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Iron-Deficiency Stress Responses in Cucumber (*Cucumis sativus* L.) Roots[1]

A Possible Role for Ethylene?

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Most dicotyledonous species respond to Fe deficiency by developing several mechanisms known as Fe-deficiency stress responses. To study the regulation of these responses, young cucumber plants (*Cucumis sativus* L. cv Ashley) were grown in nutrient solution for 11 d, being deprived of Fe during the last 4 or 5 d. Inhibitors of ethylene synthesis (2 or 10 μM aminoethoxyvinylglycine; 10 or 20 μM aminooxyacetic acid; 1, 2, 5, or 10 μM Co^{2+} as $CoCl_2$) or action (50, 200, or 800 μM Ag^+ as silver thiosulfate) were added to the nutrient solution at different times during this period of growth with no Fe. After this period, the reduction of Fe^{3+} ethylenediaminetetraacetate by the roots of entire plants was measured with ferrozine by reading the absorbance at 562 nm after 2 h. The presence of the ethylene inhibitors in the nutrient solution inhibited the Fe-deficiency stress responses ferric-reducing capacity and subapical root swelling. In another experiment, the addition of 1 μM 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor of ethylene synthesis, to the nutrient solution of plants having low ferric-reducing activity increased notably the ferric-reducing capacity and subapical root swelling. Here we show evidence that ethylene plays a role in the development of Fe-deficiency stress responses, since when ethylene synthesis or action was inhibited, the responses were also inhibited, and when a precursor of ethylene (ACC) was added, the responses were increased.

Fe deficiency induces several morphological and physiological responses in many plants. In dicots, the subapical root tip generally exhibits a characteristic swelling with extensive root hair development; in these regions other physiological responses such as an increase in root ferric-reducing capacity and an acidification of the extracellular medium occur (see Römheld and Marschner, 1986; Bienfait, 1988; Kochian, 1991, for reviews). The mechanisms by which these responses are regulated are not well understood, although a low root Fe content (Bienfait et al., 1987) and hormonal changes have been implicated (Landsberg, 1984; De la Guardia et al., 1988; Romera et al., 1992).

Ethylene could be one of the hormones involved in Fe regulation. This hypothesis is supported by three circumstantial pieces of evidence. First, in sorghum, it has been shown that Fe-deficient plants exhibiting leaf chlorosis (a typical symptom of Fe deficiency) produced almost 5 times as much ethylene as Fe-sufficient plants (Morgan and Hall, 1962). Second, interactions among ethylene, auxin, and cytokinins have been implicated in root morphological changes such as inhibition of root elongation and swelling of root tips (Eliasson et al., 1989; Bertell et al., 1990; Bertell and Eliasson, 1992); similar root morphological changes are induced by Fe deficiency (Römheld and Marschner, 1981). Finally, when roots were exposed to Co^{2+}, an inhibitor of ethylene synthesis, some of the responses commonly associated with Fe deficiency, such as induction of transfer cells, acidification, reductant excretion, and enhanced Fe translocation, were shown to be inhibited (Landsberg, 1982; Blaylock et al., 1985).

Ethylene is synthesized from Met via the following metabolic sequence: Met → SAM → ACC → ethylene (Yang and Hoffman, 1984). One approach to the study of the role of ethylene in different stress responses has been the use of inhibitors of ethylene synthesis or action. These inhibitors include AOA and AVG, which have been shown to inhibit ACC synthase, the enzyme that converts SAM to ACC (Yang and Hoffman, 1984; Reid, 1988). Additionally, Co^{2+} is an inhibitor of ACC oxidase, which converts ACC to ethylene (Grover and Purves, 1976; Lau and Yang, 1976; Yu and Yang, 1979), and Ag^+ has been used as an inhibitor of ethylene action (Beyer, 1976; Veen, 1983; Atta-Aly et al., 1987; Liu et al., 1990). A complementary approach to the use of ethylene synthesis and site of action inhibitors has been to artificially increase the tissue ethylene level by treatments with the precursor of ethylene, ACC, or the ethylene-releasing substance ethephon (Yang, 1969; Lavee and Martin, 1981; Reid, 1988; Yip et al., 1988).

