Is There a Relationship between Ferric-chelate Reductase Activity in Roots of *Poncirus trifoliata* and Leaf Chlorophyll Contents?

Pedro J. Correia[1], Dora Lopes[2], Amílcar Duarte[1], Florinda Gama[1], Teresa Saavedra[1] and Maribela Pestana[1]

[1]Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Universidade do Algarve, Faro, Portugal , [2]Faculdade de Ciências e Tecnologia, Universidade do Algarve, Faro, Portugal.

*Email corresponding author: aduarte@ualg.pt

Abstract

***Poncirus trifoliata* is a citrus rootstock very sensitive to Fe deficiency. This deficiency is very common in crops grown in calcareous soils due to the detrimental effect of bicarbonate ion. Higher plants have distinct behaviours when faced with Fe chlorosis, and several mechanisms may be activated under Fe shortage. The aim of this study was to investigate the activity of ferric-chelate reductase (FC-R), a key enzyme in Fe uptake, and to verify whether relationships with leaf chlorophyll contents could be established. Plants were grown in nutrient solutions without Fe (0 µM Fe), with 1 µM Fe, with 120 µM Fe and with 120 µM Fe plus $CaCO_3$ (1 g L^{-1}). Total leaf chlorophyll in young and mature leaves was determined using a calibration model based on a relationship between SPAD readings and concentration of chlorophyll (r^2=0.95; P<0.01). The activity of FC-R was determined in roots apexes and several biomass parameters in shoots (number of leaves, height, dry and fresh weight) and roots (dry and fresh weight) were evaluated at the end of the experiment. The activity of FC-R increased in plants grown without iron (0 µM Fe). The results about the relations between root FC-R and leaf chlorophyll are discussed.**

Keywords: Biomass, calcareous soils, Citrus rootstock tolerance, chlorosis, SPAD.

INTRODUCTION

In Mediterranean soils, large concentrations of bicarbonate ions result in iron (Fe) deficiency in several fruit crops (including citrus). The outcome of this nutritional disorder is the decreased content of photosynthetic pigments, which led to the typical yellow colour of young leaves (Pestana et al., 2004). Plants developed several morphological and physiological mechanisms to better adapt to this stress (Schmidt, 1999). One of those mechanisms is the induction of a ferric chelate-reductase (FC-R) in roots that converts Fe (III) to Fe (II) which then can be taken up by a transporter. Fe is then incorporated into heme and non-heme proteins. Although Fe is not present in the chlorophyll molecule, it is required for the formation of protochlorophyllide from Mg-protoporphyrin (Miller et al., 1995). In root, FC-R may be indicative of different degrees of tolerance to Fe deficiency, since its activity increases in plants exhibiting Fe chlorosis symptoms. Indeed, an increase in the activity of the FC-R related to Fe chlorosis has been shown to occur in several fruit crops, including *Citrus* and other related genera (Castle et al., 2009; Pestana et al., 2001; 2005). However, in kiwifruit, Rombolà et al. (2002) showed less activity in Fe stressed plants. These contradictory findings may be due to multiple reasons but the high affinity ligands to Fe(II) frequently used to assess the root activity of FC-R may display much higher activities than those occurred *in vivo* (Abadía et al. 2011).

The rootstock *Poncirus trifoliata* (L.) Raf. is very susceptible to Fe deficiency and in the absence of Fe, it shows low chlorophyll content and low FC-R activity (Llosá et al., 2009; Pestana et al., 2012). The aim of this study was to investigate in this genotype the activity of FC-R and to establish relationships with leaf chlorophyll contents or chlorosis degree, and to access a possible effect of calcium carbonate on the activity of this key enzyme of Fe metabolism.

MATERIAL AND METHODS

The experiment was conducted in a glasshouse where *P. trifoliata* plants, acquired in a commercial nursery, were transferred to 20 L containers with Hoagland's nutrient solution (composition in mM: 5 Ca $(NO_3)_2.4H_2O$, 5 KNO_3, 1 KH_2PO_4, 2 $MgSO_4.7H_2O$, and (in µM) 46 H_3BO_3, 0.8 $ZnSO_4.7H_2O$, 0.4 $CuSO_4.5H_2O$, 9 $MnCl_2.4H_2O$ and 2 MoO_3).

