Rhizosphere Acidification as a Response to Iron Deficiency in Bean Plants

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ABSTRACT

Iron deficiency in higher plants causes accumulation of salts of organic acids in the roots, the most characteristic being citrate. We show that citrate and malate accumulate in beans (Phaseolus vulgaris L. var. Prélude), not because of a lack of the iron-containing enzyme aconitate (EC 4.2.1.3), but in close coupling to the extrusion of protons during rhizosphere acidification, one of the 'Fe-efficiency' reactions of dicotyledons. When proton excretion is induced in roots of control bean plants by addition of fuscloacin, only malate, not citrate, is accumulated. We propose that iron deficiency induces production of organic acids in the roots, which in beans leads to both proton excretion and an increased capacity to reduce ferric chelates via the induced electron transfer system in the root epidermis cells.

Plants growing under iron deficiency may employ different strategies to increase iron uptake: (a) dicotyledons and non-grass monocotyledons develop a strong ferric reduction activity at the root surface (2); moreover, they may acidify the rhizosphere by active proton extrusion (23); and (b) grasses excrete highly effective siderophores, which are structurally related to the cellular iron carrier nicotianamine (27). Both mono- and dicotyledons are known to accumulate organic acid salts, especially citrate, in roots (6, 14, 31) and leaves (10, 26).

Evidence has accumulated which shows that the ferric reduction activity in the roots of Fe-efficient dicotyledons resides in the plasma membrane of the root epidermis cells, where an enzyme system can transfer electrons from cytosolic NADPH to extracellular ferric chelates, with low substrate specificity (2). This enzyme system has not been found in grasses (19).

We recently proposed that accumulated citrate, via the activities of aconitate and isocitrate dehydrogenase, is responsible for the strongly reduced redox poise of NADP in cells with high ferric reduction capacity (4). Citrate accumulation, in response to iron deficiency, has been suggested to be caused by lowered levels of the iron-containing enzyme aconitate (1, 8, 31). If this were true, citrate could not drive the reduction of NADP via the aconitase pathway described above. Landsberg (14), on the other hand, proposed that a hormonal unbalance caused by iron deficiency would induce the roots to excrete protons, and that organic acid production would follow from the functioning of the cellular pH-stat.

We tested the old hypothesis that low aconitate levels cause organic acid accumulation during iron deficiency. Our results show that citrate and malate accumulation in iron-deficient dicotyledons are not caused by lack of aconitate, but that they are coupled to the induced proton extrusion activity. The citrate formed may then cause the high NADPH levels found in the roots of iron-deficient dicotyledons and provide electrons for the induced ('Turbo,' Ref. 2) ferric chelate reduction capacity (4).

MATERIALS AND METHODS

Bean plants (Phaseolus vulgaris L. var. Prélude, from Sluis Enkhuizen, the Netherlands) were grown on a modified Knop nutrient solution as described before (24), with or without 40 μM Fe-EDTA as source of iron. The nutrient solution was replaced at 3 and 6 d after the plants were put on nutrient solution. In plants grown without iron, chlorosis symptoms started to appear at d 5 or 6. All plants were used at d 7 or 8.

Enzyme Assays. Tissue extracts were made by grinding the tissue (roots after washing with 0.5 mm CaSO4 with 50% (w/w) polyvinylpolypyrrolidone, washed sand and an extraction buffer at 0°C in a porcelain mortar. The resulting brei was centrifuged at 11 000 g for 5 min and the supernatant at 50 000 g for 10 min. From the last supernatant 2.5 ml was brought over 4 ml Sephadex G-25 and the eluate was used for determination of enzyme activity. The extraction buffer for aconitate (EC 4.2.1.3) was 0.1 M Hepes, 10 mM tricarballylate, pH 7.5. The activity was determined in two ways: (a) by following the formation of cis-aconitate from citrate at 240 nm (1 mM cis-aconitate: A240 = 3.55) (18). The reaction mixture contained, in a final volume of 500 μl, 0.1 M Hepes, 10 mM citrate, pH 7.5, and up to 100 μl enzyme preparation. The reaction was started by addition of citrate; and (b) by monitoring the reduction of NADP at 340 nm in a coupled reaction with isocitrate dehydrogenase (EC 1.1.1.42) using citrate as substrate. The cuvette contained, in a final volume of 500 μl, 0.1 M Hepes, 0.5 mM MgSO4, 0.6 mM NADP*, 10 mM citrate, pH 7.5, and up to 100 μl enzyme preparation. Isocitrate dehydrogenase was already sufficiently present in all preparations. All solutions in this assay contained Na+ instead of K+ ions (cf. Ref. 32).

