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Modulation of NO_3^- uptake by water-extractable humic substances: involvement of root plasma membrane H^+ATPase

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Abstract

The effect of a water extractable humic substances fraction (WEHS) on nitrate uptake and plasma membrane (pm) H^+-ATPase activity of maize roots was investigated. Four days old maize root seedlings were exposed for 4 to 24 h to a nutrient solution containing 200 μM nitrate in the absence or presence of 5 mg org. $C \cdot L^{-1}$ WEHS. Plants exposed to nitrate developed a higher capacity to absorb the anion (induction): the net uptake rate progressively increased up to 12 h of contact with the solution; thereafter, a decline was observed. When WEHS was present together with nitrate in the nutrient solution, the induction of nitrate uptake was evident and maximal already 4 h after starting the treatment. The rate of net nitrate uptake decreased only slightly during the remaining period (4-24 h). Stimulation of net nitrate uptake rate was also observed when WEHS was added to a nitrogen- or nitrate-free nutrient solution or to a 5 mM $CaSO_4$ solution. The activity of pmH^+-ATPase raised upon exposure of the roots to nitrate with the same pattern observed for nitrate uptake. The contemporary presence of nitrate and WEHS caused a further stimulation of the pmH^+-ATPase activity after 4 h treatment. An increase in the enzyme activity was also observed when plants were treated for 4 h in the presence of WEHS in $CaSO_4$, nitrogen- or nitrate-free solutions. However, when nitrate was present the enhancement was even greater. Results support the idea that the plasma membrane proton pump might be one of the primary targets of the action of humic substances on plant nutrient acquisition. A role of WEHS in the modulation of nitrate uptake via an interaction with the pm H^+-ATPase is also discussed.

Introduction

Root apparatus growing in the soil are exposed to a wide array of organic and inorganic compounds. A considerable part of organic compounds is represented by humic substances; these molecules are characterized by large differences in molecular size and solubility. It has been estimated that in the soil solutions these substances can be present at a concentration as high as 250 mg L^{-1} (Gerke, 1993). Several evidences (Vaughan and Ord, 1981; Pinton et al., 1998) suggest that low molecular size humic molecules could reach root surface and flow in the apoplast thus rendering plausible the interaction with the plasma membrane. Using an in *vitro* approach we could demonstrate that low molecular weight humic substances can stimulate the H^+-ATPase activity of plasma membrane vesicles isolated from oat roots (Varanini et al., 1993). In addition, humic substances have been proven to enhance active proton extrusion from roots of oat (Pinton et al., 1997).

The stimulation of plasma membrane (pm) H^+-ATPase activity could, at least in part, explain the positive effect of these soil molecules on nutrition and growth of plants. Various studies (Guminski et al., 1983; Maggioni et al., 1987) showed that uptake of cationic and anionic macronutrients is increased when

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the roots are placed in contact with suitable concentrations of humic substances. In particular, Albuzio et al. (1986) demonstrated that the uptake of nitrate was about 40% stimulated in barley roots after 8 h contact with the humic molecules.

Nitrate is one of the major anionic macronutrients present in the soil solution. Its uptake is substrate-inducible and energy-requiring. Recently we showed that the activity and amount of pmH^+-ATPase were increased in maize roots induced for nitrate uptake (Santi et al., 1995).

In the present work we attempt to define the role of a water extractable humic substances fraction (WEHS) on the induction of nitrate uptake via an interaction with the pmH^+-ATPase.

Materials and methods

Humic substances

Water extractable humic substances (WEHS) were obtained as reported by Pinton et al. (1998). Briefly, the WEHS were extracted from finely ground sphagnum peat (2.5 g) by adding 50 mL of distilled water and shaking for 15 h at room temperature. Thereafter, the suspension was centrifuged at 8000 RPM for 30 min and the supernatant filtered on a Whatman WCN 0.2 μM membrane filter. The resulting solution was acidified to pH 2 with H_2SO_4 and loaded onto a column (Ø20 mM, height 200 mM) of Amberlite XAD-8 resin (Aiken et al., 1979). Adsorbed humic substances were washed with 100 mL of distilled water and eluted from the column with 0.1 *N* NaOH. The solution was treated with Amberlite IR-120 (H^+ form) down to pH 1-2, and then adjusted to neutrality with 0.1 *N* NaOH. The humified organic fraction was freeze dried before storage and immediately dissolved before use at a concentration of 2 g organic $C \cdot L^{-1}$.

