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Iron requirement for and effects of promoters and inhibitors of ethylene action on stimulation of Fe(III)-chelate reductase in roots of strategy I species

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Stimulation of root Fe(III) reductase activity by iron additions to iron-deficient growth media may be the result of iron activation of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase required for ethylene biosynthesis. Two different ethylene inhibitors, aminooxyacetic acid (AOA) (20 μM; ACC synthase inhibitor) and cobalt (3 μM $CoCl_2$; ACC oxidase inhibitor), were used to study the effects of iron supply and cobalt inhibition on ethylene action in controlling the activity of Fe(III)-chelate reductase in pea (*Pisum sativum* L.) roots. Supplying 20 μM Fe(III)–*N*,*N*'-ethylenebis[2-(2-hydroxypheyl)-glycine [Fe(III)–EDDHA] to either cobalt-treated, iron-deficient *Sparkle* (normal parent) or *E107* (*brz* mutant genotype) pea seedlings reversed the negative effects of cobalt on root Fe(III)-reductase activity. Re-supplying 20 μM Fe(III)–EDDHA to iron-deficient, AOA-treated seedlings did not enhance root (Fe(III)-reductase. Apparently, cobalt competes with iron for the active site in ACC oxidase during ethylene synthesis. Inhibition of root reductase activity by cobalt treatment lowered manganese, zinc, magnesium and potassium content of mutant *E107* pea seedlings. In contrast, iron enhancement of root reductase activity in iron-deficient, cobalt-treated *E107* seedlings resulted in higher seedling accumulations of manganese, zinc, magnesium and potassium. These results support the hypothesis that root cell plasma membrane reductase activity plays a role in cation uptake by root cells.

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

Introduction

Iron-deficiency stress responses in dicots and non-grass monocots (i.e. strategy I species) include growth media acidification and increased root Fe(III) reducing capacity (Römheld & Marschner 1986). Iron-deficiency stress responses to inadequate iron supply allow roots to accumulate more iron from normally insoluble rhizosphere Fe(III) pools (Römheld & Marschner 1986, Bienfait 1988). Iron-sufficient plants repress these iron-deficiency stress responses (Römheld & Marschner 1981, Maas *et al.* 1988). Some studies report stimulation of iron-deficiency stress responses, at least transitorily, when a small amount of iron is supplied to iron-deficient plants (Chaney *et al.* 1972, De Vos *et al.* 1986, Jolley *et al.* 1986, Grusak *et al.* 1990, Romera *et al.* 1992).

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Chaney *et al.* (1972) reported higher Fe(III) reducing capacity in soybeans supplied 0.32 μM Fe when compared with those supplied 0.1 μM Fe. Grusak *et al.* (1990) reported higher Fe(III) reducing capacity in *E107* pea mutant seedlings supplied 2 μM Fe(III)–EDDHA than in the same mutants grown without iron additions to their nutrient solutions. Additionally, they found a transitory increase of Fe(III) reducing capacity when they supplied 2 μM Fe(III)-EDDHA to iron-deficient *Sparkle* pea plants (Grusak *et al.* 1990). Romera *et al.* (1992) reported higher Fe(III) reducing capacity in sunflower plants grown with bicarbonate and 2 μM Fe than in the plants grown with bicarbonate but without iron. Jolley *et al.* (1986) found differences in the time course of the acidification response by the roots of soybeans, depending on the presence or absence of iron in the nutrient solution. In HA soybeans with no added iron, 10 days were required to detect a pH decrease equivalent to that obtained in 5 day old HA soybeans supplied 0.05 mg $Fe\ l^{-1}$ (Jolley *et al.* 1986). De Vos

et al. 1986) reported induction of an acidification cycle by roots of iron-deficient bean plants when they supplied 3 μM Fe(III)–EDTA to their nutrient solution.

