0.2 M sodium acetate) and 0.8 ml of 0.1 M sodium acetate (pH 4.8). The mixture was incubated for 2 h in a 30 °C water bath to digest sucrose to glucose and fructose. 5 ml of colour reagent (0.03 M hydroxybenzoic acid hydrazide, 0.05 M trisodium citrate, 0.01 M calcium di-chloride, and 0.5 M sodium hydroxide) was added to the sample solutions (sucrose and reducing sugars) and boiled for 4 min in a water bath. The cooled coloured solution was measured spectrophotometrically at 415 nm (AA spectrometer U-3300, Hitachi, Tokyo, Japan). Starch was determined in the residual pellet (according to Blakeney and Matheson, 1984). Starch was dissolved in 2 ml di-methyl sulphoxide by boiling for 10 min in a water bath. The sample was centrifuged at 2500 g (Minifuge, Hereaus GmbH, Stuttgart, Germany) and the pellet was washed with 8 ml 0.1 mM sodium acetate (pH 4.8) solution. 1 ml of the sample solution was mixed with 2 ml amyloglucosidase solution (1.2 U ml⁻¹ buffered in 0.2 M sodium acetate) and incubated for 12 h at 37 °C in a water bath. After incubation, 5 ml of colour reagent (4000 U glucose oxidase, 1000 U peroxidase, 0.07 M di-sodium hydrogen phosphate, 0.04 M sodium di-hydrogen phosphate, 0.016 M benzoic acid, 0.5 mM 4-amino antipyrin, 0.01 M p-hydroxybenzoic acid) was added to each 1 ml sample solution and kept in a water bath at 40 °C for 15 min. The cooled coloured solution was measured spectrophotometrically at 510 nm (AA spectrometer U-3300, Hitachi, Tokyo, Japan).

Nitrogen analysis

Total N was estimated in freeze-dried plant material with a NCS 2500 Elemental Analyser (CE Instruments, Milan, Italy) using Dumas combustion. The sample is energetically oxidized yielding a gas mixture in which N is detected by a thermoconductivity detector. Nitrate was determined in a water extract of the
was calculated from the di
the pots was recorded after complete draining of nutrient
from the culture vessels (1 plant per pot), and the weight of
with NH

Water uptake was determined gravimetrically after a preculture
Determination of water uptake

is the apparent hydraulic conductivity,

where \( L_p \) is the apparent hydraulic conductivity, \( J_x \) is the water
flux (ml h\(^{-1}\)), \( \alpha \) is the reflection coefficient, \( P_e \) is the pressure
of xylem exudate (MPa), and \( P_a \) is the pressure of external
solution (MPa). It was assumed that \( \alpha \) was not affected
by the form of N supply, and for the calculation of \( L_p \) \( \alpha \) was set = 1.

Determination of water uptake

Water uptake was determined gravimetrically after a preculture
with NH\(_4\) or NH\(_4\)NO\(_3\) (2 mM N) followed by 2 d of NH\(_4\) or
NO\(_3\) treatments (2 mM N), respectively. Plants were removed
from the culture vessels (1 plant per pot), and the weight of
the pots was recorded after complete draining of nutrient
solution from the root systems. The daily rate of water uptake
was calculated from the differences in weight compared to
the start of the experiment. Background evaporation was negligible
since the pots were kept completely closed with lids throughout
the entire experimental period.

Analysis of cytokinins

Xylem exudate was collected as described above and stored at
-80 °C. Cytokinins (CKs) were determined (according to
Bangerth, 1994). The exudate was purified by adjusting the pH
to 8.5 and passing it first over a polyvinylpyrrolidone column
and then, after adjusting the pH to 3.0, over a Waters C-18 Sep
Pak cartridge (Waters, Milford, Mass., USA). The cartridge was
then washed with 0.1 M acetic acid and zeatin (Z) and
zeatinriboside (ZR) were eluted with 4 ml of 25% (v/v) methanol
in 0.1 M acetic acid; finally isopentenyladenine + isopentenyl-
adenosine (i-Ado + i-Ado) were eluted with 70% (v/v) methanol.
After vacuum evaporation, the purified CKs were quantified by
a radioimmunoassay (as described by Bohnert and Bangerth,
1988). All immunoassays were performed in triplicate.

