Effect of NO\textsubscript{3}\textsuperscript{-} transport and reduction on intracellular pH: an \textit{in vivo} NMR study in maize roots

Luca Espen, Fabio F. Nocito and Maurizio Cocucci*

Dipartimento di Produzione Vegetale, University of Milan, Via Celoria 2, I-20133 Milano, Italy

Received 8 January 2004; Accepted 18 June 2004

Abstract

The effect of NO\textsubscript{3}\textsuperscript{-} uptake on cellular pH was studied in maize roots by an \textit{in vivo}\textsuperscript{31}P-NMR technique. In order to separate the effects on cytoplasmic pH due to NO\textsubscript{3}\textsuperscript{-} uptake from those due to NO\textsubscript{3}\textsuperscript{-} reduction, tungstate was used to inhibit nitrate reductase (NR). The results confirm that in maize roots tungstate inhibited NR activity. \textsuperscript{15}N-NMR \textit{in vivo} experiments demonstrated the cessation of nitrogen flux from nitrate to organic compounds. Tungstate affected neither NO\textsubscript{3}\textsuperscript{-} uptake nor the levels of the main phosphorylated compounds. Slight changes in cytoplasmic pH were observed during NO\textsubscript{3}\textsuperscript{-} uptake and reduction (i.e. control). By contrast, in the presence of tungstate, a consistent decrease in cytoplasmic pH occurred. The vacuolar pH did not change in any of the conditions tested. These data show that NO\textsubscript{3}\textsuperscript{-} uptake is an acidifying process and suggest a possible involvement of NO\textsubscript{3}\textsuperscript{-} reduction in pH homeostasis. In the presence of NO\textsubscript{3}\textsuperscript{-}, a transient depolarization of transmembrane electric potential difference (\(E_m\)) was observed in all the conditions analysed. However, in tungstate-treated roots, a lesser depolarization accompanied by a greater ability to recover \(E_m\) was found. This was related to a higher activity of the plasma membrane (PM) H\textsuperscript{+}-ATPase. When NO\textsubscript{3}\textsuperscript{-} was administered as potassium salt, its uptake increased and a greater depolarization of \(E_m\) took place, whilst the changes in cytoplasmic pH were remarkably reduced, according to the central role played by K\textsuperscript{+} in the control of plasma membrane activities and cell pH homeostasis. A possible involvement of cytoplasmic pH in the control of PM H\textsuperscript{+}-ATPase expression during nitrate exposure is suggested.

Key words: Cytoplasmic pH, maize, nitrate reductase, nitrate uptake, NMR spectroscopy, tungstate.

Introduction

Absorption of nitrate (NO\textsubscript{3}\textsuperscript{-}) by roots occurs by means of at least three types of NO\textsubscript{3}\textsuperscript{-} transport systems operating at the plasma membrane, which consist of an inducible high-affinity (IHATS), a constitutive high-affinity (CHATS), and a low-affinity (LATS) transport system (Glass and Siddiqi, 1995; Crawford and Glass, 1998; Forde and Clarkson, 1999; Forde, 2000; Glass \textit{et al.}, 2001). Current evidence strongly suggests that all these systems use the H\textsuperscript{+} electrochemical gradient to drive the uptake of NO\textsubscript{3}\textsuperscript{-} into the cells by means of a cotransport mechanism. According to this model, nitrate uptake increases as external pH decreases, with transient depolarization of the plasma membrane (McClure \textit{et al.}, 1990; Ullrich and Novacky, 1990). In this context, one should therefore expect that such a transport mechanism would lead to acidification of the cytoplasm, at least in the earlier period after the addition of nitrate into the medium. Nevertheless, experimental evidence is controversial. It has been observed that, in roots of maize seedlings growing in nutrient solutions at different pH and supplemented with nitrate, only small changes in cytoplasmic pH occurred (Gerendás \textit{et al.}, 1990). These results are attributed by the authors to the presence of a tight regulatory mechanism of intracellular pH. Moreover, in \textit{Limnobia stolonifera} root hairs, nitrate uptake leads to an increase in cytoplasmic pH (Ulrich and Novacky, 1990). The authors speculate that this could be ascribed to nitrate assimilation which is a proton-consuming process (Raven, 1985, 1986; Ulrich and Novacky, 1990).

