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Ethylene Production by Fe-deficient Roots and its Involvement in the Regulation of Fe-deficiency Stress Responses by Strategy I Plants

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Species that showed marked morphological and physiological responses by their roots to Fe-deficiency (Strategy I plants) were compared with others that do not exhibit these responses (Strategy II plants). Roots from Fe-deficient cucumber (*Cucumis sativus* L. 'Ashley'), tomato (*Lycopersicon esculentum* Mill. T3238FER) and pea (*Pisum sativum* L. 'Sparkle') plants produced more ethylene than those of Fe-sufficient plants. The higher production of ethylene in Fe-deficient cucumber and pea plants occurred before Fe-deficient plants showed chlorosis symptoms and was parallel to the occurrence of Fe-deficiency stress responses. The addition of either the ethylene precursor ACC, 1-aminocyclopropane-1-carboxylic acid, or the ethylene releasing substance, Ethephon, to several Fe-sufficient Strategy I plants [cucumber, tomato, pea, sugar beet (*Beta vulgaris* L.), *Arabidopsis* (*Arabidopsis thaliana* (L.) Heynh 'Columbia'), plantago (*Plantago lanceolata* L.)] promoted some of their Fe-deficiency stress responses: enhanced root ferric-reducing capacity and swollen root tips. By contrast, Fe-deficient roots from several Strategy II plants [maize (*Zea mays* L. 'Funo'), wheat (*Triticum aestivum* L. 'Yécora'), barley (*Hordeum vulgare* L. 'Barbarrosa')] did not produce more ethylene than the Fe-sufficient ones. Furthermore, ACC had no effect on the reducing capacity of these Strategy II plants and, except in barley, did not promote swelling of root tips. In conclusion, results suggest that ethylene is involved in the regulation of Fe-deficiency stress responses by Strategy I plants.

Key words: *Arabidopsis* (*Arabidopsis thaliana* (L.) Heynch), barley (*Hordeum vulgare* L.), cucumber (*Cucumis sativus* L.), ethylene, iron deficiency, maize (*Zea mays* L.), pea (*Pisum sativum* L.), plantago (*Plantago lanceolata* L.), ferric-reducing capacity, sugar beet (*Beta vulgaris* L.), tomato (*Lycopersicon esculentum* Mill.), wheat (*Triticum aestivum* L.).

INTRODUCTION

Iron deficiency induces several morphological and physiological responses in dicotyledonous species (Strategy I plants) while in other species, these responses are largely absent (Strategy II plants). Strategy I plants exhibit subapical root swelling with abundant root hairs, rhizodermal transfer cells, enhanced root ferric-reducing capacity and acidification of the extracellular medium (Römheld and Marschner, 1986; Bienfait, 1988; Kochian, 1991). Several hypotheses have been proposed to explain their regulation. According to Bienfait (1988), there is an activating protein in the root that turns on the responses, i.e. up regulates their respective genes when not bound to Fe. Scholz *et al.* (1992) suggested that the complex nicotianamine-Fe participates in the repression of the responses in such a way that, when nicotianamine is not complexed with Fe, the responses are turned on.