Statistical analysis

The statistical software Sigma Stat Version 2.03 (SPSS Inc.)
was used for analysis of variance. Comparisons among the
means were conducted using Student–Newman–Keuls test.

Results

N form effects on growth

Tobacco plants examined in this study did not exhibit
any visible symptoms of NH\(_4\) toxicity such as marginal
decay and interveinal chlorosis on the leaves, wilting,
stunted root growth or brownish roots (Goyal et al.,
1982; Maynard and Barker, 1969; Barker et al.,
1966) (Figs 1, 2).

The fresh weights of shoot and roots in two cultivars
of tobacco (cv. Samsun and cv. Gatersleben) were signi-
ificantly reduced when NH\(_4\) was supplied instead of NO\(_3\)
as the N source (Table 1; Fig. 1). Shoot biomass produ-
c tion was mainly affected by inhibition of leaf growth,
whereas the number of unfolding and unfolded leaves
was only slightly decreased (Table 1). Microscopic anal-
ysis of the epidermis of fully expanded leaves revealed a
50% reduction of cell number and a 30% reduction of
cell size in NH\(_4\)-treated plants (Table 2; Fig. 3).

Fig. 1. Effect of N form on shoot growth of tobacco (cv. Samsun)
cultivated in nutrient solution at 2.5 mM N supplied with NH\(_4\) or
NO\(_3\) for 11 d before harvest (46 DAS).

Collection of xylem exudate and determination of apparent
hydraulic conductivity

For collection of xylem exudate and the determination of water
flux, plant shoots were cut 2 cm above the root
shoot interface. After at least 15 min, the cut stem was cleaned with paper
tissue to avoid contamination with contents of wounded cells
and phloem exudate. Thereafter, xylem exudate was collected
with a Pasteur pipette for approximately 1 h (storage on ice)
and subsequently stored at -20 °C. Water flux was calculated
from the quotient of collected volume of xylem exudate and
the collecting time. The osmotic potentials of xylem exudate
and nutrient solution were determined using an osmometer.

The apparent hydraulic conductivity was calculated after the
formula of Fiscus (Fiscus, 1975):

\[
L_p = J_x \times (\alpha \times (P_e - P_a))^{-1}
\]

\[
\text{[ml MPa}^{-1}\text{ h}^{-1}\text{ g}^{-1}\text{ root fresh weight]}
\]

where \( L_p \) is the apparent hydraulic conductivity, \( J_x \) is the water
flux (ml h\(^{-1}\)), \( \alpha \) is the reflection coefficient, \( P_e \) is the pressure
of xylem exudate (MPa), and \( P_a \) is the pressure of external
solution (MPa). It was assumed that \( \alpha \) was not affected
by the form of N supply, and for the calculation of \( L_p \) \( \alpha \) was set = 1.

Press sap analysis

Press sap of young, expanding and fully expanded leaves was
analysed for K\(^+\) and Ca\(^{2+}\) by flame emission photometry
(ELEX 6361, Eppendorf, Hamburg, Germany), Mg\(^{2+}\) by
atomic absorption spectrometry (AAspectrometer Unicam 939,
ATI UNICAM, Kassel, Germany), Cl\(^-\) using a chloridemeter
(chloridimeter 6610, Eppendorf, Hamburg, Germany) and
NO\(_3\) using the auto analyser procedure as described above, or
by using a RQflex reflectometer (Merck, Darmstadt, Germany).
The osmotic potential of leaf press sap was determined using
an osmometer (automatisches Halbmikro-Osmometer Typ
Digital, Knauer, Berlin, Germany).

