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Responses of 'Newhall' Orange Trees to Iron Deficiency in Hydroponics: Effects on Leaf Chlorophyll, Photosynthetic Efficiency and Root Ferric Chelate Reductase Activity

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ABSTRACT

Orange (*Citrus sinensis* L. Osb. cv. 'Newhall') plants grafted on *Citrange troyer* rootstock were grown in nutrient solution with 0, 5, 10 or 20 μM Fe, with and without calcium carbonate. Calcium carbonate was added in order to mimic the natural conditions in calcareous soils. Leaf chlorophyll concentration was estimated every 3–4 days using the portable instrument SPAD-502 meter. Chlorophyll fluorescence parameters, photosynthetic capacity estimated from oxygen

evolution, leaf Fe concentrations, and root tip ferric chelate reductase activity were measured at the end of the experiment. Plants from the 0 and 5 μM Fe treatments showed leaf chlorosis and had decreased leaf chlorophyll concentrations. Leaves of plants grown in the absence of Fe in the solution had smaller rates of oxygen evolution both in the presence and absence of calcium carbonate, compared with plants grown in the presence of 10 μM Fe. In the absence of calcium carbonate the photosystem II efficiency, estimated from fluorescence parameters, was similar in all treatments. A slight decrease in photosystem II efficiency was observed in plants grown without Fe and in the presence of calcium carbonate. A 2.5-fold increase in root tip ferric chelate reductase activity over the control values was found only when plants were grown with low levels of Fe and in the presence of calcium carbonate.

INTRODUCTION

Iron availability in calcareous soils does not fulfill plant needs in spite of the high total soil Fe concentrations (Chen and Barak, 1982; Marschner and Römheld, 1995). The high level of bicarbonate ion present in theses soils, seems to affect metabolic processes in roots and leaves, decreasing Fe availability within the plant at the root and shoot levels (Mengel, 1995). In these situations, when Fe is in short supply, Fe-efficient genotypes develop shoot and/or root controlled responses, which include physiological, biochemical and morphological changes (Marschner et al., 1988; Mengel, 1995; Terry and Zayed, 1995; Schmidt, 1999).

The activity of the plasma membrane-bound ferric chelate-reductase (FC-R) is essential for Fe absorption. In the presence of bicarbonate the activity of the enzyme is inhibited due to high pH (Susín et al., 1996). Some plants can increase the reducing power of roots by inducing the activity of a special plasma membrane reductase enzyme, known as "turbo-reductase", which could play a role in avoiding iron deficiency (Bienfait et al., 1983, 1985; Römheld, 1987; Brüggemann et al., 1990; Moog and Brüggemann, 1994; Mengel, 1995).

At the shoot level, Fe is particularly important in chlorophyll (Chl) synthesis and thylakoid stabilization (Terry, 1980; Abadía et al., 1991; Abadía, 1992; Abadía and Abadía, 1993). The conversion of photosynthetic energy is largely affected by Fe chlorosis and may be assessed by fluorescence parameters (Lichtenthaler and Rinderle, 1988; Morales et al., 1991).

The objective of this project was to study the effects of Fe deficiency on i) leaf chlorophyll concentration, ii) photosynthetic efficiency estimated by gas exchange and chlorophyll fluorescence parameters, and iii) root tip ferric chelate reductase on 'Newhall' orange plants grafted in *Citrange troyer* and grown in hydroponics.

MATERIALS AND METHODS

Orange plants (*Citrus sinensis* L. Osb. cv. 'Newhall') grafted on *Citrange troyer* (*Citrus sinensis* (L.) Osb. *x Poncirus trifoliata* (L.) Raf.) were used. Three months after grafting, plants (20 cm shoot size, approximately 10-12 leaves) were transferred to nutrient solutions containing

