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Ethylene involvement in the over-expression of Fe(III)-chelate reductase by roots of *E107* pea [*Pisum sativum* L. (*brz*, *brz*)] and *chloronerva* tomato (*Lycopersicon esculentum* L.) mutant genotypes

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Recently, ethylene was reported to be involved in the regulation of Fe(III)-chelate reducing capacity by cucumber (*Cucumis sativus* L.) roots. Here, we studied the effect of two ethylene inhibitors, aminooxyacetic acid (AOA) and cobalt, on the Fe(III) reducing capacity in roots of mutant genotypes [*E107* pea [*Pisum sativum* L. (*brz*, *brz*)] and *chloronerva* tomato (*Lycopersicon esculentum* L.] that exhibit high rates of Fe(III)-chelate reduction and excessive iron accumulation. The ethylene inhibitors, AOA and cobalt, markedly inhibited Fe(III)-chelate reducing capacity in roots of both genotypes. Over-expression of root Fe(III) reductase activity by both mutants appears to be related to ethylene. Possibly, both mutants are genetically defective in their ability to regulate root ethylene production. The large inhibitory effect of both ethylene inhibitors on Fe(III)-chelate reducing capacity in roots of the mutant tomato genotype, *chloronerva*, disputes the contention that the nicotianamine–Fe(II) complex is the repressior of the gene responsible for Fe(III)-chelate reductase activity, as previously suggested by others. However, since nicotianamine shares the same biosynthetic precursor as ethylene, i.e. *S*-adenosyl methionine, nicotianamine may affect Fe(III)-chelate reductase activity in dicot and non-grass monocot roots by influencing ethylene biosynthesis.

Keywords: ethylene, ethylene function, inducible reductase, iron-chelate reductase, iron deficiency, iron-deficiency, roots

Introduction

Iron uptake by roots of dicots and non-graminaceous monocots is regulated by the activity of a root-cell plasma membrane Fe(III)-chelate reductase, which reduces Fe(III) in various Fe(III)-chelates or complexes to Fe(II) before subsequent Fe^{2+} transport across the plasma membrane into the cytosol (Grusak *et al.* 1990, Kochian & Lucas 1991, Welch 1995). Root Fe(III)-chelate reductase activity is stimulated by iron-deficiency growth conditions (Bienfait & Lüttge 1988, Bienfait 1988).

The pea mutant *E107* [*Pisum sativum* L. (*brz*, *brz*)] is a pleiotropic mutant of its parent genotype *Sparkle*. This mutant is characterized by low nodulation, leaf necrosis, excessive mineral ion accumulation, shorter roots and decreased shoot size. *E107* continuously displays iron-deficiency stress as characteristically high rates of root Fe(III)-chelate reduction and excessive iron uptake, even when supplied sufficient levels of available iron. As a result, *E107* accumulates toxic levels of iron in its older leaves even when supplied levels of iron that would be adequate for growth of normal genotypes (Grusak *et al.* 1990, Welch & La Rue 1990).

Chloronerva is a tomato mutant (parent genotype, *Lycopersicon esculentum* Mill. cv '*Bonner Beste*') that lacks the facility to synthesize nicotianamine (Scholz *et al.* 1988, 1992). Nicotianamine forms stable complexes with Fe(II) and other transition metal ions, and has been implicated in the intra and intercellular transport of iron in plants (Scholz *et al.* 1988, 1992). *Chloronerva* accumulates excessive amounts of iron in its leaves, and exhibits retarded growth, shorter roots and inter-veinal chlorosis of young leaves

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(Scholz *et al.* 1992). In addition, *chloronerva* shows several other characteristics of iron-deficiency stress including elevated rates of root Fe(III)-chelate reducing capacity and high rates of root H^+ efflux, even when propagated under iron-sufficient conditions (Scholz *et al.* 1992). The growth and development of *chloronerva*, as well as its physiological behaviour, is mostly restored to normal upon application of small amounts (i.e. 1 μmol) of nicotianamine either to its leaves or to its growth medium (Scholz *et al.* 1992). This normalizing effect of nicotianamine led Scholz and his group to suggest that nicotianamine is involved in the genetic regulation of iron uptake processes. They proposed a model in which nicotianamine–Fe(II) is the repressor of the genes responsible for iron-deficiency stress responses. According to their model, nicotianamine would function similarly to the *FUR* protein in microorganisms (Scholz *et al.* 1992).

