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BRIEF COMMUNICATION

# Allelochemical effects on net nitrate uptake and plasma membrane $H^+$-ATPase activity in maize seedlings

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## Abstract

Seven-day-old maize seedlings grown in a nitrogen-free hydroponic culture were exposed for 48 h to 0, 100 and 300 μM *trans*-cinnamic, *p*-coumaric, ferulic, caffeic acids, umbelliferone and 200 μM $KNO_3$. Net nitrate uptake was affected by *trans*-cinnamic, ferulic and *p*-coumaric acids in a concentration-dependent manner, and *trans*-cinnamic acid appeared to be the strongest inhibitor. Conversely, at low concentrations, caffeic acid stimulated net nitrate uptake while umbelliferone did not influence it. After 24 h of treatment, plasma membrane $H^+$-ATPase activity significantly decreased in a concentration-dependent manner in response to *trans*-cinnamic, ferulic and *p*-coumaric acids, while umbelliferone and caffeic acid had no effect on $H^+$-ATPase activity.

*Additional key words*: caffeic acid, ferulic acid, *p*-coumaric acid, *trans*-cinnamic acid, umbelliferone.

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*Trans*-cinnamic acid and some derivates such as ferulic, caffeic and *p*-coumaric acids inhibit the root growth of many different species (Fujita and Kubo 2003, Abenavoli *et al*. 2004) and interfere with water and nutrient uptake (Pospíšil *et al*. 1987, Booker *et al*. 1992, Yu and Matsui 1997). In particular, ferulic acid promotes the net $K^+$ efflux from cucumber roots (Booker *et al*. 1992), and inhibits the $K^+$ uptake in oat and lettuce roots (Jénsen *et al*. 1994), while *trans*-cinnamic acid, the main component of cucumber root exudates, mainly affects sulphate and magnesium uptake (Yu and Matsui 1997). Despite evidence of allelochemical effects on nitrate uptake (Booker *et al*. 1992, Yu and Matsui 1997), little information about the effect of these compounds on the regulation of this process has been reported. The nitrate uptake into roots, at low external concentrations, is regulated by two high activity transport systems (HATS), the constitutive and inducible, cHATS and iHATS, respectively (Aslam *et al*. 1992). The cHATS is constitutively expressed in nitrate-starved roots and exhibits a constant nitrate uptake rate, while the iHATS, the dynamic component, is induced by nitrate reaching a maximum uptake rate (full induction) after several hours of exposure to the anion (Aslam *et al*. 1992) and it is feedback-regulated by downstream of N metabolites such as glutamate, glutamine and arginine (Forde 2000, Li *et al.* 2009). Previous studies have shown that ferulic acid and coumarin do not affect cHATS but interfere with iHATS activity (Bergmark *et al*. 1992, Abenavoli *et al*. 2001). In particular, 250 μM ferulic acid prevented development of the $NO_3^-$ inducible accelerated uptake rate in maize roots, while 100 μM coumarin stimulated the net nitrate uptake upon full induction in wheat roots (Abenavoli *et al*. 2001).

Changes in net nitrate uptake could be closely correlated with variations in plasma membrane $H^+$-ATPase activity ($pmH^+$-ATPase). Santi *et al*. (1995) demonstrated that, in maize plants, the $pmH^+$-ATPase activity was regulated by the nitrate, and its activity increased to sustain the increased nitrate uptake. At present, no evidence concerning the parallel effect of individual allelochemicals on nitrate uptake regulation and $pmH^+$-ATPase has been reported. The only information about the allelochemical effects on $pmH^+$-ATPase

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*Abbreviations*: BPT - 1,3-bis[tris (hydroxymethyl)-methylamino]-propane; DDT - 1,4-dithioerythritol; EDTA - ethylenediaminetetraacetic acid; EGTA - ethyleneglycoltetraacetic acid); $IC_{50}$ - the dose causing 50 % inhibition of the plasma membrane $H^+$-ATPase activity; MES - 2-[N-morpholino]ethanesulphonic acid; NNUR - net nitrate uptake rate; PMSF - phenylmethylsulphonyl fluoride.

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with regard to nutrient solution used, concerned the allelochemical sorgoleone (Hejl and Koster 2004).

The aim of the present paper, therefore, was to evaluate the simultaneous effect of several simple phenolic compounds on net nitrate uptake regulation and $pmH^+$-ATPase in maize roots.