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Table 1. Growth characteristics of tobacco as affected by the N form: (a) cv. Samsun supplied with 2.5 mM N for 11 d before harvest (DAS 46), (b) cv. Gatersleben supplied with 1.5 mM N for 10 d before harvest (DAS 32)

Unfolded and unfolding leaves were counted starting from the oldest leaf (first). Leaf area of the youngest expanded leaf (10th) is shown. Data are mean values ± se of four replicate plants. Differences between N treatments were statistically tested by ANOVA and means in the same column with different letters are significantly different (P<0.05, Student–Newman–Keuls test).

<table>
<thead>
<tr>
<th>N supply</th>
<th>Biomass (g fresh weight)</th>
<th>Leaf number</th>
<th>Leaf area (cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shoot</td>
<td>Roots</td>
<td></td>
</tr>
<tr>
<td>(a) cv. Samsun</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>175.1 ± 7.9 a</td>
<td>61.7 ± 2.5 a</td>
<td>405 ± 15.5 a</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>67.5 ± 8.4 b</td>
<td>36.7 ± 2.5 b</td>
<td>181 ± 11.3 b</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>156.8 ± 16.2 a</td>
<td>42.1 ± 4.7 b</td>
<td>407 ± 36.5 a</td>
</tr>
<tr>
<td>(b) cv. Gatersleben</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>76.9 ± 3.3 a</td>
<td>25.5 ± 0.8 a</td>
<td>264 ± 4.7 a</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>23.4 ± 4.3 b</td>
<td>14.8 ± 1.3 b</td>
<td>100 ± 4.8 b</td>
</tr>
</tbody>
</table>

*: Not determined.

Table 2. Effect of N form on leaf area (cm²), epidermal cell size (mm²) and cell number per leaf of the youngest fully expanded leaf of tobacco (cv. Samsun) cultivated in nutrient solution at 2 mM N supplied with NO₃⁻ or NH₄⁺ for 12 d before harvest (40 DAS)

Data are mean values ± se of four replicate plants. Differences between N treatments were statistically tested by ANOVA and means in the same column with different letters are significantly different (P<0.05, Student–Newman–Keuls test).

<table>
<thead>
<tr>
<th>N supply</th>
<th>Leaf area (cm²)</th>
<th>Cell number per leaf (× 10⁶)</th>
<th>Cell size (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO₃⁻</td>
<td>399 ± 29 a</td>
<td>70.6 ± 5.8 a</td>
<td>0.57 ± 0.01 a</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>145 ± 15 b</td>
<td>36.2 ± 4.8 b</td>
<td>0.40 ± 0.01 b</td>
</tr>
</tbody>
</table>

Growth inhibition exhibited a rapid response to NH₄⁺ application. Transferring the plants to a nutrient solution with NH₄⁺ as the sole N source, after a preculture with NO₃⁻–N, resulted in a significant reduction of leaf growth within 24 h. Similarly, stimulation of leaf growth was detectable 24 h after reapplication of NO₃⁻–N to plants formerly grown with a sole NH₄⁺ supply (Table 3).

No inhibition of leaf growth was detectable when NH₄⁺ and NO₃⁻ were applied simultaneously at concentration levels of 2.5 mM (Table 1; Fig. 2). ¹⁵N-uptake studies revealed similar net uptake rates of total N for plants supplied with NO₃⁻ or NH₄⁺NO₃ (Fig. 4). Mixed N supply resulted in a 6:4 net uptake ratio for NO₃⁻ and NH₄⁺ (Fig. 4). Obviously, there was no complete discrimination of NH₄⁺ net uptake by the presence of NO₃⁻ at the supplied 1:1 ratio.

N form effects on plant concentrations of carbohydrates and mineral nutrients

To assess putative effects of NH₄⁺ toxicity at the physiological level, the responses of various physiological parameters known to be affected by NH₄⁺ toxicity were recorded in NH₄⁺-treated tobacco plants. Concentrations of N and soluble sugars, which are the main substrates for leaf growth and expansion, were not reduced by supply of NH₄⁺ instead of NO₃⁻ as the sole N source.