In previous work, Romera & Alcántara (1993, 1994) suggested that the plant hormone, ethylene, was involved in the regulation of Fe(III) reducing capacity by cucumber (*Cucumis sativus* L.) roots. The increase in Fe(III) reducing capacity in iron-deficient roots of cucumber plants was markedly depressed by treatment with inhibitors of ethylene synthesis or site of action [aminooxyacetic acid (AOA), aminoethoxyvinylglycine (AVG), cobalt or silver]. Additionally, Fe(III)-chelate reducing capacity of cucumber roots was greatly enhanced in iron-sufficient cucumber plants treated with ethylene promoters [1-aminocyclopropane-1-carboxylic acid (ACC) or ethephon].

The reduced growth and shorter lateral roots of *E107* and *chloronerva* suggest links between their metabolic disorders and the possibility that ethylene action might be involved in both because ethylene is an inhibitor of root growth (Bertell *et al.* 1990). Furthermore, *E107* exhibits low root nodulation ability, which is partly restored when *E107* roots are treated with the ethylene inhibitors AVG or Ag^+ (Guinel & La Rue 1992). Because of these similarities between *E107* and *chloronerva* root growth characteristics, and the known effects of ethylene on root growth, possibly, *E107* and *chloronerva* contain a genetic alteration that interfers with their ability to regulate ethylene production or in their sensitivity to ethylene.

Ethylene is synthesized from L-methionine via the pathway shown schematically in Figure 1. The conversion of SAM to ACC is catalysed by the enzyme ACC synthase, whose activity is inhibited by a number of chemicals, including AOA (Yang & Hoffman 1984). The conversion of ACC to ethylene is catalysed by ACC oxidase, whose activity is dependent upon Fe^{2+} and is competitively inhibited by Co^{2+} ions (Dilley *et al.* 1993). SAM is a common biosynthetic precursor of ethylene (Yang & Hoffman 1984), polyamines (Even-chet *et al.* 1982) and nicotianamine (Shojima *et al.* 1990). Additionally, nicotianamine is an intermediate in the biosynthesis of several other phytometallophores in the metabolic pathway from L-methionine via SAM in graminaceous species (Shojima *et al.* 1990, Scholz *et al.* 1992).

The objective of this research was to determine if ethylene plays a role in the over-expression of Fe(III)-chelate reductase activity by roots of *E107* pea and *chloronerva* tomato mutants which also could have implications for the role of ethylene in iron-deficiency stress responses in dicots and non-grass monocots.

Materials and methods

Plant culture

Seeds of pea [*P. sativum* L. (*brz*, *brz*)] and of its parental line '*Sparkle*' were imbibed overnight in aerated deionized water (designated day 0). Seeds were then placed between sheets of moistened filter paper in plastic trays covered with plastic wrap and germinated in the dark at room temperature. On day 4, seedlings radicals were inserted through the plastic-mesh bottom of black polyethylene seedling cups. The seedlings cups were inserted into holes in lids covering black plastic pots containing 5 l of continuously aerated nutrient solution (see below for solution composition) without micronutrients. The seedlings cups were covered with black plastic caps. The caps were removed on day 7 and black polyethylene beads were added to a depth of about 1.0 cm to prevent the entry of light into the nutrient solution. Micronutrients were added on day 8. Each pot contained four seedlings.

Seeds of tomato (*L. esculentum* Mill. *chloronerva*) and of its parental line '*Bonner Beste*' were sown in vermiculite moistened with water. Seedlings (11 days old) were transferred to nutrient solution as described above for the pea seedlings.