The objective of this work was to study the possible role of ethylene in the regulation of Fe-deficiency stress responses by either applying different inhibitors of ethylene to cucumber (*Cucumis sativus* L.) seedlings during the onset of Fe deficiency or by studying the effect of increasing tissue ethylene on some of the Fe-deficiency responses in plants not

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Abbreviations: AOA, aminooxyacetic acid; AVG, aminoethoxyvinylglycine; EDDHA, *N,N'*-ethylenebis[2-(2-hydroxyphenyl)-glycine]; SAM, *S*-adenosylmethionine; STS, silver thiosulfate.

normally expressing these responses. Our results suggest that ethylene is involved not only in the root morphological changes induced by Fe deficiency, but also in the increase in ferric-reducing capacity by root cells.

MATERIALS AND METHODS

Growth of Plants and Treatments

Cucumber (*Cucumis sativus* L. cv Ashley) seeds were germinated in perlite moistened with 5 mM $CaCl_2$. After 2 d they were transferred to a plastic mesh held over diluted nutrient solution (one-half concentration) and kept in the dark for 2 additional days. Seedlings were then transplanted individually to plastic vessels containing 80 mL of a continuously aerated nutrient solution with the following composition: (mM) 2 $Ca(NO_3)_2$, 0.75 K_2SO_4, 0.65 $MgSO_4$, 0.5 KH_2PO_4; (μM) 50 KCl, 10 H_3BO_3, 1 $MnSO_4$, 0.5 $CuSO_4$, 0.5 $ZnSO_4$, 0.05 $(NH_4)_6Mo_7O_{24}$, and 20 Fe-EDDHA. The pH was adjusted to 6.0 with 0.1 N KOH. After 3 d the solution was replaced with a new nutrient solution containing either no Fe or different concentrations of Fe-EDDHA, depending on the experiment. Plants were grown for an additional period of 4 to 6 d. During this period the different inhibitors (Co^{2+}, AOA, AVG, Ag^+) or promoters (ACC, ethephon) of ethylene synthesis were added to the nutrient solution bathing the roots from aqueous stock solutions of each chemical. A stock solution of STS, which was used as a source of Ag^+ ions, was prepared by mixing equal volumes of 0.01 M $AgNO_3$ and 0.04 M $Na_2S_2O_3$ (Liu et al., 1990). Plants were grown in a growth chamber at 22°C day/18°C night with an RH between 50 and 70% and a 14-h photoperiod at a photosynthetic irradiance of 350 μmol m^{-2} s^{-1} provided by fluorescent tubes (Sylvania Cool White VHO).

Measurement of Root Fe(III) Reduction

Plants were pretreated for 30 min in nutrient solution without microelements, pH 6.0, and then transferred for 2 h to a similar nutrient solution that also contained 100 μM Fe^{3+} EDTA and 300 μM ferrozine, pH 5.0 (assay solution). The environmental conditions during the measurement of Fe(III) reduction were the same as for the growth conditions described above. The ferric-reducing capacity was determined by measuring the concentration of Fe^{2+}-ferrozine complex formed via measurements of A_{562} in a spectrophotometer. Visualization and localization of root reducing capacity was also determined by using the nitroblue tetrazolium technique reported by Sijmons and Bienfait (1983).

Each experiment was repeated at least twice and representative results are presented. Data are given as means ± SE.

RESULTS

Treatment of Plants Deprived of Fe with Inhibitors of Ethylene Synthesis or Action

Under our experimental conditions, plants began to show Fe-deficiency stress responses after 3 or 4 d of growth without Fe, reaching a maximum ferric-reducing capacity and rhizosphere acidification on the 4th d. When Co^{2+} was included in the nutrient solution for the 3rd and 4th d of growth under −Fe conditions, at concentrations from 1 to 10 μM, there was a dramatic and progressive inhibition of the ferric-reducing capacity that is normally induced during Fe deficiency (Fig. 1A). It is not likely that the Co^{2+} inhibition of induced ferric reductase activity is due to Co^{2+} toxicity, because the lower Co^{2+} concentrations (1–3 μM) had no effect on root fresh weight, whereas the higher Co^{2+} levels caused only small decreases in root fresh weight (data not shown). Continuing the Co^{2+} exposure for 2 additional days caused a greater inhibition of the ferric-reducing capacity (Fig. 1B).