Fig. 2. Effect of N form on the shoot growth of tobacco (cv. Samsun) cultivated in nutrient solution at 2.5 mM N supplied with NH₄NO₃ or NO₃⁻ for 11 d before harvest (46 DAS).

Fig. 3. Effect of N form on epidermis cells (cellulose acetate imprints) of fully expanded leaves of tobacco (cv. Samsun) cultivated in buffered nutrient solution at 2.5 mM N supplied with NO₃⁻ (A) or NH₄⁺ (B) for 11 d before harvest (42 DAS). The scale-bar is 100 μm.
Leaves were counted starting with the oldest (first). Data are mean values ± se with four (cv. Samsun) or five (cv. Gatersleben) replicate plants. Differences between N treatment within each group (genotype and leaf number) were tested by ANOVA and means in the same column within each group (genotype and leaf number) with different letters are significantly different (P<0.05, Student–Newman–Keuls test).

<table>
<thead>
<tr>
<th>N form during preculture</th>
<th>N form after transfer</th>
<th>Leaf expansion rate (cm² h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) cv. Samsun</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11th leaf</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>NH₄⁺</td>
<td>2.2 ± 0.18 a</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>NO₃⁻</td>
<td>1.6 ± 0.18 b</td>
</tr>
<tr>
<td>10th leaf</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>NO₃⁻</td>
<td>1.6 ± 0.11 a</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>NH₄⁺</td>
<td>1.2 ± 0.09 b</td>
</tr>
<tr>
<td>(b) cv. Gatersleben</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7th leaf</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>NO₃⁻</td>
<td>5.5 ± 0.47 a</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>NH₄⁺</td>
<td>3.5 ± 0.35 b</td>
</tr>
</tbody>
</table>

Fig. 4. Effect of N form on net uptake rates of NO₃⁻ (open columns) and NH₄⁺ (hatched columns) of tobacco (cv. Gatersleben). Nitrogen was supplied at 2 mM either as K¹⁵NO₃ or NH₄¹⁵NO₃ or NH₄¹⁵NO₃, and NH₄¹⁵NO₃. Columns represent mean uptake rates of plants 3 and 5 d after transfer from preculture with NH₄NO₃ (28 and 30 DAS) to solutions containing either NO₃⁻ or NH₄NO₃. Vertical bars represent the standard error of the mean (n = 10).

Discussion

Effects of NH₄⁺ supply on leaf expansion

Ammonium induced inhibition of shoot growth has been reported for various plant species such as Phaseolus vulgaris L., Beta vulgaris L., Lycopersicon esculentum Mill., and Zea mays L. (Raab and Terry, 1994; Chaillou et al., 1986; Cramer and Lewis, 1993; Magalhães and Huber, 1989). However, there are also contradictory reports for some species (Oryza sativa L., Calluna vulgaris...
L., *Potentilla erecta* L.), which were found to be tolerant to sole *NH₄⁺* nutrition (Troelstra et al., 1995; Magalhães and Huber, 1989).

In this study with tobacco, inhibition of shoot growth in response to *NH₄⁺* application could be attributed mainly to a reduction in leaf area and not to a decreased number of leaves (Table 1). Similar results have been reported for *Beta vulgaris* L. (Raab and Terry, 1994). Reduced leaf area may be due to reduced cell number (MacAdam et al., 1989) and/or smaller cell size (Snir and Neumann, 1997; Palmer et al., 1996; Taylor et al., 1993). Leaf growth of *NH₄⁺*-treated tobacco was found to be limited by both reduced cell number and cell expansion (Table 2). Most remarkably, growth inhibition was already detectable 24 h after the start of the *NH₄⁺* treatment, and was quickly reversible within 24 h of reapplication of *NO₃⁻*.