Plants were grown for 87 days in nutrient solutions without Fe (Fe0); with Fe added as Fe(III)-EDDHA using the following concentrations: 1 µM Fe (Fe1); 120 µM Fe (Fe120) and 120 µM Fe plus $CaCO_3$ (1 g L^{-1}) (Fe120+), in a total of four treatments. Addition of $CaCO_3$ aimed to mimic calcareous soils conditions.

Total leaf chlorophyll in young leaves was determined using a calibration curve (Fig. 1). This curve was obtained by analysing leaf disks that presented different degrees of Fe deficiency with the SPAD-502 apparatus (Minolta 502, Japan) and 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 (Lichtenthaler, 1987).

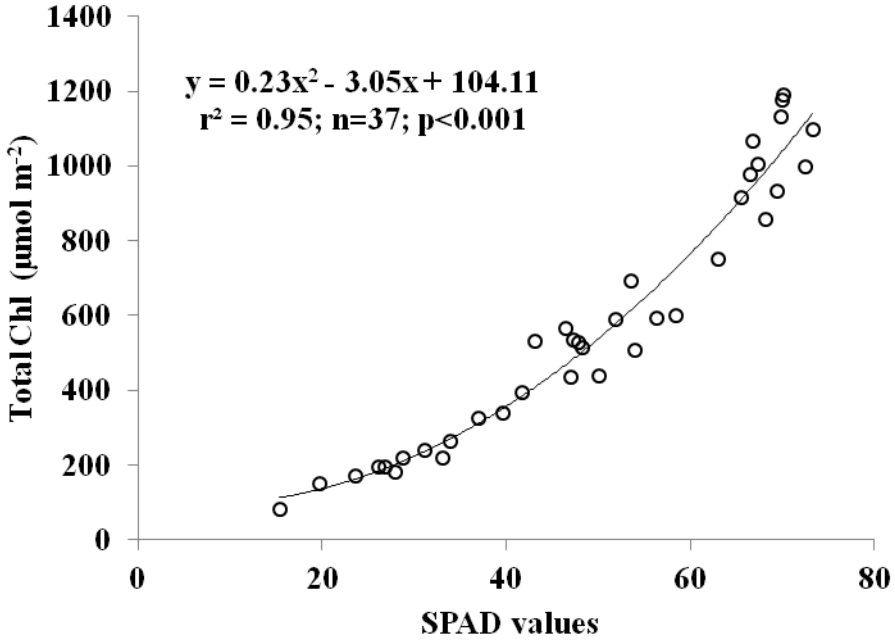

Fig. 1 Relationship between total leaf chlorophyll concentration (µmol m^{-2}) and SPAD values in *P. trifoliata* plants grown in hydroponics.

The activity of root FC-R in each treatment was measured by the formation of the Fe(II)-bathophenantrolinedisulfonate (BPDS) complex from Fe(III)-EDTA (Bienfait et al., 1983). Root tips (approximately 2 cm long) were harvested 87 days after the beginning of the experiment and incubated in an Eppendorf tube in the dark with 900 µL of micronutrient-free half Hoagland's nutrient solution, containing 300 µM BPDS, 500 µM Fe(III)-EDTA and 5 mM MES (pH 6.0). Measurements were done after centrifugation, one hour after starting the incubation. An extinction coefficient of 22.14 mM cm^{-1} was used.

Biomass parameters were assessed at the end of the experiment. Number of leaves, plant height, and fresh and dry weight of shoots and roots were registered.

The containers were positioned in a complete randomized design. Treatments effects were evaluated by one-way ANOVA and means were compared using the Duncan Multiple Range Test. The data were subjected to statistical analysis using SPSS software (v. 16.0).

RESULTS AND DISCUSSION

Typical Fe chlorosis symptoms were identified in Fe0 and Fe1 treatments (Fig. 2).