The nutrient solution had the following composition (in mM): KNO_3, 1.2; $Ca(NO_3)_2$, 0.8; $NH_4H_2PO_4$ 0.2; $MgSO_4$, 0.2; and (in μM) KCl, 50; H_3BO_3, 12.5; $ZnSO_4$, 1; $CuSO_4$, 0.5; H_2MoO_4, 0.1; $NiSO_4$, 0.1. Additionally, to buffer nutrient solution pH the nutrient solutions contained

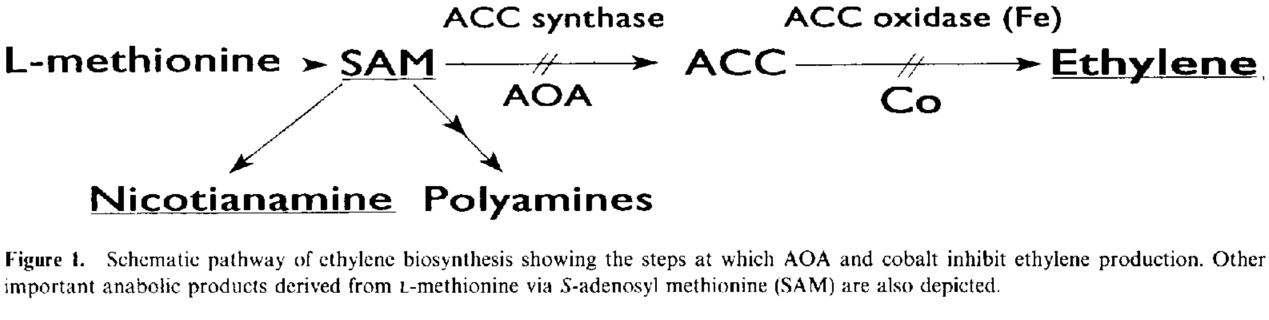

Figure 1. Schematic pathway of ethylene biosynthesis showing the steps at which AOA and cobalt inhibit ethylene production. Other important anabolic products derived from L-methionine via *S*-adenosyl methionine (SAM) are also depicted.

5 mM MES buffer (adjusted to pH 5.5 with KOH). After day 8, Fe(III)-EDDHA (*N,N'*-ethylenebis[2-(2-hydroxyphenyl)-glycine) and, when appropriate, the particular ethylene inhibitors (cobalt or AOA), were added to the nutrient solution from concentrated aqueous solutions. In experiments longer than 14 days, nutrient solutions were replaced every week after day 14. Plants were grown in a controlled environmental chamber with a 16 h, 20°C/8 h, 18°C, day–night regime under artificial lights (combination of fluorescent and incandescent lights; 580 μE m^{-2} s^{-1} at plant height).

Fe(III)-chelate reductase assays

Fe(III) reducing capacity of the roots was quantified spectrophotometrically using the chromophore Fe(II)-BPDS. Just prior to initiation of the root reductase assays, pea roots were excised from the shoot and rinsed for 5 min in 0.2 mM $CaSO_4$ solution. Then, the entire excised pea root system was submerged in an assay solution consisting of 0.2 mM $CaSO_4$, 5 mM MES buffer (pH 5.5), 0.1 mM Fe(III)-EDTA and 0.2 mM Na_2-BPDS. In *chloronerva*, the reduction was determined for root systems of entire plants without excision. Previous studies had demonstrated that excision of the root system in peas did not effect Fe-(III)-chelate reducing capacity for approximately 6 h (Welch *et al.* 1993). The pH of the MES-buffered reductase solution remained constant during the 30 min assay. The reductase assays were conducted under low light conditions in a temperature-controlled water bath at 23°C. After 30 min, the absorbance at 535 nm of the assay solutions was determined spectrophotometrically; an aliquot of assay solution containing no roots was used as a blank. The concentration of Fe(II)-BPDS was calculated using a molar extinction coefficient of 22.14 mM^{-1} cm^{-1}.

Results

Effects of cobalt and AOA on root Fe(III)-chelate reductase activity in iron-deficient seedlings

The addition of Co^{2+} ions (as 3 μM $CoSO_4$) to the nutrient solution of iron-deficient *E107* pea plants resulted in a drastic inhibition of root Fe(III)-chelate reducing capacity, even when cobalt was supplied for only 1 day (Figure 2). At this low cobalt concentration, neither the root fresh weight nor the shoot fresh weight were affected (data not shown), indicating that inhibition of root Fe(III)-chelate reducing capacity was not the result of cobalt toxicity. Apparently, iron seed stores supplied enough iron to suppress the induction of root-cell Fe(III)-chelate reductase until day 13 because enhanced Fe(III) reduction was not observed until days 13–14 from germination in control seedlings.