In response to Fe deficiency, dicot roots exhibit a stimulated H^+ extrusion in addition to increased ferric reduction. The time-dependent expression of both of these processes in response to Fe deficiency can vary from plant to plant. Therefore, in another experiment, on the 3rd d of growth without Fe, seedlings were assayed and separated into those that acidified and those that did not acidify the external medium. These two groups represented seedlings that were either expressing or not expressing the Fe-deficiency stress responses. It was found that when 5 μM Co^{2+} was applied to the growth medium after the 3rd d of growth on −Fe medium, the inhibition of the ferric-reducing capacity, measured 1 d later, was clearly greater in plants that were not acidifying at that time (Fig. 2). These results indicate that Co^{2+} did not block the Fe-deficiency-stimulated expression of the ferric reductase in seedlings in which Fe-deficiency stress responses were already expressed.

Treatments with other inhibitors of ethylene synthesis (AOA and AVG) and an inhibitor of ethylene action (Ag^+, as STS), at different concentrations, also resulted in an inhibition of ferric-reducing capacity (Fig. 3). It should be noted that inclusion of Co^{2+} (5 μM), AVG (10 μM), AOA (20 μM), or STS (50 μM) in the Fe(III) reduction assay solution did not affect rates of Fe(III) reduction in Fe-deficient seedlings that were not exposed to these treatments during their growth on −Fe medium (data not shown). This result indicates that these

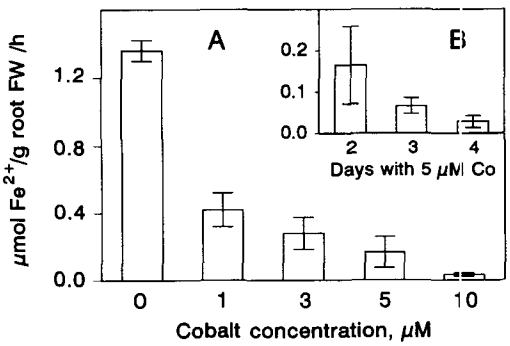

Figure 1. A, Ferric-reducing capacity of roots of cucumber plants at the end of a 4-d growth period with no Fe. For the last 2 d of this period, $CoCl_2$ at different concentrations (0, 1, 3, 5, and 10 μM) was present in the nutrient solution. B, Reducing capacity at the end of 4, 5, or 6 d of growth with no Fe and with 5 μM $CoCl_2$ during the last 2, 3, or 4 d, respectively. Shown are means ± SE (n = 5). FW, Fresh weight.

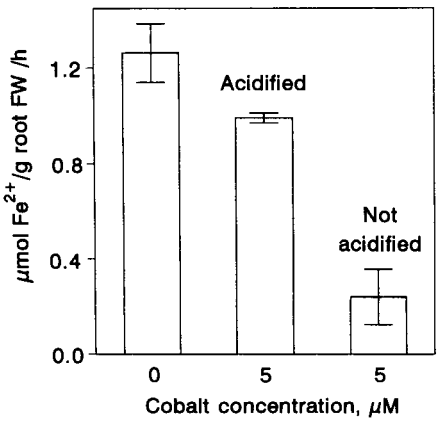

Figure 2. Ferric-reducing capacity of cucumber plants at the end of a 4-d growth period with no Fe. On the 3rd d of growth with no Fe, 5 μM $CoCl_2$ was supplied to plants that had either started or not started to acidify the nutrient solution. Shown are means ± SE (n = 5). FW, Fresh weight.

inhibitors of ethylene synthesis/action do not directly affect the activity of the root ferric reductase.

In addition to the effect of these inhibitors on the development of ferric-reducing capacity, other Fe-deficiency responses were studied. The development of subapical swollen root tips was suppressed by all four inhibitors. The capacity to acidify the nutrient solution was also inhibited, although to a lesser extent in the treatments with STS and AVG (data not presented). Root growth was affected differentially depending on the inhibitor and the concentration used. STS at any of the concentration, Co^{2+} at concentrations of 3 μM or lower, and AVG at 2 μM did not produce significant differences in the root fresh weight with respect to the control. In the other cases, there was a mild to moderate reduction in root fresh weight to between 60 and 80% of the controls.