Other authors propose that Fe deficiency increases the levels of some hormones (auxin, ethylene), and that these trigger the responses (Landsberg, 1984, 1996; Romera and Alcántara, 1994; Schmidt and Bartels, 1996). Results from Romera, Alcántara and de la Guardia (1992), obtained in split-root experiments, and Grusak and Pezeshgi (1996), obtained using grafted plants, suggest that a signal compound that acts as a promoter of the responses could move within the plant. The application of hormonal compounds, such as indoleacetic acid (IAA), 2,4-dichloro phenoxyacetic acid (2,4-D) (synthetic auxin), 1-amino-cyclopropane-1-carboxylic acid (ACC) (an ethylene precursor) or Ethephon (an ethylene releasing substance), to roots of several Fe-sufficient Strategy I plants promoted the formation of subapical root swelling with abundant root hairs and rhizodermal transfer cells, similar to those produced by Fe deficiency (Romera and Alcántara, 1994; Landsberg, 1996; Schmidt and Bartels, 1996). Furthermore, the application of ACC, or Ethephon, to Fe-sufficient cucumber plants greatly enhanced their root ferric-reducing capacity (Romera and Alcántara, 1994). Similarly, the addition of 2,4-D to Fe-sufficient plantago plants increased their ferricyanide reducing capacity (Schmidt, 1994; Schmidt and Bartels, 1996). By contrast, the addition of ethylene inhibitors, such as Co^{2+}, Ag^{1+}, aminoethyoxyvinylglycine (AVG) or aminooxyacetic acid (AOA), to Fe-deficient cucumber, pea or tomato plants abolished some of their Fe-deficiency stress responses (Romera and Alcántara, 1994; Romera *et al.*, 1996*a*, *b*). Ethylene is involved in the promotion of root hairs and other morphological changes in roots (Chadwick and Burg, 1967; Baskin and Williamson, 1992, Finlayson and Reid, 1996; Dolan, 1997; Kieber, 1997), and in the stimulation of nitrate reductase activity in *Agrostemma* embryos (Schmerder and Borriss, 1986).

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Other hormones involved in the regulation of Fe-deficiency stress responses were reported by Jolley, Brown and Terry (1995), who found higher ferric-reducing capacity in sunflower-stem tissue inoculated with *Agrobacterium tumefaciens* than in uninoculated tissue, and attributed this to changes in plant hormones. In yeast, which has Fe-uptake mechanisms similar to those in plants, some authors have suggested that ferrireductase activity is not simply related to the Fe status of the cells (Lesuisse *et al.*, 1991; Eide *et al.*, 1992; Lesuisse and Labbe, 1992).

Here, we provide new evidence in support of the hormonal control of Fe-deficiency stress responses by presenting data on ethylene production by roots of several Fe-sufficient and Fe-deficient Strategy I plants, together with confirmation that ethylene promotes the ferric-reducing capacity of roots of different Strategy I plant species.

MATERIALS AND METHODS

Growth of plants and treatments

Seeds of cucumber (*Cucumis sativus* L. 'Ashley'), pea (*Pisum sativum* L. 'Sparkle'), barley (*Hordeum vulgare* L. 'Barbarrosa'), wheat (*Triticum aestivum* L. 'Yécora') and maize (*Zea mays* L. 'Funo') were germinated within moistened papers. Seeds of *Arabidopsis* (*Arabidopsis thaliana* (L.) Heynh. 'Columbia') were germinated in perlite, and those of sugar beet (*Beta vulgaris* L.), plantago (*Plantago lanceolata* L.) and tomato (*Lycopersicon esculentum* Mill. T3238FER) in sand. In all cases, seeds were moistened with 5 mM $CaCl_2$ and kept in the dark until germination. Seedlings were transferred to plastic pots containing a continuously aerated nutrient solution (solution A) 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 Fe-EDDHA (or Fe-HEDTA for grasses). Levels of Fe-EDDHA (or Fe-HEDTA) used varied between experiments. The pH was adjusted to 6·0 with 0·1 N KOH. In some experiments with tomato, the following solution (solution B) was used (mM): 2 $Ca(NO_3)_2$; 3 KNO_3; 3 $(NH_4)_2SO_4$; 1 $MgSO_4$, 0·2 $NH_4H_2PO_4$; micronutrients as above but with 45 μM H_3BO_3 (the cultivar used was B-inefficient; Brown, Chaney and Ambler, 1971), Fe as Fe-HEDTA, and pH 5·5. Nutrient solutions were periodically renewed. In some experiments, ethylene production by roots and root ferric-reducing capacity were determined as described below. In other experiments, ACC or Ethephon were added to the nutrient solution bathing the roots from stock solutions of each chemical. After several hours in these treatments, root ferric-reducing capacity was determined as described below. Stock solutions of ACC and Ethephon were prepared in deionized water. Plants were grown in a growth chamber at 22 °C day/18 °C night temperatures, with relative humidity between 50 and 70% and a 14 h photoperiod at a photosynthetic irradiance of 250 μmol m^{-2} s^{-1} provided by fluorescent tubes (Sylvania Cool White VHO). For *Arabidopsis*, the growth conditions were similar except that temperatures were 24 °C day/22 °C night and the photoperiod was 8 h, to postpone flowering.

