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Metabolic Implications in the Biochemical Responses to Iron Deficiency in Cucumber (*Cucumis sativus* L.) Roots[1]

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Strategy I plants respond to Fe deficiency by inducing morphological and biochemical modifications at the root level that are apt to make iron available for uptake. Cucumber (*Cucumis sativus* L.) grown in the absence of Fe has been shown to increase the capacity to acidify the rhizosphere and Fe^{3+} reduction activity. We have determined in these roots some metabolic activities that might be correlated with the increased proton extrusion. Proton efflux from roots may be followed by a mechanism regulating the cytosolic pH according to the pH-stat theory. Roots grown in the absence of Fe showed an increase in dark $^{14}CO_2$ fixation and organic acid synthesis and a 6-fold increase in the extractable phospho*enol*pyruvate carboxylase activity with respect to the control roots. Dehydrogenase activities producing cytosolic NAD(P)H were also increased under Fe deficiency. The presence of Fe^{2+}, but not Fe^{3+}, inhibited dark $^{14}CO_2$ fixation in a range between 24 and 52% but did not show any effect on the in vitro phospho*enol*pyruvate carboxylase activity.

Plants have evolved different adaptive mechanisms to mobilize and increase the availability of Fe at the root-soil interface. The adaptive processes exhibited by plant species classified as strategy I under Fe-deficiency growth conditions are characterized by: (a) a higher active proton extrusion, which decreases the pH in the free space of the roots and in the rhizosphere and influences the availability of Fe compounds and possibly the reduction of Fe^{3+} to Fe^{2+} (Brown, 1978; Olsen and Brown, 1980; Wallace and Wallace, 1984; Zocchi and Cocucci, 1990); (b) an enhancement of Fe^{3+} reduction to Fe^{2+} at the plasmalemma level with a preferential uptake for Fe^{2+} (Chaney et al., 1972); (c) a release of reducing and/or chelating substances such as dihydroxyphenols, which could increase the concentration of soluble Fe by the roots under certain conditions (Hether et al., 1984); and (d) morphological changes such as root hair enhancement (Landsberg, 1982) and inhibition of root extension accompanied by additional cell division in the rhizodermal layer and formation of rhizodermal transfer cells (Kramer et al., 1980; Landsberg, 1982; Römheld, 1987).

Proton extrusion and Fe^{3+} reduction are linked, respectively, to the activity of an H^+-ATPase and to an NADH: Fe^{3+}-chelate reductase localized on the plasma membrane and presumably confined to the apical root zone (Römheld and Marschner, 1981; Landsberg, 1982; Römheld et al., 1984; Zocchi and Cocucci, 1990; Alcantara et al., 1991; Rabotti and Zocchi, 1994, and refs. therein).

Active proton extrusion induced by Fe-deficiency stress is a sophisticated and important mechanism that involves other biochemical and physiological processes in plants and chemical processes in soil.

The pH decrease in the rhizosphere favors an increase in the mobilization of sparingly soluble inorganic Fe^{3+}. A lower rhizosphere pH was in fact demonstrated to be responsible for solubilization of Fe from Fe oxides at the root surface (Chapman, 1939). The availability of Fe^{3+} depends on the pH: its decrease from 8.0 to 4.0 will increase the concentration of Fe^{3+} from 10^{-20} to 10^{-8} M (Römheld and Marschner, 1986).

Many authors have found that a high proton extrusion by Fe-deficient roots is linked to an increase in the synthesis and in the accumulation of organic acids, in particular citric and malic acids (Landsberg, 1981; de Vos et al., 1986). The link between higher proton extrusion and enhanced synthesis of organic acids could be explained by the fact that the proton efflux causes cytoplasm alkalinization, which in turn activates the dark fixation of CO_2 (Smith and Raven, 1979; Bienfait et al., 1989; Guern et al., 1991), a reaction that is catalyzed by the PEP carboxylase (pH-stat theory) (Davies, 1973).

In the present investigation we have used a well-characterized strategy I plant to better define the relationships among the biochemical mechanisms involved in the response to the Fe-deficiency stress and the possible link between active proton extrusion and the proton-generating metabolism.