Treatments with ACC

Either Fe-sufficient plants or Fe-deficient plants in which the expression of stimulated Fe(III) reduction was blocked by application with AVG were treated with ACC, an immediate precursor of ethylene. This was done to determine if ethylene precursors could stimulate the development of ferric-reducing capacity. Plants were either grown with no Fe for 4 d in the presence or absence of 10 μM AVG (for the last 3 d) or they were grown with sufficient concentrations of Fe (40 or 80 μM as Fe-EDDHA). In both cases, roots were exposed to ACC in the nutrient solution for 7 h and root ferric reduction was determined. As shown in Figure 4, ACC treatment increased ferric-reducing capacity in Fe-sufficient roots to rates comparable to those observed in Fe-deficient roots; also, ACC caused a similar increase in ferric reduction in Fe-deficient roots that had been exposed to AVG. Furthermore, ACC treatment caused the appearance of numerous swollen root tips (Fig. 5).

Another experimental approach was to apply ethephon (ethylene-releasing substance) to plants grown in −Fe medium in which the expression of the ferric reductase had been blocked by treatment with 10 μM AOA. In some cases, such as when ethephon (80 μM) was applied for 7 h, it produced an increase in ferric-reducing capacity, but these results were more difficult to reproduce than those with AVG and ACC. In any case, supplying ethephon resulted in the appearance of numerous swollen root tips. It is worth mentioning that the increase of ferric-reducing capacity caused by either ACC or ethephon was located in the region of swollen root tips (Fig. 5).

DISCUSSION

In this work we have found that the development of Fe-deficiency stress responses such as ferric-reducing capacity (Fig. 1A), acidification, and root morphological changes can be inhibited by Co^{2+}, a known inhibitor of ethylene synthesis. In the treatments with 1 and 3 μM Co^{2+}, plant growth was similar to that in the control seedlings, indicating that inhibition of the ferric-reducing capacity was not caused by Co^{2+} toxicity. When Co^{2+} concentrations were higher or the period of treatment was prolonged, the moderate growth inhibitions observed could contribute to the lower rates of Fe(III) reduction (Fig. 1, A and B).

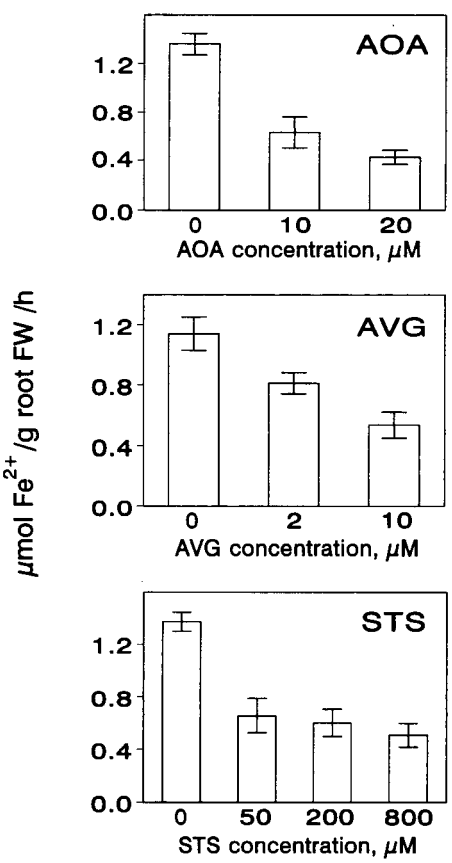

Figure 3. Ferric-reducing capacity of cucumber plants at the end of a 4-d growth period with no Fe and with different treatments. AOA (10 and 20 μM) and AVG (2 and 10 μM) were present during the last 2 d, and STS (50, 200, and 800 μM) was present during the full 4 d. Shown are means ± SE (n = 5). FW, Fresh weight.