The pH changes occurring in root cells during nitrate exposure could be directly related to the activities of both NO\textsubscript{3}\textsuperscript{-} transport and reduction and N assimilation, as well as to the effectiveness of the intracellular pH regulatory mechanisms (Kurkdjian and Guern, 1989; Li and Oaks, 1993; Sivansankar \textit{et al.}, 1996; Crawford and Glass, 1998). It is interesting to observe that in the \textit{chl1} mutant of
Arabidopsis thaliana, impaired in nitrate transport but not defective in nitrate reduction, the cytoplasmic pH changed with respect to the wild type (Romani et al., 1996). Moreover, the balance between NO\textsubscript{3}\textsuperscript{-} uptake and reduction and N assimilation radically alters under different growing conditions. In fact, NO\textsubscript{3}\textsuperscript{-} and NH\textsubscript{3} availability, as well as the levels of some metabolites, such as amino acids, directly influence both NO\textsubscript{3}\textsuperscript{-} transport systems and NO\textsubscript{3}\textsuperscript{-} reduction (Forde and Clarkson, 1999; Stitt, 1999; Vidmar et al., 2000; Wang et al., 2000).

In order to gain more information on the separate effects of NO\textsubscript{3}\textsuperscript{-} transport and NO\textsubscript{3}\textsuperscript{-} reduction and assimilation on cytoplasmic pH, it would be useful to carry out a study using plants in which only one of these processes is operating. In this view, tungstate, a nitrate reductase (NR) inhibitor, could be used to study in vivo the effect of NO\textsubscript{3}\textsuperscript{-} transport on cytoplasmic pH (Heimer et al., 1969; Deng et al., 1989). Under these conditions, the cytoplasmic proton balance should only be related to the NO\textsubscript{3}\textsuperscript{-} uptake mechanisms. To clarify these aspects, it would also be important to evaluate the influence of K\textsuperscript{+}, a cation that plays a central role in the regulation of plasma membrane activities (i.e. transmembrane electric potential difference and H\textsuperscript{+}-ATPase) and in the homeostasis of cell pH (Kurkdjian and Guern, 1989; Ullrich and Novacky, 1990; Briskin and Hanson, 1992; Sacchi and Cocucci, 1992; Guern et al., 1991; Nocito et al., 2002).

With these aims, a study has been carried out in roots of maize seedlings using the nuclear magnetic resonance (NMR) technique to study in vivo cell pH changes. Moreover, to obtain more information about the mechanism of NO\textsubscript{3}\textsuperscript{-} uptake, the changes in the transmembrane electric potential difference (E\textsubscript{m}) and the activity and expression of the plasma membrane H\textsuperscript{+}-ATPase were also studied. To study the influences of K\textsuperscript{+}, some experiments were carried out in the presence or in the absence of this cation.

## Materials and methods

### Plant material and growing conditions

Maize (Zea mays L. cv. Cecilia, Pioneer) seeds were germinated in the dark for 72 h at 26 °C on paper saturated with distilled water and then transferred to a hydroponic system with the following solution: (i) 4 mM CaSO\textsubscript{4} for 48 h; (ii) 0.2 mM K(NO\textsubscript{3})\textsubscript{2}, 0.025 mM NH\textsubscript{4}H\textsubscript{2}PO\textsubscript{4}, 0.1 mM K\textsubscript{2}SO\textsubscript{4}, 0.1 mM MgSO\textsubscript{4}, 0.4 mM CaSO\textsubscript{4}, 0.175 mM KH\textsubscript{2}PO\textsubscript{4}, 20 mM Fe-EDTA, 2.5 mM H\textsubscript{2}BO\textsubscript{3}, 0.2 mM MnSO\textsubscript{4}, 0.2 µM CuSO\textsubscript{4}, 0.2 µM ZnSO\textsubscript{4}, and 0.05 µM H\textsubscript{3}MoO\textsubscript{4} (complete nutrient solution) for 72 h; (iii) complete nutrient solution without K(NO\textsubscript{3})\textsubscript{2} and NH\textsubscript{4}H\textsubscript{2}PO\textsubscript{4} and with 0.2 mM K\textsubscript{2}SO\textsubscript{4} for 48 h (−N solution). The plants were then incubated for a further 6 h in the complete solution modified for KNO\textsubscript{3} (0.1 mM instead of 0.2 mM) or in an identical solution deprived of H\textsubscript{2}MoO\textsubscript{4} and containing 50 or 100 µM Na\textsubscript{2}WO\textsubscript{4}. The pH of all solutions used was adjusted to the value of 6.1 with NaOH. Hydroponic cultures were maintained in a growth chamber with a day/night regime of 16/8 h and a photosynthetic photon flux density (PPFD) of 200 µmol m\textsuperscript{-2} s\textsuperscript{-1} at the plant level. The temperature was 22 °C in the dark and 26 °C in the light.

