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Leaf and Root Responses to Iron Deficiency in Avocado[1]

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ABSTRACT

Physiological responses to iron (Fe) deficiency were characterized in the roots and leaves of avocado plants. These responses included sharply higher catecholase activity, but slightly lower peroxidase activity in leaves and roots of avocado seedlings grown in minus Fe nutrient solutions, and in Fe-deficient leaves of field trees. Iron deficiency in avocado seedlings resulted in much higher rates of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction on the root surfaces. Similarly, the initial rates of FeHEDTA reduction were greater for roots of Fe-deficient avocado seedlings. In contrast, root respiration rates were relatively unaffected by Fe deficiency. Large differences in the manganese (Mn) and zinc (Zn) concentrations in the

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leaves of the Fe-deficient and Fe-sufficient avocado seedlings suggested that the physiological responses that occurred in the roots during Fe deficiency influenced the uptake of other metal micronutrients.

INTRODUCTION

The high carbonate soils of many avocado-growing regions result in frequent metal micronutrient deficiencies in avocado (*Persea americana* Mill.) (Wallihan and Miller, 1968; Kadman and Lahav, 1982; Gregoriou et al., 1983; Crowley, 1992). Avocado trees grown under these field conditions are typically affected by more than one micronutrient deficiency, the physiological effects of which negatively impact tree productivity, growth, and disease resistance. Yet, in spite of exhaustive attempts to develop effective means of supplementing avocado trees with metal micronutrients, the deficiencies in many cases remain. Much of this difficulty arises from a poor understanding of the uptake and translocation of different metal ions by avocado, and of the complex chemistry of metal micronutrients in the plant rhizosphere (Laurie and Manthey, 1994; Reisenauer, 1994).

In an attempt to understand the biochemistry of micronutrient deficiencies in avocado, we characterized a number of responses to Fe deficiency that are expressed in leaf and root tissue. In this study, the changes in the levels of several metalloenzymes, known to be inducible during plant stress, were measured in leaves of field-grown trees and in avocado seedlings grown in minus-Fe nutrient solutions. Iron deficiency induces in certain dicots a number of physiological responses which increase the solubility and availability of Fe in the rhizosphere (Bienfait, 1987). One of these responses, increased electron transfer rates on root surfaces, is reported for Fe-deficient avocado seedlings.

METHODS

Growth Conditions

Avocado (*P. americana* Mill. cv 'Hass') seeds were germinated in potting soil mix and grown to approximately 30 cm in height in a greenhouse with natural lighting. Sour orange (*C. aurantium* L.) seeds were similarly germinated, and grown under identical conditions to a height of 15 cm. Avocado and sour orange seedlings were transferred to complete nutrient solutions containing: 1.3 mM calcium nitrate [$Ca(NO_3)_2$], 1.0 mM potassium nitrate (KNO_3), 0.8 mM magnesium sulfate ($MgSO_4$), 0.1 mM potassium monohydrogen phosphate (K_2HPO_4), 0.56 µM zinc sulfate ($ZnSO_4$), 6.7 µM manganese sulfate (MnSO4), 35 µM Fe^{3+}-N-hydroxy-ethylethylenediaminetriacetic acid (FeHEDTA), 0.24 µM copper sulfate ($CuSO_4$), and 33 µM boric acid (H_3BO_3). All solutions were aerated and maintained at pH 7.5 to 8.0. The seedlings were grown in 30 L of nutrient solution for four

weeks or until new root growth commenced. Half of the plants were transferred to minus Fe nutrient solutions. Fresh nutrient solutions were provided every third week.

Leaves were collected from 'Hass' avocado trees grown on Mexican rootstock in a commercial orchard in Ventura County, California. The orchard (>15 years old) was located on a moderately sloping (15-30%) hillside with soil characterized as a Soper loam containing free calcium carbonate ($CaCO_3$) which was 60 to 150 cm deep over conglomerate rock. Surface soil (0-20 cm) pH values ranged from 7.8 to 8.0 and were buffered by 0.1 to 3% soil carbonate content. Patchy areas containing free $CaCO_3$ were associated with the visibly chlorotic trees. Leaves were selected at the early season, fully expanded development stage from trees exhibiting moderate-to-severe foliar deficiencies. Leaves were washed with detergent and sequentially rinsed with 0.1N hydrochloric acid (HCl) and distilled water to remove trace metal contamination. Levels of Mn, Fe, and Zn in acid-digested leaf tissue were measured in triplicate by atomic absorption spectrometry.