Elementary composition, element contents and molecular sizes distribution were reported elsewhere (Pinton et al., 1998). The fraction contained essentially humic compounds with a molecular weight lower than 1kDa.

Plant growth

Maize (*Zea mays* L. cv Cecilia, Pioneer Hybrid Italia S.p.A.) seeds, previously steeped in water for 24 h, were germinated over aerated deionized water, in the dark at 27 °C and 90% relative humidity. After 72 h, seedlings were transferred to 5 L plastic vessels containing 5 m*M* $CaSO_4$ aerated solution and grown for 24 h under a 16/8 light/dark regime at 25 °C, 65-70% relative humidity and a light intensity of 300 μE m^{-2} s^{-1}. Seedlings (4-d old) were transferred to a nutrient solution having the following composition: (μM) $MgSO_4$ 100, KCl 5, H_3BO_3 2.5, $MnSO_4$ 0.2, $ZnSO_4$ 0.2, $CuSO_4$ 0.05, $NaMoO_4$ 0.05, Fe-EDTA 2, K_2SO_4 200, KH_2PO_4 175 and $CaSO_4$ 400 and, when added, 25 μM $NH_4H_2PO_4$. In order to induce nitrate uptake, 200 μM KNO_3 was added to the solution. The effect of WEHS was evaluated adding to the nutrient solution the humic fraction at 5 mg org. $C \cdot L^{-1}$.

In some experiments 4-d-old seedlings were transferred to a 5 m*M* $CaSO_4$ solution in the presence or absence of 5 mg org. $C \cdot L^{-1}$ WEHS.

Net nitrate uptake

After exposure to the different treatments, intact roots of 2 maize plants (about 0.4 g f.w.), were rinsed for 2 min in 1 m*M* $CaSO_4$ and subsequently immersed in 20 mL of solution containing 200 μM KNO_3 and 4 m*M* $CaSO_4$ at pH 6.0 (uptake solution). Nitrate depletion from the solution was measured over a 10 min period collecting 0.2 mL of uptake solution at 2 min intervals. Nitrate was determined spectrophotometrically at 410 nm according to Cataldo et al. (1975).

In the period of time considered the consumption of nitrate was linear allowing the calculation of the rate of nitrate uptake by linear regression analysis.

Isolation of plasma membrane vesicles

Plasma membrane vesicles were isolated from maize roots by following the small-scale procedure described by Giannini et al. (1988) with minor changes. Briefly, 2.5 g of root apical segments (3-4 cm) were homogenized in a mortar and pestle with 2.5-fold volumes of a freshly prepared ice-cold medium containing 250 m*M* sucrose, 10% (v/v) glycerol, 10 m*M* glycerol-1-phosphate, 2 m*M* $MgSO_4$, 2 m*M* EDTA, 2 m*M* EGTA, 2 m*M* ATP, 2 m*M* DTT, 5.7% (w/v) choline-iodide, 1 m*M* PMSF, 20 μg/mL chymostatin and 25 m*M* BTP titrated to pH 7.6 with solid Mes.

The breis were filtered through six layers of cheese-cloth and then centrifuged at 13,000g for 3 min in an Eppendorf 5402 microcentrifuge at 4 °C. The recovered surnatant was then centrifuged at 13,000g for 25 min in the microcentrifuge in order to obtain a microsomal membrane pellet. Microsomes were

gently resuspended in 0.4 mL of homogenization medium and loaded onto a discontinuous sucrose gradient made by layering 700 μL of sucrose solution (1.13 g $\cdot$ cm^{-3}) onto 300 μL of sucrose solution (1.17 g $\cdot$ cm^{-3}) cushion and centrifuged at 13,000g for 1 h. The sucrose solution were prepared in 5 m*M* BTP-Mes, pH 7.4, and contained all the protectants present in the homogenization medium.

Vescicles banding at the 1.13/1.17 g $\cdot$ cm^{-3} interface were collected, diluted with homogenization medium and used for the enzyme assays immediately.