(in mM) 5.0 $Ca(NO_3)_2$, 1.4 KNO_3, 0.6 K_2SO_4, 1.0 $MgSO_4$, 0.9 NaCl, 0.6 $(NH_4)_2HPO_4$, 3.0 $(NH_4)_2SO_4$, 0.2 $MgCl_2$, and (in µM) 41.8 H_3BO_3, 3.8 $ZnSO_4$, 3.9 $CuSO_4$, 6.9 $MnSO_4$ and 1.0 $(NH_4)_6Mo_7O_{24}$ (Carpena, 1983). Iron was added to the solutions as Fe(III)Na-EDTA at four different concentrations, 0, 5, 10, and 20 µM Fe. Each Fe concentration was used with and without 1 g $CaCO_3$ L^{-1}. Calcium carbonate was added in order to mimic the natural conditions in a calcareous soil. Initial pH was 6.0 ± 0.2 and 7.4 ± 0.1 in the treatments with and without carbonate, respectively. Ten plants were used per treatment, with five plants in each 20 L container, distributed in a complete randomized design. Plants were grown in a glasshouse for 7 weeks (from May 20 to July 4). The nutrient solution was replaced once when the electrical conductivity dropped to 1.7 dS m^{-1}.

Leaf chlorophyll (Chl) concentration per area (µmol m^{-2}) was estimated using the portable instrument SPAD-502 meter (Minolta, Osaka, Japan). SPAD readings were taken every 3-4 days in the second and third fully expanded leaves, in five plants per treatment. Previously, a calibration curve was made by reading samples with the SPAD (Figure 1), extracting the pigments from the same leaf area with 100% acetone in the presence of Na ascorbate (Abadía and Abadía, 1993) and measuring Chl spectrophotometrically according to Lichtenthaler (1987).

All other estimations were carried out at the end of the experiment. Chlorophyll fluorescence parameters (F_0, basal fluorescence; F_m, maximum fluorescence; $F_v = F_m-F_0$, variable fluorescence,) were measured with a portable fluorimeter (PEA, Plant Efficiency Analyser, Hansatech, Kyngs Lynn, U.K.) in the second fully developed leaf. After 20 min of dark adaptation, leaves were illuminated with a saturating pulse of 2100 µmol quanta m^{-2} s^{-1} for 5 s.

Photosynthetic capacity was determined as O_2 evolution on an area basis in leaf discs randomly collected from the second and third fully developed leaves in 3 plants per treatment, using a leaf disc O_2 electrode (Hansatech, Kyngs Lynn, U.K.). Oxygen evolution could be used to estimate relative photosynthetic rates (Morales et al., 1991). Measurements were made with 9 leaf discs (total area 7 cm^2) at 25 °C, using a 5% CO_2 concentration and a PPFD (PAR) of 1625 µmol m^{-2} s^{-1}. This PPFD was saturating, as estimated from irradiance response curves (not shown).

Root ferric chelate-reductase activity (FC-R) was measured by the formation of the Fe(II)-BPDS (bathophenantrolinedisulfonate) complex from Fe(III)-EDTA (Bienfait et al., 1983). Measurements were performed with root tips excised with a razor blade from each of three plants randomly selected per treatment. Five excised root tips (approximately 2 cm, 64 ± 23 mg fresh mass) were incubated in an Eppendorf tube in the dark with 1 mL of micronutrient-free half Hoagland´s nutrient solution, containing 300 µM BPDS, 500 µM Fe(III)-EDTA and 5 mM Tris, pH 6.5. Readings were done after centrifugation, 3 h after starting the incubation. An extinction coefficient of 22.14 mM cm^{-1} was used. Blank controls without root tips were also used in order to correct for any unspecific Fe reduction.

To determine leaf Fe concentration, three plants were randomly selected in each treatment at the end of the experiment. The second and third fully developed leaves were sampled. After removal of the petiole and the main vein, leaves were washed with tap water, distilled water containing a non-ionic detergent, 10 mM HCl and finally with rinsed three times with distilled water. Leaves were dried at 70 °C for 48 h, ashed, digested and analyzed following standard methods (A.O.A.C., 1990; Belkhodja et al., 1998). Iron was determined by atomic absorption spectrometry.

The effects of treatments were evaluated by analysis of variance and the means compared using the Duncan Multiple Range Test (DMRT) at 0.05, using the SAS software.