### Nitrate reductase activity

NR was extracted from apical root segments (4 cm) at 4 °C with 50 mM MOPS-KOH buffer (pH 7.8), 5 mM EDTA, 2 mM MSH, 1 mM PPSM, 10 µM leupeptin, and 10 µM chymostatin (dissolved in DMSO). The homogenate was centrifuged at 12,000 g for 15 min at 4 °C. NR activity was measured as previously described by Ferrario-Méry et al. (1998) using a reaction mixture containing 50 mM MOPS-KOH buffer (pH 7.8), 1 mM NaF, 10 mM KNO\textsubscript{3}, 0.17 mM NADH, and 5 mM EDTA. After 10 or 20 min of incubation at 26 °C, the reaction was stopped by the addition of an equal volume of sulfanilamide (1%, w/v in 3 N HCl). Afterwards, N-naphthylethylenediamine dihydrochloride (0.02%, w/v) was added and after 30 min the concentration of NO\textsubscript{2} was measured spectrophotometrically at 540 nm. Proteins were determined by the Bradford (1976) procedure using γ-globulin as a standard.

### Nitrate uptake

Nitrate uptake was determined at 26 °C as depletion from the incubation medium. Two seedlings were placed in a vessel and washed twice for 10 min with 30 ml of an aerated solution (basal medium) containing 0.1 mM MES-Ca (pH 6.1) and 0.5 mM CaSO\textsubscript{4}. After this washing, the seedlings were incubated in the same basal medium containing 0.1 mM Ca(NO\textsubscript{3})\textsubscript{2} or 0.2 mM KNO\textsubscript{3} and aliquots of 2 ml were collected at 2 min intervals. Two ml of 20 mM MES-BTP buffer (pH 6.1) containing 25 mM K\textsubscript{2}SO\textsubscript{4} were added to these aliquots and the concentration of nitrate was measured by means of a NO\textsubscript{3} selective electrode (ISO25N03, Radiometer, Copenhagen), using a double bridge reference electrode (REF251, Radiometer, Copenhagen). The values expressed in mV were converted into concentrations using a calibration curve obtained with standard solutions of KNO\textsubscript{3} in 10 mM MES-BTP buffer (pH 6.1) and 12.5 mM K\textsubscript{2}SO\textsubscript{4}.

### NMR spectroscopy

\textsuperscript{15}N-NMR and \textsuperscript{31}P-NMR spectra were recorded on a standard broadband 10 mm probe on a Bruker AMX 600 spectrometer (Bruker Analytische Messtechnik GmbH, Rheinstetten-Forschheim, Germany) equipped with an Indy computer running XWIN-NMR version 2.6.

The \textsuperscript{15}N-Nitrate assimilation was monitored both in in vivo experiments and in vitro, with extracts obtained from apical root segments incubated in \textsuperscript{15}N-Nitrate.

For in vivo experiments, 5 mm root tips of maize seedlings were packed in a 10 mm diameter NMR tube equipped with a perfusion system connected to a peristaltic pump in which the aerated, thermo-regulated (26 °C) medium [12.5 mM Ca(\textsuperscript{15}NO\textsubscript{3})\textsubscript{2}, 0.5 mM CaSO\textsubscript{4}, 5 mM sucrose, 1 mM MES-BTP pH 6.1 with or without 100 µM Na\textsubscript{2}WO\textsubscript{4} flowed (10 ml min\textsuperscript{-1}).

The extracts were prepared as described by Amâncio et al. (1993) using apical root segments (4 cm) incubated for 10 h in the same conditions used for in vivo experiments.

H-decoupled \textsuperscript{15}N-NMR spectra were recorded at 60.8 MHz using a 90° pulse angle, a spectral window of 8 KHz, a 2 s recycle time with low power broadband decoupling to maintain the nuclear Overhauser effect and normal decoupling during the 0.25 s acquisition. Chemical shifts were measured relative to the signal from a glass capillary containing 400 mM \textsuperscript{15}Nurea which is at −299.3 ppm relative to the signal from \textsuperscript{15}N-nitrate which is at 0 ppm.