Ferric-Iron and MTT Reduction Reactions

Assays for Fe and MTT reduction by excised avocado root tips were performed as described by Manthey et al. (1994a). Each Fe and MTT reduction reaction involved approximately 20 to 30 actively growing white root tip segments (3-4 cm) randomly selected from 10 or more seedlings of the same Fe status. The MTT reduction at the root surfaces resulted in the precipitation of a purple-colored formazan product. Amounts of formazan formation on the root surfaces were measured by the absorbance at 504 nm of the formazan quantitatively solubilized into 20 mL dimethylformamide.

Plant Enzyme Extracts

Soluble protein extracts were prepared at 4°C from Fe-sufficient and Fe-deficient avocado leaves from field-grown trees and from hydroponically-grown seedlings. Actively growing root tip segments (3-4 cm) were collected from avocado seedlings grown in hydroponics. Leaves and roots were homogenized in cold 0.1M potassium phosphate buffer (pH 6.8) containing 0.02 mM protease inhibitor AEBSF [4-(2-aminoethyl)-benzynesulfonyl fluoride] and 5% polyvinyl-polypyrtolidone. The homogenate was filtered through cheesecloth and centrifuged for 40 min at 17,000 g_{max} The proteins were precipitated from the resulting supernatants with addition of ammonium sulfate $[(NH_4)_2SO_4]$ to 80% saturation, followed by centrifugation for one hour at 17,000 g_{max}. The protein-containing pellets were resolubilized in cold homogenization buffer and subsequently centrifuged at 4°C for 20 min for clarification. The protein fractions were concentrated using Amicon Centriprep and Centricon concentrators (10K

molecular weight cutoff). The concentrated samples were desalted using Bio Rad PD-10 columns. Protein concentrations were measured according to Bradford (1976).

Enzyme Assays

Peroxidase (EC 1.11.1.7) assays of root and leaf homogenates were run in 2.6 mL of 0.10M potassium phosphate buffer (pH 6.8), 0.35 mM p-phenylenediamine, and 0.35 mM hydrogen peroxide (H_3O_3). The peroxidase assays, run in triplicate, were monitored at 540 nm, and initiated by the addition of 25-100 µL of enzyme solution.

Root respiration rates were measured as the rates of oxygen (O_2) consumption by excised root segments in 5 mL of 0.075M potassium phosphate/10 mM EDTA buffer (pH 5.8) at 26°C. Oxygen consumption was measured with a Yellow Springs Oxygen Monitor. Electrode responses were calibrated by monitoring the levels of O_2 production from standardized amounts of H_2O_2 by separately added catalase. Assays, run in triplicate, contained 10 to 15 1.5-cm actively growing root tip segments.

Catecholase (EC 1.10.3.2) activity levels were measured by monitoring the increase in absorbance at 390 nm accompanying the oxidation of catechol. Catecholase assays contained 2.4 mL 0.05M potassium phosphate buffer (pH 7.0) with 0.8 mM catechol. Each sample was run in duplicate using a Cary 15 dual-beam spectrophotometer.

Electrophoresis

The catecholase isozymes were detected by activity-staining the leaf protein fractions resolved by slab gel polyacrylamide electrophoresis (7.5% total acrylamide polymer) according to Laemmli (1970) minus SDS and ß-mercaptoethanol. To visualize protein bands with catecholase activity, the resolved gels were immediately placed in 50 mL of catecholase assay solution. Rapid brown band formation occurred where catecholase activity occurred on the gel.

Phenol Analysis

Phenolic compounds in avocado leaves were extracted into dimethyl sulfoxide and analyzed by high-performance liquid chromatography as described by Kanes et al. (1993).

RESULTS AND DISCUSSION

Leaf Responses to Iron Deficiency

Among the hydroponically-grown avocado seedlings the chlorotic (Fe-deficient) plants contained less Fe than the control plants, but the Mn and Zn levels were, respectively, 7.7 and 2.8 times higher in the chlorotic plants. In contrast, similarly

grown sour orange seedlings had no significant difference in Mn content between the control and chlorotic plants. Zinc levels were only 1.5 times higher in the chlorotic plants. This suggests that the unusual metal uptake of the chlorotic avocado seedlings reflected physiological properties of the avocado root systems and not the composition of the nutrient solutions.