MATERIALS AND METHODS

Plant Material

Cucumber seeds (*Cucumis sativus* L. cv Marketer from F.lli Ingegnoli, Milan, Italy) were germinated in Agriperlite moistened with 0.1 mM $CaSO_4$ for 4 d in the dark at 26°C and then transferred for hydroponic culture as already reported (Zocchi and Cocucci, 1990; Rabotti and Zocchi, 1994). Culture medium was supplied (Fe-sufficient plants) or not (Fe-deficient plants) with 0.1 mM Fe^{3+}-EDTA. A

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Abbreviations: PD, transmembrane electrical potential; pH_c, cytosolic pH.

day/night regime of 16/8 h and a PPFD of 200 μmol m^{-2} s^{-1} at the plant level, measured with a Li-Cor (Lincoln, NE) quantum radiometer model LI-1000, were maintained in a growth chamber. The temperature was 18°C in the dark with an RH of 60% and 24°C in the light with an RH of 80%. Roots of 10- to 12-d-old plants were used throughout the work (i.e. after acidification of the culture medium occurred).

Dark $^{14}CO_2$ Fixation and Acidification Capacity

Batches of 25 subapical root segments from Fe-deficient and Fe-sufficient plants were preincubated in 10 mL of a buffer containing 5 mM Suc and 0.5 mM $CaSO_4$ (pH 6.0 with NaOH) in Erlenmeyer flasks in a shaking water bath at 26°C. After 1 h of incubation, the buffer was substituted with 10 mL of the same fresh solution, and the pH change of the medium was recorded with a Radiometer (Copenhagen, Denmark) pHM 64 pH-meter (acidification capacity) or with 10 mL of the same fresh solution supplemented with $NaH^{14}CO_3$ (37 kBq/mL). In the latter case the incubation was performed in sealed flasks to avoid loss of $^{14}CO_2$, and root segments were incubated for the desired time to determine the $^{14}CO_2$ incorporation capacity (time course). At the end of the incubation period, root segments were rinsed in distilled water, blotted on a paper towel, weighed, and homogenized in 0.1 mL of 0.1 N HNO_3 to eliminate the $^{14}CO_2$ not incorporated. Ten milliliters of Ready Solv MP (Beckman) scintillation cocktail were added and the radioactivity was measured in a liquid scintillation counter (Beckman LS 7500). Alternatively, after the desired time of incubation, 0.1 mL of 4.0 mM $NaH^{14}CO_3$ (37 kBq/mL) was added to 9.9 mL of the solution, the flasks were hermetically sealed with a rubber cap, and the incubation was continued for 15 min (Morgutti et al., 1984) in the presence or in the absence of Fe^{2+} or Fe^{3+} (as sulfate salts and both at 0.1 mM). At the end of the labeling period the radioactive medium was sucked off and the segments were rinsed and treated as reported above for the time course.

Enzyme Assays

Roots from plants grown in the presence or in the absence of Fe were excised, rinsed in distilled water, and homogenized in a mortar at 2 to 4°C with 4 volumes of a buffer containing 330 mM Suc, 50 mM Mops-1,3-bis(tris [hydroxymethyl]methylamino)-propane (pH 7.5), 3 mM EGTA, 5 mM DTT, and 2 μg mL^{-1} PMSF.

The homogenate was filtered through four layers of gauze and centrifuged at 13,000*g* for 15 min. The supernatant was again centrifuged at 100,000*g* for 60 min. Pellets were discarded, soluble proteins in the supernatant were concentrated by precipitation with $(NH_4)_2SO_4$ at 80% saturation, and the precipitate was collected by centrifugation at 13,000*g* for 15 min. Proteins were resuspended in 3 mL of buffer containing 20 mM Tris-HCl (pH 8.0), 10 mM KCl and dialyzed against 2 L of the same buffer for 3 h at 4°C with two changes. The extracts were clarified by centrifugation at 13,000*g* for 15 min and used for the enzymatic assays.

