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Fe deficiency induction in *Poncirus trifoliata* rootstock growing in nutrient solution changes its performance after transplant to soil

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ABSTRACT

The absence of iron (Fe) in the nutrient solution induces several physiological and morphological adaptations in the roots of *Poncirus trifoliata*, a citrus rootstock, thereby modifying its overall nutritional status. Whether these changes are advantageous when plants are transplanted to calcareous soils needs to be assessed. To achieve this objective a two-phase experiment was established, first in nutrient solution (phase I) then in pots containing different soils (phase II). In phase I, *P. trifoliata* L. Raf. plants were grown in Hoagland's solution with 120 μM of Fe (Fe120 treatment) or without (Fe0 treatment). At the end of phase I (87 days), Fe-chlorotic plants had less chlorophyll in apical younger leaves, root tips were swollen and their FC-R activity was enhanced, typical responses to Fe-stress. Chlorotic plants had less Fe compared to control plants, but accumulated more Cu and Zn. In contrast the root to shoot ratio (dry weight) and the amounts of macronutrients were not affected by Fe chlorosis. In phase II, plants of both treatments were transplanted to pots containing a calcareous (C) or a non-calcareous (nC) soil resulting in four treatments: Fe0nC, Fe120nC, Fe0C and Fe120C. From the end of phase I until the end of the experiment (353 days), the calcareous soil negatively affected the overall nutritional balance in both Fe0 and Fe120 treatments. Apparently, the ability to change metal homeostasis in particular Cu, as a Fe-stress response was maintained in plants grown in non-calcareous soil. Moreover, the previous induction of physiological and morphological adaptations to Fe depletion alleviated the iron chlorosis symptoms caused by soil carbonates. These results may point to the utilization of internal stress signalling as a tool to cope with different soil conditions.

1. Introduction

Iron (Fe) chlorosis is an important nutritional disorder in fruit trees that results from a poor uptake and transport of Fe within the plant. It normally occurs in calcareous soils due to the large amount of bicarbonate ion and high pH which varies between 7.5 and 8.5. In the Mediterranean region, between 20% and 50% of fruit trees are affected by this deficiency resulting in a low photosynthetic rate, limited plant growth, nutritional imbalances, poor fruit quality and yield (Pestana et al., 2003; Rombolà and Tagliavini, 2006; Álvarez-Fernández et al., 2011). Visible symptoms start in young leaves as an interveinal chlorosis and then progress into an overall chlorosis with sharp decreases in leaf chlorophyll. To solve this problem in orchards, farmers apply different types of Fe salts: inorganic Fe-compounds, natural Fe-complexes and Fe-chelates (Abadía et al., 2011), but the latter are the most used and effective (Lucena, 2009). Every year large amounts of synthetic chelates are applied to soil or leaves, even in the nursery, to increase the availability of Fe in plant tissues in high valuable crops, like peach, pear, apple, grapevines and citrus.

In citrus, tolerance to Fe chlorosis is highly variable (Castle et al., 2009). For example, sour orange (*Citrus aurantium* L.), rough lemon

Abbreviations: ACCE, active calcium-carbonate equivalent; BPDS, bathophenantrolinedisulfonate; C, calcareous soil; CEC_c, cation exchange capacity expressed in centimoles of positive charge per unit exchange; Chl, chlorophyll; EC, electrical conductivity; FC-R, ferric chelate redutase; Fe_d, dithionite-extractable iron; Fe_{ox}, acid ammonium oxalate extractable iron; nC, non-calcareous soil; OM, organic matter.

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(*Citrus jambhiri* Lush.), Rangpur lime (*Citrus limonia* Osb.), Volkamer lemon (*Citrus volkameriana* Ten. and Pasq.) and Cleopatra mandarin (*Citrus reshni hort ex Tanaka*) are tolerant while trifoliate orange (*Poncirus trifoliata* L. Raf.), and its hybrids like Troyer and Carrizo citranges (*Citrus sinensis* L. Osb. × *P. trifoliata* L. Raf.) are susceptible to this deficiency (Pestana et al., 2011a). However, citranges are the most widely used rootstocks even in calcareous soils due to the agronomic interest such as tolerance to several pests and diseases such as Tristeza. Some genotypes are able to increase Fe uptake by enhancing proton extrusion, which acidifies the root apoplast, and the activity of the ferric chelate reductase (FC-R) as found in Strategy I plants (Abadía et al., 2011). Several studies have focussed on the differences in root responses to Fe deficiency among citrus species (Chouliaras et al., 2004; Pestana et al., 2005, 2011a) and concluded that the rootstock *P. trifoliata* is one of the most susceptible to Fe chlorosis. In experiments conducted in nutrient solutions, the activity of the root FC-R was low in the absence of Fe but it was incremented if small amounts of Fe were added to the nutrient solution (Pestana et al., 2012), as also reported for *Prunus* sp. (Gogorcena et al., 2004; Jiménez et al., 2008). However, the activation of the FC-R may revert if more Fe is added, resulting in a regreening of chlorotic plants (Pestana et al., 2011b). This "on-off" regulation has direct implications on root Fe uptake but also on the balance of macro and micronutrients among different plant organs, in particular on Fe homeostasis with other metals like Cu and Zn (Pestana et al., 2013), and Mn, as found in leaves of the *Prunus* rootstock GF 677 (Jiménez et al., 2009).

