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Rhizosphere Acidification by Iron Deficient Bean Plants: The Role of Trace Amounts of Divalent Metal Ions

A Study on Roots of Intact Plants with the Use of ^{11}C- and ^{31}P-NMR

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ABSTRACT

Rhizosphere acidification by Fe-deficient bean (*Phaseolus vulgaris* L.) plants was induced by trace amounts of divalent metal ions (Zn, Mn). The induction of this Fe-efficiency reaction was studied by $^{14}CO_2$ and $^{11}CO_2$ fixation experiments, and with ^{31}P-NMR on roots of whole plants. The starting and ending of an acidification cycle was closely coupled to parallel changes in CO_2 fixation, within the maximal resolution capacity of 20 min. ^{31}P-NMR experiments on intact root systems showed one peak which was ascribed to vacuolar free phosphate. At the onset of proton extrusion this peak shifted, indicating increase of pH in the cells. Proton extrusion was inhibited, with a lag period of 2 hours, by the protein synthesis inhibitors cycloheximide and hygromycin. It is assumed that Zn and Mn induce proton extrusion in Fe-deficient bean roots by activating the synthesis of a short-living polypeptide; the NMR data suggest a role for this peptide in the functioning of a proton pumping ATPase in the plasma membrane.

Under Fe deficiency, dicotyledonous plants develop a high capacity in the roots to excrete protons (1, 23). The resulting acidification of the rhizosphere helps to dissolve sparingly soluble ferric deposits in the soil.

At the same time, these plants also accumulate salts of organic acids, most notably citrate and malate (8, 13). This accumulation is not necessarily coupled to proton extrusion, because Fe deficient grasses also accumulate malate and citrate (13) even though they fail to acidify the rhizosphere upon Fe shortage (19, 23, 28). In general, Fe-deficient plants accumulate organic acids, and in dicots this formation is stimulated during proton extrusion. The cause of malate and citrate accumulation in the absence of proton extrusion is not known.

Roots of Fe-deficient bean plants can only acidify the nutrient solution in the presence of micromolar concentrations of Fe(III)EDTA or Zn^{2+}, Mn^{2+}, or Co^{2+} salts (in decreasing order of effectivity) (6, 25). Ferric chelates are taken up at very low rates (22, 26), but they are rapidly reduced to ferrous in presence of Fe-deficient dicot roots (2), and we therefore assume that in all cases the divalent metal ions are the effective agents. On a molar basis up to 1000-fold more protons are excreted than there are metal ions in the nutrient solution (6) which argues against exchange and for a role as catalyst.

When Zn and Mn are constantly present in the nutrient solution, proton extrusion is also continuous, but showing a daily rhythm with a minimum during the night (25). Removal of the divalent metal ions, either by uptake or by replacement of the nutrient solution, stops proton excretion. Upon resupply of Zn, Mn, or Fe to the nutrient solution the plant resumes proton excretion but with a lag of 2 to 6 h (6).

To understand how divalent metal ions induce proton excretion by Fe-deficient bean plants we undertook to compare the kinetics of proton excretion with those of accompanying cellular processes.

MATERIALS AND METHODS

Phaseolus vulgaris L., var Prélude (Sluis, Enkhuizen, The Netherlands), was used throughout.

Growth Conditions

Experiments on CO_2 Fixation

Plants were grown as described (2) on Knop nutrient solution containing Zn^{2+} at 0.7 μM, Mn^{2+} at 0.9 μM, and Co^{2+} at 0.12 μM, with or without 40 μM Fe-Na-EDTA. The medium was changed at d 9 and 12, the plants were used at d 13 or 14.

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Experiments with ^{31}P-NMR

After 6 d germination, the plants were transferred to Knop nutrient solution containing 0.1 μM phosphate instead of 2 mM, and Fe as 0.1 μM Fe-Na-EDTA. At d 9, the leaves were sprayed with 1 mM giberellic acid in water. This led to strong elongation of the internodes in the 2 following weeks. The medium was changed every 2 to 3 d. When the leaves at 70 cm stem height or higher had fully developed, all lower leaves were cut off; this was also done with leaves and tillers emerging later in that region.

