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The Citrus Rootstocks Cleopatra Mandarin, *Poncirus trifoliata,* Forner-Alcaide 5 and Forner-Alcaide 13 Vary in Susceptibility to Iron Deficiency Chlorosis

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Abstract

We have studied the tolerance to iron chlorosis of four citrus seedling rootstocks: the two hybrids Forner-Alcaide 5 (F-A 5) and Forner-Alcaide 13 (F-A 13), as well as their parents [Cleopatra mandarin (*Citrus reshni* Hort. ex Tan.) and *Poncirus trifoliata* (L.) Raf.]. Six-month-old potted plants were grown in four different iron concentrations (0, 9, 18 and 36 μM) and leaf iron content, dry weight, total chlorophyll content, catalase activity and root ferric chelate reductase (FCR) activity were measured after 60 days. F-A 5 had the highest FCR activity, total chlorophyll content, total dry weight and iron content in roots. *P. trifoliata*, a genus susceptible to iron chlorosis, showed the lowest total chlorophyll content, FCR activity and total dry weight. F-A 5 is an iron-chlorosis-tolerant rootstock, which may be suitable for use in soils that cause this problem.

Calcareous soils with high pH and restricted iron availability for plants are commonly found in the Mediterranean basin. The genus *Citrus* and related rootstock species are considered to be susceptible to iron chlorosis (6). Iron deficiency tolerance is determined by the rootstock (30) so rootstocks onto which citrus trees are grafted display differences in susceptibility. Previous field studies on the iron-deficiency tolerance of rootstocks resulted in more citrus trees being planted worldwide on Cleopatra mandarin in high pH soils, because of its high tolerance to chlorosis (7, 8). However, Cleopatra mandarin rootstock has some drawbacks in that it takes a long time to start bearing and fruits are smaller than on other rootstocks (7). Carrizo citrange is the most widespread rootstock in Spain and trees on Carrizo yield more than trees on Cleopatra mandarin. However the susceptibility of Carrizo to iron chlorosis necessitates expensive Fe chelate treatments to improve its performance, and consequently, production costs increase.

To date, there are no rootstocks available combining tolerance to CTV (citrus tristeza virus), *Phytophthora* spp., and iron chlorosis; hence it is essential to search for new rootstocks that cover all these three characteristics. Conventional breeding is very slow for woody plant species such as citrus, so the search for new citrus rootstocks using physiological screening methods is an important topic for research. One of the goals of the citrus-rootstock breeding program at the Instituto Valenciano de Investigaciones Agrarias (IVIA) in Valencia, Spain, is to develop genetically improved rootstock with enhanced iron chlorosis tolerance.

Field trials have been carried out to select chlorosis-tolerant genotypes, however it takes a long time to obtain results and only a limited number of hybrids can be evaluated. New screening techniques are needed to identify chlorosis-tolerant genotypes, which can be used in breeding programs (24). We have over five hundred hybrid citrus rootstocks and we are developing greenhouse screening tests that are easier to implement than field trials in order to evaluate the iron chlorosis tolerance of the different rootstocks.

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There are several ways to assess a plant's iron nutritional status, such as measuring the total iron concentration in leaves (12, 29), the activity of two heme-proteins in leaves: peroxidase (EC 1.11.1.7) and catalase (EC 1.11.1.6) (2, 12), or measuring the root ferric chelate reductase activity; all have been used as tools for rootstock screening in *Citrus* and *Prunus* (12, 20, 23). We have included catalase and ferric chelate reductase activity in the present screening work.

The two citrus rootstocks Forner-Alcaide 5 (F-A 5) and Forner-Alcaide 13 (F-A 13) are both hybrids of "Cleopatra" mandarin x *P. trifoliata* obtained through traditional hybridization by J. B. Forner at IVIA. Field evaluation showed that F-A 5 is more tolerant to iron chlorosis than Carrizo citrange, although F-A 13 is less tolerant than F-A 5 and Carrizo (16).