Protein was determined according to Bradford (5), using BSA as a standard.

Extraction of Organic Acids. Roots or leaf blades were ground in a mortar and pestle with liquid N2 and lyophilized. Fifty mg of the resulting powder was suspended in 5 ml water, boiled for 30 min, and the extract was filtered over a 10 μm nylon filter. Citrate and malate were determined enzymically with commercial kits (Boehringer).

Xylem exudates were collected by cutting the stems of bean plants below the marks of the cotyledons. A closely fitting piece of plastic tubing was put on the stump and the accumulating sap was collected after 2 h. Citrate and malate were determined enzymically on this sap without further purification.

Phloem sap was collected by the method of King and Zeevaart (11). The stem of a bean plant was cut below the cotyledon marks, while submerged under 20 mm K-EDTA, pH 7.5, and the end with the leaves attached was hung in an Eppendorf
The ratio composition and with 100% plants bean leaves decreased in and control tissues. The length of the acidification period depended on the added Zn²⁺ + Mn²⁺ concentration; 0.7 μM Zn²⁺ + 9 μM Mn²⁺ gave reproducible acidification periods of at least 16 h and we used this combination routinely for the induction of acidification. Addition of 3 μM Fe⁶⁺-EDTA resulted in an acidification cycle which was as rapid and pronounced as that with Zn²⁺ and Mn²⁺, but it lasted for only 1 d. The plants started to regress by then. Iron-sufficient control plants never acidified the nutrient solution.

The correlation between proton extrusion and organic acid accumulation was investigated by inducing an acidification cycle as described, and measuring citrate and malate levels in the roots during the acidification cycle. Iron-deficient plants without added Zn and Mn and iron-sufficient plants were used for comparison (Fig. 1). Table III shows that levels of citrate and malate in roots of iron-deficient plants did not increase significantly during an acidification period. However, the xylem sap collected from the same plants showed a strong increase in citrate and malate upon acidification (Fig. 1). Measurements on the phloem sap, collected from other plants in a parallel experiment showed a slight increase of citrate and malate in the collected sap from acidifying plants 24 h after induction of medium acidification. When related to the amount of collected sugar, however, the differences were not significant (data not shown).

Fuscocin was used to induce proton extrusion by roots of iron-sufficient bean plants. Acidification of the medium was coupled to accumulation of malate, not of citrate, in the roots (Table IV).

**DISCUSSION**

The well-known accumulation of citrate in tissues of iron-deficient plants has been ascribed to (a) decreased levels of the iron-containing enzyme aconitase (1, 8, 31), or (b) the functioning of the cellular pH-stat upon proton extrusion (14). Our work shows that citrate accumulation was not correlated with lowered aconitase levels in bean and barley tissues.

The property of iron-deficient bean plants, that they can only acidify an iron-free nutrient solution in the presence of low concentrations of Zn²⁺ and Mn²⁺, and that they lose this capacity within 8 h upon withholding these ions, enabled us to study proton excretion in comparison with control iron-deficient plants, without apparent occurrence of Zn or Mn deficiencies.

In this way we could show, that in bean plants proton extrusion was clearly coupled to production of citric and malic acids, as proposed by Landsberg (14). Malate and citrate are the only acids that accumulate significantly in roots of iron-deficient beans (14) (PC Sijmons, unpublished data). Table III shows that the citrate level in the roots was already increased before the induced acidification cycle. This increased level probably resulted from previous cycles. The citrate and malate which were produced during the experiment apparently were completely exported via the xylem to the shoot (Table III, Fig. 1). In the experiment of Table III, the amounts of citrate and malate accumulated as a consequence of one acidification cycle were 4.4 ± 0.4 μmol and 10.4 ± 3.9 μmol, respectively. When it is assumed that citrate may yield 2.5 and malate 2.0 protons/mol during rhizosphere acidification, the amounts accumulated may have yielded 33 ± 14 μmol H⁺ for extrusion by the roots. The amounts of protons needed to reach pH 3.6 in 170 ml medium
Brown (6) had already shown that roots of iron-deficient plants accumulate citrate. Upon transfer of these plants to iron-containing nutrient solution, fast iron uptake was coupled to a greatly increased export of citrate and iron from the roots to the shoots (28, 29). Such a mobilization of citrate from the roots by iron that needs citrate as a carrier in the xylem can now be explained. Added Fe-EDTA, like Zn²⁺ and Mn²⁺, induces an acidification cycle in iron-deficient bean roots which lasts for 1 d. By then, the beginning recovery from iron-deficiency has apparently taken away the impulse for acidification (cf. Ref. 20). However, the acidification is strong enough to permit both production and release into the xylem of the amount of citrate reported by Tiffin (28, 29).