Maize (*Zea mays* L. cvs. Cecilia, provided by *Pioneer*, Italy) seeds, surface sterilized for 20 min in 20 % (v/v) sodium hypochlorite solution and rinsed with deionized water, were germinated for 72 h under dark at 24 °C in a plastic container filled with 0.5 mM $CaSO_4$. Individual seedlings, selected by uniform size, were transferred into a growing unit containing 4.3 $dm^3$ of aerated one-fourth-strength Hoagland solution (Hoagland and Arnon 1950) without nitrogen. The pH was adjusted to 6.0 with 0.1 M KOH. The growing units were placed in a growth chamber at 24 °C with a 14-h photoperiod, a photon flux density of 300 µmol $m^{-2}$ $s^{-1}$ and 70 % RH. The hydroponic solution was renewed every 2 d. Seedlings (7 d-old) were transferred into a single growing unit containing the same nutrient solution with *trans*-cinnamic, *p*-coumaric, ferulic, or caffeic acids, or umbelliferone, at final concentrations of 0, 10, 100 and 300 µM and 200 µM nitrate ($NO_3^-$). Seedlings exposed only to $NO_3^-$ were referred to as control plants. The hydroponic solution was renewed every 2 d. All phenolic compound, purchased from *Sigma* (St. Louis, MO, USA), were freshly prepared as stock solutions in hot distilled water for each experiment.

For each treatment, three maize seedlings (7-d-old) were collected after 0, 8, 24 and 48 h and their intact roots were carefully rinsed with 0.5 mM $CaSO_4$. The seedlings were then immersed in 80 $cm^3$ of continuously aerated nutrient solution (uptake solution) containing 200 µM $KNO_3$ at pH 6.0. Sample solutions (1 $cm^3$) were taken from the uptake solution at 5 min intervals over a 50 min period and nitrate concentration was measured spectrophotometrically at 210 nm (Goldsmith *et al.* 1973) using a UV-Vis spectrophotometer (*Perkin-Elmer*, Norwalk, CT, USA). The NNUR was calculated from the linear phase of the nitrate depletion curve and expressed as nmol ($NO_3^-$) $cm^{-1}$ (root length) $h^{-1}$.

Plasma membrane vesicles were isolated from maize roots using a small-scale procedure from Giannini *et al.* (1988) modified by Santi *et al*. (1995). Treated and control maize roots (2 - 3 g) were homogenized in extraction buffer (250 mM sucrose, 10 % (v/v) glycerol, 10 mM glycerol-1-phosphate, 2 mM $MgSO_4$, 2 mM EDTA, 2 mM EGTA, 2 mM ATP, 2 mM DTT, 5.7 % (m/v) choline chloride, and 25 mM BTP buffered to pH 7.6 with MES, and 1 mM PMSF, and 20 mg $dm^{-3}$ chimostatin freshly added before homogenization), filtered and centrifugated twice at 12 700 *g* for 3 and 25 min, at 4 °C. The suspension was layered over a 25/38 % discontinuous sucrose gradient (10 mM DL-α-glycerol-1-phosphate, 2 mM $MgSO_4 . 7 H_2O$, 2 mM EGTA, 2 mM ATP, 1 mM PMSF, 2 mM DTT, 20 µg $cm^{-3}$ chimostatin, 5.7 % choline chloride, 5 mM BTP buffered at pH 7.4 with MES) and centrifugated at 12 700 *g* for 60 min at 4 °C. The vesicles, banding at the 25/38 % interface layers, were collected and centrifuged at 14 000 *g* for 45 min at 4 °C. The pellets, resuspended in a medium (20 % glycerol (v/v), 2 mM EGTA, 2 mM EDTA, 0.5 mM ATP, 1 mM PMSF, 2 mM DTT, 20 mg $dm^{-3}$, 5.7 % choline chloride, 5 mM BTP buffered at pH 7 with MES), were immediately frozen in liquid $N_2$ and stored at -80 °C until use. Total soluble protein was estimated according to the Bradford (1976) using bovine serum albumin as standard.

ATP-hydrolyzing activity was determined by measuring the release of inorganic phosphate, as described by Forbusch (1983) at 38 °C. The assay medium (0.6 $cm^3$) contained 50 mM BTP-MES, pH 6.5, 5 mM $MgSO_4$, 5 mM ATP, 0.6 mM $Na_2MoO_4$, 100 mM $KNO_3$, 1.5 mM $NaN_3$, 0.01 % (m/v) $Brij_{58}$, with or without 100 µM vanadate ($V_2O_5$), an inhibitor of P-type $H^+$-ATPase (Sze 1985). Sodium azide and $KNO_3$ were used as selective inhibitors of mitochondria and tonoplast $H^+$-ATPase, respectively. The difference between these two activities was attributed to the $pmH^+$-ATPase. The reaction was initiated by the addition 0.5 - 1.5 µg of membrane protein and was stopped after 30 min with a solution containing: 0.6 M HCl, 3 % SDS, 3 % ascorbic acid and 0.5 % ammonium molybdate at 2 °C. The inhibitory effects on $pmH^+$-ATPase activity in microsomal fractions of maize roots treated with each allelochemical for 24 h, were evaluated by the best-fit equation based on the coefficient of determination ($r^2$) and estimation $IC_{50}$ values (Reigosa and Pazas-Malvido 2007).