In spite of the current knowledge on the response mechanisms to Fe deficiency exhibited by *P. trifoliata* grown in nutrient solutions, there is no information on how these plants react when transplanted to soils. Such study may allow the optimization of nutritional inputs at the nursery stage and anticipate nutritional constraints which might occur in calcareous soils. It may also provide new insights on the effectiveness of internal Fe pools and the impact of physiological and morphological stress-adaptations when facing a new environment. In this work, we examine the behaviour of plants with contrasting levels of Fe (grown in nutrient solutions with and without Fe) when transplanted to two different soils: non-calcareous and calcareous.

2. Materials and methods

2.1. Growth of plants in nutrient solution (phase I)

One-year old *P. trifoliata* (L.) Raf. rootstocks were acquired in a commercial nursery. Plants were removed from the substrate and the roots were thoroughly washed and disinfected by immersion in a solution with 2 g L^{-1} fosethyl-aluminium for 2 h.

The experiment started on the 12th of April and at this stage, the plants had the following characteristics (mean ± standard error): height 31.3 ± 1.6 cm; number of leaves 20 ± 2; shoot dry weight 2.2 ± 0.1 g; root dry weight 6.5 ± 0.3 g Forty plants were grown for 87 days in full-strength Hoagland's nutrient solution with the following composition (in mM): 5Ca $(NO_3)_2{\cdot}4H_2O$, $5KNO_3$, $1KH_2PO_4$, $2MgSO_4{\cdot}7H_2O$, and (in μM): $46H_3BO_3$, $0.8ZnSO_4{\cdot}7H_2O$, $0.4CuSO_4{\cdot}5H_2O$, $9MnCl_2{\cdot}4H_2O$ and 0.02 $(NH_4)6Mo_7O_{27}{\cdot}H_2O$, without Fe (Fe0 treatment) or with 120 μM of Fe (Fe120 treatment). Iron was added to the solutions as Fe (III)-EDDHA. Each Fe treatment consisted of 20 plants, in a total of 40 plants distributed in eight containers (20 L) which were placed in a complete randomized design.

During this experimental period, plants were grown in a glasshouse under natural photoperiod conditions and air temperature ≤25 °C. The nutrient solutions were constantly aerated and the pH adjusted to 6.0 ± 0.1. At the beginning of the experiment the electrical conductivity (EC) was 2.2 dS m^{-1}. The solutions were monitored periodically, every two days and replaced when the EC value was less than 2.0 dS m^{-1}.

2.2. Growth of plants in soils (phase II)

At the end of phase I, plants were around 36 ± 3 cm tall, irrespective of treatment. Ten plants from each Fe treatment were transplanted to 21 cm-diameter pots containing 4600 g of a calcareous soil (C) and a non-calcareous soil (nC), both mixed with vermiculite and organic matter (OM) in a 2:1:1 proportion to improve their properties. A NPK fertilizer (7:21:21) was also applied to ensure non-limiting amounts of these elements throughout the experimental period of phase II.

Four treatments were imposed. The Fe0nC and Fe120nC, corresponding to plants that grew in 0 or 120 μM Fe in phase I and then on the non-calcareous soil (nC). The Fe0C and Fe120C treatments refers to plants that grew in 0 or 120 μM Fe in phase I and then were transplanted to the calcareous soil (C) in phase II.

To characterize the soils, three random samples of each soil mixed with amendments were oven-dried for 48 h at 40 °C then passed through a 2-mm sieve. The pH was evaluated in 1:2.5 soil–water suspensions, and EC was measured with a conductivity meter (portable WTW conductivity meter) in 1:5 soil–water suspensions. Organic carbon was analysed by oxidation using dichromate (Walkley and Black, 1934). Active Ca-carbonate equivalent (ACCE) or active lime was extracted with ammonium oxalate and quantified by titration with potassium permanganate (Drouineau, 1942). Phosphorus (P) was extracted using a solution of sodium bicarbonate pH 8.5 (Olsen and Sommers, 1982) and the content in the extracts was quantified colorimetrically. Potassium (K) was extracted using a solution of ammonium acetate (Riehm, 1958) and determined by flame spectrometry. Cation exchange capacity was determined by the barium chloride-triethanolamine method (Mehlich, 1984). The citrate/bicarbonate/dithionite-extractable Fe (Fe_d) was determined according to Mehra and Jackson (1960) except that extraction was carried out at 25 °C for 16 h and provides a measure of the Fe in all Fe oxides. The acid NH_4 oxalate extractable Fe (Fe_{ox}) which provides an estimate for the Fe in poorly crystalline Fe oxides, was determined according to Schwertmann (1964) except that the soil: solution ratio was 1:200 in order to prevent a significant pH increase in the extractant due to the presence of carbonate. Soil texture was determined by the hydrometer method (Bouyoucos, 1962). The calcareous soil (C) had significantly higher pH value, ACCE and also higher levels of P and K compared to the non-calcareous soil (Table 1). Extractable Fe, measured by Fe_{ox} was similar in both soils; however the ratio Fe_{ox}/ACCE was significantly lower in C, confirming its ability to induce iron chlorosis in plants.