**Growth depression due to *NH₄⁺* toxicity?**

Growth reduction in response to application of *NH₄⁺* as the sole N source has been frequently related to *NH₄⁺* toxicity associated with acidification, uncoupling of photophosphorylation, lack of carbohydrates or mineral nutrients and impairment of the water status. Excess uptake of cations as a consequence of *NH₄⁺* nutrition is balanced by an increased net efflux of protons. The resulting acidification of the root environment can lead to inhibition of root growth and even to a destruction of the root tissue (Claussen and Lenz, 1995; Goyal et al., 1982; Maynard and Barker, 1969). To prevent over-acidification of the growth medium in our experiments, the nutrient solution was continuously buffered around pH 7.0 by addition of CaCO₃. At neutral or alkaline pH, however, the *NH₃/NH₄⁺* ratio in the nutrient solution increases. In contrast to *NH₄⁺* ions, plasmalemma permeation of ammonia (NH₃) is mediated by diffusion, which cannot be controlled by the plant. As a consequence, accumulation of toxic intracellular NH₃ levels has been reported in response to *NH₄⁺* nutrition (Tillberg et al., 1977; Avron, 1960), which was related to disturbances in the regulation of the intracellular pH, uncoupling of photophosphorylation and a reduction of photosynthesis (Raab and Terry, 1994). However, recent studies have

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**Table 4. Effect of N form on the concentrations of N, sugars (glucose, fructose, sucrose) and starch in shoot and roots of tobacco (cv. Samsun).** Data are means (± se, n=8) of plants cultivated at 0.5 mM or 5 mM N supplied as *NO₃⁻* or *NH₄⁺* for 12 d before harvest (42 DAS)

<table>
<thead>
<tr>
<th>N supply</th>
<th>Shoot</th>
<th>Roots</th>
<th>Shoot</th>
<th>Roots</th>
<th>Shoot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mol N g⁻¹ fresh weight</td>
<td>g⁻¹ fresh weight</td>
<td>g⁻¹ fresh weight</td>
<td>g⁻¹ fresh weight</td>
<td>g⁻¹ fresh weight</td>
</tr>
<tr>
<td><em>NO₃⁻</em></td>
<td>288 ± 6.2 a</td>
<td>214 ± 0.8 a</td>
<td>21.2 ± 1.9 a</td>
<td>13.0 ± 1.1 a</td>
<td>2.9 ± 0.4 a</td>
</tr>
<tr>
<td><em>NH₄⁺</em></td>
<td>310 ± 12.3 a</td>
<td>198 ± 9.0 a</td>
<td>39.0 ± 3.5 b</td>
<td>20.2 ± 2.5 a</td>
<td>9.4 ± 1.4 b</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N supply</th>
<th>Shoot</th>
<th>Roots</th>
<th>Shoot</th>
<th>Roots</th>
<th>Shoot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(MPa)</td>
<td>K⁺</td>
<td>Mg²⁺</td>
<td>Ca²⁺</td>
<td>NO₃⁻</td>
</tr>
<tr>
<td>Old leaves</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>NH₄⁺</em></td>
<td>−1.2 ± 0.05 a</td>
<td>136 ± 8.3 a</td>
<td>22 ± 1.4 a</td>
<td>24 ± 2.6 a</td>
<td>n.d.</td>
</tr>
<tr>
<td><em>NO₃⁻</em></td>
<td>−1.4 ± 0.07 b</td>
<td>218 ± 11 b</td>
<td>31 ± 1.7 b</td>
<td>51 ± 6.7 b</td>
<td>35 ± 4.5 a</td>
</tr>
<tr>
<td><em>NH₄NO₃</em></td>
<td>−1.4 ± 0.03 b</td>
<td>222 ± 8.5b</td>
<td>36 ± 1.1 c</td>
<td>49 ± 3.9 b</td>
<td>38 ± 6.7 a</td>
</tr>
<tr>
<td>Expanding leaves</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>NH₄⁺</em></td>
<td>−1.2 ± 0.04 a</td>
<td>167 ± 11 a</td>
<td>17 ± 1.1 a</td>
<td>11 ± 1.0 a</td>
<td>n.d.</td>
</tr>
<tr>
<td><em>NO₃⁻</em></td>
<td>−1.2 ± 0.08 a</td>
<td>203 ± 8.1 b</td>
<td>19 ± 1.5 a</td>
<td>17 ± 3.1 a</td>
<td>31 ± 6.4 a</td>
</tr>
<tr>
<td><em>NH₄NO₃</em></td>
<td>−1.2 ± 0.04 a</td>
<td>185 ± 1.9 ab</td>
<td>18 ± 1.1 a</td>
<td>16 ± 1.3 a</td>
<td>34 ± 5.0 a</td>
</tr>
<tr>
<td>Young leaves</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>NH₄⁺</em></td>
<td>−1.1 ± 0.03 a</td>
<td>144 ± 9.0 a</td>
<td>15 ± 0.6 a</td>
<td>8 ± 0.5 a</td>
<td>n.d.</td>
</tr>
<tr>
<td><em>NO₃⁻</em></td>
<td>−1.1 ± 0.07 a</td>
<td>173 ± 7.7 a</td>
<td>13 ± 0.9 b</td>
<td>9 ± 0.8 a</td>
<td>30 ± 3.4 a</td>
</tr>
<tr>
<td><em>NH₄NO₃</em></td>
<td>−1.1 ± 0.05 a</td>
<td>172 ± 6.3 a</td>
<td>13 ± 0.5 b</td>
<td>7 ± 0.7 a</td>
<td>23 ± 1.2 a</td>
</tr>
</tbody>
</table>