Some of the results cited in the previous paragraph have been explained by considering that plants grown with a small amount of iron in the nutrient solution were healther than the plants grown without iron (Grusak *et al.* 1990). Additionally, it has been suggested that iron stimulates iron-deficiency stress responses because iron is required for enzymes and electron transporters implicated in Fe(III) reduction and other iron-deficiency stress responses (Grusak *et al.* 1990).

Previously, we reported evidence that ethylene plays a role in regulating root Fe(III)-chelate reductase activity (Romera & Alcántara 1993, 1994, Romera *et al.* 1995). The inhibition of either ethylene biosynthesis or action by ethylene inhibitors [amino oxyacetic acid (AOA), aminoethoxyvinylglycine (AVG), Co^{2+} or Ag^{+}] drastically decreased the development of root Fe(III) reductase activity in iron-deficient cucumber (Romera & Alcántara 1993, 1994), tomato and pea seedlings (Romera *et al.* 1995). The addition of ethylene promoters (ACC or ethephon) to iron-sufficient cucumber plants greatly increased their root Fe(III) reducing capacity (Romera & Alcántara, 1994).

Ethylene is synthesized from L-methionine via the pathway shown in Figure 1 (Yang & Hoffman 1984). The conversion of *S*-adenosyl methionine (SAM) to ACC is catalysed by ACC synthase which is inhibited by AOA, as well as by other substances (Yang & Hoffman 1984). The conversion of ACC to ethylene is catalysed by ACC oxidase. ACC oxidase requires iron for activation and is competitively inhibited by Co^{2+} (Dilley *et al.* 1993). Accordingly, the stimulatory effect of iron treatment on root iron-deficiency stress responses could be the result of the activation of ACC oxidase by iron followed by the stimulation of ethylene production and subsequent induction of iron-deficiency root stress responses (Romera & Alcántara 1993, 1994, Romera *et al.* 1995).

We employed the ethylene inhibitors, i.e. AOA (ACC synthase inhibitor) and cobalt (ACC oxidase inhibitor), to study the affects of iron supply on ethylene action in stimulating Fe(III)-chelate reductase activity in roots of iron-deficient seedlings. Additionally, we studied the relationship between root Fe(III)-chelate reductase activity and uptake of cations by the iron accumulating peat mutant, *E107*. This mutant continuously reduces Fe(III)-chelates, even when grown under iron-sufficient conditions (Welch & LaRue 1990), and as a result, accumulates iron to toxic levels in its older leaves. Also, manganese, zinc, magnesium and potassium are consistently accumulated to high levels in *E107* seedlings (Grusak *et al.* 1990, Welch & LaRue 1990, Welch *et al.* 1993, Welch 1995).

Materials and methods

Growth of plants and experimental treatments

Seedlings of pea (*Pisum sativum* L. *E107*) and its parental line, *Sparkle*, were grown in aerated nutrient solution without iron as previously described (Romera *et al.* 1995). On day 12, either 3 μM $CoCl_2$ or 20 μM AOA was added to the nutrient solution of the cobalt and AOA treatments, respectively, to test the effects of ethylene inhibitors on Fe(III)-chelate reductase activity in seedling roots. On day 13, 20 μM Fe–EDDHA was added to half of the seedlings in the cobalt and AOA treatments. There were four to six replicate pots per treatment.

We tested the effects of iron supply, as well as manganese, zinc and copper supplies on the reduction of Fe(III)-chelates by iron-deficient, cobalt-treated pea seedlings. Here, *Sparkle* pea seedlings received not only 20 μM Fe–EDDHA, but also either 10 μM $MnSO_4$, 5 μM $ZnSO_4$ or 5 μM $CuSO_4$ in their iron-deficient nutrient solutions on day 13 after germination. Iron(III)-chelate reductase activity in roots was determined on day 12–19 for individual seedlings via procedures previously described (Romera *et al.* 1995).