The Mn and Zn status was determined in Fe-deficient leaves of 34 field-grown trees where Mn and Zn deficiencies also occurred. Unlike the hydroponically-grown seedlings, there were no correlations between Mn or Zn levels with the Fe status of the leaf tissue (data not shown). This is attributed to the severely limited Zn and Mn bioavailability in the high carbonate soils in which the trees were grown as compared to the sufficient levels of Mn and Zn maintained in the hydroponic solutions.

Earlier work showed that Fe and Mn deficiencies could be readily distinguished in citrus leaf tissue by the effects of these specific deficiencies on peroxidase activity levels in chlorotic leaves (Bar-Akiva and Lavon, 1969). Iron deficiency in citrus leaves resulted in decreased peroxidase levels, whereas the peroxidase levels were higher in the Mn-deficient leaf tissue. Similar analyses of leaf peroxidase activities in field-grown 'Hass' avocado trees showed that the dependence of peroxidase activity levels on Fe concentrations was statistically significant ($P=0.002$) (Figure 1). However, no correlation occurred between the peroxidase activity levels and leaf Mn content (data not shown).

The peroxidase activity levels in the leaves of hydroponically grown avocado seedlings were much lower than in the field-grown leaves, and in contrast to the regression relationship shown in Figure 1, the peroxidase activity levels in leaf extracts of the control and chlorotic hydroponically-grown 'Hass' avocado seedlings, based on leaf fresh weights, showed very little difference (1.73 and 1.98 dA_{540} $min^{-1} \cdot \mu g^{-1}$ FW, respectively, Table 1). However, when the rates were based on μg^{-1} soluble protein, the chlorotic leaf rates (25 dA_{540} $min^{-1} \cdot \mu g^{-1}$) were nearly six times lower than the control leaf rates (161 dA_{540} $min^{-1} \cdot \mu g^{-1}$). Measurements of leaf disk peroxidase activity (Bar-Akiva and Lavon, 1969) in equally mature leaves of hydroponically-grown seedlings routinely showed no significant difference between control and chlorotic avocado leaves (data not shown).

The effects of Fe deficiency in 'Hass' avocado leaves were further studied by measuring the differences in the catecholase activity levels in leaves from avocado trees with and without foliar Fe-deficiency symptoms. Similar measurements were made with control and chlorotic hydroponically-grown seedlings. These studies were based on the routine observation that aqueous extracts (pH 6.8) of the chlorotic leaves of hydroponically-grown avocado seedlings rapidly turned dark brown, whereas extracts of the control leaves showed no color formation. The browning of the chlorotic leaf extracts suggested rapid phenol oxidation, possibly by the catalytic activities of catecholase enzymes similar to those that are found in high concentrations in avocado fruit (Golan et al., 1977). High-performance liquid chromatography analysis of the phenols extracted from control

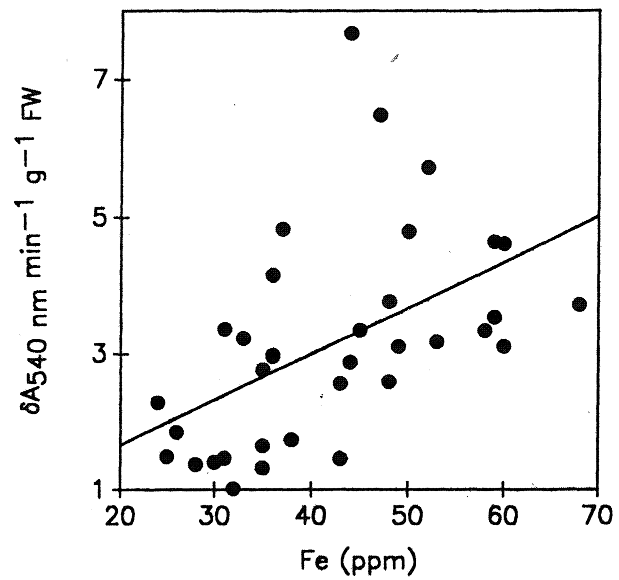

FIGURE 1. Peroxidase activity (dA_{540} $min^{-1} \cdot g^{-1}$ FW) of fully-expanded leaves of field-grown trees. Peroxidase activities of individual trees are plotted against leaf Fe content. Line represents linear regression analysis of the dependence (y=3.0402 + 0.6744x) of leaf peroxidase activity on Fe content, significant at P=0.002.

