Plasmalemma Redox Activity and $H^+$ Extrusion in Roots of Fe-Deficient Cucumber Plants

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ABSTRACT
Cucumber plants (Cucumis sativus L.) with incipient Fe deficiency showed increased root capacity to reduce chelated Fe$^{3+}$ compared to Fe-sufficient plants. When Fe-ethylenediaminetraacetate was added to the root medium of the Fe-deficient plants, the reductase activity was associated with acidification of the medium and an increase in the net apparent K$^+$ efflux. In the presence of the H$^+$-ATPase inhibitor N$^{N'}$-dicyclohexylcarbodiimide the net apparent H$^+$ efflux was completely suppressed, though some reductase activity was preserved, and the net apparent K$^+$ efflux was significantly increased. The inhibition of the reductase activity by H$^+$-ATPase was similar whether the pH of the medium was buffered or not. Anoxia and the protonophore carbonyl cyanide m-chlorophenyl hydrazone also caused a similar inhibition of the reductase activity. It is proposed that this redox system transports electrons only and that its activity is inhibited by plasmalemma depolarization and anoxia. The H$^+$ and K$^+$ efflux associated with the reductase activity may be a result of the plasmalemma depolarization it causes.

Iron deficiency induces morphological and physiological changes in many dicotyledoneous species that make the Fe in the rhizosphere more readily available to the plant. Such changes occur in subapical root regions and include transformation of epidermal root cells into transfer cells (13), acidification of the rhizosphere (20, 24), and increased ferric reducing capacity of the roots (4, 6, 10, 18, 19). Recent advances on this subject are the localization of the reductase activity to the plasmalemma (9) and the finding of a close correlation between acidification of the external medium, hyperpolarization of the transmembrane electrical potential, and increased H$^+$-ATPase activity (24).

Bienfait (5) suggested the occurrence of two different plasmalemma redox systems in plant cells, namely the standard system, which can reduce ferricyanide and is not related to Fe uptake, and the turbo system, which is capable of reducing a variety of ferric chelates and ferricyanide and is induced by Fe shortage. Brüggemann et al. (8), however, concluded that iron deficiency in tomato induces increased expression of a ferric chelate reductase that is already present in iron-sufficient plants. Whether or not this reductase is the same as the standard has not yet been established. In any case, the presence of a suitable extracellular e$^{-}$ acceptor causes an acidification of the medium. The standard system features two possible linkages between e$^{-}$ and H$^+$ efflux: (a) both e$^{-}$ and H$^+$ are transported by the redox system (7, 16); (b) only e$^{-}$ are transported by the redox system, while H$^+$ are transported by H$^+$-ATPase (14, 15, 21, 23). The redox system increased by iron deficiency has been suggested to transfer e$^{-}$ and H$^+$ coupled across the plasmalemma and at a ratio of 2 e$^{-}$/1 H$^+$ (22); however, their results do not rule out the possibility of these H$^+$ being transported by H$^+$-ATPase.

The aim of the present work was to study the linkage between e$^{-}$ and H$^+$ efflux in the redox system increased by iron deficiency in the roots of cucumber plants (Cucumis sativus L.), and the effects of DCCD, CCCP, a fixed pH, and anoxia on the redox system. Some preliminary results in this respect were published elsewhere (1).

MATERIALS AND METHODS

Growth of Plants
Cucumber seeds (Cucumis sativus L. cv Burpee pickler) were germinated in perlite moistened with 5 mM CaCl$_2$. After 2 d, they were transferred to a plastic mesh held over one-half strength nutrient solution kept in the dark for two more days. Seedlings were transplanted individually to plastic vessels containing 75 mL of a continuously aerated standard solution with the following composition (mm): 2 Ca(NO$_3$)$_2$, 0.75 K$_2$SO$_4$, 0.65 MgSO$_4$, 0.5 KH$_2$PO$_4$; (uM): 50 KCl, 10 H$_2$BO$_3$, 1 MnSO$_4$, 0.5 CuSO$_4$, 0.5 ZnSO$_4$, 0.05 (NH$_4$)$_6$Mo$_7$O$_{24}$, and 5 Fe-EDDHA. The pH was adjusted to 6.0 with 0.1 N KOH. After 5 d, the solution was replaced with a new standard solution containing no Fe (deficient plants) or 20 uM Fe-EDDHA (control plants). Two days later the experiments were started. Plants were grown in a growth chamber at 22°C day/18°C night with a 14 h photoperiod, a RH between 60 and 80%, and a photosynthetic irradiance of 350 µmol m$^{-2}$ s$^{-1}$ provided by fluorescent tubes (Sylvania Cool White VHO).