PEP carboxylase was determined by coupling its activity to malate dehydrogenase-catalyzed NADH oxidation in a buffer containing 100 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 4 mM Na_3PEP, 0.2 mM NADH, 10 mM $NaHCO_3$, and 15 μg mL^{-1} malate dehydrogenase (Boehringer-Mannheim) at 26°C. The reaction was started by adding aliquots of the protein extracts.

Glyceraldehyde-3-P dehydrogenase was determined in a buffer containing 82.5 mM triethanolamine-NaOH (pH 7.6), 2 mM $MgSO_4$, 6 mM 3-phosphoglycerate, 1 mM ATP, 0.2 mM NADH, 1 mM EDTA, and different aliquots of protein extracts. The reaction was started by adding 1 μg/mL 3-phosphoglycerate kinase (Boehringer-Mannheim).

Glc-6-P dehydrogenase was assayed in a buffer containing 82.5 mM triethanolamine-NaOH (pH 7.6), 6.2 mM $MgCl_2$, 1.2 mM Glc-6-P, and 0.4 mM $NADP^+$. The reaction was started by adding aliquots of the protein extracts.

$NADP^+$-dependent malic enzyme was determined in a buffer containing 65 mM triethanolamine-NaOH (pH 7.4), 4 mM $MnCl_2$, 0.5 mM L-malate, and 0.225 mM $NADP^+$, according to the method of Hsu and Lardy (1969). The reaction was started by adding aliquots of the protein extracts.

All enzymatic assays were performed at 26°C and in a 1-mL final volume. Oxidation of NADH or reduction of $NADP^+$ were followed spectrophotometrically at 340 nm with a Beckman DU-50.

Proteins in the extracts were determined by the Bradford (1976) procedure using γ-globulin as the standard.

Organic Acids Assay

Roots of plants grown in the presence or in the absence of Fe were excised, rinsed in distilled water, homogenized with 5 mL of 10% (v/v) perchloric acid, and centrifuged for 15 min at 10,000*g*. The pH of the supernatant was brought to pH 7.5 with 0.5 M K_2CO_3 to neutralize the acidity and to precipitate the perchlorate. The extract was clarified with another centrifugation at 15,000*g* for 15 min. Citric and malic acid contents were determined enzymatically, using specific kits from Boehringer-Mannheim; the assay was performed according to the manufacturer's instructions. Recovery of both organic acids was more than 90% as determined by use of an internal standard.

RESULTS

Organic Acid Synthesis in Fe-Deficient Cucumber Roots

It has been previously shown that Fe-deficient cucumber roots are able to acidify the external medium to a greater extent with respect to the control (Zocchi and Cocucci, 1990). This major capacity of acidification is linked to the activation of proton extrusion brought about by an H^+-ATPase localized on the plasmalemma (Zocchi and Cocucci, 1990; Rabotti and Zocchi, 1994). As a consequence, the result is an alkalinization of the cytosol and a more negative charge inside (PD more negative) (Zocchi and Cocucci, 1990). The possible involvement of the metabolism in the control of proton extrusion and in the re-equilibration of pH_c has been assessed by determining the activity of the intracellular proton-generating system(s).

Table I. *Concentration of malic and citric acids in roots of plants grown in the absence (−Fe) or in the presence (+Fe) of 0.1 mM Fe^{3+}EDTA*

Values are the means ± SE of three experiments in triplicate.

Malic Acid		Citric Acid	
+Fe	−Fe	+Fe	−Fe
μg g^{-1} fresh wt			
598 ± 42	1608 ± 111	85.5 ± 4.3	407.5 ± 26

Table I shows that under Fe deficiency in cucumber roots there was a remarkable increase in the organic acid concentration, in particular citric and malic acids. These data confirm previous findings by other authors (Brown, 1978; Landsberg, 1981) who showed that in this nutritional stress condition strategy I plants increase the synthesis of these compounds.

Dark $^{14}CO_2$ Fixation and Effect of Different Forms of Fe

The greatest active proton extrusion and, therefore, the hyperpolarization of the membrane potential found in Fe-deficient roots and the accumulation of malic and citric acids could be regulated at a metabolic level owing to the activation of an organic-acid-producing mechanism, through an increase in the dark fixation of CO_2, with a consequent increase in cytosolic proton concentration, according to the pH-stat theory (Davies, 1973).