TABLE 1. Enzyme levels in leaves of control and chlorotic hydroponically-grown 'Hass' avocado seedlings. Rates (g^{-1} FW) are total activity levels in leaf extracts. Assays were run in triplicate.

Fe-status	peroxidase (dA_{540} min^{-1} g^{-1} FW)	catecholase (10^{-3} dA_{390} min^{-1} g^{-1} FW)
Control	1.73±0.05[1]	7.13±1.72
Chlorotic	1.98±0.04	0.24±0.03

[1]Standard deviation.

and chlorotic leaves showed no differences in the levels of phenols in the two sets of leaves (data not shown). In contrast, the catecholase activity levels were nearly 30 times higher in the chlorotic avocado leaves than in control leaves (Table 1).

In a similar experiment, the catecholase activity levels measured individually for six equal-sized control and chlorotic leaves from hydroponically-grown seedlings yielded rates of 0.1381±0.102 and 2.911±1.133 dA_{390} $min^{-1}{\cdot}g^{-1}$ FW, respectively. Although catecholase activity levels in the mature, field-grown leaves were substantially lower than in the younger leaves of the hydroponically-grown seedlings, similar differences were observed in the catecholase activity levels. Evidence of the strong induction of catecholases during Fe deficiency in avocado leaves is shown in the catecholase activity-stained native gels (Figure 2) of the soluble protein fractions of the leaves of control and chlorotic seedlings. Three pairs of protein samples (each pair containing equal amounts of protein) were resolved on native gels. Due to the different rates of staining of the individual catecholase isozymes, increasing amounts of protein were loaded in the lane pairs, A/B, C/D, and E/F. In these gels, no catecholase bands appeared in the lanes containing the soluble protein fraction of control hydroponically-grown avocado leaves (Figure 2). In contrast, five bands with catecholase activity were found in the chlorotic leaf protein fraction.

Root Responses to Iron Deficiency

Similar to chlorotic avocado leaf tissue, there were increased catecholase and decreased peroxidase activities in the roots of the chlorotic hydroponically-grown seedlings (Table 2). No differences were detected in the respiration rates of the control and chlorotic roots (Table 3).

Iron deficiency in certain dicots and nongraminaceous monocots is known to result in a number of physiological responses which ultimately lead to greater Fe solubility in the rhizosphere and increased Fe uptake by the roots (Bienfait, 1987; Korcak, 1987; Manthey et al., 1994b). One of the main root responses is increased rates of reduction of extracellular electron acceptors, including Fe chelates. In this study, we monitored the occurrence of Fe-deficiency-stimulated electron transfer on the root surfaces of hydroponically-grown 'Hass' avocado seedlings by measuring the rates of reduction of FeHEDTA and of the tetrazolium substrate MTT. The roots of chlorotic 'Hass' avocado seedlings reduced much more MTT than the roots of the control seedlings (Table 3). Reduction of MTT by the chlorotic roots did not occur at the root tips meristem, but did occur between 2-10 mm from the tip, probably in the zones of elongation and differentiation.

Much smaller differences occurred in the rates of FeHEDTA reduction. The time course of FeHEDTA reduction by the control roots was linear (Figure 3), whereas the time course for the chlorotic roots showed a rapid initial rate followed by a slower linear rate. Very little difference occurred between the slower linear rate of the chlorotic roots and of the linear rate of the control roots (Table 3).

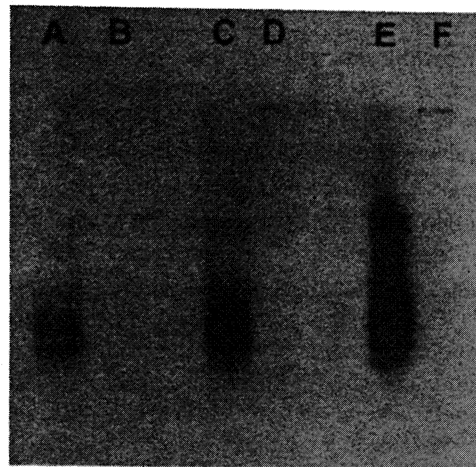

FIGURE 2. Catecholase-stained polyacrylamide electrophoresis gel resolving control and chlorotic leaf protein extracts. Lanes A, C, and E contain 5, 10, and 15 µg total protein from chlorotic avocado leaf extracts. Lanes B, D, and F contain 5, 10, and 15 µg total protein from control avocado leaf extracts.