Plants were transplanted by the end of July (after 87 days in the nutrient solutions), and the pots placed outdoors at *Campus* de Gambelas, Faro, Portugal (37°02′40′N, 7°58′27′W). The site has a typical Mediterranean climate, with hot dry summers and mild winters. Mean air temperature ranges between 12 °C in January and 24.2 °C in July and mean precipitation ranges between 114.1 mm in December and 1.8 mm in July. Drip irrigation took place daily at a rate of 0.4 L per pot. The pH of the irrigation water was 6.5 and the electrical conductivity 0.3 dS m^{-1}.

As *P. trifoliata* is a deciduous rootstock, leaves were gradually shed during winter and a spring flush started approximately 253 days (end of March) after transplant from the nutrient solutions. At the beginning of April, a clear differentiation could be made between young apical and mature basal leaves according to their position in the stem.

Table 1
Characteristics of the two soils used in phase II.

Parameter[a]	Soil		*P*
	Non-calcareous	Calcareous	
ACCE (g kg^{-1})	7 ± 0.40	104 ± 0.40	***
pH	6.18 ± 0.02	7.79 ± 0.04	***
EC (dS m^{-1})	0.64 ± 0.24	0.48 ± 0.01	ns
OM (g kg^{-1})	11.7 ± 1.05	15.2 ± 1.99	ns
Clay (g kg^{-1})	222 ± 6.65	203 ± 13.3	ns
CEC_c (cmol kg^{-1})	133 ± 4.17	154 ± 8.33	ns
Olsen P (mg kg^{-1})	34 ± 3.56	54 ± 4.25	**
K (mg kg^{-1})	261 ± 1.25	292 ± 4.26	**
Extractable Fe:			
Fe_d (mg kg^{-1})	12500 ± 650.6	6400 ± 131.8	**
Fe_{ox} (mg kg^{-1})	837 ± 29.9	998 ± 56.5	ns
(Fe_{ox}/ACCE)* 10^4	1260 ± 41.8	96 ± 5.65	***

[a] Mean ± standard error.
ACCE, active Ca-carbonate equivalent; CEC_c, cation exchange capacity expressed in centimoles of positive charge per unit exchange; EC, electrical conductivity of the 1:5 soil: water extract; Fe_d, citrate/bicarbonate/dithionite-extractable Fe; Fe_{ox}, acid NH_4 oxalate extractable Fe; ns: not significant; OM, organic matter; ** or *** indicate, respectively: significant for $P < 0.01$ or $P < 0.001$ (ANOVA; *F* test); $n = 3$.

2.3. Plant growth and degree of chlorosis

The degree of chlorosis was determined in fully expanded young (apical) leaves and in mature (basal) leaves using a SPAD-502 apparatus (Minolta Corp., Osaka, Japan). SPAD values were converted to total leaf chlorophyll (Chl) concentration, using a calibration curve previously obtained (Correia et al., 2014).

At the end of phase I, ten plants per treatment were separated into roots and shoots and after phase II (as stems were bigger) plants were also separated into roots, stems and leaves. Each plant material was washed with tap water, followed by distilled water containing a non-ionic detergent, then with 0.01 M HCl and finally rinsed three times with distilled water. Samples were dried to constant weight at 60 °C. Height, dry weights and root/shoot ratios were registered at the end of each phase.

2.4. Mineral composition

Dried plant material was ground and ashed at 450 °C followed by digestion in an acidic solution (HCl 1 M). The concentration of K, Ca, Mg, Mn, Zn, Cu and Fe was determined by atomic absorption spectrophotometry (Pye Unicam, Cambridge, UK) following standard methods (A.O.A.C., 1990). Phosphorus was analysed colorimetrically by the molybdo-vanadate method at 375 nm Nitrogen was analysed by the Kjeldahl method. Nutrient concentrations are expressed on a dry weight (DW) basis for each plant material. The nutrient contents were calculated by multiplying the DW by the concentrations.

2.5. Activity of the root FC-R

The activity of the root FC-R was measured by the formation of the Fe(II)-bathophenantrolinedisulfonate (BPDS) complex from Fe(III)-EDTA (Bienfait et al., 1983). Measurements were performed at days 48, 78 and 87 of phase I and at the end of phase II. For this purpose, roots were gently washed with distilled water. At least nine root tips were excised (approximately 2 cm each) with a razor blade from each of three plants per treatment. The root tips with about 20.8 ± 5 mg fresh weight (FW) were incubated in an Eppendorf tube in the dark with 900 μL of micronutrient-free half strength Hoagland's nutrient solution, containing 300 μM BPDS, 500 μM Fe(III)-EDTA and 2 mM MES at pH 6.0. Readings were done after 2 h of incubation. The reducing capacity was determined by measuring the concentration of the Fe (II)-BPDS complex at 520 nm in a spectrophotometer (Bienfait et al., 1983). An extinction coefficient of 22.14 mM cm^{-1} was used. Blank controls without root segments were also used to correct for any unspecific Fe reduction. The FC-R activity was expressed on a root FW basis.

Table 2
Plant height, biomass determined at the end of phase I (87 days in nutrient solution). Ferric chelate reductase (FC-R) activity was also measured 48 and 78 days after the beginning of the experiment.