To study the physiological basis of iron-chlorosis tolerance, a greenhouse experiment was carried out to evaluate the contribution of catalase and root ferric chelate reductase activity to iron-chlorosis tolerance of F-A 5 and F-A 13 compared to their parents, Cleopatra mandarin and *P. trifoliata.*

Materials and Methods

Experimental conditions. Seeds of all rootstocks were harvested from the mother seed trees held in the germplasm collection at IVIA (Moncada) Valencia, Spain. Seeds were sown on 55 x 40 cm trays containing a mixture of peat and siliceous sand (3:2 vol:vol) in an aphid-proof greenhouse with a cooling system that kept temperatures between 15 ºC and 28 ºC and 80% relative humidity. Plants were grown with supplementary light (< 50 mol m^{-2} s^{-1}, 400-700 nm) to extend the photoperiod to 16 h. Five-month-old plants were transplanted into 5-L plastic pots containing siliceous sand (peat remains were previously eliminated from the roots) and kept in the greenhouse. After transplanting, the plants were irrigated three times a week for 2 wk to acclimatize them to the new substrate, and after 2 wk a nutrient solution (3 mM $Ca(NO_3)_2$, 3 mM KNO_3, 2 mM $MgSO_4$, 3 mM H_3PO_4, 18 µM Fe and trace elements according to Hoagland and Arnon (22)) was added to the irrigation water. Nutrient solution pH was adjusted to 6.0 with 1M KOH or 1M H_2SO_4. After this period, the plants were divided into four groups that received either 0, 9, 18 or 36 µM Fe with six replicate plants per 4 Fe levels x 4 rootstocks. All iron was applied as Fe-EDDHA added to the watering with nutrient solution and applied three times per week for 60 days. Plants were then randomly distributed and a row of buffer plants, not included in the experiment, was placed around the perimeter. At the end of the experiment, the plants were uprooted and after separating the different organs, their fresh weight was determined. After taking a leaf sample for analysis, young leaves, stems and roots were harvested, dried in an oven at 60°C for 72 h and their dry weight determined again. Total dry weight was determined as the sum of the total dry weight of leaves, stems and roots.

Iron analysis. Iron content was determined in young leaves by humid digestion and resolution by atomic absorption analysis (10) with an atomic absorption spectrometer (Perkin-Elmer Aanalyst 200, Madrid, Spain)

Catalase activity. A fresh sample of 2 g of recently fully expanded young leaves was homogenized in a Polytron 3100 (Kinematica, Lucerne, Swizerland) using 10 ml phosphate buffer (10 mM, pH 6.5) containing 0.25 g of insoluble polyvinylpolypyrrolidone (PVPP, Sigma, Barcelona, Spain). The crude extract was centrifuged at 12000 rpm at 4ºC for 30 min, and the supernatant was used for the catalase assay.

The reaction medium (2 ml) contained 50 mM phosphate buffer pH 7.0, and 100 µl of the supernatant obtained after centrifugation of the crude extract. The reaction was started by adding 100 µL of 10 mM H_2O_2. Catalase activity was spectrophotometrically determined by the decrease in hydrogen peroxide (H_2O_2) (3). The reaction was monitored at 240 nm in a spectrophotometer UV-1610 (Shimadzu Corp., Kyoto Japan), at room temperature. The molar extinction coefficient used was 43.6 $M^{-1}cm^{-1}$. Catalase activity was expressed as mmol H_2O_2 consumed x (min x g of protein)$^{-1}$.

Ferric chelate reductase activity. Ferric

chelate reductase (FCR) activity was determined in young roots (20 mg fresh weight) (9). They were washed with 0.2 mM $CaS0_4 \cdot 2H_2O$ solution for 5-10 min, and subsequently placed in a 50 ml Falcon tube containing 0.2 mM $CaSO_4 \cdot 2H_2O$, 0.1 mM Fe(III)-EDTA and 0.3 mM bathophenanthrolinedisulphonate (BPDS) in 10 ml of 5 mM (N-morpholino)-ethanesulphonic acid (MES) pH 5.5 for 24 h at room temperature in the dark. Color development due to the generation of the Fe(II)-$BPDS_3$ complex was determined in a microplate reader UVM340 (AsysHitech GmbH, Eugendorf, Austria) at 535 nm. A calibration curve for Fe(II)-$BPDS_3$ was used to determine Fe(III) reduction (R^2=0.998). FCR activity was expressed as µmol Fe(II) x (hour x g fresh weight)$^{-1}$.

Chlorophyll content. Five young leaves per plant were randomly collected and three 8 mm diameter discs excised per leaf were used for the chlorophyll content determination. Chlorophylls were extracted with N,N-dimethylformamide for 72 h in the dark at 4ºC and quantified by measuring the absorbance at 647 and 664 nm (32) using a Shimadzu UV-1601 spectrophotometer (Shimadzu Corp., Kyoto, Japan).