A completely randomized design was used for nitrate uptake. All data were evaluated for normality by a Kolmogorov-Smirnov test, and for homogeneity of variances by a Levene median test. For each allelochemical, the NNUR was subject to two-way analysis of variance. A one-way analysis of variance was performed on $pmH^+$-ATPase data. Differences between means were determined using Tukey HSD test ($P < 0.05$).

In plants exposed to 200 µM nitrate (control plants), the net nitrate uptake rate (NNUR) occurred immediately and progressively increased reaching a peak of maximum activity after 8 h. Thereafter, a subsequent decline of NNUR was observed (Fig. 1).

*Trans*-cinnamic acid (10 and 100 µM) caused a shift of the maximum activity which was achieved after 24 h of treatment and, in particular 10 µM *trans*-cinnamic acid significantly increased the maximum NNUR (by 185 % compared to control plants). After 48 h, 10 and 100 µM *trans*-cinnamic acid increased the NNUR by 289 and 261 %, respectively. On the contrary, 300 µM *trans*-cinnamic acid decreased NNUR at 4 and 8 h compared to the control plants (Fig. 1*A*).

After 8 h of treatment, 10 µM *p*-coumaric acid slightly reduced the NNUR (22 %), while 100 and 300 µM markedly decrease it (of 67 and 96 %, respectively compared with the control plants). Furthermore, 300 µM *p*-coumaric acid displayed a negative effect on NNUR also after 24 and 48 h of treatment (Fig. 1*B*).

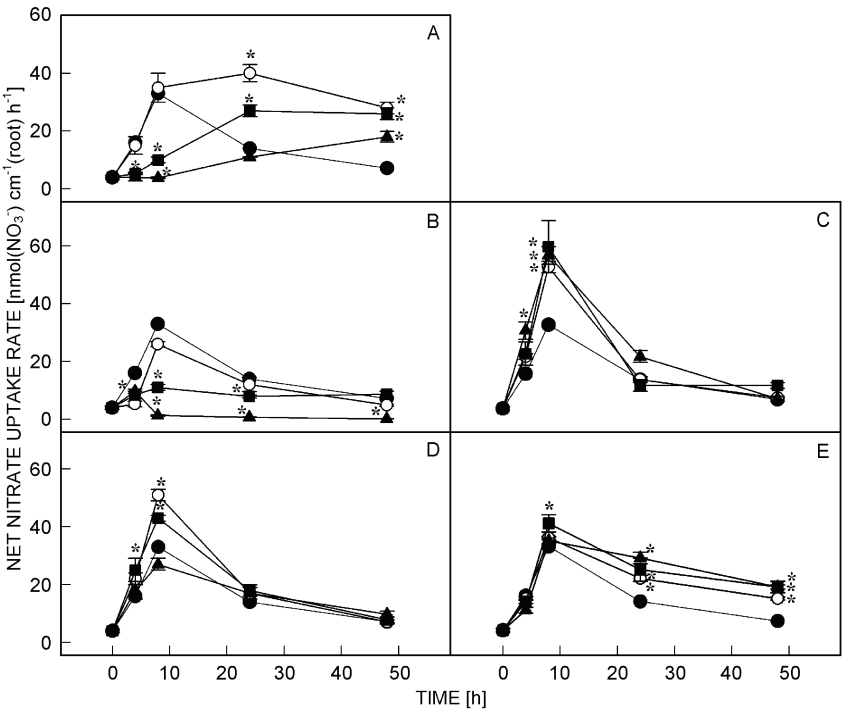


Fig. 1. Time-course of net nitrate uptake rate by 7-d-old maize seedlings exposed to 0 μM (*closed circles*), 10 μM (*open circles*), 100 μM (*squares*) and 300 μM (triagles) *trans*-cinnamic acid (*A*), *p*-coumaric acid (*B*), caffeic acid (*C*), ferulic acid (*D*) or umbelliferone (*E*) and 200 μM nitrate for 0, 4, 8, 24 and 48 h. Data are the mean of three replicates and bars indicate the standard error (*ANOVA*, Tukey's test, * = $P < 0.05$).