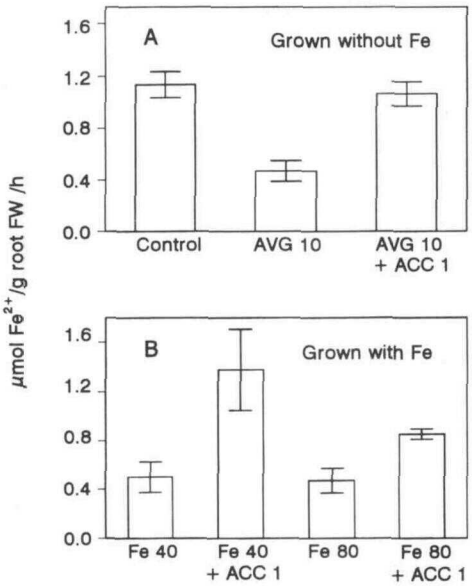

Figure 4. Effect of ACC on the ferric-reducing capacity of cucumber plants pretreated in different ways. A, Plants were deprived of Fe during 4 d before determination of ferric-reducing capacity. On the 2nd d, 10 μM AVG was applied to two of the treatments, one of which also received 10 μM ACC for 7 h; after that plants of all treatments were changed to new nutrient solutions that again contained 10 μM AVG in the treatments that previously had it. B, Plants were grown with 40 or 80 μM Fe during 3 d before determination of ferric-reducing capacity. On the 2nd d 1 μM ACC was applied for 7 h to some treatments, and after that plants of all treatments were changed to new nutrient solutions corresponding to their initial treatments. Shown are means ± SE ($n = 5$). FW, Fresh weight.

Under our experimental conditions, the different responses to Fe deficiency were observed after 3 or 4 d of growth without Fe, a period during which the endogenous level of Fe is presumably reduced to a threshold value. Generally, the root acidification response slightly preceded the increase of ferric-reducing capacity. The application of 5 μM Co^{2+} for just 1 d was enough to inhibit the responses of those plants that had not started to acidify the medium. However, there was almost no inhibition in the plants that had started to acidify the nutrient solution (Fig. 2). This indicates that Co^{2+} possibly affects a determined point in the sequence of events involved in the regulation of the Fe-deficiency stress responses, but once these responses have already been induced, Co^{2+} has no effect.

The role of ethylene in the regulation of Fe-deficiency stress responses was further supported by experiments with other inhibitors of ethylene and with precursors of ethylene. The inhibitors AOA, AVG, and STS prevented the appearance of root swelling and the development of ferric-reducing capacity (Fig. 3). Treatments with 2 μM AVG and with 50 to 800 μM STS had no effect on root growth, which suggests that these treatments were not injurious to the plant. We also used Ag^+ as $AgNO_3$, but we could not find a concentration that inhibited the responses without producing toxic effects on plant growth. The advantages of using STS rather than $AgNO_3$ have been described by others and are related to a lower free concentration of Ag^+ in STS solutions and a lower phytotoxicity of this compound (Veen, 1983; Liu et al., 1990).

If an increase in the level of ethylene is involved in regulating Fe-deficiency stress responses, then ACC and ethephon should induce the responses in plants grown with Fe or in plants grown without Fe in which the synthesis of ethylene has been inhibited. Indeed, we found these effects of ACC on ferric-reducing capacity (Fig. 4) and on root morphological changes (Fig. 5). Reversion of AVG effects by ACC have also been found by Jackson et al. (1985) in aerenchyma formation in roots of maize and by Liu et al. (1990) in adventitious rooting in hypocotyls of sunflower. When ethephon was applied to Fe-deficient plants treated with AOA, the effect on ferric-reducing capacity was more difficult to reproduce. This may be explained by difficulties in obtaining appropriate endogenous ethylene concentrations during a determinate period by ethephon exposure. Liu et al. (1990) showed that ACC and ethephon had different effects depending on the concentration used. Lavee and Martin (1981) found that the production of ethylene caused by ACC was more rapid and less prolonged than that caused by ethephon.

It is interesting to note that Fe is required for the synthesis of ethylene via its function as a cofactor of ACC oxidase (Bouzayen et al., 1991; Dong et al., 1992). Apparently, even in Fe-deficient plants in which the ferric reductase is induced, there is still enough tissue Fe to allow for the synthesis of functional ACC oxidase. As noted in the introduction, it has been shown by Morgan and Hall (1962) that Fe deficiency does cause a dramatic stimulation of ethylene production in