In vivo \textsuperscript{31}P-NMR experiments were carried out by packing about 20 apical root segments (4 cm) in the 10 mm diameter NMR tube using the same experimental conditions described above and a medium containing 0.5 mM CaSO\textsubscript{4}, 1 mM MES-BTP, pH 6.1 (minimal basal medium). Prior to starting the experiments, the root segments were washed in the minimal basal medium for 1 h. \textsuperscript{31}P-NMR spectra were recorded at 242.9 MHz without lock, with a waltz-based broadband proton decoupling and a spectral window of 16 kHz. Chemical shifts
were measured relative to the signal from a glass capillary containing 33 mM MDP, which is at 18.5 ppm. The spectra used for the quantitative in vivo analysis of metabolites were determined using a 90° pulse angle and a 6 s recycle time to give fully relaxed resonance (except for vascular phosphate). Resonance assignments were performed according to Roberts et al. (1980) and Kime et al. (1982). Metabolite concentrations in the tissue were determined according to Espen et al. (2000) by comparing the resonance intensities with that of a glass capillary containing 33 mM MDP previously calibrated against standard solutions. The area of the $^{31}$P-peaks was measured by Lorentz line-shape analysis and the values obtained were referred to the percentage volume of the tissue in the NMR tube (Spickett et al., 1992; Espen et al., 2000). The effect of the addition of 0.2 mM KNO$_3$ or 0.1 mM Ca(NO$_3$)$_2$ on cytoplasmic and vacuolar pH was evaluated using fast acquisition conditions with a recycle time of 1 s (Kime et al., 1982). Cytoplasmic and vacuolar pH values were estimated from the chemical shift of P$^i$ resonance after construction of a standard titration curve (Roberts et al., 1982).

**Measurement of the transmembrane electric potential differences**

The transmembrane electric potential differences ($E_m$) were measured using a WPI K5-700 high-impedance electrometer amplifier (World Precision Instrument, New Haven, CT, USA) and microelectrodes, pulled from single-barrelled borosilicate glass tubing (World Precision Instrument, New Haven, CT, USA), and filled with 3 M KCl (adjusted to pH 2 to reduce tip potential). Electrode resistances ranged between 10 and 15 MΩ.

Briefly, one maize seedling was placed in a 300 ml Plexiglass vessel connected to a horizontal chamber, where two or three roots were fixed on its base and impaled for $E_m$ recording. The whole system was perfused at 300 ml h$^{-1}$ for 1 h with the aerated and thermoregulated (26°C) basal medium. The experiments were then started by adding 0.1 mM Ca(NO$_3$)$_2$ or 0.2 mM KNO$_3$ to the basal medium. The electrodes were inserted, perpendicular to the root axis, into the cortical cells of the subapical (2–6 mm from the tip) root zone and the $E_m$ values of the third to the fifth layers of the cortical cells were recorded.

**Influx of potassium**

K$^+$ influx was measured using $^{86}$Rb$^+$ as a radiotracer. Two seedlings were placed in a vessel and washed twice for 30 min with 400 ml of aerated basal medium. Seedlings were then incubated for 10 min in 400 ml of the same basal medium to which 0.2 mM ($^{86}$Rb)KNO$_3$ (14.0 kBq μmol$^{-1}$) had been added. At the end of the radioactive pulse, the roots were washed twice at 4°C for 15 min with the corresponding non-radioactive medium. Apical root segments (4 cm) were excised and heated for 20 min at 80°C in 0.1 N HNO$_3$ (10 ml g$^{-1}$FW). Radioactivity was detected in aliquots of the supernatant by liquid scintillation counting in a Beckman LS 6000SC (Beckman Coulter Inc., Fullerton, CA, USA).

**Plasma membrane vesicle isolation and PM H$^+$-ATPase assay**

Plasma membrane vesicles were prepared, essentially as described by Palmgren et al. (1990), from about 20 g of maize roots. The root microsomal fractions were processed, at 4°C, by the three-step batch procedure described by Larson et al. (1987) using a phase system consisting of 6.5% (w/v) dextran T500, 6.5% (w/v) PEG 3350, 330 mM sorbitol, 5 mM K-phosphate (pH 7.8) buffer, 5 mM KCl, 1 mM DTT, and 0.1 mM EDTA.

Plasma membrane H$^+$-ATPase activity was measured by following the oxidation of NADH coupled to the hydrolysis of ATP as described by Palmgren et al. (1990). Oxidation of NADH was followed at 340 nm with a Jasco V-550 spectrophotometer.

Protein was estimated by the Bio-Rad microassay procedure, with γ-globulin as a standard (Bradford, 1976).