n.d.: Not detected.

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**Table 5. Effect of N form on the osmotic potential and ion concentrations in the press sap of old (fully expanded), expanding (about 40% of full size) and young leaves (about 15% of full size) of tobacco (cv. Samsun) cultivated in nutrient solution at 2.5 mM N supplied as *NO₃⁻* or *NH₄⁺* or both for 14 d before harvest (53 DAS)**

Data are mean values ± se (n=8). Differences between the N forms within each group (leaf stage) were tested statistically by ANOVA and means in the same column with different letters are significantly different (P<0.05, Student–Newman–Keuls test).

<table>
<thead>
<tr>
<th>N supply</th>
<th>Osmotic potential (MPa)</th>
<th>Ion concentration (mM)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>K⁺</td>
<td>Mg²⁺</td>
<td>Ca²⁺</td>
<td>NO₃⁻</td>
</tr>
<tr>
<td>Old leaves</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>NH₄⁺</em></td>
<td>−1.2 ± 0.05 a</td>
<td>136 ± 8.3 a</td>
<td>22 ± 1.4 a</td>
<td>24 ± 2.6 a</td>
<td>n.d.</td>
</tr>
<tr>
<td><em>NO₃⁻</em></td>
<td>−1.4 ± 0.07 b</td>
<td>218 ± 11 b</td>
<td>31 ± 1.7 b</td>
<td>51 ± 6.7 b</td>
<td>35 ± 4.5 a</td>
</tr>
<tr>
<td><em>NH₄NO₃</em></td>
<td>−1.4 ± 0.03 b</td>
<td>222 ± 8.5b</td>
<td>36 ± 1.1 c</td>
<td>49 ± 3.9 b</td>
<td>38 ± 6.7 a</td>
</tr>
<tr>
<td>Expanding leaves</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>NH₄⁺</em></td>
<td>−1.2 ± 0.04 a</td>
<td>167 ± 11 a</td>
<td>17 ± 1.1 a</td>
<td>11 ± 1.0 a</td>
<td>n.d.</td>
</tr>
<tr>
<td><em>NO₃⁻</em></td>
<td>−1.2 ± 0.08 a</td>
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<td>−1.2 ± 0.04 a</td>
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<td>34 ± 5.0 a</td>
</tr>
<tr>
<td>Young leaves</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>NH₄⁺</em></td>
<td>−1.1 ± 0.03 a</td>
<td>144 ± 9.0 a</td>
<td>15 ± 0.6 a</td>
<td>8 ± 0.5 a</td>
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<td><em>NO₃⁻</em></td>
<td>−1.1 ± 0.07 a</td>
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</tr>
</tbody>
</table>