Statistical analyses. All the data correspond to the mean of at least six replicate plants. ANOVA and regression analysis were performed with Statgraphics Plus for Windows, version 5.1 (Statistical Graphics, Englewood Cliffs, NJ, USA).

Results

Growth. Cleopatra mandarin exhibited higher leaf dry weight per plant than the trifoliate leaved *P. trifoliata*, F-A 5 or F-A 13 (Table 1). F-A 5 and F-A 13 had a higher leaf weight than *P. trifoliata*. None of the leaf dry weights were affected by the iron treatments regardless of rootstock except *P. trifoliata* (R^2=0.49). F-A 5 and Cleopatra mandarin had a more developed root system than F-A 13 and *P. trifoliata*. The absence of iron from the nutrient solution (0 µM) reduced root dry weight of *P. trifoliata* (R^2=0.34) and F-A 5 (R^2=0.32).

F-A 5 and Cleopatra mandarin had the greatest total dry weight and *P. trifoliata* the lowest. *P. trifoliata* dry weight was affected by Fe concentration (R^2=0.52). The ratio of root dry weight:shoot dry weight was not affected by Fe concentration (data not shown).

Table 1. Dry matter and shoot length of four citrus rootstocks grown with different levels of Fe in the nutrient solutions (values are means of six replicates)[z].

Rootstock	Treatment	Dry wt leaves (g)		Dry wt roots (g)		Total dry wt (g)	
Cleopatra m.	0 µM Fe	9.32		5.39		24.36	
	9 µM Fe	9.63		6.05		26.53	
	18 µM Fe	8.65		6.05		23.45	
	36 µM Fe	9.68	ns	6.48	ns	26.14	ns
P. trifoliata	0 µM Fe	1.19		2.65		8.24	
	9 µM Fe	2.21		4.71		17.21	
	18 µM Fe	2.50		4.64		17.17	
	36 µM Fe	2.50	Q(R^2=0.49)	4.07	Q(R^2=0.34)	17.53	Q(R^2=0.52)
F-A 5	0 µM Fe	3.47		5.11		21.04	
	9 µM Fe	3.51		6.36		25.82	
	18 µM Fe	3.89		6.31		24.45	
	36 µM Fe	3.80	ns	6.84	L(R^2=0.32)	24.80	ns
F-A 13	0 µM Fe	3.81		4.55		19.45	
	9 µM Fe	4.49		5.07		23.10	
	18 µM Fe	3.83		3.70		21.54	
	36 µM Fe	4.31	ns	5.01	ns	21.31	ns

[z] (L) linear or (Q) quadradic effect of Fe treatment, (ns) no significant quadratic or linear regression

Table 2. Iron content in leaves and roots, and catalase activity (mmol H_2O_2 consumed x (min x g protein)$^{-1}$ in leaves of four citrus rootstocks grown with different levels of Fe in the solutions (values are means of six replications)[z].

Rootstock	Treatment	Fe leaves (mg)		Fe roots (mg)		Catalase	
Cleopatra m.	0 µM Fe	252.3 def		1394.3 fgh		22.8	
	9 µM Fe	375.1 bc		3370.7 cde		32.9	
	18 µM Fe	389.3 b		4994.8 ab		52.5	
	36 µM Fe	535.9 a	L (R^2=0.65)	5406.7 a	L (R^2=0.39)	46.4	ns
P. trifoliata	0 µM Fe	94.9 h		984.5 h		25.4	
	9 µM Fe	164.4 fgh		2060.5 efgh		34.6	
	18 µM Fe	217.8 efg		2924.7 def		49.6	
	36 µM Fe	297.6 cde	L (R^2=0.68)	4615.2 abc	L (R^2=0.72)	41.7	ns
F-A 5	0 µM Fe	168.0 gh		1379.0 fgh		27.3	
	9 µM Fe	225.5 efg		2785.3 def		27.5	
	18 µM Fe	269.1 de		3621.0 bcd		40.4	
	36 µM Fe	296.8 cde	L (R^2=0.46)	5422.0 a	L (R^2=0.75)	34.5	ns
F-A 13	0 µM Fe	162.8 gh		1321.3 gh		28.6	
	9 µM Fe	282.1 de		2821.1 def		31.0	
	18 µM Fe	309.0 bcde		2609.2 defg		33.9	
	36 µM Fe	333.7 bcd	Q (R^2=0.60)	4968.8 ab	L (R^2=0.71)	31.2	ns

[z] Within each column, values with the same letter are not significantly different at 5%. (L) linear or (Q) quadradic effect of Fe concentration, (ns) no significant quadratic or linear regression.