**Western blot analysis**

The plasma membrane vesicles (corresponding to 10 μg of proteins) were diluted with an equal volume of 2× SDS-PAGE buffer [50 mM TRIS-HCl (pH 6.8), 4% (w/v) SDS, 12% (w/v) glycerol, 2% (v/v) β-mercaptoethanol, and 0.01% (w/v) bromophenol blue] and heated for 5 min at 90°C. Denatured proteins were then separated by tricine-SDS-PAGE (Schägger and von Jagow, 1987) in a Protein II TM-cell (Bio-Rad Laboratories, Richmond, CA, USA) and electrophoretically transferred to a polyvinylidene difluoride (PVDF) filter using a semi-dry blotting system (Novablot, Pharmacia, Sweden) with a buffer containing 10 mM 3-cyclohexylamino-1-propanesulphonic acid (CAPS, pH 11 with NaOH) and 10% methanol. Filters were blocked for 1 h with TBS-T buffer [50 mM TRIS-HCl (pH 7.6), 200 mM NaCl, and 0.1% (v/v) Tween 20] supplemented with 3% (w/v) of non-fat dry milk. The TBS-T buffer was used as an incubation medium throughout the procedure. Filters were incubated overnight at 4°C with primary polyclonal antibodies against the PM H$^+$-ATPase, that were kindly given by Professor Serrano. After washing with TBS-T, the filters were incubated for a further 2 h at room temperature with a secondary antibody (alkaline-phosphatase-conjugated anti-rabbit immunoglobulin G). The blot was developed with nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (FAST BCIP/NBT, Sigma). The image of the blot was scanned and the quantification of the signal was performed by ImageQuant software (Amersham Bioscience).

**Results**

**Nitrate reductase activity, NO$_3$ assimilation and phosphorylated compounds**

Maize seedlings grown for 48 h in the absence of nitrogen were incubated for the following 6 h in a complete nutrient solution containing 0.1 mM NO$_3^-$ in the absence or in the presence of 50 or 100 μM Na$_2$WO$_4$ (see Materials and methods), and the effects on nitrate reductase (NR) were evaluated.

Figure 1 shows that treatment with 50 μM Na$_2$WO$_4$ greatly inhibited the root NR activity; the same activity became undetectable upon treatment with 100 μM Na$_2$WO$_4$. This last concentration was therefore used for all further experiments. NR activity was also evaluated in the same experimental conditions used to study the cytoplasmic pH changes (see below). For at least the first hour after the readdition of 0.1 mM Ca(NO$_3$)$_2$ or 0.2 mM KNO$_3$, (administered after 6 h of nitrate exposure plus 1 h of equilibration in minimal basal medium) NR activity did not show any change in the control samples and was undetectable in the tungstate-treated ones (data not shown). The inhibiting effect of tungstate on NR was also studied by in vivo $^{15}$N-NMR experiments. To improve the detection of labelled resonances, the acquisition were carried out on root tips using a higher concentration of $^{15}$NO$_3^-$ (25 mM). Figure 2 shows $^{15}$N-NMR spectra of root tips after different times of incubation with 12.5 mM Ca($^{15}$NO$_3$)$_2$ in the
absence (control) or in the presence of 100 μM Na₂WO₄. In the control, the resonance corresponding to glutamine amide N, alanine, and glutamate amino N (peaks 1, 2, and 3, respectively) appeared in the spectrum between 180 min and 270 min, and increased with time. By contrast, no peaks relative to amino acids were detected when 100 μM Na₂WO₄ was present in the incubation medium. These results were confirmed by the ¹⁵N-NMR spectra of perchloric acid extracts of maize root segments (data not shown).

To study the effect of tungstate treatment on cell energy metabolism, phosphorylated compounds were analysed by in vivo ³¹P-NMR experiments. Figure 3 shows that during the incubation with tungstate, the levels of fructose-6P (Fru6P), phosphocholine (Pcho), cytoplasmic phosphate (Pcyt), UDP-glucose (UDP-Glc), and β-NTP did not change significantly. By contrast, the glucose-6P (Glc6P) content increased (+42% and +39% after 6 h and 9 h, respectively).

**Effect of tungstate on nitrate uptake**

Table 1 shows the effect of a 6 h treatment with 100 μM Na₂WO₄ on nitrate uptake by maize root seedlings. To evaluate the effect of accompanying cations (K⁺ and Ca²⁺) on nitrate absorption, nitrate was administered as either 0.1 mM Ca(NO₃)₂ or 0.2 mM KNO₃. Treatment with tungstate did not affect nitrate uptake in all the conditions tested. In the presence of 0.2 mM K⁺, increases in nitrate uptake rate of +33% and +37%, respectively, were observed in the control and in the seedlings previously treated with tungstate.