ATPase assay

ATP-hydrolysis was routinely assayed in a 0.6 mL reaction medium containing 50 m*M* BTP-Mes pH 6.5, 5 m*M* $MgSO_4$, 5 m*M* ATP, 0.6 m*M* Na_2MoO_4, 100 m*M* KNO_3, 1.5 m*M* NaN_3, Brij 0.01%. The reaction was started by adding 0.5-1.5 μg of membrane protein and was stopped after 30 min by the addition of 1 mL of a solution containing 0.6 N HCl, 3% (w/v) SDS, 3% (w/v) ascorbic acid, and 0.5% (w/v) ammonium molybdate at 2 °C. Inorganic phosphate was determined according to Forbush (1983). The presence of plasma membrane ATPase activity was evaluated on the basis of the effect of the inhibitor vanadate at the final concentration of 0.1 m*M*. Usually, more than 90% of ATPase specific activity was vanadate-sensitive. Inhibition by Na-molibdate was less than 15%, while azide and nitrate did not affect ATP hydrolytic activity. These results were not modified by the different treatments imposed to the roots.

Protein assay

Protein contents were determined as described by Bradford (1976), using BSA as standard.

Results

Root morphology

Plate 1 (A) shows the root apparatus of 4-d-old maize seedlings exposed for 24 h to a nutrient solution containing 200 μM nitrate in the presence (WEHS) or absence (Control) of the water-extractable humic substances fraction at a final concentration of 5 mg org. C $\cdot$ L^{-1}. Root apparatus of control plants consisted of a primary and one secondary root. In addition, at the basal zone of the primary root, the presence of lateral roots at the initial phase of development was evident. When WEHS was present together with nitrate in the nutrient solution, the development of seedlings roots was modified. In fact, a higher proliferation of secondary roots (generally about 3 per plant) and a higher number of lateral roots more developed in length than those observed in the control plants, were evident.

A similar effect of WEHS on root morphology was observed when plants were put in contact with 5 m*M* $CaSO_4$ solution for 24 h (Plate 1, B) in the presence of the humic fraction.

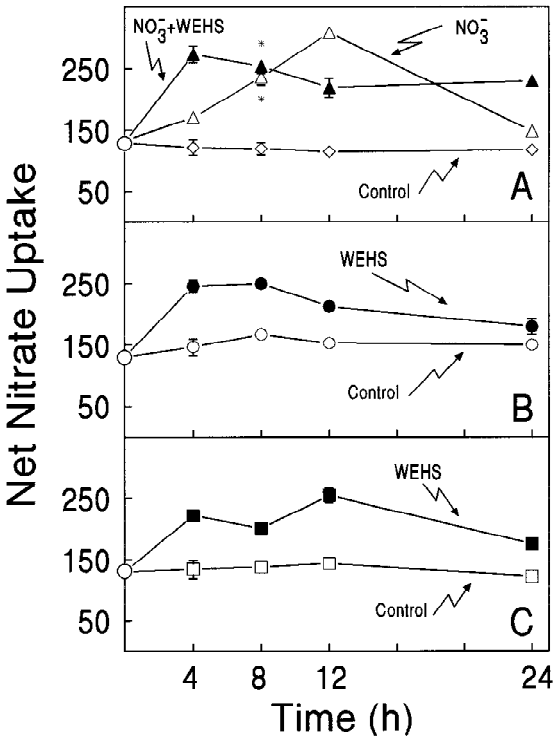

Figure 1. Net nitrate uptake (nmol NO_3^- g^{-1} f.w. min $^{-1}$) by 4-d old maize seedlings put in contact for 4, 8, 12 and 24 h with solutions having different composition: A) Control, nutrient solution containing 25 μM ammonium; NO_3^-, nutrient solution containing 25 μM ammonium plus 200 μM nitrate; NO_3^-+WEHS, nutrient solution containing ammonium plus nitrate and 5 mg org. C $\cdot$ L^{-1} WEHS; B) N-free nutrient solution with (WEHS) or without (Control) 5 mg org. C$\cdot$ L^{-1} WEHS; C) solution containing 5 m*M* $CaSO_4$ with (WEHS) or without (Control) 5 mg org. C $\cdot$ L^{-1} WEHS. Net nitrate uptake was measured as depletion from a solution containing 200 μM KNO_3 and 4 m*M* $CaSO_4$ at pH 6.0. Date are means $\pm$ SE of four independent experiments run in triplicate; SE were reported when not obscured by the symbols. Values with * are not significantly different at the Duncan's multiple range test (P= 0.05).