Measurement of ethylene produced by roots

To assay ethylene production, roots were separated from the aerial part and enclosed in either 15 or 25 ml test tubes (depending on the size of the root) containing 200 μl tap water. Tubes were sealed with rubber caps and incubated in the dark at 26 °C for 1 or 2 h. Gas samples were withdrawn from the incubation tubes with a 1 ml syringe and assayed with a Hewlett Packard gas chromatograph (Model 5890A) equipped with a 3 mm × 1 m alumina column and a flame ionization detector. The temperature of the oven was 80 °C. N_2, H_2 and O_2 flow rates were 35, 30 and 300 ml min^{-1}, respectively. Ethylene identity was based on a retention time compared to a standard. Finally, roots were taken from the tubes and their fresh weights recorded.

Measurement of root ferric reducing capacity

Intact plants (other than those used for ethylene determination) were pre-treated for 30 min in solution A without micronutrients and then transferred for 1 h to a similar solution that also contained 100 μM Fe^{3+} EDTA and 300 μM ferrozine, pH 5·0 (assay solution). Experimental conditions during the measurement of Fe(III) reduction were similar to those described above for the growth of plants. The ferric-reducing capacity was determined by measuring the concentration of Fe^{2+}-ferrozine complex formed, via absorbance measurements at 562 nm in a spectrophotometer. Reduction rates were calculated using an extinction coefficient of 29 800 M^{-1} cm^{-1}.

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

RESULTS

Ethylene production by roots and root ferric-reducing capacity

Roots from Fe-deficient cucumber (Fig. 1, Table 1), tomato (Fig. 2) and pea (Table 1) plants produced more ethylene

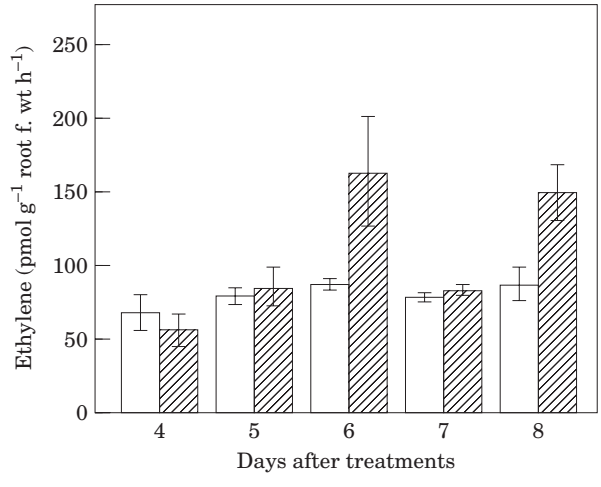

FIG. 1. Time course of ethylene production by roots of Fe-sufficient (□) and Fe-deficient (▨) cucumber plants. Values are means ± s.e. ($n = 6$).