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2 Abbreviations: e$^{-}$, electron; DCCD, N$^{N'}$-dicyclohexylcarbodiimide; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; EDDHA, ethylenediamine-dio-hydroxyphenylacetate; BPDS, bathophenanthroline disulfonate.
**Table I. Effects of Plant Fe Status and Ethanol on Fe$^{3+}$-EDTA Reduction and H$^{+}$ and K$^{+}$ Efflux**

Determinations were made at 4 h. The initial pH was 5.55 and the final pH (pHf) is given in the last column.

| Plant Fe Status | Reduction Conditions | Fe$^{3+}$ Reduced | H$^{+}$ Efflux | K$^{+}$ Efflux | pHf
\mu mol/g root fresh wt ± se
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<tbody>
<tr>
<td>+Fe Standard</td>
<td>1.9 ± 0.8</td>
<td>—b</td>
<td>6.1 ± 0.4</td>
<td>5.59 ± 0.05</td>
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<tr>
<td>−Fe Standard</td>
<td>18.4 ± 0.8</td>
<td>31.6 ± 1.6</td>
<td>10.8 ± 1.3</td>
<td>4.59 ± 0.05</td>
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<tr>
<td>−Fe Ethanol (0.5%)</td>
<td>15.5 ± 2.2</td>
<td>29.2 ± 4.8</td>
<td>9.6 ± 1.9</td>
<td>4.64 ± 0.14</td>
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*a n = 4.  b No H$^{+}$ efflux was detected as the final pH was not lower than the initial one.

**Fe$^{3+}$ Reduction by the Roots**

Roots from intact plants were preincubated for 30 min in 50 mL of 0.5 mM CaSO$_4$ at a pH of 5.5 adjusted with 0.1 N HCl, and then transferred to 50 mL of a solution containing 0.5 mM CaSO$_4$, 10$^{-4}$ M Fe-EDTA, and 3 $\times$ 10$^{-4}$ M BPDS (standard conditions), which also contained 0.5% (v/v) ethanol, 50 µM DCCD, 0.25 or 0.1 µM CCCP, and 10 mM Mes, depending on the particular experiment. Inhibitors were dissolved in ethanol and added to the solutions, the final ethanol concentration of which was 0.5% (v/v). The pH of the solutions was adjusted to the desired value with 0.1 N HCl or 0.1 N NaOH as required. Reduced ferric ion was determined by monitoring the absorbance at 535 nm. Fe-EDTA was omitted in one treatment; in another N$_2$ rather than air was bubbled through the solution. Ambient conditions throughout the reduction process were similar to the growth conditions described above in all cases.

**Net Apparent H$^{+}$ and K$^{+}$ Efflux to the Medium**

The net H$^{+}$ efflux was calculated by measuring the pH and back-titrating the medium to its initial pH with 3 mM NaOH. The net K$^{+}$ efflux was measured by atomic emission spectrophotometry.

All experiments were performed 3 or more times.

**RESULTS**

Iron-deficient plants reduced much more Fe$^{3+}$ than iron-sufficient plants did (Table I). This higher reducing capacity was associated with a net proton efflux to the medium which did not occur in iron-sufficient plants, and with a higher net potassium efflux (Table I). The presence of 0.5% (v/v) ethanol in the medium during the reduction process of iron-deficient plants did not have an important effect on the parameters determined (Table I).

All subsequent experiments were only performed on iron-deficient plants. Plants that had no added Fe-EDTA and thus did not exhibit their reducing capacity showed very slight acidification and a lower net apparent K$^{+}$ efflux than those that did receive the chelate (Table II). In the presence of Fe-EDTA, 50 µM DCCD completely suppressed the net apparent H$^{+}$ efflux, though some reducing capacity was still preserved (Table II). In addition, the net apparent K$^{+}$ efflux was significantly increased in that treatment (Table II). When the pH of the solution was kept constant by adding 10 mM Mes, the inhibitor effect of 50 µM DCCD on the reducing capacity was similar to that found in the absence of Mes, while the increase in the net apparent K$^{+}$ efflux was smaller in its presence (Tables II and III).