Mineral determinations

Shoots of replicate plants were harvested just prior to initiation of the Fe(III)-chelate reductase assays. The roots were harvested immediately after assaying for Fe(III)-chelate activity. Following reductase assays, a modification of the method of Bienfait *et al.* (1984) was used to remove apoplasmic iron adhering to roots (Grusak *et al.* 1990). Roots were transferred to a solution containing 50 ml of 0.5 mM $CaSO_4$ and 1.5 mM 2,2′-bipyridine [a Fe(II) metal chromophore], continuously N_2 purged. After 5 min, 1 ml of 250 mM $Na_2S_2O_4$ (sodium dithionite) was added. After an additional 5 min, the roots were removed from the solution and briefly rinsed with deionized water (18 MΩ quality). Shoots and roots were dried in an oven at 60°C overnight, weighed and dry-ashed overnight in quartz tubes in a muffle furnace at 550°C. The ash was dissolved in 1 ml

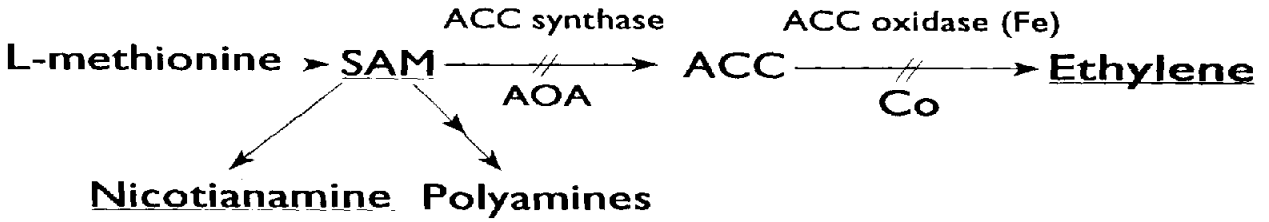

Figure 1. Schematic pathway of ethylene biosynthesis showing the steps at which AOA and cobalt inhibit ethylene production. Other important anabolic products derived from L-methionine via *S*-adenosyl methionine (SAM) are also depicted.

of concentrated HNO_3 and made to 5 ml with deionized water. Digestates were analyzed for iron, manganese, zinc, magnesium and potassium via simultaneous, inductively-coupled, argon-plasma, emission spectrometry. Statistical analyses were performed using the Statistical Analysis System Software (SAS Institute, Cary, NC).

Results

Initial iron stores in seeds of *Sparkle* seedlings grown under iron-deficient conditions were sufficient to depressed Fe(III)-chelate reductase activity in *Sparkle* roots until 13–14 days after germination. Thereafter, iron-deficient roots of Sparkle seedlings demonstrated greatly increased rates of Fe(III)-chelate reductase activity when not treated with cobalt or AOA. Addition of either 3 μM $CoCl_2$ or 20 μM AOA to nutrient solutions of 12 day old, iron-deficient *E107* and *Sparkle* pea plants for 1 day (i.e. from day 12 to 13) drastically inhibited the development of root Fe(III)-chelate reductase activity (Figures 2 and 3). The cobalt treatment did not effect root fresh weights or shoot fresh weights when determined on day 19. The AOA treatment resulted in a decrease in root fresh weights to values between 80 and 90% of the untreated controls at final harvest on day 19 (data not shown).

The addition of 20 μM Fe(III)–EDDHA to the nutrient solutions of either iron-deficient or cobalt-treated, iron-deficient *E107* pea plants on day 13 greatly increased root Fe(III)-chelate reductase activity (Figure 2A). Similarly, the