n.d.: Not detected.
shown that NH$_4^+$ supply at moderate concentrations (3 mM) had no negative effects on intracellular pH regulation (Bligny et al., 1997). Increased tissue concentrations of starch and soluble sugars reported in the present study (Table 4) also suggest that photosynthesis was not directly affected by NH$_4^+$ application. However, in the long-term, photosynthesis might be decreased by a feedback repression in response to increased sugar accumulation, which has been similarly reported for N deficiency (Paul and Driscoll, 1997).

It has been frequently stated that root growth and, therefore, whole plant growth of NH$_4^+$-fed plants is restricted by low availability of carbohydrates due to excessive consumption of soluble sugars for NH$_4^+$ assimilation (detoxification) in the root tissue (Cramer and Lewis, 1993; Kafkafi, 1990; Breteler, 1973). However, in the present work with tobacco (similar to the reports of Kandlbinder et al., 1997; Chaillou et al., 1986), NH$_4^+$ application increased the accumulation of soluble sugars both in shoot and root tissue. Furthermore, there was no effect on total N concentration in shoot and roots (Table 4). Therefore the present results suggest that growth of NH$_4^+$-fed tobacco was not limited by C and N availability. In the long-term, expanding leaves of NH$_4^+$-fed plants showed a 15% decrease in K$^+$ concentrations, but no decrease in Mg$^{2+}$ and Ca$^{2+}$ concentrations (Table 5). A decrease in cation concentrations is in accordance with results by other authors (Jungk, 1977; Wilcox et al., 1973; Barker et al., 1967). However, considering the rapid inhibition of leaf expansion within 24 h after starting the NH$_4^+$ treatment (Table 3), it is unlikely that growth was primarily restricted by NH$_4^+$-induced deficiency of mineral nutrients in the present experiment.

There are various reports postulating that growth reduction of NH$_4^+$-fed plants might be caused by a lack of NO$_3^-$ as an important osmotic anion for leaf cell expansion (Raab and Terry, 1994; Salsac et al., 1987; Chaillou et al., 1986). In the present study, however, the osmotic potential of leaf press sap was not changed in response to NH$_4^+$ application (Table 5). The absence of NO$_3^-$ was obviously compensated for by increased accumulation of chloride, which was also a quantitatively important osmotic compound even in NO$_3^-$-fed tobacco plants (Table 5). Similar results have been reported for barley supplied with NH$_4^+$ as N source (Soltani et al., 1989). The analysis of bulk leaf press sap reflects overall N form-dependent differences in the osmotic potential of the leaf tissue. However, the technique cannot account for spatial or subcellular variations of ion concentrations in different tissues and cell compartments (Fricke et al., 1994; Miller and Smith, 1996). Therefore, growth depression due to the lack of osmotica cannot be entirely excluded. Several authors suggested that reduced...
The most striking effects of sole NH$_4^+$ supply to tobacco plants was a rapid decrease in the zeatin + zeatin riboside (Z+ZR) cytokinin fraction in xylem exudates (Table 7). It is well known that xylem exudate data do not reflect translocation in intact transpiring plants (Else et al., 1995). However, as water uptake rates in intact plants were not affected by the form of N supply (Fig. 6), it can be assumed that the decrease in xylem sap Z+ZR concentration reduced Z+ZR supply from the roots to the shoot. In accordance with this assumption shoot tissue Z+ZR concentration in NH$_4^+$-fed plants dropped to approximately 30% compared with NO$_3^-$ containing nutrient solution (data not shown). Both phenomena were associated with the rapid inhibition of leaf growth. In xylem sap, a 70% decrease of Z+ZR was already detectable 24 h after starting the NH$_4^+$ treatment, and decreased to almost zero within the next 4 d. These results are in contrast to other authors (Bubán et al., 1978) who showed that cytokinins in the xylem exudate of apple rootstocks were more enhanced by NH$_4^+$ than by NO$_3^-$ supply. There are, however, obvious similarities to the rapid decline in cytokinin levels in xylem sap and leaf tissue, associated with inhibition of leaf expansion in response to N deprivation (Wagner and Michael, 1971; Sattelmacher and Marschner, 1978; Palmer et al., 1996). Nitrogen has been suggested to be an effector of cytokinin production (Samuelson et al., 1992; Parkash, 1982; Sattelmacher and Marschner, 1978), for which root meristems are an important source (Latham and Palni, 1983). Cytokinin supply to the shoot is mainly mediated via xylem transport, although root-independent cytokinin production in above-ground vegetative tissue has been demonstrated at least for transgenic plants (Fiais et al., 1997). Constant, or even slightly increased levels of the putative Z+ZR precursors isopentenyl-adenine + isopentenyl-adenosine (i-Ade + i-Ado) (Chen, 1997), in the xylem sap of NH$_4^+$-treated tobacco plants suggest that the reduction in Z+ZR levels may be either attributed to an inhibition of i-Ade + i-Ado conversion to Z+ZR or to selective inhibition of Z+ZR loading into the xylem.