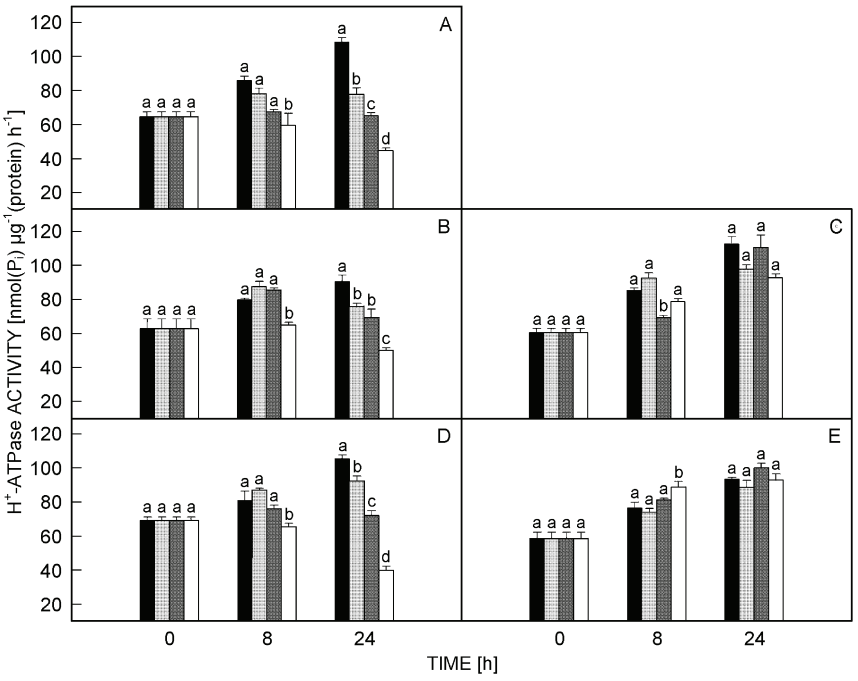


Fig. 2**.** $H^+$-ATPase activity of plasma membrane vesicles isolated from root of 7-d-old maize seedlings exposed to 0 μM (*black columns*), 10 μM (*light grey columns*), 100 μM (*dark great columns*) and 300 μM (*white columns*) *trans*-cinnamic acid (*A*), *p*-coumaric acid (*B*), caffeic acid (*C*), ferulic acid (*D*) or umbelliferone (*E*) and 200 μM nitrate for 0, 8 and 24 h. $IC_{50}$ (the dose causing 50 % of the plasma membrane $H^+$-ATPase activity inhibition) = 256.22 (*A*), 407.73 (*B*), 1650 (*C*) and 223.59 (*D*); not inhibitory (*E*). Data are the mean of three replicates and *bars* indicate SE (*ANOVA*, Tukey's test, $P < 0.05$).

Caffeic acid (10, 100 and 300 μM) significantly increased NNUR after 8 h (by 61, 82 and 73 %, respectively), compared to the control plants. After this time, caffeic acid did not significantly change NNUR (Fig. 1*C*).

At 10 and 100 μM ferulic acid, NNUR was higher (by 54 and 30 %, respectively, in comparison with the control plants) after 8-h treatment, while 300 μM ferulic acid did not cause significant change in NNUR activity (Fig. 1*D*).

The NNUR in plants exposed to 10 and 300 μM umbelliferone for 8 h did not show any significant difference from the control plants. After 24 h, 10, 100 and 300 μM umbelliferone increased NNUR by 57, 78 and 107 %, respectively, compared to the control plants. The increase was also evident after 48 h of exposure (108, 164 and 164 %, respectively). (Fig. 1*E*).

In the control plants, the $pmH^+$-ATPase activity progressively increased from 64.4 up to 108.72 nmol($P_i$) $g^{-1}$ $h^{-1}$ after 24 h. Similar behaviour was observed in maize plants treated with *trans*-cinnamic, *p*-coumaric and ferulic acids (Fig. 2*A*,*B*,*D*). In particular, after 8 h of treatment, only the highest concentration (300 μM) of these three allelochemicals reduced the $pmH^+$-ATPase activity by 31, 18.5 and 19.5 % compared to the control plants. Conversely, exposure for 24 h to 10, 100 and 300 μM of *trans*-cinnamic acid significantly inhibited $pmH^+$-ATPase activity by 29, 40 and 59 % (Fig. 2*A*), of *p*-coumaric acid by 16, 24 and 44 % (Fig. 2*B*), and ferulic acid by 12, 31 and 62 %, respectively compared to the control plants (Fig. 2*D*). Differently, caffeic acid and umbelliferone, at all concen-trations, did not significantly affect the $pmH^+$-ATPase activity with regard to the control plants (Fig. 2*C*,*E*).