Treatment of roots with AOA (either 10 or 20 μM) for 2 days resulted in a significant reduction in Fe(III)-chelate reducing capacity by the roots of iron-deficient *E107* seedlings (Figure 3). The 10 μM AOA treatment did not affect root fresh weights relative to the controls. However, treatment with 20 μM AOA reduced root fresh weight slightly to between 80 and 90% of the controls (data not shown).

Supplying the ethylene inhibitors, Co^{2+} and AOA, to 15 day old, iron-deficient, *Sparkle* pea seedlings (Figure 4) and 30 day old *Bonner Beste* tomato seedlings (Figure 5) for 2 days also inhibited the development of root Fe(III) reducing

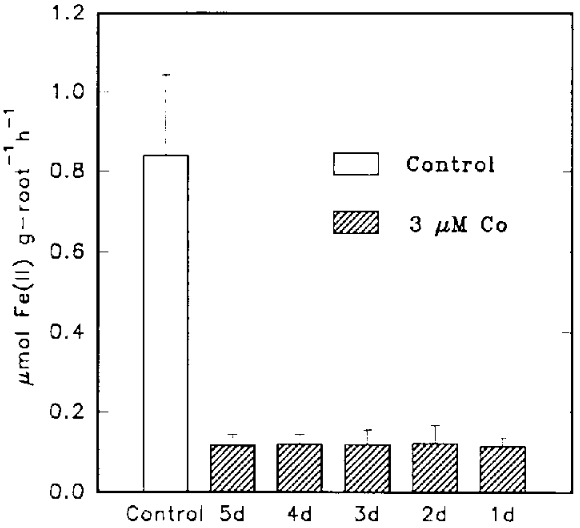

Figure 2. Fe(III)-chelate reducing capacity in roots of 14 day old iron-deficient *E107* pea plants treated with cobalt. Seedlings were grown in iron-deficient nutrient solutions from germination. Cobalt treatment (as 3 μM $CoCl_2$) was added to the nutrient solutions during the last 5, 4, 3, 2 and 1 days of growth respectively; error bars show SEM ($n = 6$).

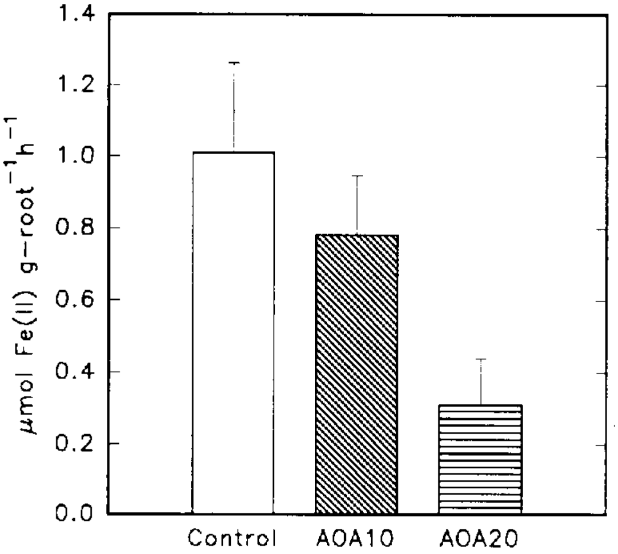

Figure 3. Fe(III)-chelate reducing capacity of 14 day old iron-deficient *E107* pea seedlings treated with AOA. On day 12, either 10 μM AOA (AOA10) or 20 μM AOA (AOA20) was added to the nutrient solutions; error bars show SEM ($n = 6$).

capacity in these parental lines of pea and tomato, respectively.

Investigation of the time course for cobalt and AOA inhibition of root Fe(III)-chelate reducing capacity after treatment of both 14 day old, iron-deficient, *E107* and *Sparkle* pea seedlings demonstrated that cobalt inhibition began between 2 and 3 h after its application. AOA became inhibitory after 3–6 h of treatment (Figure 6).