The question arises now as to what is the order of events in Fe-deficient roots. Landsberg proposed that Fe-deficiency causes a change in the roots' hormonal balance, resulting in proton extrusion (13, 14). If malate and citrate synthesis were the consequence of proton excretion, added fusicoccin, as a stimulator of the plasma membrane ATPase, would have the same effect as iron deficiency on the formation. However, addition of fusicoccin resulted in accumulation of malate only (Table IV); this response of the cytoplasmic pH-stat to cellular alkalization is well documented (17). The characteristic accumulation of citrate in iron-deficient roots can therefore not be explained on the basis of stimulated proton excretion alone. Of course, the cellular pH-stat may work differently in iron-deficient roots, and this difference may cause the production of citrate. A cause could be the lack of an Fe-enzyme, not aconitase, in the mitochondria. However, mitochondrial respiration of bean roots is not affected by iron deficiency at the stage of our studies (24) and this indicates that no serious enzyme deficiency occurs in the tricarboxylic acid cycle.

Another possibility is the possibility of iron deficiency causes, by hormonal or other effectors, primarily an increase in the production of organic acids. The resulting cellular acidosis would stimulate the

**Table II. Citrate and Malate in Phloem Exudate of Iron-Deficient and Control Bean Plants, Collected in 20 mM K-EDTA during 2 Hours**

Values given ± sd. Three experiments were performed.

<table>
<thead>
<tr>
<th>Preculture of Plants</th>
<th>Analysis of Phloem Exudate</th>
<th>Sugar</th>
<th>Citrate</th>
<th>Citrate/sugar ratio</th>
<th>Malate</th>
<th>Malate/sugar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg glucose equivalents</td>
<td>nmol</td>
<td>nmol/mg glucose equivalents</td>
<td>nmol</td>
<td>nmol/mg glucose equivalents</td>
<td></td>
</tr>
<tr>
<td>+Fe</td>
<td>300 ± 70</td>
<td>17 ± 7</td>
<td>55 ± 12</td>
<td>91 ± 22</td>
<td>304 ± 22</td>
<td></td>
</tr>
<tr>
<td>−Fe</td>
<td>620 ± 80</td>
<td>24 ± 6</td>
<td>38 ± 9</td>
<td>123 ± 5</td>
<td>201 ± 18</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1.** Induction of rhizosphere acidification by addition of Zn²⁺ + Mn²⁺ to the nutrient solution of iron-deficient bean plants, and concomitant export of citrate and malate via the xylem. Iron-deficient (○, □) and control plants (●), grown on medium without or with Fe for 6 d, were transferred to 170 ml nutrient solution each without Fe, Zn, and Mn. pH 5.3, 8 h before t = 0. At t = 0, 0.7 µM ZnCl₂ ± 9 µM MnCl₂ were added to the media of 8 iron-deficient plants (□). At the indicated points, stems of 3 (●), 2 (○), or 4 (□) plants were cut to collect xylem sap during 2 h. Citrate and malate concentrations in the xylem sap are shown as vertical bars. The values of citrate and malate at t = 0 are for iron-deficient plants.

was 43 µmol.

In bean leaves, the imported citrate and malate are only a fraction of the amounts produced by normal metabolism so that differences found during or directly after rhizosphere acidification probably disappear soon afterwards. Thus, in leaves of iron-deficient plants which were harvested randomly with respect to acidification cycles and in those of control bean plants no significant differences in citrate and malate contents were found (Table I).

**Table III. Effect of Rhizosphere Acidification on Organic Acid Contents of Roots and Leaves of Iron-Deficient Bean Plants, in Comparison with Controls**

Initial pH of nutrient solution: 5.3. Values given ± sd. Three experiments were performed in preculture with Fe, and seven without Fe.