The time courses of H^+ extrusion (Fig. 1A) and dark $^{14}CO_2$ fixation (Fig. 1B) in root segments from plants grown in the presence or in the absence of Fe were compared. Fe-deficient roots showed a greater capacity to incorporate $^{14}CO_2$ and this incorporation correlated well with proton extrusion. The close correlation between $^{14}CO_2$ incorporation and proton extrusion might be further evidence that the two mechanisms are strictly linked to each other.

Fe^{2+} and Fe^{3+} have been shown to differentially affect both medium acidification and the PD, with only Fe^{2+} showing an inhibitory effect (Zocchi and Cocucci, 1990). A decrease in proton extrusion with the consequent depolarization of PD in the presence of Fe^{2+} might be followed (or preceded) by a diminished capacity for dark CO_2 fixation if this is the mechanism producing H^+. We have tested the effect of different forms of Fe on the $^{14}CO_2$ incorporation in Fe-deficient roots. Again, Fe^{2+} but not Fe^{3+} inhibited dark $^{14}CO_2$ fixation (Table II). The inhibition exerted by Fe^{2+} increased with the time of incubation from 24 to 52%.

Activation of plasmalemma Fe^{3+}:NAD(P)H-reductase is believed to be a crucial event in strategy I plants, since only the Fe^{2+} form can be taken up (Chaney et al., 1972). Reducing equivalents (NADH and/or NADPH) necessary to sustain the Fe^{3+}-chelate reductase activity presumably come from cytosolic dehydrogenases. We have determined the activity of three cytosolic dehydrogenases; two of them, the NAD-glyceraldehyde-3-P and the NADP-Glc-6-P dehydrogenase activities, are greatly enhanced under conditions of Fe deficiency (Table III), whereas the third enzyme of this class, the NADP-dependent malic enzyme is only slightly increased by Fe deficiency (Table III).

PEP Carboxylase Activity in Vitro

Dark $^{14}CO_2$ fixation is catalyzed by the activity of the PEP carboxylase and this fixation was inhibited by Fe^{2+} (Table II). To investigate the effect of Fe deficiency and the effect of different forms of Fe on the PEP carboxylase activity we have extracted the enzyme from both Fe-deficient and Fe-sufficient plant roots. PEP carboxylase prepared from roots grown in the absence of Fe showed a 6-fold increase in the activity with respect to the control roots (Table IV). This great increase would explain the rapid and larger incorporation of $^{14}CO_2$ seen before. Furthermore, this activity was found not to be affected, unlike the dark CO_2 fixation, by the presence of the two ionic forms of Fe added at different concentrations, up to 10^{-3} M (Table IV).