**Changes in intracellular pH**

Table 2 shows the effects of the addition of either 0.1 mM Ca(NO₃)₂ or 0.2 mM KNO₃ on the cytoplasmic pH of root segments excised from seedlings grown during the last 6 h in the absence (control) or in the presence of 100 μM Na₂WO₄ (tungstate) and then equilibrated for 1 h in minimal basal medium. Before the readdition of nitrate,
the presence of K+, when no NR activity was detected, the pH, not dependent on nitrate assimilation, was operating. In the control, addition of Ca(NO₃)₂ induced a very slight change in cytoplasmic pH in the control root segments, whilst in the following period cytoplasmic pH increase was probably due to the effect of nitrate uptake, short-term (10 min) influx experiments using ⁸⁶Rb⁺ as a tracer were performed. The results showed that in tungstate-treated roots the influx of K⁺ was higher (+25%) than in the control ones (Fig. 5B). In the tungstate-treated roots, the addition of KN0₃ induced an early depolarization of Eₘ values to a lesser extent (by about 60 mV) than that observed in the control, the Eₘ then recovered, reaching more negative values than the starting values (Fig. 5C). Moreover, the addition of KN0₃ induced a depolarization of Eₘ lower than that observed in the control; the Eₘ values recovered completely over the longer periods (Fig. 5D).

Under all the conditions tested, the values of vacuolar pH did not change significantly (data not shown).

### Changes in transmembrane electric potential differences values and potassium influx

Figure 5 shows the changes in the values of transmembrane electric potential differences (Eₘ) in the cortical cells of maize seedling roots after the addition to the incubation medium of 0.1 mM Ca(NO₃)₂ or 0.2 mM KN0₃. Under all conditions tested, a similar trend of Eₘ changes was observed, but of different magnitude. In the control, the presence of Ca(NO₃)₂ induced an early depolarization of Eₘ (by about 10 mV), which was then almost completely recovered (Fig. 5A). By contrast, KN0₃ induced an early dramatic increase in Eₘ values (by about 60 mV), which was only partially recovered (by about 10 mV) over longer times (Fig. 5B). In the tungstate-treated roots, the addition of Ca(NO₃)₂ induced a depolarization of Eₘ values to a lesser extent (by about 60 mV) than that observed in the control, the Eₘ then recovered, reaching more negative values than the starting values (Fig. 5C). Moreover, the addition of KN0₃ induced a depolarization of Eₘ lower than that observed in the control; the Eₘ values recovered completely over the longer periods (Fig. 5D).

### PM H⁺-ATPase activity and western blot analysis

Figure 6 shows the values of PM H⁺-ATPase activity and PM H⁺-ATPase expression (evaluated by western blot analysis) in control or tungstate-treated maize seedling roots. Treatment with 100 μM Na₂WO₄ during the last 6 h of seedling growth induced an increase in PM H⁺-ATPase activity (+49%). Western blot analysis showed that antibodies recognized a single band of about 100 kDa related to the PM H⁺-ATPase. The intensity of the band increased in plasma membrane vesicles from tungstate-treated roots; the

---

Table 1. Net nitrate uptake by roots of maize seedlings grown in the last 6 h in the presence of 0.05 μM H₂MoO₄ (control) or 100 μM Na₂WO₄ (tungstate)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Net Uptake (μmol h⁻¹ g⁻¹ root FW) +100 μM Ca(NO₃)₂</th>
<th>Net Uptake (μmol h⁻¹ g⁻¹ root FW) +200 μM KN0₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.71±0.20</td>
<td>6.27±0.30</td>
</tr>
<tr>
<td>Tungstate</td>
<td>4.55±0.23</td>
<td>6.22±0.34</td>
</tr>
</tbody>
</table>

Nitrate uptake was determined as depletion from the incubation medium after addition of 0.1 mM Ca(NO₃)₂ or 0.2 mM KN0₃, as described in the Materials and methods. Values are the means ±SE of four experiments run in triplicate.
increase was positively related to the relative increase in ATPase activity.

Discussion

Current evidence strongly indicates that the uptake of nitrate in plants is driven by electrogenic proton cotransport. The influx of this anion into the cells is accompanied in different plant materials by a transient depolarization and is equal to \pm 0.02 pH units. Values in brackets represent the changes in comparison with the values measured at the end of the equilibration period.

Table 2. Changes in the cytoplasmic pH of apical maize root segments after the addition of 100 \mu M Ca(NO_3)\_2 or 200 \mu M KNO_3