<table>
<thead>
<tr>
<th>Preculture of Plants</th>
<th>Additions of Zn²⁺ + Mn²⁺ at t = 0</th>
<th>pH of nutrient solution</th>
<th>At 20 h:</th>
<th>Roots</th>
<th>Leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Citrate</td>
<td>Malate</td>
</tr>
<tr>
<td>+Fe</td>
<td>+</td>
<td>5.8 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>1.9 ± 0.2</td>
<td>ND*</td>
</tr>
<tr>
<td>−Fe</td>
<td>-</td>
<td>5.5 ± 0.3</td>
<td>2.0 ± 0.6</td>
<td>2.8 ± 0.6</td>
<td>12.0 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>3.6 ± 0.1</td>
<td>1.5 ± 0.6</td>
<td>2.6 ± 0.6</td>
<td>16.4 ± 3.1</td>
</tr>
</tbody>
</table>

* Not determined.
root cells to excrete protons. An example is the mould Aspergillus niger, which accumulates citric acid as a response to Fe or Mn deficiency (15, 16). In A. niger, too, there is no lowering of acitinate levels (12), but the ATP-dependent phosphorfructokinas is made insensitive to control by citrate via a rise in the endogenous ammonia level (9). The resulting increase in glycolysis rates leads to a high production of citric acid via carboxyla- tion of pyruvate (9).

An important difference between the two possible reaction sequences is their effect on cytosolic pH. Proton extrusion leads to alkalization and to malic acid formation through the action of the cellular pH-stat (25). Carboxylation of pyruvate (more likely phosphoenolpyruvate in roots) leads to acidification. In A. niger the process leads mainly to citric acid. The aerobic acidification of the cytosol may lead to production of both citrate and malate.

Landsberg (14) measured organic acid accumulation in monocotyledons and dicotyledons. His and our results show that roots of both kinds of plants accumulate citrate and malate in roughly equal amounts upon iron deficiency. Since citrate levels in the roots of normal plants are much lower than those of malate, the increase in citrate is relatively more prominent.

The effect of organic acid accumulation in dicotyledons is different from that in grasses. In dicotyledons, organic acid production is closely coupled to proton excretion (14) (this work). The resulting acidification of the rhizosphere leads to solubilization of ferric ions from precipitates in the soil. A ferric reductase, system, induced by iron deficiency, reduces ferric ions and chelates to free ferrous, which is easily taken up (7). The reduction system has a very low pH optimum (3, 21). Furthermore, the accumulated citrate will, via mitochondrial aconitase and cytosolic isocitrate dehydrogenase, increase the level of cytosolic NADPH (4), which is the electron donor for the reduction system (24). Such an inducible ('Turbo') (2) ferric reductase is only present in dicotyledons and non-grass monocotyledons (19).

In grasses, the accumulation of acids is not coupled to net proton excretion (13, 14). According to van Egmond and Aktas (30), grasses normally excrete so many hydroxyl ions, mainly as a consequence of nitrate reduction in the roots, that iron defi- ciency only results in less alkalization of the medium. Lands- berg (13) elegantly showed that iron-deficient maize, when de- void of nitrate reductase activity in the roots, did acidify the medium. This result also indicates that iron-deficiency induced proton excretion, as a secondary effect of organic acid accumulation, may or may not occur depending on other factors such as the cation/anion uptake balance and nitrate reduction. It is logical that grasses, which so strongly tend to alkalize the rhizosphere, do not have an iron uptake system based on the reduction of ferric ions: at pH values of 7 and higher this system is practically inactive (3, 21). The system used by grasses is based on the excretion of phytosiderophores (27) and uptake of their ferric complexes without prior reduction (22). This system is active at low and high pH values (22), and therefore does not depend on a low pH in the rhizosphere.

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Table IV. Effect of 10 µM Fusicoccin on the Levels of Citrate and Malate in Roots of Bean Plants after 4 Hours of Incubation

<table>
<thead>
<tr>
<th>Preculture of Plants</th>
<th>No. of Plants</th>
<th>Addition</th>
<th>pH of Nutrient Solution after 4 h Incubation</th>
<th>Content of Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.7 ± 0.1</td>
<td>Citrate</td>
</tr>
<tr>
<td>−Fe</td>
<td>4</td>
<td></td>
<td></td>
<td>Malate</td>
</tr>
<tr>
<td>+Fe</td>
<td>4</td>
<td>Fusicoccin</td>
<td>5.0 ± 0.1</td>
<td>21 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30 ± 2</td>
</tr>
</tbody>
</table>

Acknowledgment—The authors wish to thank Dr. Sijmons for stimulating discussions.

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