	Treatments		
	Fe0	Fe120	
Height (cm)	45 ± 4	49 ± 2	ns
Dry weight:			
Shoot (g)	6.27 ± 0.75	4.30 ± 0.31	**
Root (g)	3.01 ± 0.37	2.05 ± 0.10	*
Root/shoot	0.49 ± 0.04	0.49 ± 0.04	ns
FC-R:			
(nmol Fe(II) min^{-1} g^{-1} FW)			
48 days	3.85 ± 0.60	4.87 ± 0.68	ns
78 days	2.67 ± 0.12	2.49 ± 0.24	ns
87 days	4.43 ± 0.62	2.69 ± 0.24	**

Mean ± standard error; FW, fresh weight; *, ** or *** indicate, respectively: significant for $P < 0.05$ or $P < 0.01$ or $P < 0.001$ (ANOVA; F test); at least $n = 5$.

2.6. Statistical analysis

In phase I, the effects of Fe treatments were evaluated by analysis of variance (ANOVA; *F* Test). In phase II, main effects and interactions of substrate (S), Fe treatments (Fe) and plant organs (O) were also assessed. When ANOVA yielded a significant *F* value, the individual means were compared using the Duncan Multiple Range Test (DMRT) at $P < 0.05$. All the determinations were obtained with randomly chosen plants. Data were analysed statistically using SPSS® (Release 18.0, SPSS Inc, Chicago, IL) software package.

3. Results

3.1. Phase I

Plants grown without Fe (Fe0) started showing symptoms in the young leaves after 35 days of growth and Chl values decreased throughout phase I, whereas plants grown in Fe120 remained green and showed an increase in Chl during all this phase (Fig. 1). In mature leaves, Chl values were similar and they had no symptoms of Fe chlorosis.

In spite of the differences in leaf Chl, Fe0 plants grew well as shoot height (34 cm) and root dry weight (4 g) were similar in all 40 plants at the end of phase I. Shoot dry weight was even significantly higher in these plants (Table 2). The FC-R activity decreased from 4.87 to 2.69 in Fe120 plants while in Fe0, root FC-R increased from 3.85 to 4.43 nmol Fe (II) min^{-1} g^{-1} of FW between days 48 and 87, respectively. At the end of this phase, the FC-R activity was significantly higher in Fe0 plants (Table 2) than in Fe120 plants.

In roots of chlorotic plants P and Ca contents were higher than in non-chlorotic plants (Table 3). Plants of Fe120 contained significantly higher amounts of total Fe (1873 μg per plant) compared to Fe0 plants (1021 μg per plant). Conversely, Fe0 plants accumulated more Cu and Zn. As for all the remaining nutrients, contents were statistically similar (Table 3).

3.2. Phase II

After the vegetative rest (end of winter), the lowest values of leaf Chl were found in calcareous soil either Fe0 or Fe120 plants, in both kind of leaves (Fig. 1). Chl values were lower compared to those obtained in phase I, irrespective leaf type. The highest values