TABLE 1. *Ethylene production by roots and root ferric-reducing capacity of Fe-sufficient and Fe-deficient cucumber and pea plants*

Treatment	Ethylene (pmol g^{-1} root f. wt h^{-1})	Reducing capacity (nmol Fe^{2+} g^{-1} root f. wt h^{-1})
Cucumber		
40 μM Fe	72 ± 8 (100 %)	284 ± 86 (100 %)
−Fe (2d)	92 ± 6 (128 %)	682 ± 171 (240 %)
−Fe (3d)	122 ± 9 (170 %)	1099 ± 45 (387 %)
Pea		
10 μM Fe	45 ± 3 (100 %)	75 ± 4 (100 %)
−Fe (9d)	70 ± 9 (156 %)	426 ± 61 (568 %)

Values in brackets are percentages of those in Fe-sufficient plants. Values are means ± s.e. ($n = 6$).

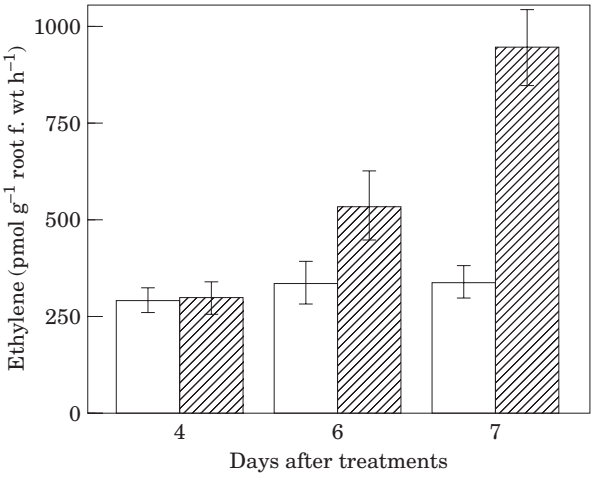

FIG. 2. Time course of ethylene production by roots of Fe-sufficient (□) and Fe-deficient (▨) tomato plants. Values are means ± s.e. ($n = 6$).

TABLE 2. *Ethylene production by roots of Fe-sufficient and Fe-deficient maize, barley and wheat plants*

Treatment μM Fe	Ethylene (pmol g^{-1} root f. wt h^{-1}) Days after treatments 6	7	8
Maize			
40	36 ± 5	37 ± 2	48 ± 1
2	39 ± 2	40 ± 4	47 ± 4
Barley			
40	78 ± 6	55 ± 2	64 ± 6
2	79 ± 7	49 ± 5	49 ± 3
Wheat			
40	146 ± 11	141 ± 14	162 ± 12
2	118 ± 4	118 ± 7	149 ± 10

Values are means ± s.e. ($n = 6$).

than those from Fe-sufficient plants. The higher production of ethylene by Fe-deficient roots was appreciable within 2 to 9 d of treatment, depending on the species, and before plants showed symptoms of leaf chlorosis. The higher ethylene production of Fe-deficient cucumber and pea plants was related to enhanced root ferric-reducing capacity (Table 1) and to the appearance of subapically swollen root tips.

Roots from Fe-deficient maize, wheat and barley plants did not produce more ethylene than those from the Fe-sufficient ones (Table 2). These plants showed mild chlorosis at the end of the experiments.

TABLE 3. *Effect of ACC on the root ferric-reducing capacity of several Fe-sufficient Strategy I plants*

	Reducing capacity (nmol Fe^{2+} g^{-1} root f. wt h^{-1}) Control	ACC treatment
Pea	75 ± 14 (100 %)	156 ± 6 (208 %)
Sugar beet	110 ± 15 (100 %)	299 ± 25 (272 %)
Tomato	290 ± 65 (100 %)	648 ± 36 (223 %)
Arabidopsis	202 ± 66 (100 %)	1095 ± 386 (542 %)

Values in brackets are percentages of those in control plants. Values are means ± s.e. ($n = 6$).

Treatments with ACC or Ethephon

Treatment of different Fe-sufficient Strategy I plants with ACC substantially increased their root ferric-reducing capacity (Table 3, Fig. 3A). Similarly, the addition of Ethephon to Fe-sufficient plantago plants also enhanced their reducing capacity (Fig. 3B). Furthermore, both ACC and Ethephon treatments caused the appearance of numerous subapically swollen root tips with abundant root hairs.