Nitrate uptake

Figure 1 shows the pattern of net nitrate uptake by 4-d-old maize seedlings put in contact with solutions having different composition, in the presence or ab-

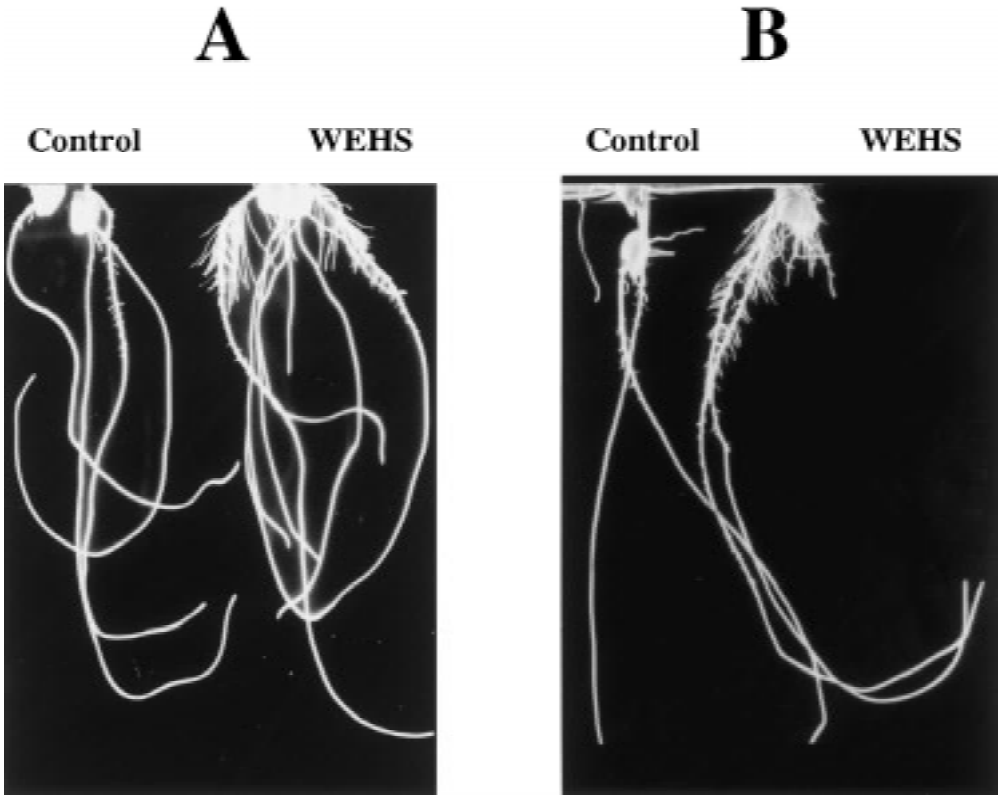

Plate 1. Root apparatus of 4-d old maize seedlings exposed for 24 h to a (NH_4^+ + NO_3^-)-containing nutrient solution (A) or to 5 m*M* $CaSO_4$ (B) in the presence or absence of 5 mg org. C $\cdot L^{-1}$ WEHS.

sence of 5 mg org. C $\cdot L^{-1}$ WEHS. As shown in panel A, plants treated with a nutrient solution without added nitrate did not modify their capacity to absorb the anion over a 24 h period. On the other hand, when plants were exposed to a nutrient solution containing 200 μM nitrate, the net nitrate uptake rate progressively increased up to 12 h of contact with the solution (induction). Thereafter, a decline in net nitrate uptake rate was observed suggesting the operation of a feedback regulatory mechanism.