Induction of Proton Excretion

One to 2 d before an acidification cycle was to be induced, a plant was transferred to nutrient solution without Fe, Zn, Mn, and Co, in the following termed 'no metal' nutrient solution. Proton excretion was induced by replacing this solution with fresh medium containing 0.7 μM $ZnCl_2$ and 9 μM $MnCl_2$ (in experiments on $^{14}CO_2$ and $^{11}CO_2$ fixation) or 0.7 μM $ZnCl_2$ and 0.9 μM $MnCl_2$ (in experiments with ^{31}P-NMR and protein synthesis inhibitors'.

$^{14}CO_2$ Fixation

$^{14}CO_2$ fixation was studied with the roots of intact plants placed in 100 mL aerated nutrient solution (pH 5.3) at 25°C. The reaction was started by adding 10 μL $NaH^{14}CO_3$ (2 mCi·mL^{-1}) to the medium. After 30 min the roots, about 1 g fresh weight, were rinsed with cold, distilled water, blotted dry, excised and ground with 2.4 mL 10% TCA in mortar and pestle. Samples of 0.1 mL were added to 5 mL scintillation fluid (Picofluor) to determine radioactivity in triplo. Data were corrected for efficiency and quenching with an external standard. $^{14}CO_2$ was obtained from Amersham (England). Xylem exudate was obtained as described (6).

Labeling with ^{11}C

CO_2 labeled with short-lived ^{11}C ($t_{½}$ = 20 min) was used for noninvasive studies. ^{11}C is a positron emitter and can be measured by external counting of the two 511 keV photons which are emitted simultaneously in opposite directions on annihilation of the β^+ particle.

Continuous production of $^{11}CO_2$ with high specific activity was achieved by means of the photonuclear reaction $^{12}C(\gamma, n)^{11}C$ on CO_2-gas as the target compound. High energy photons were produced as Bremsstrahlung with an electron beam ($E_{e^-} \leq$ 140 MeV) generated by the linear accelerator at NIKHEF (4). The primary reaction of the recoiling ^{11}C atoms proceeded with traces of oxygen yielding ^{11}CO, converted for about 50% into $^{11}CO_2$ as a result of the accompanying radiation field. The irradiated CO_2 gas containing $^{11}CO_2$ and no-carrier-added ^{11}CO was transferred with a transfer time of about 15 to 20 min, for decay of short-living ^{15}O, to on-line chemical processing. Air was added at 10 mL·min^{-1} to maintain flow rate, after which CO_2 and associated ^{11}C activity was removed by passage over NaOH pellets. The remainder of the ^{11}C activity present as ^{11}CO was oxidized in flow to $^{11}CO_2$ by CuO at 650°C. The final radiochemically pure CO_2 product was finally delivered in a 30 mL·min^{-1} stream of normal air, resulting in a 40-mL·min^{-1} stream of air containing 0.025% [^{11}C]CO_2. The production rate was about 100 kBq $^{11}CO_2$ per min.

After germination for 6 d and transfer of the plants to Fe-free hydroponic culture as usual, a plant was transferred, between d 7 and 10, with its root system in a 200 mL-chamber thermostatted at 26°C, containing 100 mL Fe-free aerated nutrient solution. Two d later, the medium was replaced by no-metal nutrient solution. An experiment was performed 1 or 2 d later. The experiment was started by refreshing the medium with fresh nutrient solution with or without 0.7 μM Zn^{2+} and 9 μM Mn^{2+}. After 1 to 2 h the aerating air stream was replaced by the $^{11}CO_2$ containing air stream. Radioactivity in the vessel was determined in 20-s collecting periods by two 2 × 2 inch Na(T1)I scintillation crystals on both sides of the chamber. A double gated-coincidence standard technique was employed for counting (7, 18). For the determination of radioactivity in the roots, every 10 min the medium was rapidly pumped into a container behind a lead wall; the air stream containing $^{11}CO_2$ was diverted to this container. Then air of 26°C and 100% RH was directed through the experiment chamber at 200 ml·min^{-1} to remove $^{11}CO_2$. Radioactivity in the roots was determined in six measurements after the first measurement in which the radioactivity was lower than in the following one. Immediately afterward, the medium was pumped back into the chamber, and aeration with the $^{11}CO_2$ containing air was resumed. Radioactivity of the medium + roots was determined in six measurements before pumping away the medium. The $t_{½}$ of radioactive decay in the medium and in the roots was always 20 ± 1 min. A titrating pH meter with 1 mM KOH was connected to the chamber.