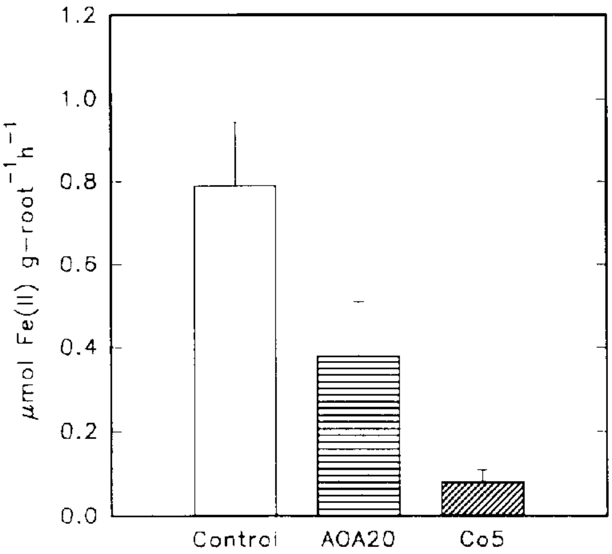

Figure 4. Fe(III)-chelate reducing capacity of 15 day old iron-deficient *Sparkle* pea seedlings treated with either AOA or cobalt. On day 13, either 20 μM AOA (AOA20) or 5 μM $CoCl_2$ (Co5) was added to the nutrient solution; error bars show SEM ($n = 6$).

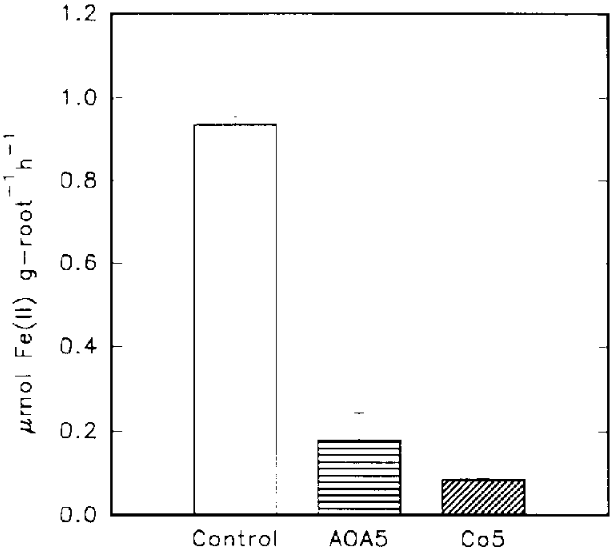

Figure 5. Fe(III)-chelate reducing capacity of 30 day old iron-deficient *Bonner Beste* tomato plants treated with either AOA or cobalt. On day 24, Fe(III)–EDDHA was withheld from their nutrient solutions. On day 28, either the 5 μM AOA (AOA5) or the 5 μM $CoCl_2$ (Co5) treatments were applied; error bars show SEM ($n = 4$).

Neither 5 μM Co nor 20 μM AOA affected root Fe(III)-chelate reducing capacity in either *E107* or *Sparkle* pea seedlings when added directly to the reductase assay solutions prior to root exposure, confirming that the effect of these inhibitors was not on the assay or the reduction of Fe(III) *per se* (data not shown).

Effects of cobalt and AOA on Fe(III)-chelate reductase activity in iron-sufficient seedlings

These experiments were conducted only with *E107* and *chloronerva* mutants because their normal parental genotypes suppress Fe(III)-chelate reducing capacity when supplied adequate iron (Scholz *et al.* 1988, Grusak *et al.* 1990, Welch & La Rue 1990). The addition of Co^{2+} ions (3 μM) to the nutrient solution of iron-sufficient *E107* pea seedlings inhibited the development of root Fe(III)-chelate reducing capacity (Table 1). On day 18, the oldest leaves of the untreated iron-sufficient *E107* pea plants showed severe iron toxicity symptoms (i.e. leaf bronzing and inter-venial necrosis) while toxicity symptoms were absent in older leaves of iron-sufficient plants treated with cobalt. Toxicity symptoms were correlated with the iron concentration in shoots, with iron concentrations substantially lower in shoots of cobalt-treated plants (Table 1). The cobalt-treated, iron-sufficient seedlings grew normally and had higher root and shoot fresh weight than those plants not treated with cobalt.