Bubbling N$_2$ through the solution completely suppressed the net apparent H$^{+}$ efflux, inhibited the reducing capacity, and increased the net apparent K$^{+}$ efflux compared with the standard conditions (Table IV). In the experiments with CCCP, the reducing capacity was not affected by 0.25 µM CCCP, but was inhibited by 1 µM CCCP, while the net apparent K$^{+}$ efflux was increased at both CCCP concentrations in relation to the control treatment (Table V).

**DISCUSSION**

The iron-deficient plants used in this work had a reducing capacity markedly increased with respect to the control plants

**Table II. Effects of Fe$^{3+}$-EDTA and DCCD on Fe$^{3+}$-EDTA Reduction and H$^{+}$ and K$^{+}$ Efflux**

Determinations were made at 1 and 2.5 h. The initial pH was 5.54 and the final pH (pHf) is given in the last column. All treatments contained ethanol 0.5% (v/v).

| Plant Fe Status | Reduction Conditions | Time (h) | Fe$^{3+}$ Reduced | H$^{+}$ Efflux | K$^{+}$ Efflux | pHf \(\mu mol/g root fresh wt ± se\)
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<tbody>
<tr>
<td>−Fe −Fe-EDTA</td>
<td>1.0</td>
<td>0.0</td>
<td>—b</td>
<td>1.3 ± 0.2</td>
<td>5.54 ± 0.02</td>
<td></td>
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<tr>
<td>−Fe Standard</td>
<td>1.0</td>
<td>4.7 ± 0.4</td>
<td>4.1 ± 0.7</td>
<td>2.6 ± 0.1</td>
<td>4.89 ± 0.10</td>
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<tr>
<td>−Fe +DCCD 50 µM</td>
<td>1.0</td>
<td>1.6 ± 0.1</td>
<td>—</td>
<td>8.4 ± 0.7</td>
<td>5.72 ± 0.02</td>
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*a n = 4.  b No H$^{+}$ efflux was detected as the final pH was not lower than the initial one.
Table III. Effects of DCCD on Fe³⁺-EDTA Reduction and K⁺ Efflux

| Plant Fe Status | Reduction Conditions | Fe³⁺ Reduced | K⁺ Efflux | μmol/g root fresh wt ± se
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<tbody>
<tr>
<td>−Fe Standard</td>
<td>6.8 ± 0.7</td>
<td>5.3 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−Fe +DCCD 50 μM</td>
<td>2.1 ± 0.2</td>
<td>21.4 ± 0.9</td>
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* n = 4.

(Table I). However, their acidification response independent of the reductase activity was very low as is shown when Fe-EDTA was not added (Table II). That means that when Fe-EDTA was added most of the acidification produced was related to the redox system activity.

The acidification of the medium associated with redox activity was supposed to originate from an activation of the H⁺-ATPase caused by the plasmalemma depolarization and cytosol acidification produced by the redox activity (14, 15, 21, 23). However, other works (7, 16) have reported that H⁺ and e⁻ are transported coupled by the redox system. The effects of DCCD found in this work (Table II) provide evidence that the redox system, whose activity is increased by iron deficiency, transports only e⁻. However, it has been suggested (7, 16) that DCCD inhibition of the redox associated acidification does not indicate that acidification is mediated by the H⁺-ATPase, because DCCD may affect H⁺ uptake. Other inhibitors, such as diethylstilbestrol, have been shown to produce an alkalinization response (16), but the same effect does not appear with 100 μM DCCD (7, 23).