RESULTS AND DISCUSSION

In the first three observation dates of the experiment the Chl concentrations of recently expanded, fully developed leaves were similar for all treatments, with an average of 747.5 ± 8.1 µmol Chl m^{-2}. Later on, leaves of plants from the 0 and 5 µM Fe treatments showed a decrease in Chl concentration (Figure 2) and developed characteristic visual symptoms of Fe chlorosis that lasted until the end of the experiment. In plants grown without Fe the decrease in Chl occurred by the fourth observation date, both in the presence and absence of $CaCO_3$. In plants grown with 5 µM Fe the decrease in Chl occurred by the fifth or sixth observation dates**,** in the absence and presence of $CaCO_3$, respectively. The decrease in leaf Chl concentration was more pronounced in the presence of $CaCO_3$, reaching values similar to those exhibited by plants growing without Fe. At the end of the experiment the leaf Chl concentration in plants growing with 0 and 5 µM Fe was similar (314 µmol Chl m^{-2}). Plants grown with 10 µM Fe had a small but significant decrease in leaf Chl concentration when compared with plants grown with 20 µM Fe in the absence, but not in the presence of $CaCO_3$.

The leaf Fe concentrations were similar at the end of the experiment in all treatments, with approximately 177 ± 10 mg Fe kg^{-1} dry weight. Therefore, leaf Fe was not correlated with total Chl. This agrees with results reported by other authors (Rashid et al., 1990, Carpena-Artes et al.,

1995) and suggests that Fe was subjected to physiological inactivation within the leaf (Abadía, 1992; Morales et al., 1998).

The Chl fluorescence parameters obtained at the end of the experiment are shown in Table 1. In the absence of $CaCO_3$ the F_v/F_m ratios were similar in all treatments. However, in plants growing with $CaCO_3$ the F_v/F_m ratio was slightly smaller in plants growing without Fe compared with plants grown with 20 µM Fe.

These data suggest that the photosystem II efficiency was only very slightly affected by Fe deficiency in orange tree leaves, as shown previously in sugar beet and pear (for a review see Abadía et al., 1999). The decrease in F_v/F_m was associated with an increase in F_0 (data not shown). It should be noted that increases in F_0 could be originated from increases in the dark reduction of the plastoquinone pool, as shown recently for sugar beet and other plant species affected by Fe deficiency (Belkhodja et al., 1998).

Leaves of plants grown without Fe had lower rates of oxygen evolution than plants grown with higher Fe supplies, both in the presence and absence of calcium carbonate (Figure 3).

There was a linear relationship between oxygen evolution and leaf chlorophyll concentration in the plants growing with $CaCO_3$ (Figure 4). These data indicate that Fe deficiency decreases moderately the leaf photosynthetic rates of plants grown in nutrient solution.

The external morphology of roots from Fe0 and Fe5 treatments with $CaCO_3$, were quite different from those of the other treatments. The roots were smaller and less ramified. Young laterals showed a subapical swelling zone of 2 to 4 mm in length that had a reddish colour after

the Fe reduction experiments. Some of these changes were similar to those reported by Landsberg (1995) in sugar beet.

The FC-R activities with Fe (III)-EDTA as substrate, measured for different treatments at the end of the experiment are shown in Figure 5. The values of the FC-R activity are similar to those found in other studies with woody plants (Schmidt, 1999). The presence of $CaCO_3$ led to a significant increase in FC-R activity (approximately 2.5-fold) for the two smallest Fe concentrations, 0 and 5 µM, when compared to plants grown with 10 and 20 µM Fe. Increases in FC-R activity are frequently observed in dicots cultivated with low levels of Fe, and this has been assumed to arise from an inducible plasma membrane-bound FC-R enzyme(s) (Bienfait et al., 1983, 1985; Moog and Brüggemann, 1994). The induction of this system in orange trees grown in nutrient solution appeared to be localized at the subapical root zone (data not shown) as reported for other Strategy I species (Marschner et al., 1982, 1988; Römheld, 1987; Schmidt, 1999). Under our working conditions, an increase of root FC-R activity was observed only when plants were grown in the presence of $CaCO_3$. This may suggest that for citrus species a low level of Fe was not sufficient by itself to induce increases in FC-R. In citrus seedlings, FC-R activity was first detected by Treeby and Uren (1993) and Manthey and McCoy (1993). Root tip FC-R activities reported were in the ranges 4.1-6.3 and 2.0-56.1 nmol Fe reduced min^{-1} g^{-1} fresh mass, for control and Fe-deficient seedlings; increases in FC-R activities with Fe deficiency ranged between less than 1 to 10-fold depending on genotypes (Manthey et al., 1994). Several reports have indicated that in other fruit tree species Fe deficiency alone does not always produce increases in FC-R rates (Romera et al., 1991; Tagliavini et al., 1995; Gogorcena et al., 2000).