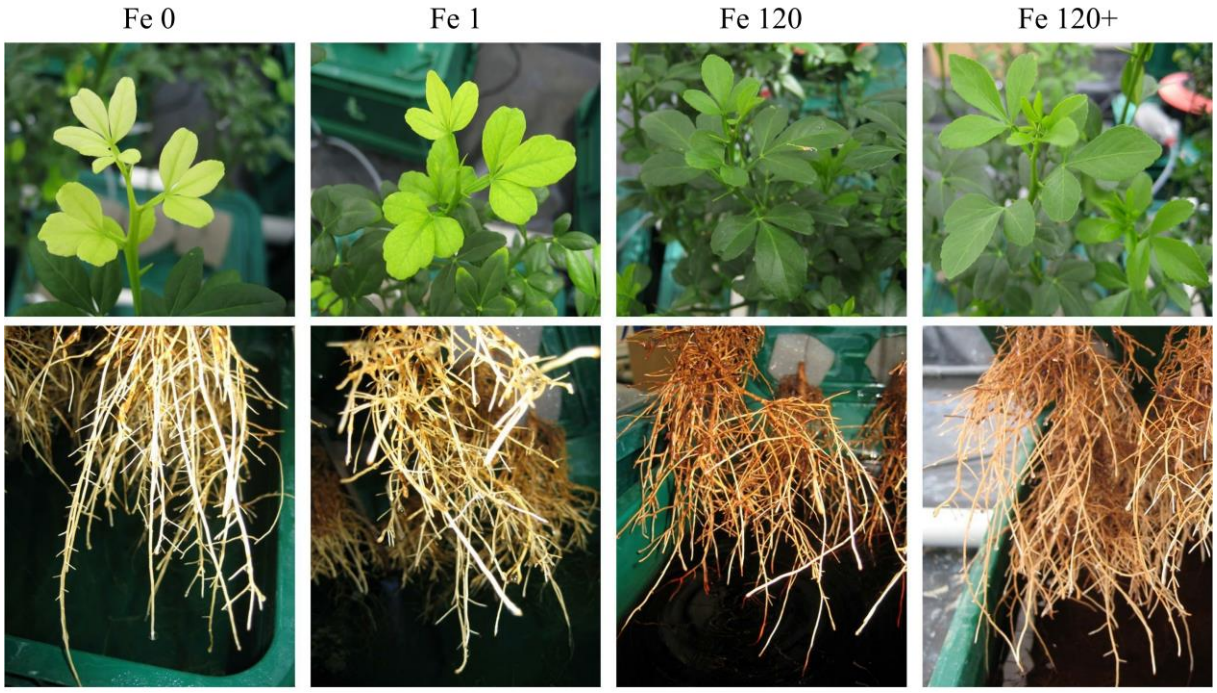

Fig. 2. Leaves and roots of *P. trifoliata* grown in hydroponics for 87 days under the following treatments: Fe0 (no Fe in the solution); Fe1 (1 µM Fe); Fe120 (120 µM Fe); Fe120+ (120 µM Fe plus $CaCO_3$). In Fe0 and Fe1 it is possible to see the white colour of young leaves which means a pale green of the leaf mesophyll.

Young apical leaves were characterized by a yellow coloured interveinal chlorosis and subapical swelling of roots was also observed, which are associated to formation of lateral roots, roots hairs and rhizodermal transfer cells (specialized cells showing infolding of the plasma membrane). Since *P. trifoliata* is highly susceptible to lime induced chlorosis (Castle et al., 2009), it was expected also a decrease in plant performance for Fe120+ treatment. Table 1 shows the effect of Fe treatments on some vegetative traits of shoots and roots, and it can be seen that no major differences occurred. Nevertheless, root DW was higher in the Fe-stressed treatments (Fe0 and Fe120+). Under Fe stress, the growth and the concentration of leaf chlorophyll in *P. trifoliata* is negatively affected (Pestana et al., 2012) but in the present work this response was not evident. Then, it is possible that the Fe endogenous pool build up during nursery stage contributed to the lack of differences in this experiment.

Table 1. Fe effects on the number of leaves, plant height, fresh and dry weight of root and shoot (FW – fresh weight; DW - dry weight). Means (n=6) with the same letter are not significantly different at $P < 0.05$ (Duncan test).

[Fe] µM	Number of Leaves	Height (cm)	Shoot		Root	
			FW (g)	DW (g)	FW (g)	DW (g)
0	57.3 a	45.4 a	14.92 a	3.14 a	10.73 ab	3.01 a
1	31.8 b	49.6 a	9.95 b	2.17 b	8.43 bc	2.71 a
120	41.0 b	49.3 a	10.62 b	2.15 b	7.62 c	2.05 b
120+	63.8 a	53.0 a	15.30 a	3.00 a	12.02 a	3.28 a

Several studies showed an increase of FC-R activity in plants grown in absence or with small amounts of Fe in the solution. However, there are other divergent reports (Pestana et al., 2004; Abadía et al., 2011). In *P. trifoliata*, Pestana et al. (2012) registered a lower activity of FC-R in Fe-deficient plants, but the activity was enhanced in plants grown with small amounts of Fe.