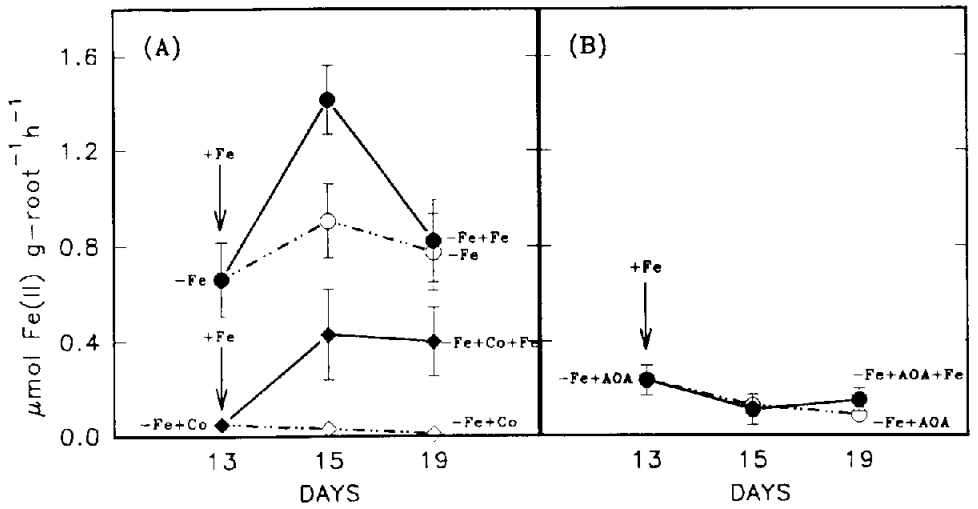

Figure 2. Effect of Fe(III) on Fe(III) reduction rates in roots of iron-deficient *E107* peas treated with either Co^{2+} (**A**) or AOA (**B**). Plants were grown in nutrient solution without Fe. On day 12, either 3 μM $CoCl_2$ or 20 μM AOA was added to the nutrient solution in the Co^{2+} and AOA treatments, respectively. On day 13, 20 μM Fe(III)–EDDHA (arrows) was added to half of the plants in each treatment ($n=6$).

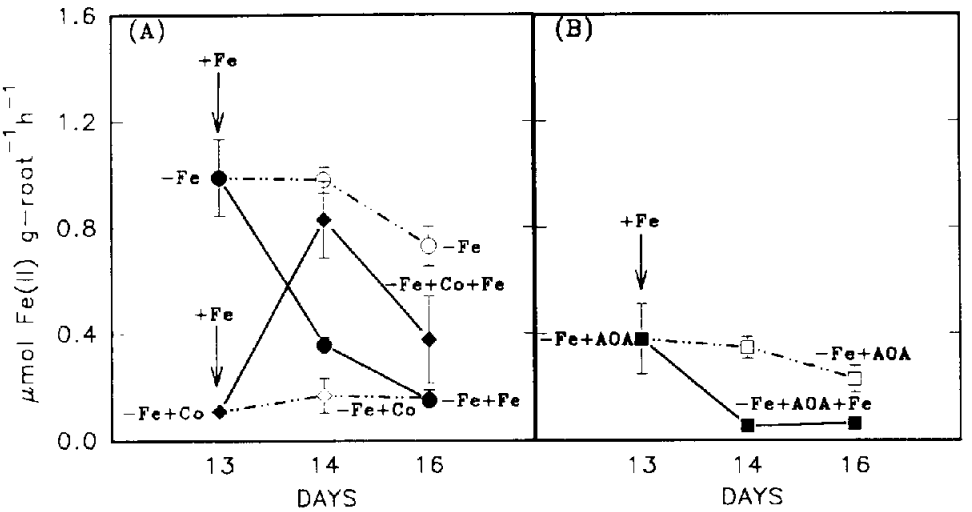

Figure 3. Effect of Fe(III) on Fe(III) reduction rates in roots of iron-deficient *Sparkle* peas treated with either Co^{2+} (**A**) or AOA (**B**). Plants were grown in nutrient solution without iron. On day 12, either 3 μM $CoCl_2$ or 20 μM AOA was added to the nutrient solution in the Co^{2+} and AOA treatments, respectively. On day 13, 20 μM Fe(III)–EDDHA (arrows) was added to half of the plants in each treatment ($n=6$).