CONCLUSIONS

Leaf chlorophyll concentration decreased significantly in orange plants grafted on *Citrange troyer* submitted to Fe deficiency. Photosynthetic capacity was decreased to a lesser extent and photosystem II efficiency was only slightly affected. Iron-deficient orange plants had increases in root FC-R activity in the presence, but not in the absence of $CaCO_3$.

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TABLE 1. Photosystem II efficiency (Fv/Fm ratio) in recently expanded fully developed leaves of 'Newhall' orange plants grown with different iron concentrations in the presence and absence of $CaCO_3$. Measurements were made after 7 weeks of imposing different Fe treatments.

	F_v/F_m	
µM Fe	Without $CaCO_3$	With $CaCO_3$
0	0.78 a	0.78 b
5	0.78 a	0.79 ab
10	0.80 a	0.80 ab
20	0.79 a	0.82 a

Means with the same letter in each column were not significantly different at 0.05, using Duncan multiple range test.

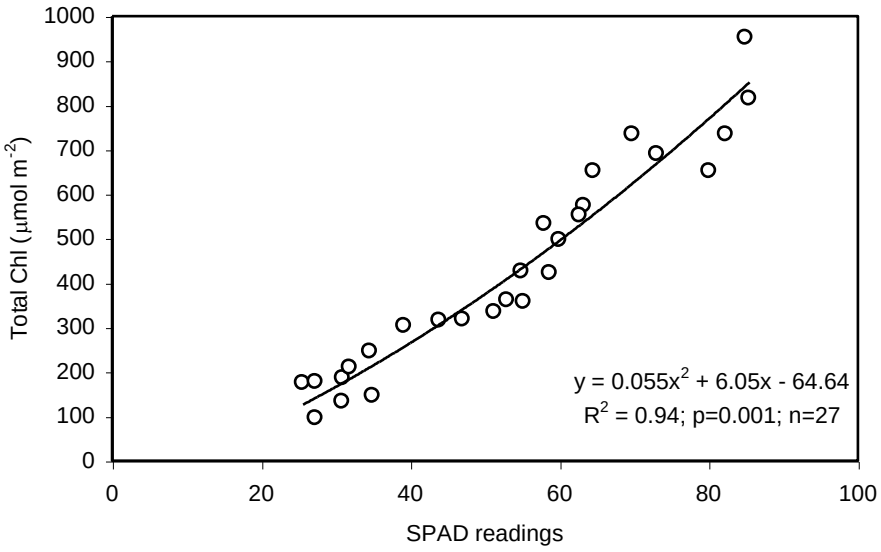

FIGURE 1. Relationship between total leaf chlorophyll (µmol m^{-2}) and SPAD readings in orange trees (cv. 'Newhall') grown in hydroponics.