The effect of ACC on the root ferric-reducing capacity of Fe-sufficient cucumber plants depended on its concentration, acting as a promoter only at the lower concentrations (Fig. 3A). Moreover, at 50 μM, ACC caused deformation and curvature of the subapical root zone. Similar results were found with Ethephon in plantago plants (Fig. 3B).

The application of ACC to different Fe-sufficient grasses (Strategy II plants) did not increase their root ferric-reducing capacity significantly (Table 4). In addition, with the exception of barley, ACC treatment did not cause swollen root tips with root hairs in these plants.

DISCUSSION

Previous results, obtained using both ethylene inhibitors and precursors, indicated an involvement of ethylene in triggering Fe-deficiency stress responses by Strategy I plants (Romera and Alcántara, 1994; Romera *et al.* 1996*a*, *b*). It was suggested that Fe deficiency could increase ethylene production by these plants, but this hormone was not assayed at that time. Here, new evidence was obtained to support this hypothesis by measuring the production of ethylene by roots of both Fe-sufficient and Fe-deficient Strategy I plants. As shown in Figs 1 and 2, and Table 1,

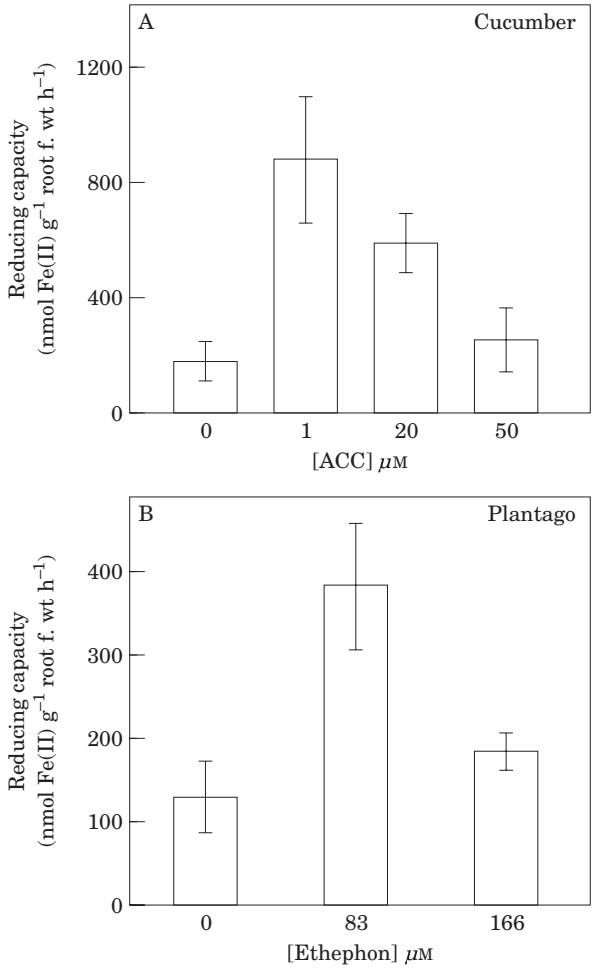

FIG. 3. Influence of ACC (A) and Ethephon (B) concentration on the root ferric-reducing capacity of cucumber and plantago plants. Values are means ± s.e. ($n = 6$).