addition of 20 μM Fe(III)–EDDHA to cobalt-treated (on day 12), iron-deficient *Sparkle* pea plants on day 13 also greatly increased root Fe(III) reducing capacity, although transitorily (Figure 3A). However, the addition of 20 μM Fe(III)–EDDHA on day 13 did not increase Fe(III)-chelate reductase activity in either *E107* or *Sparkle* pea plants already inhibited by AOA treatment on day 12 (Figures 2B and 3B). In contrast to iron-deficient *E107*, addition of 20 μM Fe(III)–EDDHA on day 13 drastically decreased Fe(III)-chelate reductase activity in roots of iron-deficient *Sparkle* pea plants (Figure 3A). In the absence of cobalt treatment, mutant *E107* roots (grown under iron-deficient or iron-adequate conditions) always express high rates of Fe(III)-chelate reduction because they lack the genetic ability to suppress their Fe(III)-chelate reductase activity (Welch & LaRue 1990).

Supplying 10 μM $MnSO_4$, 5 μM $ZnSO_4$ or 5 μM $CuSO_4$ to the nutrient solutions of cobalt-treated, iron-deficient, *Sparkle* pea seedlings on day 13 did not reverse the inhibitory effect of cobalt on Fe(III)-chelate reductase activity (Figure 4). However when 20 μM Fe(III)–EDDHA was added to the nutrient solutions on day 13 (Figure 4) of the iron-deficient, cobalt-treated seedlings, root Fe(III)-chelate reducing capacity was greatly stimulated. This was true even though the activity of Fe^{3+} in solution would be exceedingly low compared with the relatively high concentrations of Mn^{2+}, Cu^{2+} or Zn^{2+} supplied, because of the very high stability of the Fe(III)–EDDHA complex. Even at these relatively high levels of Mn^{2+}, Cu^{2+} and Zn^{2+}, these ions could not overcome the inhibitory effects of cobalt on Fe(III)-chelate reduction showing a remarkably high specificity of iron in overcoming cobalt inhibition of Fe(III) reduction.

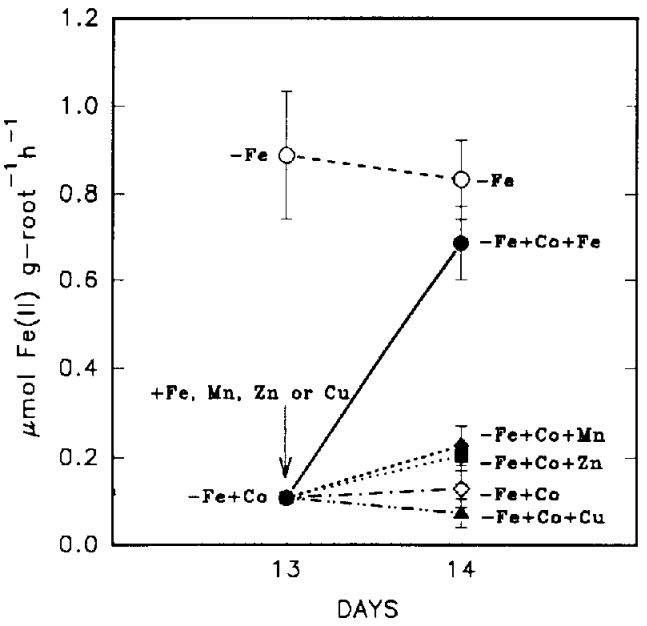

Figure 4. Effect of iron, manganese, zinc and copper treatments on Fe(III) reduction rates of iron-deficient *Sparkle* peaks treated with Co^{2+}. Plants were grown in nutrient solution without iron. On day 12, 3 μM $CoCl_2$ was added to the nutrient solution in the Co^{2+} treatment. On day 13, either 20 μM Fe–EDDHA, 10 μM $MnSO_4$, 5 μM $ZnSO_4$ or 5 μM $CuSO_4$ (arrows) was added to the nutrient solution of the Co^{2+} treated plants ($n=4$).