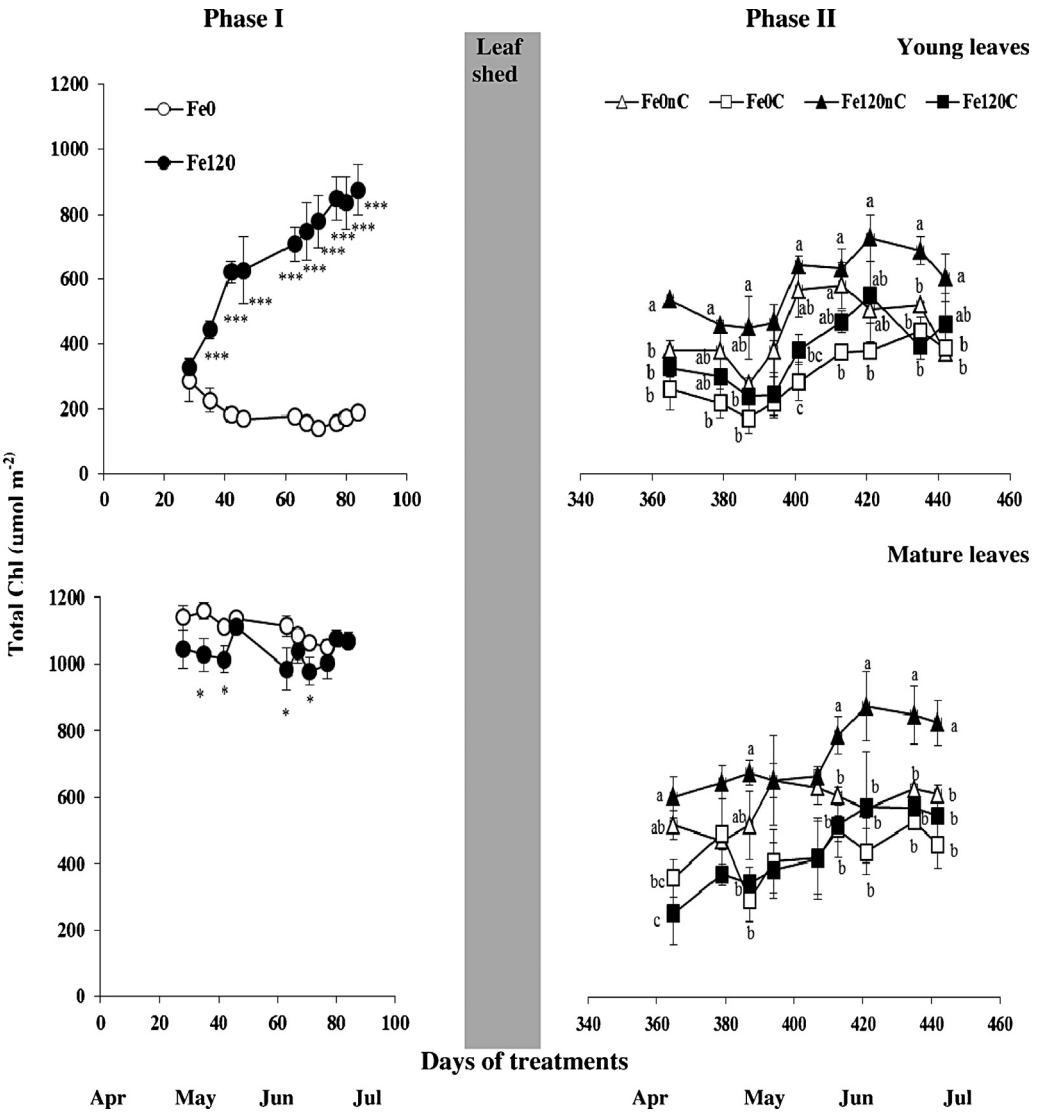

Fig. 1. Variation of total leaf chlorophyll (Chl; mean ± standard error) in young apical leaves and basal leaves in two phases: nutrient solution phase (phase I) and soil phase (phase II). Phase I ended in late July (after 87 days in nutrient solution) when plants were transplanted to pots. In the autumn, all leaves were shed (grey area) and in the following April the first measurements were done at the beginning of phase II. Different letters indicate significant differences for $P < 0.05$; $10 < n < 40$ for both phases.

of Chl were observed in mature leaves in phase I, even in Fe0 plants, and in Fe120nC plants in phase II throughout the experiment. Plants transplanted to the calcareous soil (C) had smaller shoot and root dry weights, irrespectively of the amount of Fe in nutrient solution during phase I (Table 2).

Plant height was only significantly smaller in the plants of the Fe0C treatment. As with Chl, root FC-R activities during phase II (Table 4) were in general lower than those observed in phase I (Table 2). The highest activity was registered in Fe120 plants grown in the calcareous soil (1.97 nmol Fe (II) min^{-1} g^{-1} of FW). Values in the remaining treatments were similar, with no significant differences between them. At the end of phase II (Figs. 2 and 3), the contents of macronutrients (N, P, K, Mg and Ca) were higher in stems than in roots or leaves, while the micronutrients accumulated more in the roots, thus leading to a significant effect of plant organ (O) on all nutrients contents (Table 5).

Plants grown in the non-calcareous soil (nC), either grown without or with 120 μM Fe in phase I, had consistent higher amounts of N, P, K, Mg and Ca compared to plants grown in the calcareous soil in all parts except for Ca in roots (Fig. 2). The soil effect was therefore highly significant, but not the Fe treatments effects except on Cu and Zn contents (Table 5).

The differences in the contents of N, Mg and Ca in stems were not significant but higher values of macronutrients were normally found in leaves and roots of the Fe0nC treatment. As for Zn, Mn and Fe, the responses were similar, particularly for Fe (Fig. 3). In this case, statistical differences were observed between plants grown in each soil (nC and C). The exception was Cu, since a greater accumulation was registered only in Fe0nC plants. In general, the interactions between the main factors (plant organs, soil and previous Fe level) were not significant (Table 5).

4. Discussion

Iron is not a component of Chl but it is required for the synthesis of protochlorophyllide from Mg-protoporphyrin (Miller et al., 1995). The sharp decrease in leaf Chl of young leaves during phase I and the appearance of Fe chlorosis confirms the poor tolerance of *P. trifoliata* to Fe stress (Castle et al., 2009; Forner-Giner et al., 2010). The depletion of leaf Chl, however, did not affect biomass accumulation in chlorotic plants, since the dry weight of roots as well as root to shoot ratio were not significantly different between treatments. This result was somewhat unexpected since Fe chlorosis usually reduces vegetative growth. However, Forner-Giner et al.