TABLE 2. Enzyme levels in roots of control and chlorotic hydroponically-grown 'Hass' avocado seedlings. Assays were run in duplicate and values are reported as total activity levels.

Fe-status	peroxidase (dA_{540} min^{-1} g^{-1} FW)	catecholase (10^{-3} dA_{390} min^{-1} g^{-1}FW)
Control	9.6±0.4[1]	45±5
Chlorotic	6.3±0.1	209±3

[1]Standard deviation.

TABLE 3. Rates of FeHEDTA and MTT reduction and respiration rates of control and chlorotic 'Hass' avocado roots. Levels of MTT reduction were measured as the absorbance at 504 nm of the formazan product dissolved in 19 mL dimethylformamide. FeHEDTA reduction rates are calculated from the time courses in Figure 3. FeHEDTA and MTT reduction reactions contained approximately 20 to 30 root tip segments randomly selected from 10 or more seedlings of the same Fe status.

Fe-status	FeHEDTA	MTT	respiration rate
	μmol Fe^{2+} h^{-1} g^{-1}DW	A_{504} g^{-1}FW	mmol O_2 h^{-1} g^{-1}DW
Control	1.22	0.206	1.02±0.25
Chlorotic	4.14 (initial)[1] 0.94 (final)[2]	0.764	1.03±0.19

[1]Initial rate immediately after addition of substrates.
[2]Final rate measured between 3-7 h.

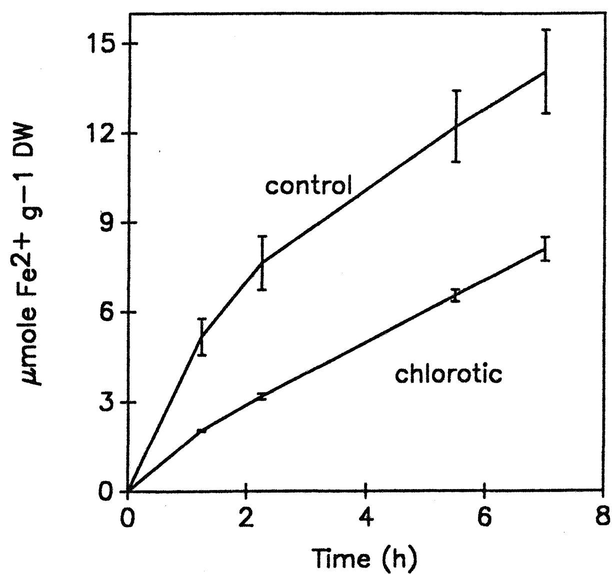

FIGURE 3. Time course of FeHEDTA reduction by control and chlorotic avocado root segments. Values represent means of 2 assays and error bars represent standard deviation.

TABLE 4. Leaf metal concentration (ppm) of hydroponically-grown avocado and sour orange seedlings. Ten to 14 leaves were selected from 15-20 Fe-sufficient (control) and Fe-deficient (chlorotic) plants. Metal ion concentrations were measured in triplicate.

Plant	Fe	Mn	Zn
Hass avocado			
Control	78±26[1]	19±3	17±7
Chlorotic	24±5	146±19	47±17
sour orange			
Control	58±1	41±1	17±1
Chlorotic	31±2	35±7	27±1

[1]Standard deviation.

The absence of much larger rate increases for the FeHEDTA reduction by the chlorotic roots is consistent with the low tolerance to Fe deficiency of 'Hass' avocado. It is likely that other more tolerant avocado varieties, such as the West Indian rootstock cultivars (Kadman and Ben-Yaacov, 1982), will show larger Fe^{3+} reduction rate increases. Nevertheless, evidence of the physiological importance of the root responses of 'Hass' avocado is shown by the unusually high levels of Mn and Zn found in the chlorotic leaf tissue (Table 4). These differences in the metal levels point to possible influences that these responses have on the uptake of other metal micronutrients as was suggested earlier by Warden and Reisenauer (1991) and Welch et al. (1993).

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