Treatment of iron-sufficient *chloronerva* plants with either 5 μM AOA or 5 μM $CoCl_2$ for 2 days resulted in a drastic reduction in root Fe(III) chelate reductase activity (Figure 7). Neither 5 μM AOA nor 5 μM $CoCl_2$ affected root or shoot fresh weight of *chloronerva* plants (data not shown). Thus, cobalt and AOA, supplied at these low levels, did not inhibit plant growth; therefore, their effects on root Fe(III) reductase activity cannot be attributed to gross toxicity of these ethylene inhibitors on plant development.

Discussion

The addition of cobalt, an inhibitor of ACC oxidase (Lau & Yang 1976, Dilley *et al.* 1993), to the nutrient solution of either iron-deficient or iron-sufficient *E107* pea seedlings inhibited the development of root Fe(III)-chelate reducing activity (Figure 2 and Table 1). Similarly, the Fe(III)-chelate reducing capacity of iron-deficient *E107* pea plants was inhibited by AOA (Figure 3), which inhibits the activity of ACC synthase (Yang & Hoffman 1984). These results suggest that ethylene is involved in the over-expression of Fe(III)-chelate reducing capacity by roots of *E107* pea mutants. The results agree with previous results obtained with cucumber, which demonstrated that the induction of root Fe(III)-chelate reducing capacity during the onset of iron deficiency was inhibited when seedlings were treated with ethylene inhibitors (Romera & Alcàntara 1993, 1994).

It is not possible to determine if ethylene directly affects root Fe(III)-chelate reductase synthesis from the data shown in Figure 2. Perhaps, ethylene influences the gene(s)