Treatment of 12 day old, iron-deficient, *E107* pea seedlings with 3 μM $CoCl_2$ decreased both their root Fe(III)-chelate reducing capacity (Figure 2A) and their total uptake (expressed as total uptake per root dry weight) of manganese, zinc, magnesium and potassium (treatments 2 and 3, Table 1). Furthermore, the addition of 3 μM $CoCl_2$ to the 12 day old, iron-treated, *E107* pea seedlings, decreased both their root Fe(III)-chelate reducing capacity (Figure 2A) and their total uptake of manganese, zinc, magnesium and potassium (treatments 1 and 4, Table 1). The addition of 20 μM Fe(III)–EDDHA to cobalt-treated, iron-deficient, *E107* seedlings on day 13 increased both their root Fe(III)-chelate reducing capacity (Figure 2A) and their uptake of manganese, zinc, magnesium and potassium when harvested on day 19, when compared with the cobalt-treated plants not receiving Fe(III)–EDDHA (treatments 3 and 4, Table 1). Thus, 3 μM Co treatment of iron-deficient seedlings on day 12 inhibited their accumulation of manganese, zinc, magnesium and potassium, while treatment of the iron-deficient seedlings with Fe(III)–EDDHA on day 13 stimulated cation accumulation in either the presence or absence of 3 μM Co applied on day 12 (Table 1).

Discussion

The addition of the ethylene inhibitors, cobalt or AOA (for discussions of ethylene inhibitors, see Lau & Yang 1976, Yang & Hoffman 1984) to the nutrient solutions of both iron-deficient *E107* and *Sparkle* peas drastically inhibited their root Fe(III) reducing capacity (Figures 2 and 3). These

Table 1. Effect of cobalt and iron treatments on the total plant uptake (total content of roots plus shoots) per g root (dry weight) of iron, manganese, zinc, magnesium and potassium in 19 day old *E107* pea seedlings

Treatments	Fe (μg g root DW^{-1})	Zn (μg g root DW^{-1})	Mn (μg g root DW^{-1})	Mg (μg g root DW^{-1})	K (μg g root DW^{-1})
−Fe, +Fe	10757^a	3054^a	2082^a	27214^a	243476^a
−Fe	198^c	1984^b	2220^a	22267^a	208675^a
−Fe, +Co	70^c	688^d	587^c	9031^b	82385^c
−Fe, +Co, +Fe	1540^b	937^c	877^b	11667^b	127314^b

Seedlings were grown in nutrient solution without iron additions. On day 12, 3 μM $CoCl_2$ was supplied to seedlings in treatments 3 and 4. On day 13, 20 μM Fe(III)–EDDHA was supplied to plants in treatments 1 and 4. Means ($n=6$) in the same column with different superscripts differ significantly ($P<0.05$).

results strongly suggest that ethylene plays a role in the regulation of root Fe(III)-chelate reductase activity in both genotypes and agree with previous results obtained with cucumber (Romera & Alcántara 1993, 1994), tomato and pea plants (Romera *et al.* 1995). In these previous reports, both cobalt and AOA greatly inhibited root Fe(III)-chelate reducing capacity of iron-deficient seedlings, suggesting that increased ethylene production is a prerequisite for stimulating root Fe(III)-chelate reductase activity (Romera & Alcántara, 1993, 1994, Romera *et al.* 1995).