Several authors have discussed the importance of cytokinins in regulating biomass partitioning between shoot and root (Fetene and Beck, 1993; Kuiper et al., 1988). Fetene and Beck found that exogenous cytokinin supply to the roots stimulated shoot growth by increasing carbon partitioning towards the shoot (Fetene and Beck, 1993). There is also evidence that exogenously supplied cytokinins promote leaf expansion independent of uptake and utilization of exogenously supplied carbohydrates.
(Nielsen and Ulvskov, 1992). Cytokinin can promote both cell division (Taiz and Zeiger, 1998) and cell expansion (Rayle et al., 1982). Passage of cells through phases of the mitotic cycle is controlled by a family of auxin-induced serine/threonine protein kinases and their regulatory subunits, the cyclins (Kende and Zeevaart, 1997). There is evidence that cytokinin increases the abundance of cyclin mRNA (Soni et al., 1995). Recently, it was reported that the product of a cytokinin-inducible soybean mRNA (Ciml1) is located to the cell wall with homology to a subfamily of expansin proteins (Downes and Crowell, 1998). Expansins are probably involved in cell expansion as catalysts, mediating loosening of cell walls (Cosgrove, 1998). There is also evidence for both synergistic and antagonistic interactions of cytokinins with auxins, which are involved in the regulation of cell expansion (Coenen and Lomax, 1997). Therefore, it seems likely that growth inhibition of tobacco leaves in response to NH$_4^+$ nutrition (Table 3), which was associated with a reduction in both cell number and cell size, is mediated by decreased root-to-shoot translocation of cytokinins (Z+ZR).

The involvement of plant growth regulators, such as cytokinins, in leaf morphogenesis is further supported by the rapid induction of the N-form responses. The striking similarities to the effects of N limitation suggest that growth inhibition by NH$_4^+$ nutrition is related rather to the absence of NO$_3^-$ than to the presence of NH$_4^+$. Thus, apart from its function as N source, NO$_3^-$ may be involved in a signal transduction chain that regulates leaf morphogenesis by supplying the shoot with cytokinins. Both root and leaf NO$_3^-$ levels rapidly decreased to almost zero within 24 h in response to NH$_4^+$ application. However, it is still an open question as to whether the primary response to the absence of NO$_3^-$ is located in root or leaf tissue. Moreover, it is also possible that other hormonal factors such as abscisic acid, which has been reported to decrease xylem sap, leaf and root tissue of NH$_4^+$-treated R. communis plants (Peuke et al., 1994), mutually interact with cytokinins in the regulation of leaf morphogenesis.

Acknowledgements

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