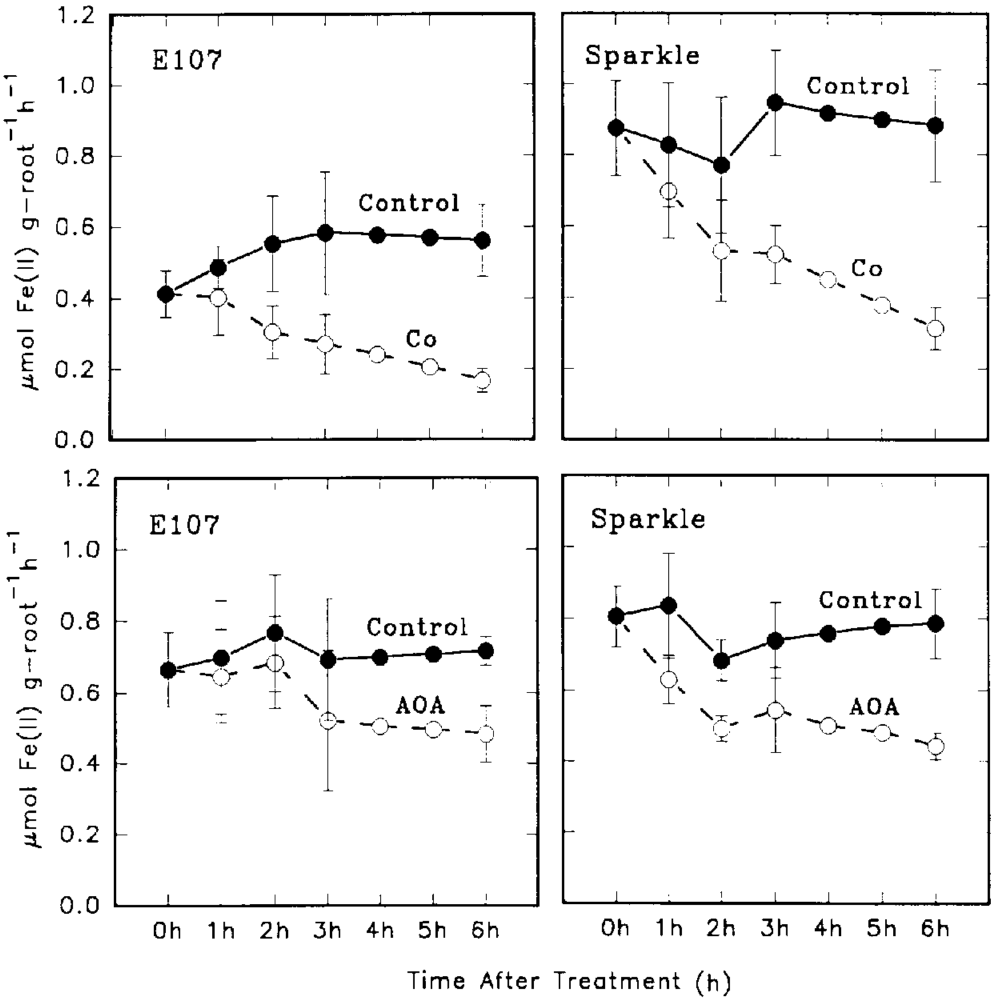

Figure 6. Effect of cobalt and AOA treatment on the root Fe(III)-chelate reductase activity of 14 day old iron-deficient *E107* (A) and *Sparkle* (B) pea seedlings. Seedlings were grown in nutrient solution without iron. On day 14, either 5 μM $CoCl_2$ or 20 μM AOA was added to the nutrient solution at time (h)=0. Subsequently, Fe(III) reducing capacity was determined at different hours during the day using different seedlings for each assay; error bars show SEM ($n=5$).

Table 1. Effects of Co^{2+} on *E107* pea root Fe(III)-chelate reductase activity, shoot iron concentration, and shoot and root fresh weights (FW) of 19 day old, iron-sufficient seedlings

Treatment	Reductase activity (μmol Fe^{2+} g RFW^{-1} h^{-1})	Shoot [Fe] (μg g DW^{-1})	Shoot FW (g)	Root FW (g)
+Fe	1.45 (±0.19)	849 (±82)	2.6 (±1.1)	2.3 (±1.3)
+Fe, +Co	0.44 (±0.20)	193 (±23)	4.5 (±1.1)	5.1 (±0.5)

E107 seedlings were grown in nutrient solution supplied with 20 μM Fe(III) EDDHA from day 11. On day 12, 3 μM $CoCl_2$ was supplied to the cobalt treatment; mean±SEM ($n=6$).

responsible for Fe(III)-chelate reductase expression directly. However, ethylene may influence the expression of other genes prior to the induction of Fe(III)-chelate reductase synthesis, such as the genes involved in root morphological changes (e.g. root hair development). We have observed that ACC promotes the development of root hairs (e.g. 'root swollen tips'), which is a prerequisite for enhanced Fe(III)-chelate reductase activity in roots of plants grown under iron-deficient conditions. This observation supports the latter possibility.

In iron-sufficient *E107* pea seedlings, cobalt not only inhibited Fe(III)-chelate reducing activity but also eliminated some of the phenotypic characteristics of this mutant. Under cobalt treatment, iron-sufficient *E107* pea plants did not accumulate iron to toxic levels (Table 1). Moreover, the seedlings were normal in appearance, looking similar to their parent genotype, *Sparkle*. This lower iron content can be explained by considering that Fe(III) reduction to Fe(II) is required beofre Fe^{2+} is absorbed (Chaney *et al.* 1972); thus, decreasing Fe(III)-chelate reductase activity by cobalt treatment will result in decreased uptake of iron. Previously, Blaylock *et al.* (1985) reported that cobalt treatments resulted in a decrease in foliar iron in tomato and soybean plants.

The treatment of iron-sufficient *chloronerva* tomato plants with either cobalt or AOA also inhibited root Fe(III)-chelate reductase activity (Figure 7). These results further suggest that over-expression of root Fe(III)-chelate reducing capacity by *chloronerva* tomato plants is linked to ethylene action. Additionally, cobalt and AOA treatment also inhibited root Fe(III)-chelate reducing capacity in both iron-deficient *Sparkle* pea plants (Figure 4) and iron-deficient *Bonner Beste* tomato plants (Figure 6).

Taken together our results strongly support the contention that ethylene action is involved in the expression of root Fe(III)-chelate reducing capacity by plant roots as first reported by Romera & Alcántara (1993, 1994). Possibly, the differences between the normal plants and their mutant genotypes involve alterations in the regulation of ethylene synthesis or in plant sensitivity to ethylene. Perhaps, the mutants constitutively produce high levels of root ethylene, even under iron-sufficient conditions.