TABLE 4. *Effect of ACC on the root ferric-reducing capacity of several Fe-sufficient Strategy II plants*

Treatment	Reducing capacity (nmol Fe^{2+} g^{-1} root f. wt h^{-1})		
	Maize	Wheat	Barley
Control	17 ± 3	19 ± 9	9 ± 2
0·1 μM ACC	17 ± 4	8 ± 3	12 ± 4
0·5 μM ACC	21 ± 5	9 ± 2	14 ± 3
1 μM ACC	16 ± 5	19 ± 2	9 ± 2
5 μM ACC	17 ± 8	25 ± 4	17 ± 7

Values are means ± s.e. ($n = 6$).

roots from several Fe-deficient Strategy I plants produced more ethylene than those from Fe-sufficient plants. Ethylene levels obtained here are in line with those reported by other authors in pea (Lee and LaRue, 1992), tomato (Zacarias and Reid, 1992), cucumber (Rab and Saltveit, 1996) and sunflower (Finlayson and Reid, 1996; Finlayson, Liu and Reid, 1996). In grasses, which use another strategy to obtain Fe (Strategy II; Römheld and Marschner, 1986), an increase in ethylene production by Fe-deficient roots was not detectable (Table 2). Experimental conditions were not identical for the different plant species because, according to previous experience, they have distinct requirements.

Whole tomato plants with Ca or Mg deficiency (Barker and Corey, 1988; Feng and Barker, 1992), Fe-deficient sorghum plants (Morgan and Hall, 1962), and roots of bean plants with P deficiency (Lynch and Brown, 1997), also produced more ethylene than nutrient-sufficient plants. However, in some cases the determinations of ethylene production were performed when plants showed very clear symptoms of deficiency, which could lead to tissue necrosis and thereby stimulation of wound ethylene (Lynch and Brown, 1997). Here, increased production of ethylene by Fe-deficient roots was evident a few days after transferring the plants to Fe-deficient conditions (Table 1, Figs 1 and 2), before plants showed symptoms of leaf chlorosis. This implies that plants were suffering latent Fe deficiency despite still being green. Different plant species develop morphological and physiological Fe stress-response reactions while their leaves are still green (Landsberg, 1995). Increased ethylene production by Fe-deficient roots paralleled the enhancement of root ferric-reducing capacity (Table 1) and the appearance of swollen root tips.

The involvement of ethylene in the regulation of Fe-deficiency stress responses is also supported by results showing a promoting effect of ACC and Ethephon on both ferric-reducing capacity (Table 3, Fig. 3) and subapically swollen root tips of several Strategy I plants. Ethylene probably induces enhancement of the ferric-reducing capacity only within a specific concentration range. As shown in Fig. 3, ACC and Ethephon both increased the reducing capacity of cucumber and plantago plants, respectively, when applied at low rather than high concentrations.

In Strategy II plants that neither enhance their reducing capacity nor develop swollen root tips under Fe deficiency, ACC had no promoting effect on either reducing capacity, which was always very low (Table 4), or on swollen root tips, except in barley. Barlow (1976) also found no swelling of maize roots with ethylene treatment. Neither did the addition of ACC to barley seedlings induce the phytosiderophore efflux by their roots, the most important Fe-deficiency stress response by Strategy II plants (Welch *et al.*, 1997).

The induction of Fe-deficiency stress responses by Strategy I plants has also been associated with an increase in auxin in subapical root tips (Landsberg, 1984, 1996; Römheld and Marschner, 1986). In fact, Römheld and Marschner (1986) reported a higher auxin content in roots from Fe-deficient sunflower plants than in those from Fe-sufficient ones. It may be possible to mediate the effect of auxin through ethylene, since high levels of auxin promote ethylene production by inducing the synthesis of ACC synthase (Kim *et al.*, 1992).

The way ethylene influences the Fe-deficiency stress responses of Strategy I plants is not known, but it probably triggers the expression of genes responsible for physiological and morphological changes. This is supported by the fact that ethylene induces not only enhancement of ferric-

reducing capacity, but also root epidermal transfer cells and swollen root tips (Romera and Alcántara 1994; Landsberg, 1996). In some experiments, a promotion of proton extrusion by ACC was also observed (data not shown), which suggests that this response is also regulated by ethylene.

In conclusion, the results here further support the hypothesis that ethylene is involved in the regulation of different Fe-deficiency stress responses by Strategy I plants.

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