Supplying Fe(III)–EDDHA on day 13 to either cobalt-treated (day 12), iron-deficient *E107* or *Sparkle* seedlings greatly increased root Fe(III)-chelate reductase activity, partly reversing the inhibitory effect of cobalt on reductase activity (Figures 2A and 3A). Increased Fe(III)-chelate reducing capacity was also observed when iron-deficient *E107* peas, not treated with cobalt on day 12, were supplied Fe(III)–EDDHA on day 13 (Figure 2A). Supplying iron on day 13 to iron-deficient seedlings treated with AOA on day 12 did not effect root reductase activity (Figure 1B). These results suggest that different sites of ethylene biosynthesis are affected when iron-deficient seedlings are treated with iron in the presence or absence of either AOA or cobalt.

ACC oxidase catalyses the conversion of ACC to ethylene. This enzyme requires iron for activation and is inhibited competitively by Co^{2+} (Dilley *et al.* 1993). Our findings that iron reverses the effects of cobalt inhibition of Fe(III)-chelate reductase activity in roots of iron-deficient peas is consistent with the concept that iron competed with cobalt for active sites in ACC oxidase (Dilley *et al.* 1993). ACC synthase catalyses the conversion of SAM to ACC (Yang & Hoffman 1984). AOA is a known inhibitor of ACC synthase and iron is not involved in the activation of this enzyme. Figure 2(B) shows that supplying iron to iron-deficient seedlings supplied AOA on day 12 did not effect root reductase activity. Hypothetically, addition of Fe(III)–EDDHA to cobalt-treated, iron-deficient pea seedlings would be expected to increase ethylene production and thereby stimulate root Fe(III)-chelate reductase activity, while iron additions to AOA treated roots would be expected to inhibit root reductase activity. The results shown in Figures 2 and 3 support this hypothesis. Others have reported that in Co^{2+}-treated pea plants, ACC concentrations greatly increase, since Co^{2+} inhibits the conversion of ACC to ethylene (Yu & Yang 1979). Consequently, when iron is added to cobalt-treated plants, the limiting factor for ethylene synthesis would be ACC oxidase activity, not ACC substrate levels (Romera & Alcántara 1993, 1994, Romera *et al.* 1995).

In cobalt-treated, iron-deficient *Sparkle* pea seedlings, supplying Fe(III) EDDHA on day 13 greatly increased root Fe(III) reductase activity, although transitorily (Figure 3A). Initially iron may stimulate ethylene biosynthesis and subsequently Fe(III)-chelate reductase activity (Romera & Alcántara 1993, 1994; Romera *et al.* 1995), because cobalt-treated plants would already contain relatively high root ACC levels. Subsequently, ACC concentrations would decrease as ethylene is produced, allowing the added iron on day 13 to repress Fe(III)-chelate reductase activity. Accordingly, iron could play two roles in regulating Fe(III)-chelate reductase activity, i.e. by (1) iron repressing the gene(s) responsible for Fe(III)-deficiency stress responses, acting at some step prior to ethylene biosynthesis and (2) iron stimulation of Fe(III) reductase activity via direct involvement in ethylene biosynthesis. The iron levels required for ethylene biosynthesis might be much lower than that required to repress the iron-deficiency stress response mechanisms. Under iron-deficient conditions, the concentrations of iron in plant tissues might be adequate for some ethylene biosynthesis, as shown by Morgan & Hall (1962), but not for repressing the genes responsible for induction of iron stress response mechanisms.

Supplying Fe(III)–EDDHA to 13 day old, iron-deficient, *E107* seedlings increased root Fe(III)-chelate reductase activity (Figure 2A). Similar results were reported by Grusak *et al.* (1990). Iron stimulation of reductase activity under these conditions suggests that the *E107* mutant has the ability to control ethylene production but lacks the ability to repress other gene(s) controlling iron-deficienty stress response mechanisms. In the normal parental genotype *Sparkle*, Fe(III)-chelate reductase activity was inhibited when 20 μM Fe(III)–EDDHA was added to the nutrient solution (Figure 2A). In *E107*, which cannot regulate Fe(III)-chelate reductase activity, supplying iron would stimulate ethylene synthesis continuously. Consequently, iron would continue to accumulate in these seedlings. The decrease in Fe(III)-chelate reducing capacity, observed on day 19 in iron-deficient *E107* pea plants treated with iron (treatment −Fd−Fe; Figure 2A) on day 13, could be the consequence of high (and possibly toxic) iron levels in the seedling tissues (e.g. see Table 1, first row iron levels) that could have caused metabolic disturbances within the seedlings resulting in the stunted growth and necrotic older leaves observed in seedlings supplied this treatment.