When WEHS was present together with nitrate in the nutrient solution, the induction of nitrate uptake was evident and maximal already after 4 h of treatment. The rate of net nitrate uptake only slightly decreased prolonging the treatment and remained fairly constant up to the end of the experiment (24 h). Panel B shows the behavior of net nitrate uptake in plants grown in a nitrogen-free nutrient solution. As expected, control plants did not develop a higher nitrate uptake capacity. Moreover the rates were comparable to those observed for plants growth in the presence of 25 μM ammonium (Panel A, control). On the other hand, the presence of WEHS determined an increased capacity to absorb nitrate which was 70% higher than that of control plants already after 4 h treatment.

Essentially the same pattern was observed in plants treated with a 5 m*M* $CaSO_4$ solution (Panel C).

Plasma membrane H^+-ATPase activity

Plasma membrane-enriched vesicles were isolated from maize roots by the small-scale procedure described by Giannini et al. (1988) which allows the recovery of suitable amounts of membrane vesicles for biochemical assays from a relatively low root fresh weight. Based on the effect of selective inhibitors on ATPase activity, membrane preparations appeared enriched in plasma membrane vesicles at a level comparable to that of other plasma membrane preparations obtained from maize roots (Fischer-Schliebs et al., 1994). The different treatments imposed to the roots did not significantly modify the composition of the isolated membrane vesicles preparations (not shown).

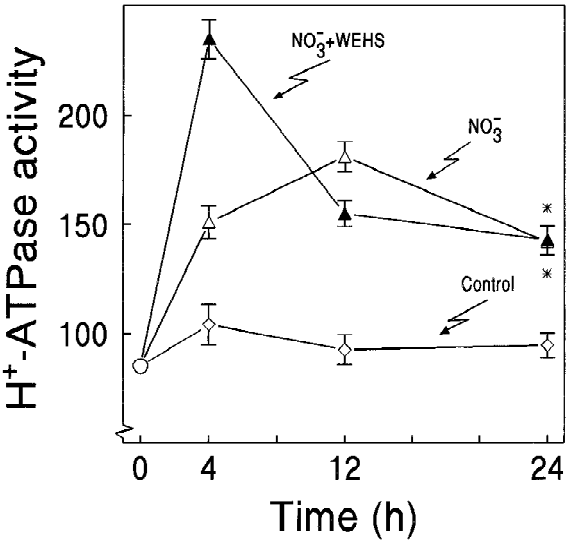

Figure 2. H^+-ATPase activity (μmol Pi mg^{-1} prot. h^{-1}) of plasma membrane vesicles isolated from roots of 4-d old maize seedlings grown as reported in Figure 1, Panel A. Each point represents the mean ± SE of three determinations of four different membrane preparations. Values with * are not significantly different at the Duncan's multiple range test (P= 0.05).

Figure 2 shows pmH^+-ATPase hydrolytic activity of membrane vesicles isolated from roots of 4 days old maize seedlings put in contact for 4 to 24 h with solutions with different composition. The activity of pmH^+-ATPase displayed by plasma membrane vesicles isolated from roots treated with ammonium-containing nutrient solution did not vary significantly over the 24 h treatment. On the other hand, addition of 200 μM nitrate to the solution bathing the roots induced the development of a higher enzyme specific activity with a maximum after 12 h treatment.

When WEHS was present in combination with nitrate in the nutrient solution, a considerable increase in the pmH^+-ATPase activity was recorded already after 4 h treatment; this effect accounted for a 57% and 135% stimulation with respect to the pmH^+-ATPase activity displayed by roots treated with or without nitrate, respectively. Prolonging the growth of roots in nutrient solution containing WEHS and nitrate, a decrease in pmH^+-ATPase activity was recorded with values at 12 and 24 h similar to those shown by nitrate-treated roots.

In order to analyze the effect of WEHS alone on pmH^+-ATPase activity, independently from that already described for nitrate (Santi et al., 1995), roots were also grown for 4 h in nutrient solutions without nitrate or without any nitrogen source or in a $CaSO_4$ solution. Table 1 shows that the presence of WEHS determined an increase of 23÷31% in pmH^+-ATPase activity of plants grown either in $CaSO_4$, nitrogen-free nutrient solution and ammonium-containing nutrient solutions. As noted above (see Figure 2), the stimulation of the enzyme activity was higher (54%) when WEHS and nitrate were concomitantly present in the nutrient solution.