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control</th>
<th>Tungstate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\delta^d) (ppm)</td>
<td>pH</td>
</tr>
<tr>
<td>0</td>
<td>2.847</td>
<td>7.66 (-)</td>
</tr>
<tr>
<td></td>
<td>+ 100 \mu M Ca(NO_3)_2</td>
<td>+ 200 \mu M KNO_3</td>
</tr>
<tr>
<td>12</td>
<td>2.847</td>
<td>7.66 (-)</td>
</tr>
<tr>
<td>24</td>
<td>2.840</td>
<td>7.65 (-0.01)</td>
</tr>
<tr>
<td>36</td>
<td>2.829</td>
<td>7.63 (-0.03)</td>
</tr>
<tr>
<td>48</td>
<td>2.832</td>
<td>7.65 (-0.03)</td>
</tr>
<tr>
<td>60</td>
<td>2.842</td>
<td>7.65 (-0.01)</td>
</tr>
<tr>
<td>72</td>
<td>2.840</td>
<td>7.65 (-0.01)</td>
</tr>
<tr>
<td>84</td>
<td>2.840</td>
<td>7.65 (-0.01)</td>
</tr>
<tr>
<td>96</td>
<td>2.845</td>
<td>7.66 (-)</td>
</tr>
<tr>
<td>108</td>
<td>2.850</td>
<td>7.67 (+0.01)</td>
</tr>
<tr>
<td>120</td>
<td>2.857</td>
<td>7.68 (+0.02)</td>
</tr>
<tr>
<td>132</td>
<td>2.855</td>
<td>7.68 (+0.02)</td>
</tr>
<tr>
<td>144</td>
<td>2.857</td>
<td>7.68 (+0.02)</td>
</tr>
<tr>
<td>156</td>
<td>2.863</td>
<td>7.69 (+0.03)</td>
</tr>
<tr>
<td>168</td>
<td>2.859</td>
<td>7.68 (+0.02)</td>
</tr>
<tr>
<td>180</td>
<td>2.857</td>
<td>7.68 (+0.02)</td>
</tr>
</tbody>
</table>

\(a\) Chemical shift of cytoplasmic phosphate.

The data do not allow the clarification of the in vivo effect of nitrate transport on cytoplasmic pH. The difficulty in studying this aspect is due to the concomitant effects of nitrate uptake and reduction and nitrogen assimilation on the proton balance in the cytoplasm (Raven, 1985, 1986; Forde and Clarkson, 1999; Meharg and Blatt, 1995).

To clarify this point, the uptake of nitrate has been studied in maize seedling roots where nitrate reduction was inhibited by tungstate, which has been shown to be an appropriate tool to inhibit NR activity (Heimer et al., 1969; Deng et al., 1989). According to these data, NR activity in maize seedling roots was completely inhibited by 100 \mu M Na_2WO_4 (Fig. 1). In vivo \(^{15}\)N-NMR experiments gave further evidence that the tungstate treatment completely inhibited the influx of nitrogen from nitrate to nitrogen-containing molecules in the roots (Fig. 2). Nitrate reduction and nitrogen assimilation are metabolic processes with high energy costs, finely tuned, and strictly related to the other major metabolic pathways (Pate and Layzell, 1990). Therefore, the dramatic effect of tungstate on nitrogen assimilation activity could induce changes in the metabolism of the root cells and a toxic effect of tungstate on the energy metabolism could not be excluded. In order to study these aspects, an in vivo \(^{31}\)P-NMR analysis was conducted. The results show that the levels of the main phosphorylated compounds in maize root segments did not change, suggesting that the major biochemical pathways are not affected under the experimental conditions adopted (Fig. 3). Only an increase in glucose-6P content occurred after tungstate treatment. This result could be consistent with a reduction of the pentose-phosphate pathway, as a direct
The consequence of the lesser demand for NADPH, which is used in non-green tissues for ferredoxin reduction (Huppe and Turpin, 1996). Data from the literature also showed that both activities and transcript levels of glucose-6-P dehydrogenase and 6-phospho-gluconate dehydrogenase increase during nitrate exposure (Redinbaugh and Campbell, 1998; Wang et al., 2000). In agreement with these results, preliminary data showed that the increase in the activity of these enzymes, which occur in maize seedling roots after nitrate addition, did not occur in tungstate-treated roots (data not shown).

In order to study the nitrate uptake mechanism, as well as its effects on intracellular pH, it is important to set up experimental conditions which do not affect NO\textsubscript{3}\textsuperscript{-} transport activities. It has been shown that tungstate treatments affect nitrate influx only in plants grown at high NO\textsubscript{3} concentration (Ingemarsson et al., 1987; Vidmar et al., 2000). This could be linked to an earlier achievement of the N status (i.e. availability of N-containing compounds) that helps to induce a down-regulation of NO\textsubscript{3} uptake (Glass and Sidiqui, 1995; Crawford and Glass, 1998; Vidmar et al., 2000). According to these suggestions, maize seedlings were grown for 48 h in the absence of nitrogen, and then transferred for a further 6 h to a low nitrate concentration.
(0.1 mM) to induce nitrate transporters. Under these experimental conditions, nitrate uptake was not affected by tungstate treatment (Table 1). When the measurement was conducted with K⁺ as the accompanying cation for NO₃⁻, nitrate uptake, compared with that observed when the counterion for NO₃⁻ was Ca²⁺, was higher by about the same extent in both control and tungstate-treated roots.