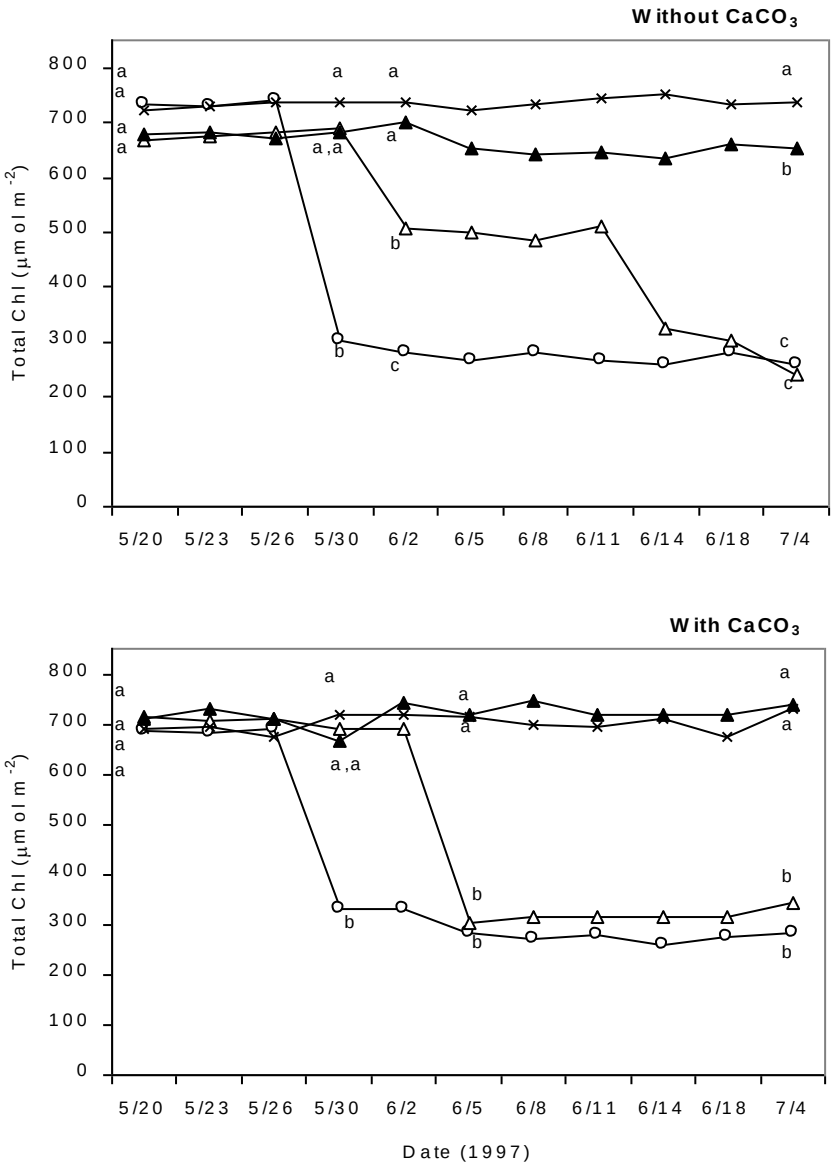

FIGURE 2. Changes in total chlorophyll concentration (µmol m^{-2}) with time in recently expanded, fully developed leaves of 'Newhall' orange plants grown with different Fe treatments. 0 µM Fe, (o); 5 µM Fe, (△); 10 µM Fe, (▲); 20 µM Fe, (×). Statistical analysis is presented only at four dates on both plots. In each date, means with the same letter were not significantly different at 0.05, using Duncan multiple range test.

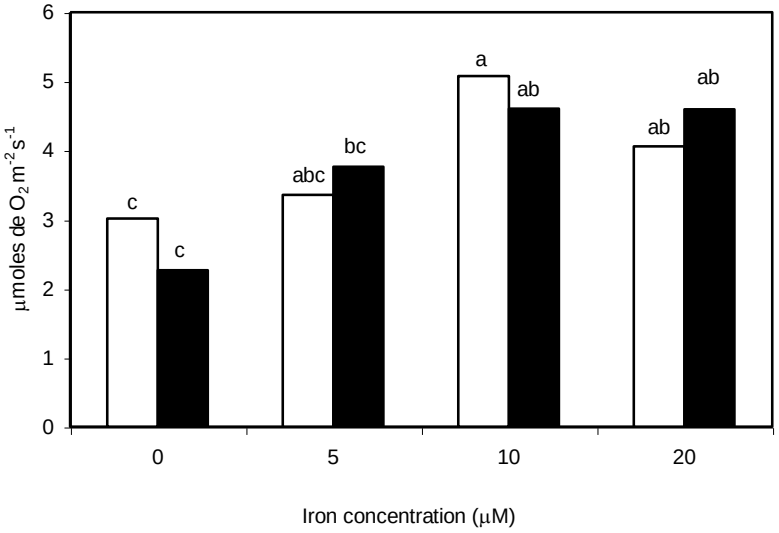

FIGURE 3. Photosynthetic capacity (µmol O_2 m^{-2} s^{-1}) of recently expanded, fully developed leaves of 'Newhall' orange plants at the end of the 7 weeks experimental period. Without $CaCO_3$, (□); with $CaCO_3$, (■). Columns with the same letter are not significantly different at 0.05, using Duncan multiple range test.