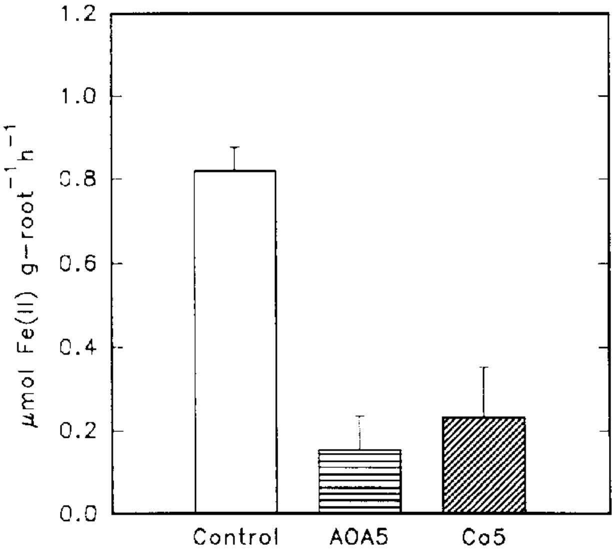

Figure 7. Root Fe(III)-chelate reductase activity of 40 day old iron-sufficient *chloronerva* tomato plants treated with either AOA or cobalt. Plants were grown in nutrient solution with 10 µM Fe(III)-EDDHA. On day 38, either 5 µM AOA (AOA5) or 5 µM $CoCl_2$ (Co5) was added to the nutrient solution; error bars show SEM ($n = 4$).

As noted above, ethylene and nicotianamine share the same precursor, SAM (Figure 1) (Yang & Hoffman 1984, Shojima *et al.* 1990). Nicotianamine Fe(II) has been postulated to be the repressor of the genes responsible for iron-deficiency stress responses (Scholz *et al.* 1992). According to this hypothesis, *chloronerva* would not be able to repress the genes for the iron-deficiency stress responses because it does not synthesize nicotianamine (Scholz *et al.* 1988, 1992). Our results suggest that this may not be the case. If nicotianamine were directly involved in the expression of the Fe(III)-chelate reductase gene, then ethylene inhibitors should not directly inhibit Fe(III)-chelate reducing capacity in *chloronerva* roots, as we have shown (Figure 7). Possibly, either nicotianamine alone or the nicotianamine–Fe(II) complex may play an indirect role in the Fe(III)-chelate reductase gene expression by influencing ethylene synthesis. Polyamines also share the same biosynthetic precursor as ethylene and nicotianamine, SAM, and have been reported to inhibit ethylene synthesis in several plant tissues (Even-chen *et al.* 1982, Li *et al.* 1992). These syntheses appear to be linked as shown by the fact that ethylene is known to inhibit the synthesis of polyamines (Li *et al.* 1992). Logically, plants should possess precise mechanisms to regulate the synthesis of these substances originating from the same precursor, in this case, SAM. Thus, *chloronerva* may not be able to regulate ethylene synthesis because it cannot synthesize one of the components required for ethylene regulation; perhaps this is nicotianamine's role.

Interestingly, Stephan & Scholz (1990) found that iron deficiency caused a drastic decrease of nicotianamine levels in root tips of sunflowers. However, in roots of iron-deficient barley, where nicotianamine is a known precursor of mugineic acid (a phytometallophore involved in iron acquisition by roots), they reported a 5- to 6-fold increase in nicotianamine levels (Stephan & Scholz 1990). Thus, iron deficiency modifies the conversion of SAM to various biosynthetic products differently in dicots and non-grass monocots, and graminaceous species where different strategies are employed for iron acquisition [Fe(III)-chelate reduction verses phytometallophore efflux, respectively] (Scholz *et al.* 1992).

Ethylene may directly affect the transcription of the gene(s) responsible for the synthesis of the root Fe(III)-chelate reductase, since ethylene has been reported to regulate the expression of other genes in plants (Woodson & Lawton 1988). Furthermore, ethylene may affect either the translation of the Fe(III)-chelate reductase gene(s) or the activity of the Fe(III)-chelate reductase already present in the root cell plasma membrane. None of these possibilities can be excluded by our results showing the time course effects of cobalt and AOA treatments on root Fe(III)-chelate reductase activity (Figure 5). If ethylene affects the transcription of the Fe(III)-chelate reductase gene(s), then possibly, the turnover time for Fe(III)-chelate reductase would not be longer than 2–3 h (Figure 5). Much remains to be learned about the function of root ethylene in regulating iron acquisition by plant roots.

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