In order to estimate if 6 h of exposure to 0.1 mM KNO₃ were sufficient to induce the nitrate transport system completely, a few measurements of the changes in $E_m$ induced by nitrate addition at different times were also conducted. The extent of $E_m$ changes might be dependent on nitrate uptake (Glass et al., 1992). Similar depolarizations induced by nitrate uptake after 0, 15, 60, 120, and 180 min of re-exposure to NO₃⁻ were observed (data not shown). These data indirectly confirm that nitrate transport did not change during the period of ³¹P-NMR experiments conducted to study the cytoplasmic pH changes (see below).

Slight changes in the cytoplasmic pH of the control roots were observed after addition of Ca(NO₃)₂, whilst in the tungstate-treated root segments a consistent decrease in cytoplasmic pH occurred (Table 2). These results suggest that NO₃⁻ uptake in maize roots involves a transport mechanism potentially able to induce acidification of the cytoplasmic pH. This acidification became apparent when nitrate reduction was inhibited by tungstate, supporting the hypothesis of Ulrich and Novacky (1990) that the tendency of the cytoplasm to become more alkaline after the addition of nitrate observed in Limnobium stoloniferum could be due to the activity of NR.

The acidification of cytoplasmic pH which occurred in the root cells of plants where nitrate reduction was inhibited could also suggest an involvement of NO₃⁻ reduction in the homeostasis of cytoplasmic pH, as suggested by Crawford and Glass (1998).

In all the conditions tested, the addition of NO₃⁻ into the incubation medium induced an initial depolarization of $E_m$ (Fig. 5). This result is consistent with a NO₃⁻ transport by an H⁺ cotransport mechanism where more than one H⁺ moves across the plasma membrane with each NO₃⁻ (McClure et al., 1990; Ulrich and Novacky, 1990; Meharg and Blatt, 1995; Mistrik and Ulrich, 1996).

The extent of the $E_m$ depolarization was greater when NO₃⁻ was added as the K⁺ salt (Fig. 5B, D), a condition in which a higher uptake of nitrate occurred (Table 1) and the changes in cytoplasmic pH diminished remarkably (Table 2). The smaller changes in cytoplasmic pH in the presence of K⁺ reflect the greater effectiveness of the pH regulatory mechanisms under this condition; these results are consistent with the depolarization of $E_m$ in the presence of K⁺ and the well-known effect of K⁺ on the PM H⁺-ATPase activity which is involved in the biophysical mechanism of intracellular pH regulation (Kurkdjian and Guern, 1989; Guern et al., 1991; Briskin and Hanson, 1992; Sacchi and Cocucci, 1992; Nocito et al., 2002).

In roots of tungstate-treated plants the initial depolarization of $E_m$ was lower, and the subsequent recovery greater (Fig. 5C, D). This was particularly evident when KNO₃ was added, even if the influx of K⁺ increased (+25%; inset in Fig. 5). These different trends seem to be related to the higher activity of the PM H⁺-ATPase (+49%), which may depend, at least in part, on de novo synthesis of this enzyme (Fig. 6). It has been suggested that the increase in net nitrate uptake in the presence of potassium could depend on plasma membrane depolarization induced by this cation (Ivashikina and Feyziev-Ya, 1998). In tungstate-treated seedling roots, after the addition of KNO₃, a lower depolarization took place, but nitrate uptake was not affected, suggesting that the stimulating effect of K⁺ on nitrate uptake could involve $E_m$ independent mechanism(s), which may lead to a better pH homeostasis.

An increase in PM H⁺-ATPase activity as well as expression of specific isoforms has been reported after nitrate supply (Santi et al., 1995, 2003). In the roots of seedlings exposed to nitrate, a transient decrease in cytoplasmic pH occurred. This effect was more evident after tungstate treatment and was accompanied by an increase in the PM H⁺-ATPase activity (Table 2; Fig. 6). These results suggest that the increased PM H⁺-ATPase activity observed during nitrate exposure could be mediated by a mechanism involving cytoplasmic pH changes as an intracellular signal.

Acknowledgement

This work was supported by grants from the Italian Ministry of Education, University and Research (MIUR-PRIN 2000 and 2002). We thank Dr Silvia Morgutti for the precious contributions given during the writing of this paper.

References


The effects of nitrate on the membrane potential.

Evidence for cotransport of nitrate and protons in maize roots. I. Studies of the uptake of nitrate in maize roots: quantitative evaluation of H\(^+\) = H\(^2\)PO\(_4\)\(^–\) transport across the plasma membrane.