^{31}P-NMR Experiments

One to 2 d before an experiment, a plant of 25 to 30 d was placed with its roots in 0.35 M mannitol, 0.5 mM $CaSO_4$. This resulted in complete loss of root turgor within 5 min. The roots could then be carefully inserted in a 15 mm NMR tube filled with no-metal nutrient solution containing 0.1 mM phosphate. Perfusion of the tube was immediately started, simultaneously with medium at 5 ml·min^{-1} and air at 100 ml·h^{-1}, through glass capillaries reaching to the bottom (inlet) and the top of the tube. The roots regained their original turgor within 30 min. The stem was encased in a 25 mm diameter glass tube 70 cm long, which was taped to the spinner on top of the NMR tube. During transport of the plant to the NMR experiment station (7 h), perfusion was only with aerated no-metal nutrient solution, but at an elevated rate of at least 500 ml·h^{-1}, in order to prevent anaerobiosis in the roots. Light during transport was from a car headlamp at about 8 W·m^{-2}. From 1 d before the start of an experiment, P-free perfusion medium was used. After insertion of the plant with roots and stem in the NMR instrument (Bruker HX-360), the leaves were illuminated with mixed fluorescent and incandescent light of about 10 W·m^{-2}. For induction of proton excretion, 0.9 μM $ZnCl_2$ and 0.9 μM $MnCl_2$ were added to the perfusion medium, and the rate was increased to 80 mL·h^{-1} for 30 min, the air perfusion rate remaining constant

at 100 mL·h^{-1}, after which the medium perfusion rate was readjusted to 5 mL·h^{-1}. The volume of medium in the NMR tube was 10 mL, the roots were 2 to 3 g fresh weight. The temperature in the NMR probe was kept at 25°C. The ^{31}P-NMR measurements were carried out at 145 MHz in the pulse Fourier transform mode. Free induction decays were accumulated in a data table of 2048 words. The spectral width was 8064 Hz; the repetition time was 2 s.

RESULTS

Divalent metal ions can activate proton extrusion by Fe-deficient roots at two kinds of sites: (a) in the metabolic pathway leading to production of carboxylates (15); (b) in the plasma membrane of the rhizodermal transfer cells, where the protons are excreted (15).

These sites may be distinguished by comparing the kinetics of CO_2 fixation and proton excretion, and by monitoring changes in the cellular pH values with ^{31}P-NMR.

Intact plants had to be used, because fast extrusion of protons at the roots requires a steady flow of carbohydrates from the leaves (14). Both techniques permit a comparison of the induced state (+Zn, Mn) with the uninduced state (−Zn, Mn) in the same plant on two consecutive days, providing an extra advantage considering the high degree of variability shown by the plants.

Effect of Proton Excretion of CO_2 Fixation in Roots of Intact Plants

Labeling with $^{14}CO_2$ was used to determine the influence of proton excretion on overall CO_2 fixation at the roots. Figure 1 shows that CO_2 fixation was increased in Fe-deficient roots. Addition of Zn + Mn resulted in a threefold stimulation of this already elevated rate, and Fe-sufficient roots did not respond.

The onset of proton excretion and the increase in CO_2 fixation showed the same lag period (about 3 h in this experiment). Citrate and malate appeared in the xylem exudate (Fig. 2) at the same time as proton excretion and increased CO_2 fixation.

For each measurement either of CO_2 fixation or of malate and citrate a plant had to be sacrificed. The plants showed considerable variation in the lag period and rate of medium acidification. It was therefore impossible to correlate the kinetics of carboxylation and proton excretion with a precision better than down to 1 to 2 h. This was insufficient to observe a significant difference, though the main conclusion remains that both extra organic acid formation and proton excretion were induced within 2 to 6 h.

Simultaneous Monitoring of $^{11}CO_2$ Fixation and H^+ Excretion with Single Plants

In roots of Fe-sufficient plants standing in a solution which was aerated with $^{11}CO_2$ containing air, radioactivity in the roots reached a plateau within 30 min after medium was at equilibrium with $^{11}CO_2$. Root radioactivity (3–5% of medium

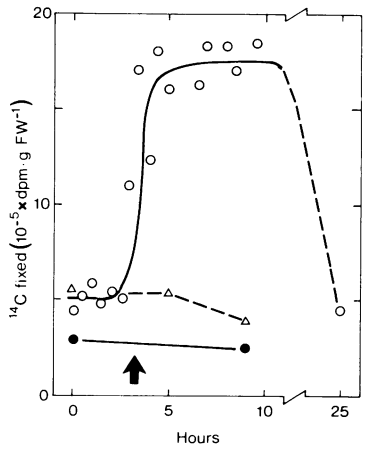

Figure 1. $^{14}CO_2$ fixation by roots of bean plants. At t = 0, 0.7 μM Zn^{2+} + 9 μM Mn^{2+} were added to the nutrient solution of 2 Fe-sufficient (●——●), and 18 Fe-deficient (○——○) plants; the mean starting point of medium acidification by the Fe-deficient plants harvested after t = 3 h is indicated (arrow). To three Fe-deficient plants, no Zn and Mn were added (△ – – – △).