Neither the addition of Zn^{2+}, Mn^{2+} nor Cu^{2+} to Co^{2+}-treated, iron-deficient *Sparkle* peaks reversed the inhibitory effect of Co^{2+} on Fe(III) reduction, as was observed for Fe(III)–EDDHA additions on day 13 (Figure 4). These results suggest that the function of iron in reversing the inhibitory effect of Co^{2+} on Fe(III) reduction is rather specific, possibly related to the role of iron in ACC oxidase enzyme (Dilley *et al.* 1993), as discussed previously.

Supplying Co^{2+} to either iron-deficient or iron-sufficient *E107* seedlings decreased root Fe(III)-chelate reduction rates (Figure 2A). Furthermore, Co^{2+} treatment also decreased the total seedling accumulation of iron, manganese, zinc, magnesium and potassium (Table 1). When iron was added to cobalt-treated, iron-deficient *E107* plants on day 13, the root Fe(III) reduction rates increased (Figure 2A) as did the accumulation of manganese, zinc, magnesium and potassium (Table 1). Thus, the effect of Co^{2+} was not the result of competition in absorption between Co^{2+} and the other divalent cations tested. Moreover, it would be difficult to explain how Co^{2+}, supplied at 3 μM, could compete with the macronutrient cations K^+ and Mg^{2+}, that were supplied at millimolar levels in the nutrient solutions. Possibly, the inhibitory effect of Co^{2+} on cation accumulation is linked

to its role as an ethylene inhibitor, through a role of ethylene in root cell plasma membrane transport processes. Decreased iron uptake under conditions of lower Fe(III) reducing capacity (Table 1) is logical since Fe(III) reduction is a requisite for absorption of iron as Fe^{2+} (Chaney *et al.* 1972). Possibly, decreased uptake of manganese, zinc, magnesium and potassium, under low Fe(III) reducing conditions, is the result of decreased Fe(III)-chelate reductase activity as suggested by Welch *et al.* (1993). They hypothesized that root cell plasma membrane reductases play a role in gating plasma membrane cation channels and through this role these reductases perform a function in regulating cation transport processes. Also, cobalt treatment could have inhibited the activity of the root cell plasma membrane H^+-translocating ATPase, thus lowering the driving force for cation uptake (Welch 1995). Interestingly, we have reported that cobalt treatment of iron-deficient cucumber roots inhibited growth media acidification (Romera & Alcántara 1994). Both of these speculations are possible and each would complement the other in their effects on cation accumulation.

Various authors have speculated that the sulphydryl groups of ion channels in the plasma membrane have to be in a reduced state to allow the opening of the channels (Bienfait & Lüttge 1988, Kochian & Lucas 1991, Welch *et al.* 1993, Welch 1995). Bienfait & Lüttge (1988) proposed that this role may be performed by the 'Standard' reductase in root cell plasma membranes. As stated above, Welch *et al.* (1993) also suggested that the Fe(III)-chelate reductase (i.e. 'Turbo' reductase) could be implicated in the gating of ion channels.

If the Fe(III)-chelate reductase affects the gating of cation channels, as suggested by our data, then it could be possible to establish a relationship among root stress, ethylene action, reductase activity and cation absorption processes. Perhaps, some of the ethylene-mediated responses of plants to different rhizosphere stresses involve increased uptake of cations. Clearly, much remains to be learned about the action of ethylene on root reductase activity, root stress responses and root cell ion transport processes in higher plants.

Acknowledgments

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