Table 1. Effect of WEHS on H^+-ATPase activity of plasma membrane vesicles isolated from roots of 4-d-old maize seedlings grown for 4 h in: 5mM $CaSO_4$; N-free nutrient solution; (NH_4^+)- or (NH_4^+ + NO_3^-)-containing nutrient solution in the presence or absence of 5 mg org. C · L^{-1} WEHS. Data are means ± SE of three determinations of four different membrane preparations; means reported within each row were significantly different at the Duncan's multiple range test (P= 0.05).

Growth conditions	H^+-ATPase activity (μmol Pi mg^{-1} prot. h^{-1})	
	Control	WEHS
$CaSO_4$	95 ± 4 (100)	122 ± 4 (128)
N.S. (-Nitrogen)	84 ± 7 (100)	110 ± 5 (131)
N.S. ($+NH_4^+$)	85 ± 6 (100)	104 ± 5 (123)
N.S. ($+NH_4^+$ + NO_3^-)	146 ± 6 (100)	225 ± 8 (154)

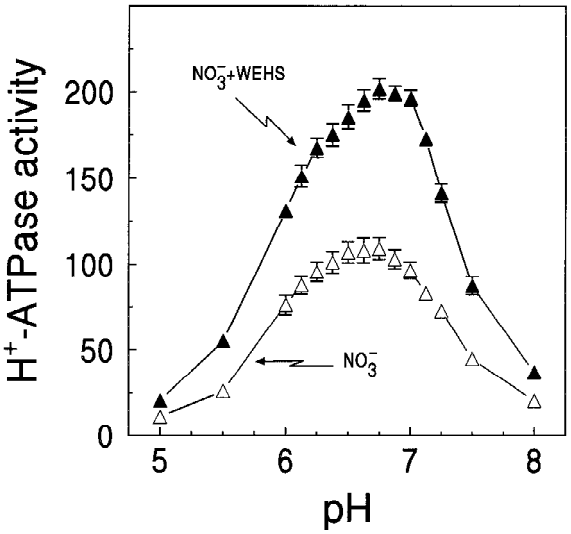

Figure 3. The effect of pH on the H^+-ATPase activity (μmol Pi · mg^{-1} prot. h^{-1}) of plasma membrane vesicles isolated from roots of 4-d old maize seedlings exposed for 4 h to a (NH_4^+ + NO_3^-)-containing nutrient solution in the presence or absence of 5 mg org. C · L^{-1} WEHS. Each point represents the mean ± SE of three determinations of four different membrane preparations. SE were reported when not obscured by the symbols.

Figure 3 shows the pH dependency of pmH^+-ATPase activity of 4 d old maize seedlings roots grown for 4 h in nutrient solution containing nitrate in the presence or absence of WEHS. The presence of WEHS caused a clear shift in pH optimum of the enzyme activity from 6.50÷6.75 to 6.75÷7.00.