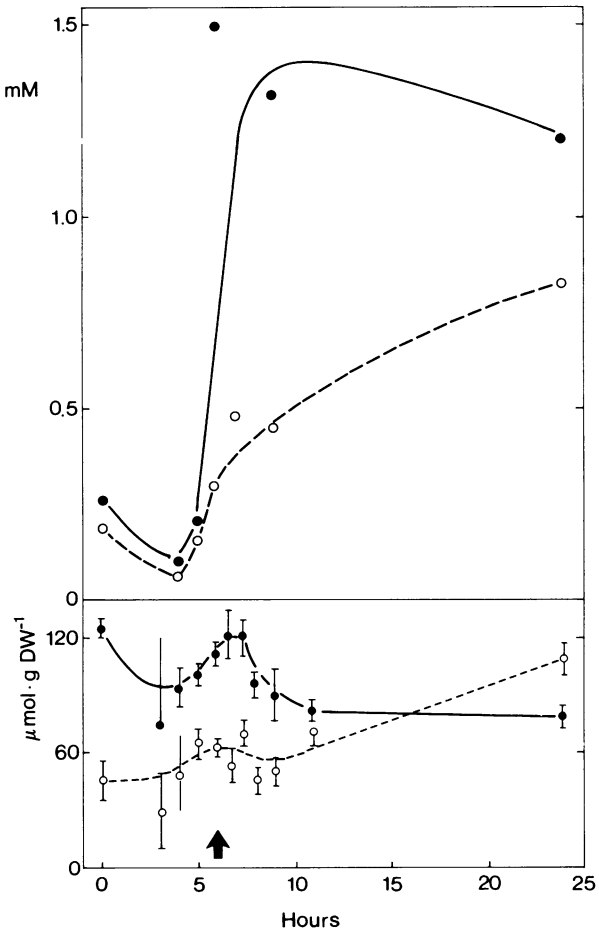

Figure 2. Citrate (○) and malate (●) in roots (bottom) and xylem sap (top) of Fe-deficient bean plants after addition of 0.7 μM Zn^{2+} + 9 μM Mn^{2+} to the nutrient solution. The mean starting point of medium acidification of plants harvested after t = 6 h is indicated (arrow). Bottom, each determination is the mean of results from three plants; bars indicate SD.

+ roots) represented a steady state resulting from uptake and fixation and storage on the one hand, and removal via the xylem, breakdown leading to freely diffusing $^{11}CO_2$ and radioactive decay ($t_{1/2}$ 20 min), on the other. The steady state level of ^{11}C in the roots was much higher in Fe-deficient plants which were actively excreting protons (10–15% of the medium + roots). Since organic acid anions formed during proton excretion were largely exported via the xylem (Fig. 2; *cf.* ref. 6), this increase is most likely caused by increased CO_2 fixation rate (see Fig. 1). It took more than 1 h to reach the steady state in roots of Fe-deficient plants.

Figure 3 shows results obtained with a single plant with increasing Fe-deficiency. At d 10, the steady state level of $^{11}CO_2$ fixation was low (4% of medium + roots level) and this was reached within 30 min. One d later, the steady state level had risen to 8%, an increase which could not be explained by growth of the roots (about 10%·d^{-1} under those conditions). A short cycle of proton excretion started 2 h after induction with Zn + Mn, reaching a maximum 2 h later. The steady state ^{11}C level in the roots closely followed the proton excretion rate, within the resolving power of this experiment (20 min). In duplicate experiments the kinetics of H^+ extrusion rates and ^{11}C fixation rates showed the same close coupling.