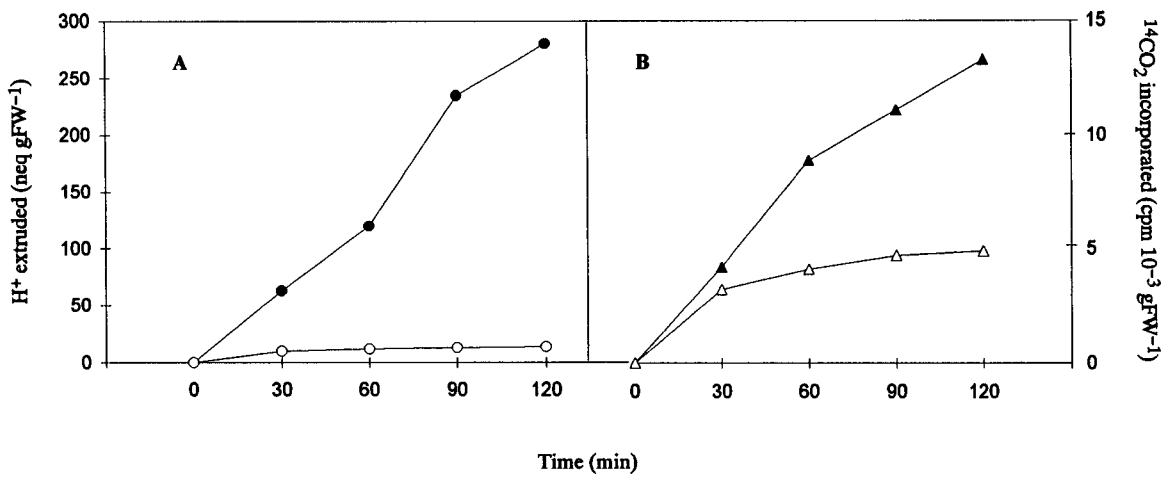

Figure 1. Time course of medium acidification (A) and dark $^{14}CO_2$ fixation (B) in root segments of plants grown in the presence (○, △) or in the absence of Fe (●, ▲). Each point is the mean of four independent experiments. SE did not exceed ±7%. FW, Fresh weight.

Table II. *Dark $^{14}CO_2$ fixation activity in root segments grown in the absence of Fe: Effect of different forms of Fe*

Fe was added as sulfate salts, at 0.1 mM. $^{14}CO_2$ incorporation was for 15 min. Numbers in parentheses represent the percentages of inhibition. Values are the means ± SE of three experiments in duplicate.

Treatment	Dark $^{14}CO_2$ Fixation at Interval Times		
	15–30 min	45–60 min	105–120 min
	nmol CO_2 g^{-1} fresh wt		
Control	18.1 ± 0.91	21.2 ± 1.27	24.5 ± 1.55
+Fe^{2+}	13.8 ± 0.96 (24)	11.9 ± 0.52 (44)	11.7 ± 0.57 (52)
+Fe^{3+}	18.5 ± 1.05	19.8 ± 1.18	24.1 ± 1.21

DISCUSSION

Cucumber roots have been shown to respond to Fe deficiency by greatly increasing medium acidification and Fe^{3+} reductase activities (Zocchi and Cocucci, 1990; Alcantara et al., 1991; Rabotti and Zocchi, 1994). Rhizosphere acidification, brought about by an increased plasma membrane H^+-ATPase activity, along with a higher capacity to reduce Fe^{3+} through the activation of a NADH-Fe^{3+} chelate reductase on the same membrane, poses some questions of how protons or reducing equivalents can be obtained in roots of Fe-deficient cells to sustain both of these fundamental activities. Active proton extrusion will rapidly induce alkalinization of cytosol (Felle, 1988). In agreement with the pH-stat theory (Davies, 1973) carboxylation of PEP to form organic acid will bring the pH_c back to its homeostatic value. Furthermore, oxidation of NAD(P)H necessary to sustain the rapid increase in the activity of plasma membrane reductase will release other protons inside the cell if only electrons are transferred to external acceptors, helping to decrease the pH_c and successively leading to the activation of the H^+-ATPase (Rubinstein and Stern, 1986; Marrè et al., 1988).

The data obtained through this work taken together are well in agreement with the proposed hypothesis. Fe deficiency increased extractable PEP carboxylase activity (Table IV), and this was correlated with a great increase in the dark CO_2 fixation (Fig. 1B; Table II) and organic acid synthesis (Table I). In addition, all of the dehydrogenase activities assayed showed an increase during the Fe-deficiency condition (Table III).

It might be of great interest to understand which of these events comes first, but irrespective of which is the cause and which is the effect, the final result will be the same. The tight link among these single mechanisms will guarantee a cascade of responses, which in the end will produce the proton extrusion and transmembrane electron transfer and the poise of the pH_c and the PD. Transmembrane electron transfer in the presence of external Fe^{3+} as acceptor has been shown to depolarize the electrical potential in beans (Sijmons et al., 1984) and to a lesser extent in cucumber (Zocchi and Cocucci, 1990). Activation of proton extrusion would also guarantee repolarization of the cell though K^+ leakage (seen under Fe deficiency) (Sijmons et al., 1984; Alcantara et al., 1991) and might help the cell to keep the electrical potential more negative.