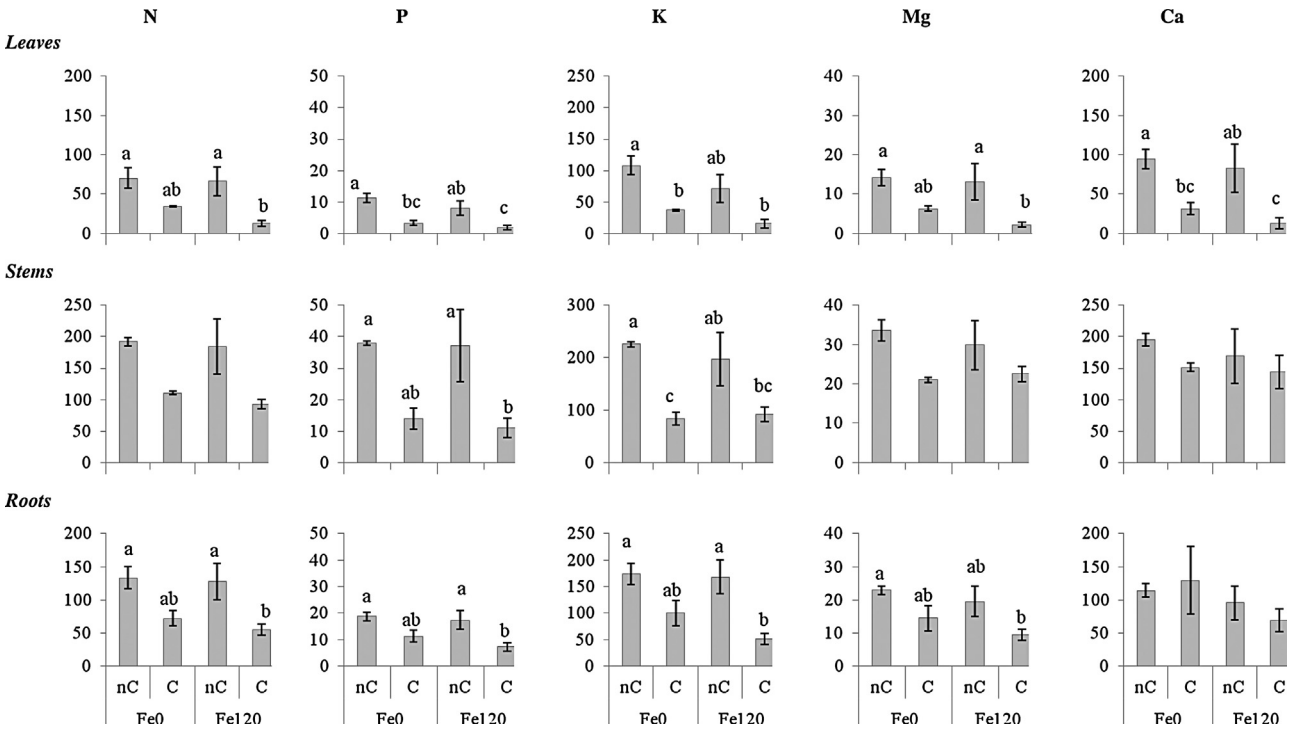

Fig. 2. Contents of macronutrients (in mg per organ) in different plant compartments at the end of phase II. Data are means ± standard error. Different letters in columns indicate significant differences for $P < 0.05$; at least $n = 5$.

(2010) also found similar values of DW of roots and leaves of *Poncirus* plants grown with and without Fe in the irrigation solution, and this lack of variation was attributed to the short term of this experiment (60 days). In our case, it is possible that Fe endogenous pools from the nursery stage (about 900 μg per plant at the beginning of phase I) and the high Chl values in basal mature leaves through the entire phase I were enough to ensure available active Fe to plants and photosynthesis rates during this phase.