Measurements with ^{51}P-NMR

So as to fit the plants on the measuring instrument, they were grown with long stems by spraying once 1 mM GA on the primary leaves 8 d after sowing. This treatment had no effect on the development of the proton excretion capacity as a response to Fe shortage. This was also the case for the short treatment (less than 10 min) of the roots with 0.35 M mannitol before insertion in the NMR tube.

Addition of 0.7 μM $ZnCl_2$ + 0.9 μM $MnCl_2$ to the perfusion medium for the roots resulted in a slow proton extrusion with a longer lag period (8–10 h) than normally found with intact plants (2–6 h). This may have been due to the tight packing of the roots in the NMR tube, which could prevent fast uptake of Zn and Mn ions. Also, the Mn^{2+} dose had to be reduced (0.9 μM instead of 9 μM) because within 1 h after adding 9 μM Mn the phosphate signal from the roots acquired a broad asymmetric line shape. This was probably due to interaction between cellular phosphate and Mn ions (17).

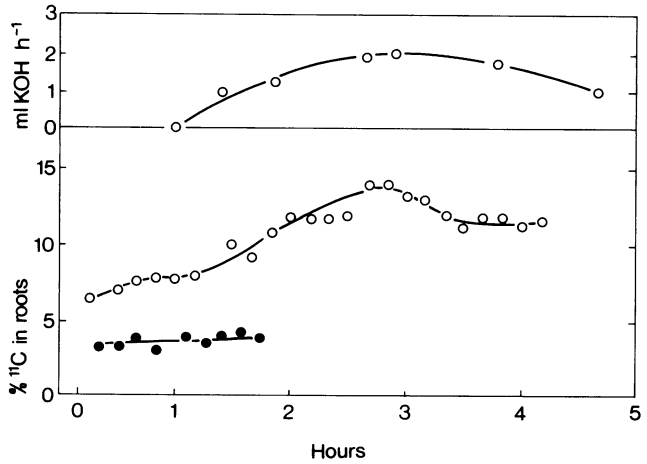

Figure 3. Proton excretion rates (top) and steady state ^{11}C levels resulting from $^{11}CO_2$ fixation, ^{11}C-decay and export in roots of a bean plant developing Fe-deficiency (bottom). (●), ^{11}C levels at d 10, before chlorosis appeared; no proton extrusion; (○), ^{11}C levels at d 11, with beginning chlorosis; proton extrusion at constant pH was induced by adding 0.7 μM Zn^{2+} + 0.9 μM Mn^{2+}, 1 h before the flux of $^{11}CO_2$ started, and measured by titration with 1 mM KOH (top). Highest proton excretion rate reached was about 1 μmol H^+·g fresh weight ·h^{-1}.

The NMR spectrum showed one peak which could not be resolved further (Fig. 4). The signal was obtained from the youngest part of the roots (apical 5 cm) so that the most apical parts, which might have given a cytoplasmic phosphate signal, probably had a vanishingly small influence on the total signal, which must have been mainly determined by vacuolar phosphate in the subapical 0.5 to 5.0 cm segments where, also, the induced proton extrusion took place (15). Subapical root segments of pea, grown on a comparable phosphate regime, showed essentially the same signal (Fig. 2b in ref. 16), which is largely due to vacuolar phosphate (27). Factors introducing noise, air bubbling and inhomogeneous packing of the roots, made it necessary to assemble spectra in 2-h periods.

When no Zn and Mn were added to the perfusion medium, the peak of the signal remained constant for 20 h. The pH of the perfusion medium increased slightly or remained constant as well. About 8 h after adding Zn and Mn, the position of the NMR peak gradually shifted, indicating an increase of pH (Fig. 5). The shape of the peak was unchanged during this transition. Starting 11 h after adding Zn + Mn, the perfusion medium was gradually acidified.

Effect of Protein Synthesis Inhibitors

The (Zn + Mn) induced proton excretion by Fe-deficient plants could be inhibited by cycloheximide (5 μg/mL) or hygromycin (25 μg/mL) (5). The effect of both compounds showed a lag period of 1.5 to 2 h (Fig. 6). This lag period was the same with hygromycin concentrations between 25 and 200 μg/mL (data not shown).