Discussion

The beneficial effect of humic substances on plant nutrition has been attributed to the promotion of root development and/or stimulation of mechanisms involved in nutrient acquisition (Nardi et al., 1996; Varanini and Pinton, 1995). As far as nitrate is concerned, an enhanced capacity to absorb the anion into roots treated with humic substances for 8 to 16 h has been demonstrated (Albuzio et al., 1986; Dell'Agnola and Nardi, 1987). In the present work a more rapid response to humic substances was observed; in fact, the induction of the highest net nitrate uptake capacity was evident already after 4 h treatment with a water extractable humic substances fraction (WEHS), having an average molecular weight lower than 1kDa. As reported for other humic substances fractions (Nardi et al., 1996) WEHS also causes the proliferation of lateral roots when put in contact for 24 h with the root apparatus of maize seedlings. Lazof et al. (1992) showed that maize lateral roots and basal primary root from which they extended were the dominant zones of ^{15}N uptake and translocation. These results might also explain the observed enhancement of ion uptake in humus-treated maize plants. However, the stimulatory effect of WEHS on net nitrate uptake appeared to be independent of the morphological changes, since no change in the root morphology was observed during the initial 4 h treatment. On the other hand, untreated roots showed the maximal uptake capacity only after 12 h of exposure to nitrate. The stimulation of nitrate uptake by WEHS was not due to the ion content of the humic fraction since the control plants (with or without nitrate in the absence of WEHS) were treated with a nutrient solution whose ion contents by far exceeded that of the added humic fraction (Pinton et al., 1998). Interestingly, a higher nitrate uptake capacity was also evident after treating for 4 h maize roots in a nitrogen-free nutrient solution or in 5 m*M* $CaSO_4$ in the presence of WEHS, indicating that the humic fraction is somehow able to modify the machinery of nitrate uptake independently from the presence of nitrate itself. It has been demonstrated the existence of two types of high-affinity nitrate transport systems: a constitutive low-capacity (CHATS) and an inducible high-capacity (IHATS) (Wang and Crawford, 1996). Both of them require protons to drive nitrate transport. The increased capacity to take up nitrate upon exposure to the anion has been explained by the upregulation of CHATS and IHATS (Crawford and Glass, 1998). Several evidences suggested that the driving force for nitrate uptake can be provided by the pmH^+-ATPase (Mc Clure et al., 1990); furthermore, Santi et al. (1995) showed that roots induced for nitrate uptake possess a higher pmH^+-ATPase activity. This result is confirmed by data presented here. In fact, the enzyme activity raised upon exposure of the roots to nitrate and showed the same pattern observed for net nitrate uptake. The contemporary presence of nitrate and WEHS caused a further stimulation of the pmH^+-ATPase activity after 4 h treatment. It has been previously shown that humic substances could stimulate active proton extrusion from oat roots (Pinton et al., 1997). In addition, Varanini et al. (1993) showed that a low molecular weight humic substances fraction incubated with isolated plasma membrane vesicles of oat roots sharply stimulated the H^+-ATPase activity, thus suggesting that the plasma membrane proton pump could be one of the main targets involved in the action of humic substances on plant nutrition. Data of the present work show that also when plants were treated with humic substances and no nitrate a higher H^+-ATPase activity was measured in isolated plasma membrane vesicles. In fact, an increase ranging from 23 to 31% in the enzyme activity was observed when plants were grown in the presence of WEHS in $CaSO_4$, nitrate- or nitrogen-free solutions. When nitrate was present, the enhancement was even greater (ca. 50%). These results indicate that the effect of WEHS on pmH^+-ATPase activity is independent of the presence of nitrate and is additive with that exerted by the anion itself. Plasma membrane H^+-ATPase is involved in fundamental processes of plant physiology (e.g. nutrient uptake, regulation of cell turgor and of intracellular and extracellular pH, extension growth). This stimulation of the proton pump by WEHS can affect H^+ fluxes across the plasma membrane and, consequently, ion movement, intracellular and extracellular pH with relevant changes of root cell metabolism. Our data do not allow to rule out a possible action of WEHS at the level of nitrate transporters. Interestingly, in *Arabidopsis* (Tsay et al., 1993) it has been shown a relationship between external pH and the expression of nitrate transporter gene *CHL1*: the more acidic the pH of the media the higher the *CHL1* steady-state mRNA

level. Furthermore, *CHL1* expression was also enhanced within two hours by acidifying the media even though nitrate was not added. Based on these observation we might hypothesize a link between the increase in net nitrate uptake and pmH^+-ATPase activity in roots of maize plants treated with a nitrate-free solution in the presence of WEHS (Figure 1B, C; Figure 2).

The enhancement in pmH^+-ATPase activity observed when plants were treated for 4 h with WEHS in addition to nitrate appeared not to be due to an increased steady-state level of the enzyme, as evaluated by western blot analysis of plasma membrane proteins performed using polyclonal antibodies raised against the major (97 kDa) SDS-PAGE separated polypeptide of maize pmH^+-ATPase (data not shown). Rather a modulation of the enzyme could occur since the shift in the pH optimum resembles that described for other effectors like fusicoccin or proteolytic treatment (Lanfermeijer and Pins, 1994). In conclusion our results strongly support the idea that WEHS can modulate nitrate uptake via an interaction with pmH^+-ATPase. Molecular approaches will further deepen our knowledge of the regulatory aspects of this phenomenon.

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