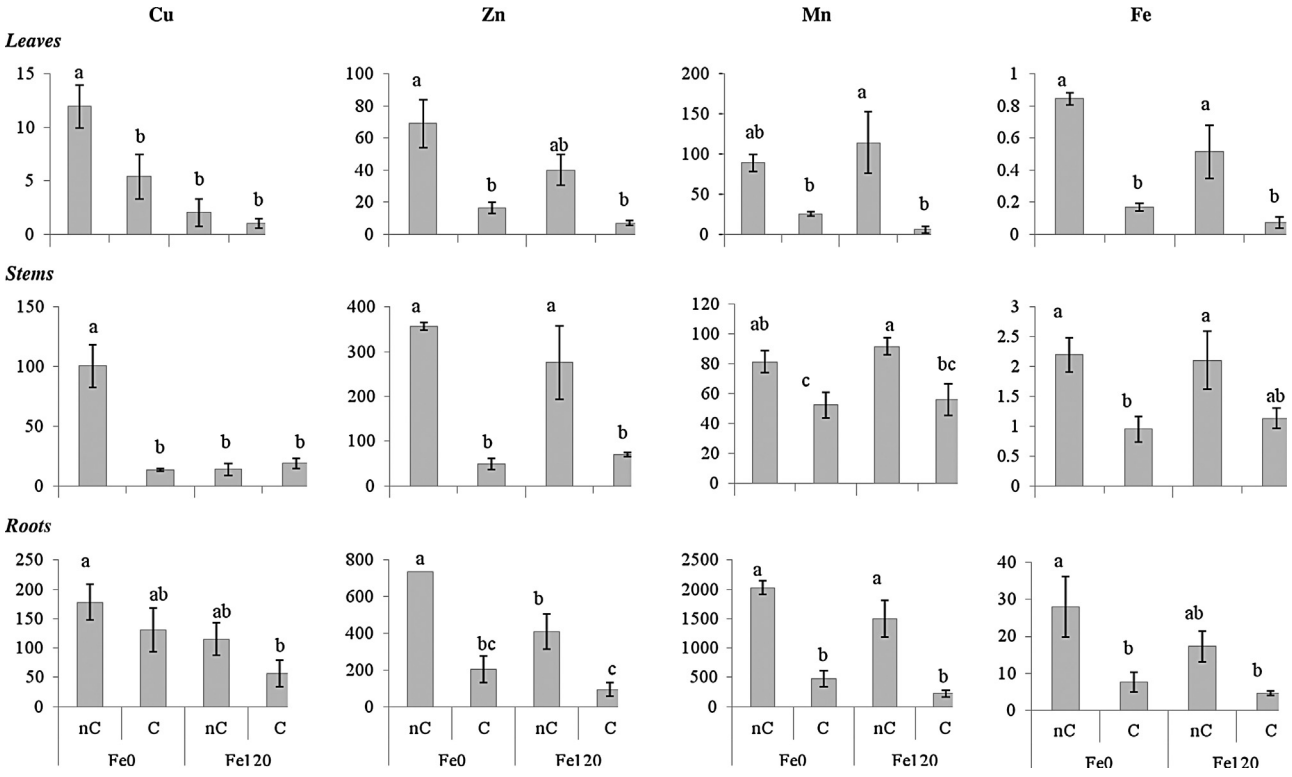

Fig. 3. Contents of micronutrients (in μg per organ) in different plant compartments at the end of phase II. For Fe values are in mg. Data are means ± standard error. Different letters in columns indicate significant differences for $P < 0.05$; at least $n = 5$.

Table 3
Nutrient content determined in roots and shoots of each treatment at the end of phase I (87 days in nutrient solution).

	Treatments		
	Fe0	Fe120	
(mg per organ)			
N			
Shoot	165 ± 22	110 ± 6	ns
Root	81 ± 11	56 ± 1	ns
P			
Shoot	20 ± 3	15 ± 0	ns
Root	19 ± 2	12 ± 1	*
K			
Shoot	106 ± 14	76 ± 6	ns
Root	49 ± 7	34 ± 0	ns
Mg			
Shoot	15 ± 2	10 ± 0	ns
Root	6 ± 1	4 ± 0	ns
Ca			
Shoot	69 ± 12	44 ± 2	ns
Root	21 ± 2	14 ± 0	*
(μg per organ)			
Cu			
Shoot	59 ± 4	25 ± 2	**
Root	75 ± 4	19 ± 3	***
Zn			
Shoot	86 ± 17	54 ± 2	ns
Root	131 ± 4	76 ± 6	**
Mn			
Shoot	138 ± 31	96 ± 6	ns
Root	694 ± 145	437 ± 51	ns
Fe			
Shoot	439 ± 109	281 ± 14	ns
Root	582 ± 47	1592 ± 27	***

Mean ± standard error; *, ** or *** indicate, respectively: significant for $P < 0.05$, 0.01 or $P < 0.001$ (ANOVA; *F* test); at least $n = 5$.

Table 5
Significance levels of each main factor and interactions for nutrient contents at the end of phase II.

Main factors	N	P	K	Mg	Ca	Cu	Zn	Mn	Fe
Substrate (S)	**	**	**	**	*	*	**	**	**
Fe treatments (Fe)	ns	ns	ns	ns	ns	*	*	ns	ns
Plant organs (O)	**	**	**	**	**	**	**	**	**
S × Fe	ns	ns	ns	ns	ns	ns	ns	ns	ns
S × O	ns	*	ns	ns	ns	ns	**	**	**
Fe × O	ns	ns	ns	ns	ns	ns	*	ns	ns
S × Fe × O	ns	ns	ns	ns	ns	ns	ns	ns	ns

ns—Not significant, (*) significant for $P < 0.05$ or (**) $P < 0.001$ (ANOVA; *F* test).

During phase II, Chl values in both leaf types were lower compared to phase I, possibly due to the physiological effort made by plants at the spring flush.

In general, the previous imposition of Fe stress triggered less Fe in roots tissues and our results confirm those reported in the literature for citrus (Pestana et al., 2004; Martínez-Cuenca et al., 2013), since total Fe in Fe0 roots (582 ± 81 μg Fe) was significantly lower than in Fe120 plants (1592 ± 47 μg Fe). The absence of Fe did not reduce the uptake of N, K, Mg or Mn in chlorotic plants, suggesting that under our experimental conditions major root uptake mechanisms were not severely affected which may also explain why the biomass did not decrease significantly. Interestingly, we found an increase of P and Ca in roots of Fe deficient plants at the end of phase II, which may be related to the accumulation of Fe-phosphate and P compounds like phytate. In peach trees grown in the field and in sugar beet in hydroponics, the concentrations of all nutrients, with the exception of Fe, was also similar between chlorotic and green leaves after foliar application of $FeSO_4$ (El-Jendoubi et al., 2011). In accordance, in the *Prunus* GF677 hybrid, the levels of Mn, Zn and Cu in leaves were higher in Fe deficient plants compared to controls (Jiménez et al., 2008), a response attributed to the role of non-specific transporters. The lack of Fe in the growing solution led to an increase of root Cu in strawberry (Pestana et al., 2013) and it has been reported that Cu and Fe have similar affinity to different enzymatic systems (Cohu and Pilon, 2007). Hence, under Fe deficiency, a metabolic shift occurs to enhance the reduction capacity resulting in a greater uptake of Cu.

The activity of the FC-R also increased in chlorotic plants compared to control plants in phase I. This is a well-known response found in Strategy I dicots plants (Walker and Connolly, 2008) to increase the metabolically active Fe (II) in roots. In a different but complementary experiment, Pestana et al. (2012) registered an increase of root FC-R in *Poncirus* plants if a small concentration of Fe (1 μM Fe) was added to the growing solution suggesting that the activation of this enzyme occurs under total absence or with a small Fe amount. This result should be interpreted with care as it may not be extrapolated to other crops. For example, in some *Prunus* rootstocks like 'Barrier' classified as Fe sensitive, *in vivo* enhancement of root FC-R activity was not observed under Fe deficiency conditions (Jiménez et al., 2008).