DISCUSSION

$^{14}CO_2$ fixation in bean roots was stimulated upon Fe-deficiency (Fig. 1; *cf.* ref. 21), even in the absence of proton

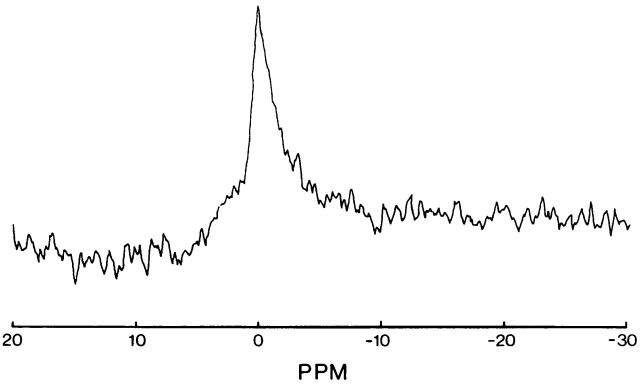

Figure 4. ^{31}P-NMR spectrum of the young part of the roots of an Fe-deficient bean plant (1800 scans).

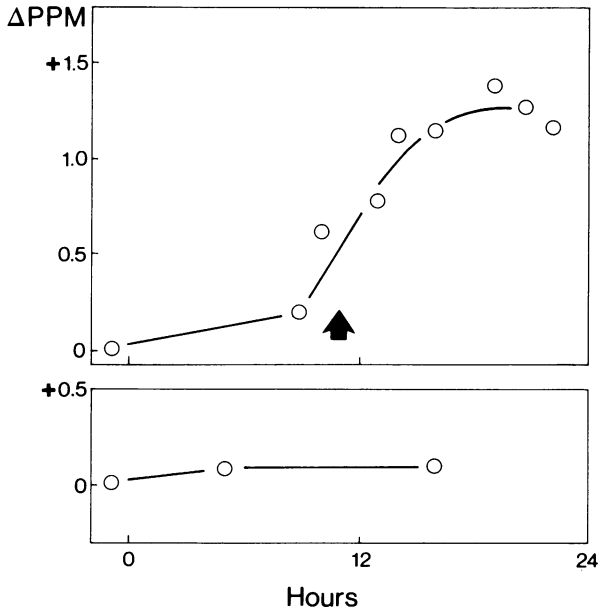

Figure 5. Shift in the main peak in a ^{31}P-NMR spectrum of an Fe-deficient bean plant after induction of proton extrusion by addition of 0.7 μM Zn^{2+} + 0.9 μM Mn^{2+} at t = 0 (top). Arrow, start of medium acidification. Bottom, the same plant 2 d later, no addition of Zn + Mn.

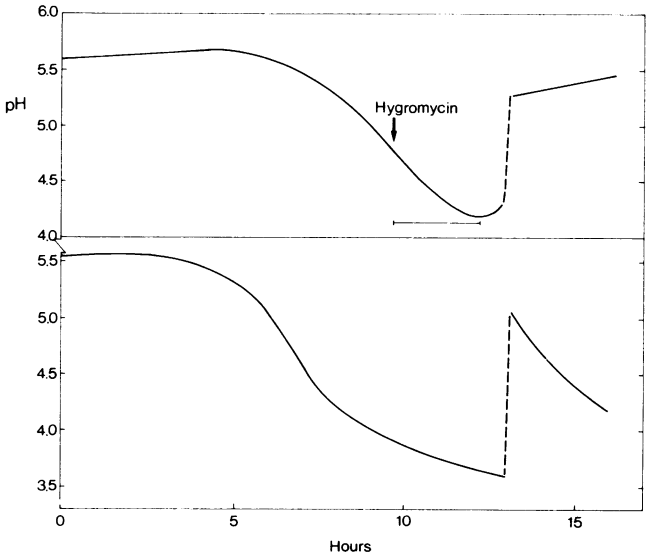

Figure 6. Proton extrusion by roots of an Fe-deficient bean plant after induction by 0.7 μM Zn^{2+} + 0.9 μM Mn^{2+} at t = 0 (bottom), and inhibition by hygromycin (top). In both incubations the pH was adjusted to 5.3 at t = 13 h. The lag period for inhibition was determined as indicated.

extrusion. This situation is comparable to that in Fe-deficient grasses, where malate and citrate are accumulated, without protons excreting from the roots (13, 28). After induction with Zn + Mn, CO_2 fixation was so greatly increased and closely coupled to H^+ excretion, that the main pathway of CO_2 in the roots must at that time have been into malate and citrate, via PEP carboxylase (9, 15).