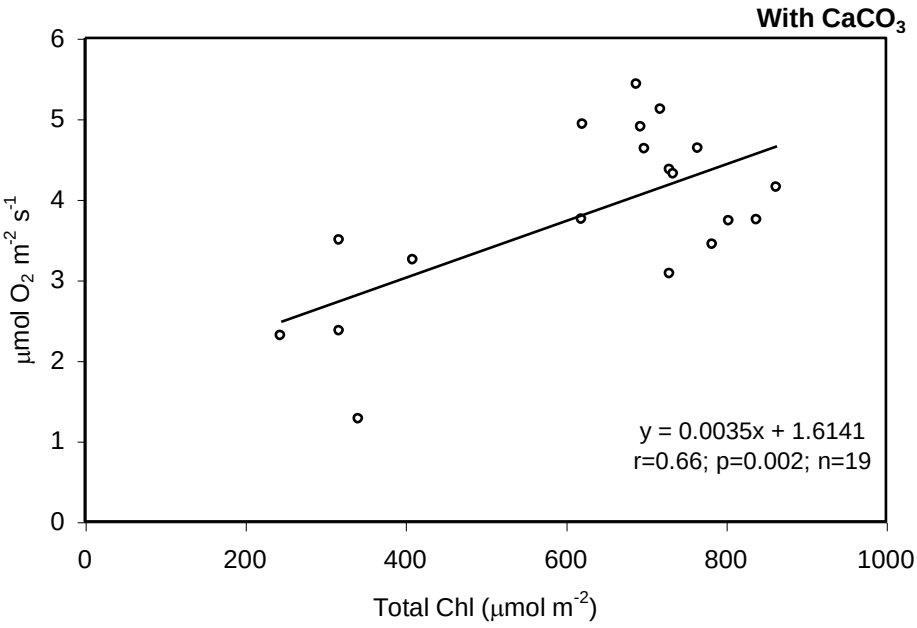

FIGURE 4. Correlation between photosynthetic rates and chlorophyll in recently expanded fully developed leaves of 'Newhall' orange plants grown for 7 weeks with different Fe concentrations.

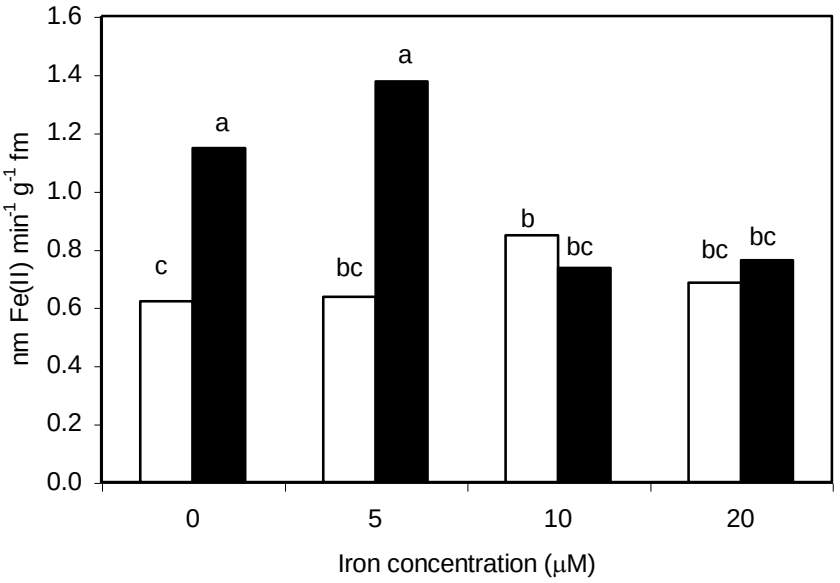

FIGURE 5. Root tips ferric chelate reductase activities (nmol Fe reduced min^{-1} g^{-1} fresh mass) of 'Newhall' orange plants grown for 7 weeks with different Fe concentrations. Without $CaCO_3$, (□); with $CaCO_3$, (■). Columns with the same letter are not significantly different at 0.05, using Duncan multiple range test.