Taken together, the results indicate that plants of the Fe0 treatment activated well-known mechanisms and could thus be used to follow changes when transplanted to the two soils. Phase II was used to evaluate how plants would behave in a non-calcareous (slightly acid soil) and an alkaline calcareous soil. Is it possible to assume that the physiological adaptations of chlorotic plants would be an advantage in calcareous soils? *P. trifoliata* is a deciduous species (e.g. Agustí et al., 2002) and all leaves were shed during winter. Thus, the retranslocation of nutrients from leaves to stems or roots and its influence on the subsequent behaviour of both chlorotic and non-chlorotic plants must be taken into consideration. Nutrients that are very mobile in the phloem, like K and P, are retranslocated in deciduous trees (Shi et al., 2011) and also in evergreen trees (Correia and Martins-Loução, 1997) before leaf senescence and a considerable pool of nutrients become available for the spring flush. A different situation can be expected for nutrients that are not very mobile. In this respect, Shi et al. (2011) observed that leaf senescence did not lead to retranslocation of Fe and other micronutrients in deciduous trees, although these findings do not agree with results reported for oak (Abadía et al., 1996).

Table 4
Plant height, dry weight and ferric chelate-reductase (FC-R) activity (nmol Fe(II) min^{-1} g^{-1} FW) after the end of phase II.

	Non-Calcareous soil				Calcareous soil			
	Fe0		Fe120		Fe0		Fe120	
Height (cm)	78 ± 4	a	93 ± 12	a	47 ± 1	b	66 ± 6	ab
Dry weight:								
Shoot (g)	33 ± 1	a	32 ± 10	a	10 ± 2	b	10 ± 2	b
Root (g)	12 ± 2	a	11 ± 3	ab	5 ± 1	bc	4 ± 1	c
Root/Shoot	0.35 ± 0.03	b	0.35 ± 0.03	b	0.48 ± 0.04	a	0.38 ± 0.06	ab
FC-R	0.64 ± 0.04	b	0.82 ± 0.10	b	0.69 ± 0.19	b	1.97 ± 0.26	a

Mean ± standard error; Different letters in a row indicate significant differences for $P < 0.05$; at least $n = 5$.

In our experiment, the presence of carbonate in the soil was a decisive factor for plant response. This was supported by the trends of leaf Chl, in young and mature leaves which showed higher values in both Fe0nC and Fe120nC treatments. In grapevine vegetative growth also decrease with increasing proportion soil carbonate irrespective of the Fe substrate availability (Díaz et al., 2009). At the end of our experiment (440 days), the adequate conditions of the non-calcareous soil resulted in a good vegetative growth, a down regulation of the FC-R activity in roots, and a high uptake of macro and micronutrients. The risk of Fe chlorosis in different crops (Reyes et al., 2006; Díaz et al., 2010) may be estimated by labile soil Fe and carbonates. Both soils had similar levels of Fe oxides (Fe_{ox}) but the ratio (Fe_{ox}/ACCE) $\times 10^4$ seemed a good indicator of Fe chlorosis in both Fe0 and Fe120 plants, as it was related with a major depression of nutrient uptake by plants which grew previously with a high supply of Fe (Fe120 in phase I). Even with this soil effect, we must consider the hypothesis of a "selective stress memory" present in plants of Fe0 from phase I. Is it possible to take advantage from it? In the absence of carbonates (nC) plants responded positively for most of the macro and micronutrients contents. Those that grew under Fe deficiency (Fe0) during phase I were able to accumulate at least similar amount of Fe from the soil in phase II comparing to Fe120 plants, which may support the existence of a stress memory in Fe0 plants. This response was also observed for the other metals (Zn, Mn and in particular Cu). This means that some internal signalling was operating during the entire phase II. Nevertheless, the Fe accumulated in plants of Fe0 was probably physiologically inactive, thus explaining the low Chl in leaves of Fe0nC treatments.

As pointed out by Abadía et al. (2011), signalling pathways and Fe sensors in regulatory mechanisms are still poorly understood. The knowledge of these mechanisms would open up the possibility of using non-tolerant genotypes in calcareous soils, without the need to apply Fe fertilizers.

5. Conclusions

Plants grown without Fe in the solution developed well-known adaptation mechanisms. Root FC-R was enhanced and there was an uptake of metals (Cu, Zn and Mn) as an alternative to Fe at the end of phase I. The hypothesis that this multiple response mechanisms may be a part of a selective stress memory that could help the plants to cope with adverse field conditions was assessed in our study. It seems that metal homeostasis was changed, particularly in what concerns to the uptake of Fe and Cu, and their mobilization in all plant compartments. A high Fe supply during the nutrient solution phase and the enrichment of the Fe pools were apparently, not an advantage to plants when facing the conditions of a calcareous soil. Ultimately, those plants did not exhibit a better performance comparing to Fe0 plants.

The adaptation mechanisms and the nutritional hardening in the low Fe supply might be used as a tool to cope with different soil constraints. Management of specific nutritional balance at nursery level may be a tool for a successful field and may potentiate the performance of rootstocks in different types of soils. Further experiments must be carried out with other tolerant and non-tolerant citrus rootstocks to validate these results.

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