By comparing the $IC_{50}$-values of $pmH^+$-ATPase activity, the following order of phytotoxicity among allelopathic compounds was established: ferulic > *trans*-cinnamic > *p*-coumaric > caffeic acids. Umbelliferone did not exhibit any effect on $pmH^+$-ATPase activity.

The results of this study evidenced the complexity of effects of phenols on the NNUR, which can be inhibitory as observed for *trans*-cinnamic, *p*-coumaric and ferulic acids or ineffective or stimulatory as reported for umbelliferone and caffeic acid, respectively. However, the activity of allelochemicals was largely dependent on the concentration but, to an even greater extent, on the chemical structure of the compound. Indeed, previous results have demonstrated that the presence of substituents in the aromatic ring changed allelopathic phytotoxicity, *i.e.* the hydroxylation of *trans*-cinnamic acid decreased the inhibitory activity on ion uptake (Yu and Matsui 1997) and reduced the inhibitory activity of coumarin on root elongation (Kupidlovska *et al*. 1994). Also the position and/or the isomerization of the substitution group defined allelopathic activity, *i.e. cis*-cinnamic acid was ten times more active than *trans*-cinnamic acid in root growth inhibition of *Arabidopsis thaliana* (Wong *et al*. 2005). Further, lactonization increased the inhibitory effect, *i.e*. coumarin was a stronger inhibitor on the germination compared to *trans*-cinnamic acid (Reynolds 1989).

In contrast with Yu and Matsui (1997), our results showed lower inhibitory activity of *trans*-cinnamic acid than *p*-coumaric acid on NNUR. The simultaneous presence of a methyl and a hydroxyl group in ferulic acid reduced the negative effect on NNUR in comparison with *trans*-cinnamic and *p*-coumaric acids. These results contrast with Bergmark *et al*. (1992) and Booker *et al*. (1992) who reported the strong inhibition of NNUR by ferulic acid. The contrast with Bergmark's results can be explained by the different species and root size. Indeed, Rubio *et al*. (2005) demonstrated that the phosphate uptake pattern changed in relation to the root size and morphology.

The positive effect of substitution in the aromatic ring on the NNUR was more evident with caffeic acid, characterized by two hydroxyl groups. Caffeic acid showed not only a less inhibition of NNUR compared to previous compounds, but even a stimulation after 8 h of treatment. Finally, the lactonization of *trans*-cinnamic acid and the simultaneous presence of a hydroxyl group in the umbelliferone reduced its inhibitory activity. In fact, umbelliferone increased NNUR at all times and concentrations applied. The presence of OH-group(s), as suggested by Glass (1974), probably nullifies the negative effect of lactonization.

An important role in NNUR is played by $pmH^+$-ATPase, which provides the driving force for nitrate transport across the membrane in maize roots (Santi *et al.* 1995). According to the above-mentioned authors, $pmH^+$-ATPase activity in membrane vesicles of maize roots increased after exposure to nitrate. Treatment with *trans*-cinnamic, ferulic and *p*-coumaric acids significantly reduced this enzyme activity. The presence of a hydroxyl group in the aromatic ring of *p*-coumaric acid increased its $IC_{50}$-value compared to that of *trans*-cinnamic acid. Conversely, the presence of two hydroxyl groups of caffeic acid decreased the phytotoxicity compared with *p*-coumaric acid. The simultaneous presence of a methyl and hydroxyl group, in ferulic acid, caused the strongest inhibitory effect. However, this order of phytotoxicity, associated with the position and number of substitution groups, changed in respect to that observed on NNUR, except for caffeic acid, which was less inhibitory in both processes. These results suggest that the allelochemical effects on NNUR and $pmH^+$-ATPase were independent. Moreover, besides sustaining nutrient uptake, $pmH^+$-ATPase is involved in other physiological processes of plant growth and development (Serrano 1989) such as the regulation of cell pressure potential, osmoregulation, cell expansion, and pH homeostasis (Palmgren 2001).

In conclusion, the results suggest that the effects of allelochemicals have many targets and their effects are complex depending on the chemical structure, the plant species, and the physiological processes considered. Furthermore, considering the importance of nitrate as nutrient and signal on plant growth and development (Crawford 1995), these results, from the ecological point of view, could explain the influence of allelochemicals on the composition of plant communities.

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