The experiments with $^{11}CO_2$ showed a strong increase of steady state ^{11}C levels in the roots during proton excretion. Moreover, the time needed to reach this steady state was considerably lengthened (From 30 min to 1–2 h). During normal metabolism most of the carbon which is fixed into malate is rapidly released by oxidative degradation. During proton excretion, the carbon incorporated concomitantly into citrate and malate is exported via the xylem (6; Fig. 2). To minimize disturbance of the cytosolic pH, carboxylation must take place in the rhizodermal transfer cells that secrete the protons (15). Thus, the newly formed malate and citrate, on their way to the xylem, must pass along the vacuoles of the cortex cells, which contain a pool of malate and citrate (8, 13). The long period needed to reach steady state in the roots could therefore reflect equilibration with vacuolar contents.

The experiments with $^{11}CO_2$ fixation showed a tight coupling of CO_2 fixation and proton excretion, no difference being observable in the kinetics within the resolving power of the technique (20 min).

The experiments with ^{31}P-NMR were hampered by technical restraints that arose from the system studied. Whole root systems connected to stems carrying photosynthesizing leaves had to be used (3, 15). The resulting inhomogeneity in the tube caused a low signal to noise ratio which made long acquisition periods necessary. The signal must have been mainly from vacuolar phosphate (16, 27) in the cells behind the elongation zone, where induced proton excretion takes place. The results (Fig. 5) then imply that, when proton extrusion started, the vacuolar pH shifted to more alkaline.

Reid *et al.* (20) reported an increase in cytosolic pH of 3-mm barley root tips upon incubation with fusicoccin within 1 h. In longer incubations of *Acer pseudoplatanus* cells (up to 10 h) with fusicoccin, a large increase of vacuolar pH was observed (12). The simplest explanation for our results then is that here, too, the vacuolar pH increased as a response to cytosolic alkalization. In that case, Zn + Mn induce by activating a proton pump. Theoretically, Zn + Mn might induce by inhibiting the tonoplast proton pump (heavy metal intoxication), which might then lead to cytosol acidification and proton extrusion. We cannot exclude this possibility as we could not obtain a signal of subapical root cytosol phosphate, but we think that, at the low concentrations used, this is not very probable. Heavy metal intoxication would interfere with more cellular processes, leading to inhibition of active proton excretion.

One possibility of Zn + Mn induced proton removal from the cell is that the metal ions directly activate a proton pumping ATPase in the plasma membrane. This enzyme activity, located in the Fe-deficiency induced rhizodermal transfer cells, is different from the proton pump which is stimulated by fusicoccin (24).

For a direct activation of a proton pumping ATPase by divalent metal ions, the lag period after adding of Zn^{2+} + Mn^{2+} is long (3–6 h under normal conditions). This is remarkable since the cells with the proton pumping ATPase to be activated are localized at the root surface (11), and since Fe-deficient plants absorb Zn and Mn ions at an increased rate (22). The long lag suggests that Zn and Mn do not act

directly on the proton pump, but indirectly via a time-consuming cellular process.

The protein synthesis inhibitors cycloheximide and hygromycin (5) both acted with a lag period of about 2 h (Fig. 6). If these compounds would interact directly with the plasma membrane ATPase, the lag period until inhibition would have been determined by the rate of uptake by the roots. However, since the lag period was the same over an eightfold range of hygromycin concentrations, this could not be the case. The simplest explanation is that cycloheximide and hygromycin stop proton extrusion indirectly, by inhibiting protein synthesis. This would mean that for proton extrusion to proceed, continuous synthesis of a polypeptide is necessary. The lag period for inhibition then reflects a short lifetime of the polypeptide. The lag period for activation by divalent metal ions would reflect the time needed for synthesis of the polypeptide.

Recently (1), Fe-deficiency induced proteins in plasma membrane enriched fractions from tomato roots were found, and a model for the regulation of Fe-efficiency reactions in dicotyledons was postulated. According to this model, an iron-sensing protein controls the transcription of genes that are involved in Fe-efficiency reactions by reversible binding to *cis*-regulating elements. Zinc has been shown to play an essential role in the recognition of nucleic acid by such controlling proteins in a large number of cases (10).

In conclusion, the present results indicate that in roots of Fe-deficient bean plants, divalent metal ions stimulate proton extrusion by activating the synthesis of a labile polypeptide. On the basis of the NMR measurements, we suggest that the peptide plays a role in the plasma membrane, *e.g.* as a component or an activator for a proton pumping ATPase in the rhizodermal transfer cells.

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