From these data it is clear that these mechanisms must be metabolically strictly correlated with each other. The biochemical responses of Fe-deficient plants reflect a strategy that has as the ultimate result the mobilization of rhizodermal Fe, its reduction, and its uptake. Activation of proton extrusion through the plasmalemma H^+-ATPase, which in Fe-deficient cucumber plants is increased by 2-fold (Rabotti and Zocchi, 1994), will result in the acidification of the rhizodermal space with an increased solubilization of Fe compounds. In addition, the increased PD (negative inside) would guarantee the driving force for the uptake of Fe^{2+} after it has been reduced.

An interesting point to comment on is the different effect that the Fe forms have on the activities assayed. Only Fe^{2+} showed some influences. Its effect seems to be principally on the proton-generating metabolism. In fact, proton extrusion and PD are negatively affected by Fe^{2+} (Zocchi and Cocucci, 1990). The effect of Fe^{2+} seems to be specific, since other divalent cations such as Ni^{2+}, Co^{2+}, Zn^{2+}, and Mn^{2+} stimulate proton extrusion in maize root segments (Marrè et al., 1982; Morgutti et al., 1984; Cocucci and Morgutti, 1986). This is in good agreement with the inhibitory effect shown by Fe^{2+} on the H^+-ATPase activity (Rabotti and Zocchi, 1994). Also in this case the effects of other divalent cations are quite different. Among them only Ni^{2+} and Zn^{2+} have been shown to have an effect on an Mg^{2+}-stimulated ATPase from maize root microsomes, although the concentration used in that work was 20-fold higher (Marrè et al., 1982). On the contrary, whereas dark CO_2 fixation is sharply decreased by the presence of Fe^{2+}, in this case the enzyme directly involved in the reaction, i.e.

Table III. *Glyceraldehyde-3-P dehydrogenase (G3P-DH), Glc-6-P dehydrogenase (G6P-DH), and malic enzyme activities in roots grown in the absence (−Fe) or in the presence (+Fe) of 0.1 mM Fe^{3+}EDTA*

Values are the means ± SE of four experiments in triplicate.

Enzyme	+Fe	−Fe	Percentage Increase
	nmol NAD(P)H mg^{-1} protein min^{-1}		
G3P-DH	3.3 ± 0.18	7.1 ± 0.67	+115
G6P-DH	79.9 ± 6.18	135.0 ± 12.1	+76
Malic enzyme	46.6 ± 2.6	64.7 ± 4.4	+39

Table IV. *PEP carboxylase activity extracted from roots grown in the absence (−Fe) or in the presence (+Fe) of 0.1 mM Fe^{3+}EDTA*

Effect of Fe^{2+} and Fe^{3+} forms added at different concentrations as sulfate salts. Values are the means ± SE of four different experiments in triplicate.

Treatment	Concentration	PEP Carboxylase Activity	
		+Fe	−Fe
	M	*μmol NADH mg^{-1} protein min^{-1}*	
Control	—	0.22 ± 0.014	1.35 ± 0.08
Fe^{2+}	10^{-5}	0.21 ± 0.012	1.25 ± 0.07
	10^{-4}	0.20 ± 0.012	1.24 ± 0.07
	10^{-3}	0.21 ± 0.012	1.30 ± 0.08
Fe^{3+}	10^{-5}	0.21 ± 0.011	1.24 ± 0.06
	10^{-4}	0.21 ± 0.012	1.28 ± 0.06
	10^{-3}	0.20 ± 0.011	1.26 ± 0.06

the PEP carboxylase, is not affected even at higher Fe^{2+} concentrations (up to 1 mM). In maize root segments PEP carboxylase activity was already inhibited by Ni^{2+} at 0.5 mM (Morgutti et al., 1984), showing a difference in behavior between Fe^{2+} and Ni^{2+}. Other divalent cations are able to affect dark CO_2 fixation to the same extent, although their concentrations were 10-fold higher (Cocucci and Morgutti, 1986), but nothing is known about their effect on the PEP carboxylase activity. The effect on the H^+-ATPase and the lack of effect on the PEP carboxylase activity might support the hypothesis that dark CO_2 fixation and the consequent organic acid synthesis are the effect of the pH_c alkalinization brought about by the primary active proton extrusion.

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