The redox system whose activity is increased by iron deficiency has been studied to much less extent. Siemons et al. (22) found both ferricyanide and Fe³⁺-EDTA to cause rapid plasmalemma depolarization in Phaseolus vulgaris. This effect was more marked in Fe-deficient than in Fe-sufficient plants. In the former, ferricyanide was reported to cause excretion of H⁺ (1 H⁺/2 e⁻) and K⁺ ions, and it was suggested that H⁺ and e⁻ transports were coupled through the redox system (22). Our results disagree with that suggestion, perhaps because we used a different e⁻ acceptor: Fe³⁺-EDTA instead of ferricyanide. Lass et al. (14) have found different responses in Lemna gibba depending on the e⁻ acceptor used. When ferricyanide was added, the membrane depolarization induced was similar in Fe-sufficient and Fe-deficient plants, but the Fe³⁺ reduction and H⁺ and K⁺ efflux were slightly greater in Fe-deficient plants. When Fe³⁺-EDTA was added, both membrane depolarization and Fe³⁺ reduction were much larger in Fe-deficient plants, but no H⁺ efflux was detected nor was K⁺ efflux stimulated.

Anoxia has been found to influence redox system activity. In iron-sufficient plants, anoxia inhibited the reductase activity slightly and the net apparent H⁺ efflux much more strongly (12). Other researchers (3, 4) found anoxia to clearly inhibit the reductase activity increased by iron deficiency. In the present work anoxia treatment effects have been compared to those of DCCD. In both treatments, e⁻ were transported with no net apparent H⁺ efflux, but both the decrease in Fe³⁺ reduction and the increase in net apparent K⁺ efflux were larger in the presence of DCCD (Tables II and IV).

The partial inhibition of the Fe³⁺ reduction by DCCD (Table II) could be attributed to an effect of the external pH since the activity of the redox system is sensitive to the pH (3, 18, 19) and the external pH was higher in the DCCD treatment than in the control (Table II). However, the effect of DCCD was similar regardless of whether the medium used had pH buffered with Mes or contained no Mes (Tables II and III). An alternative hypothesis involves the assumption that the inhibition might be caused by plasmalemma depolarization, as suggested by Marré et al. (15). Both DCCD and the protonophore CCCP are known to induce plasmalemma depolarization (11, 17). We found 1 μM CCCP to have similar effects to those of DCCD on Fe³⁺ reduction and K⁺ efflux (Tables III and V), which is consistent with the above hypothesis. Inhibition of the reductase activity by CCCP has also been reported in connection with the standard system (14) and with the iron deficiency increased system (2, 4).

Table IV. Effects of Anoxia on Fe³⁺-EDTA Reduction and H⁺ and K⁺ Efflux

| Plant Fe Status | Reduction Conditions | Fe³⁺ Reduced | H⁺ Efflux | K⁺ Efflux | μmol/g root fresh wt ± se
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<tr>
<td>−Fe Standard</td>
<td>7.9 ± 0.5</td>
<td>6.9 ± 0.5</td>
<td>4.4 ± 0.7</td>
<td>5.05 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>−Fe Anoxia</td>
<td>4.3 ± 0.3</td>
<td>-b</td>
<td>7.0 ± 1.2</td>
<td>6.07 ± 0.07</td>
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*n = 5.  
*b No H⁺ efflux was detected as the final pH was not lower than the initial one.

Table V. Effects of CCCP on Fe³⁺-EDTA Reduction and K⁺ Efflux

| Plant Fe Status | Reduction Conditions | Fe³⁺ Reduced | K⁺ Efflux | μmol/g root fresh wt ± se
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<tbody>
<tr>
<td>−Fe Standard</td>
<td>+CCCP 0.25 μM</td>
<td>6.4 ± 0.4</td>
<td>10.0 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>−Fe +CCCP 1.00 μM</td>
<td>2.9 ± 0.8</td>
<td>15.6 ± 0.8</td>
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* n = 4.
Results of net apparent K+ efflux found here are also consistent with those obtained by other authors (14, 15, 22, 23). The K+ efflux in the different treatments seems to be related to changes in the plasmalemma depolarization. A higher K+ efflux is associated with a higher reductase activity as a result of either the Fe status of the plants (Table I) or the presence of Fe-EDTA in the medium (Table II). In the presence of DCCD, CCCP, and anoxia, the K+ efflux was much greater (Tables II–V).

In conclusion, our results appear to indicate that the redox system whose activity is increased by Fe deficiency in cucumber roots transports e– only, while acidification is caused by a H+ efflux through H+-ATPase as a side effect. The reductase activity is partially inhibited by DCCD, anoxia, and CCCP, and it is suggested that plasmalemma depolarization that takes place under these conditions may be an important factor in inhibiting the reductase activity.

ACKNOWLEDGMENT

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LITERATURE CITED