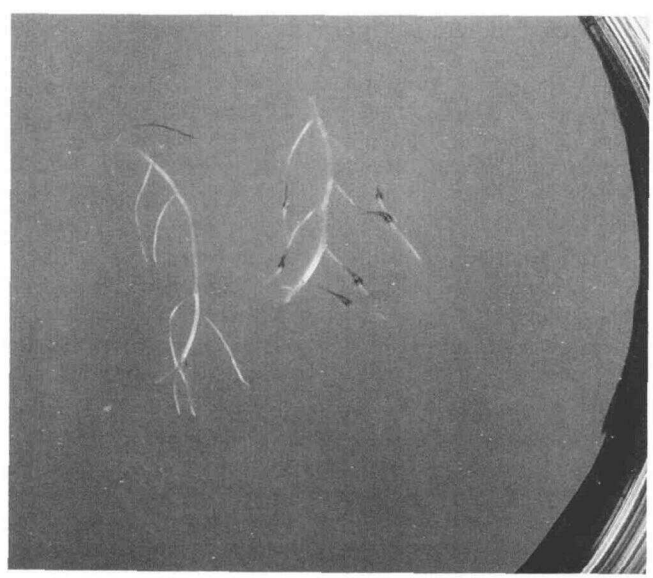

Figure 5. Location of ferric-reducing capacity by the nitroblue tetrazolium technique in cucumber roots treated with ACC. Left, Treatment with Fe; right, treatment with Fe plus ACC. Black areas correspond to sites of active reduction. Plants (9 d old) were grown with 40 μM Fe. On the 8th d 1 μM ACC was applied for 7 h to the plants in the Fe plus ACC treatment, and after that plants of both treatments were changed to their initial nutrient solution. Pictures were taken on d 9.

sorghum, which certainly supports the model being presented here.

The induction of Fe-deficiency stress responses has been linked to an increase of auxin in subapical root tips (Landsberg, 1984; Römheld and Marschner, 1986; De la Guardia et al., 1988). Possibly, the effect of auxin could be through ethylene, since high levels of auxin promote ethylene production by inducing the synthesis of ACC synthase (Yu and Yang, 1979; Kim et al., 1992). In previous work (Romera et al., 1992), we found that when a portion of the root system was supplied with Fe while another portion was grown without Fe, roots supplied with Fe exhibited Fe-deficiency responses and this was induced by substances coming from the roots growing without Fe. Results from the present work suggest that ACC could be the translocatable substance, because it has been shown to be mobile in both xylem (Bradford and Yang, 1980) and phloem (Amrheim et al., 1982). Ethylene itself, because it is a gas, is not very mobile within the plant. Bienfait et al. (1987) found that a low concentration of root Fe could be the initial signal for inducing Fe-deficiency stress responses. They proposed that a gene-activating protein may be involved in the induction of Fe-deficiency stress responses by roots. This protein might activate the genes when it is not bound to Fe (Bienfait, 1988). Results from our present study show that ethylene is implicated in the regulation of the Fe-deficiency stress responses and that it could be the regulator of gene expression, because it does perform similar functions in other systems (Woodson and Lawton, 1988).

Ethylene has been implicated in many other plant stress responses (Yang and Hoffman, 1984; Reid, 1988), including other nutritive stresses (Feng and Barker, 1992; He et al., 1992). Because ferric reduction and Fe absorption are believed to be highly regulated by tissue Fe levels (Römheld and Marschner, 1986) to prevent the accumulation of toxic levels of Fe in the plant, how might this reductase be regulated by a hormone that is implicated in many different stress responses? Possibly, ethylene could induce Fe-deficiency stress responses only within a specific concentration range, and for a specific period of time and at a specific location within the plant. When we supplied either ACC or ethephon for more than 1 d to plants with low root reducing capacity, we did not observe the increase in ferric reduction that was observed when these compounds were supplied for 7 h (data not shown).

Alternatively, it is quite possible that the ferric reductase is not as tightly regulated by Fe as previously thought. Information has recently been presented in the literature indicating that the ferric reductase can be induced by deficiencies of mineral nutrients other than Fe. Welch et al. (1993) showed that Cu deficiency induced the pea root ferric reductase as effectively as Fe deficiency did. Jolley and Brown (1991) presented data suggesting that the ferric reductase could also be stimulated by Zn deficiency in navy bean roots. It has been proposed by Welch et al. (1993) that the reductase might play a more general role in regulating cation absorption in plants. Therefore, the response of this reductase to a number of mineral deficiencies could be mediated via involvement of ethylene. This could mean that the ferric reductase could be activated under conditions in which the roots are exposed to luxury levels of Fe. Presumably, when this occurs other internal detoxifying mechanisms would function to prevent Fe toxicity, or possibly the activation of the reductase would occur for brief periods of time.

Experiments to determine ethylene production and ACC synthase and ACC oxidase activities by both Fe-deficient and Fe-sufficient plants are in progress. Possibly, we will be able to determine which step in ethylene synthesis is affected by Fe deficiency and gain a better understanding of the regulation of Fe reduction and absorption in plants.

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