In Figure 3, the activity of FC-R in each plant is plotted as a function of SPAD values or chlorophyll to understand if the plant is able to reduce more Fe at root level, as a response to the increment in leaf chlorophyll content. Only 11% or 10% of the variation of FC-R is explained by chlorosis degree measured using the SPAD apparatus or leaf chlorophyll, respectively. Since the variability among treatments is quite high, it is difficult to get a clear feature for all the results. Nevertheless, some plants that were grown in the absence of Fe (Fe0) showed higher FC-R activity leading to negative trend in the relationship between the activity of the enzyme and chlorophyll.

No significant effect of $CaCO_3$ addition to the nutrient solution was observed. Also small amount of Fe in the nutrient solution (Fe1) did no enhance FC-R activity as previously reported by Pestana et al. (2012).

As conclusion, Fe depletion leads to leaf chlorosis and root development. However, under our experimental conditions, shoot growth was not markedly affected. Higher values of FC-R were related to low SPAD values and low chlorophyll contents, which indicated a response to Fe-stress conditions. However, the variability among treatments was high. Also, the addition of $CaCO_3$ did not induce a clear change in the mechanism of FC-R induction.

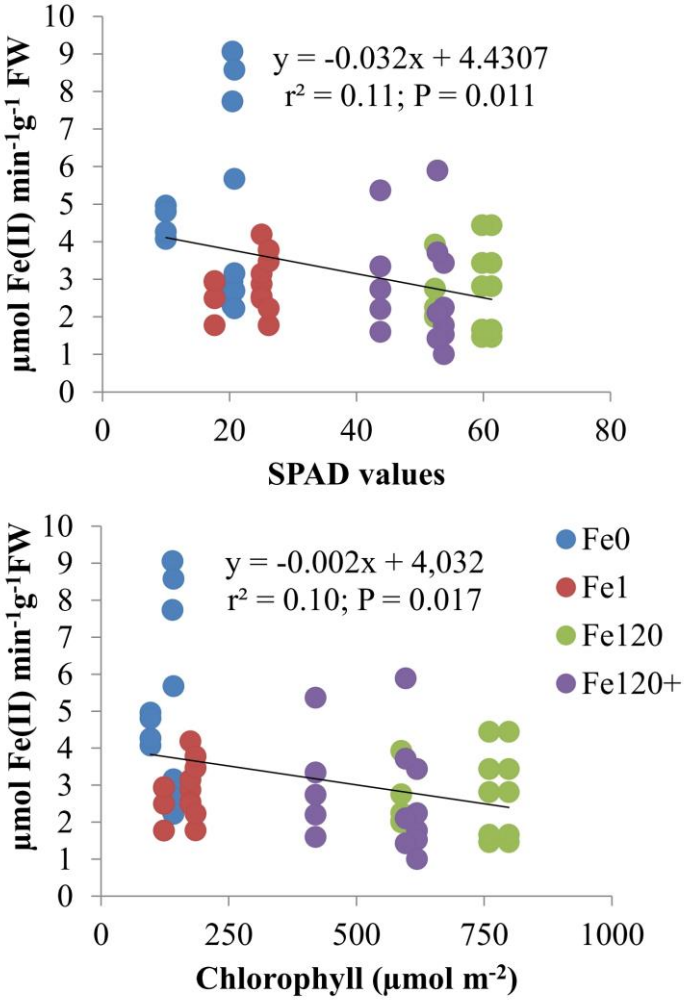

Fig. 3. Regression relationships between root ferric-chelate reductase activities (nmol Fe(II) $min^{-1}g^{-1}$ FW) and SPAD units (above) or total chlorophyll concentration (µmol m^{-2}, below) considering all the treatments at the end of the experiment.

ACKNOWLEDGEMENTS

This study was funded by the national Project from the FCT (PTDC/AGR-AAM/100115/2008).

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Citation:

Correia P.J., Lopes D., Duarte A., Gama F., Saavedra T., & Pestana M. 2015. Is there a Relationship between Ferric-Chelate Reductase Activity in Roots of *Poncirus trifoliata* and Leaf Chlorophyll Contents? *Acta Horticulturae (ISHS)* 1065 (vol. 1):373-377.

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