Iron concentration. The iron concentration in young leaves after 60 days of Fe treatments increased significantly as applied Fe increased (Table 2). F-A 5 and F-A 13 did not show any changes in foliar iron concentration among Fe treatments above 0 µM. Cleopatra mandarin generally had higher iron concentrations in leaves for any given treatment than *P. trifoliata*, F-A 5 or F-A 13. The Fe treatments had a significant effect on root iron concentration in all rootstocks (Table 2). At any given level of Fe, there were no significant differences in root Fe content among the rootstocks.

Catalase activity. The different concentrations of iron did not affect the catalase activity in any of the rootstocks (Table 2).

Chlorophylls. The two hybrids, F-A 13 and F-A 5, had the highest levels of total chlorophyll in the 0, 9 and 18 µM Fe treatments, while for 36 µM treatment F-A 13 was significantly higher than F-A 5 (Fig. 1). Total chlorophyll in leaves of Cleopatra mandarin was lower than in the F-A rootstocks, but significantly higher than *P. trifoliata*, except at 18 µM Fe in nutrient solution, where the two were not significantly different. Despite its much higher leaf DW (Table 1), Cleopatra mandarin had a lower concentration of chlorophyll in those leaves per unit of DW than F-A 5 or F-A 13 (Fig.1).

Root ferric chelate reductase activity. In plants grown without iron in the nutrient solution, the four rootstocks differed significantly in FCR activity (i.e. differed in y-intercepts in Fig. 2). F-A 13 had high FCR activity in the treatment without any iron in solution, similar to Cleopatra mandarin but lower than F-A 5. *P. trifoliata* was very low compared to all other rootstocks. In the case of F-A 13, FCR activity increased (R^2=0.939) in line with an increase in iron concentration in solution. F-A 5 had the highest FCR activity for any given iron treatment and activity increased (R^2=0.929) linearly with increasing iron in solution. The treatments did not affect all rootstocks equally (i.e. the slopes of the regressions differed). Cleopatra mandarin displayed no significant difference in FCR activity as iron increased, at least in the range tested here, whereas *P. trifoliata,* F-A 5 and F-A 13 all showed significant linear increases in FCR activity in roots as iron in the medium rose.

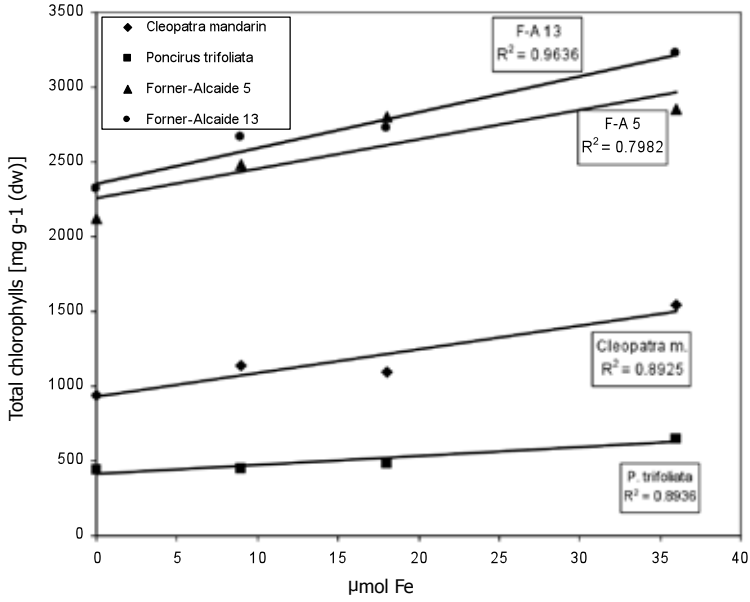

Fig. 1. Total chlorophylls (chla+Chlb) (mg•g^{-1} dry weight) of different rootstocks grown for 60 days with four different Fe concentrations.

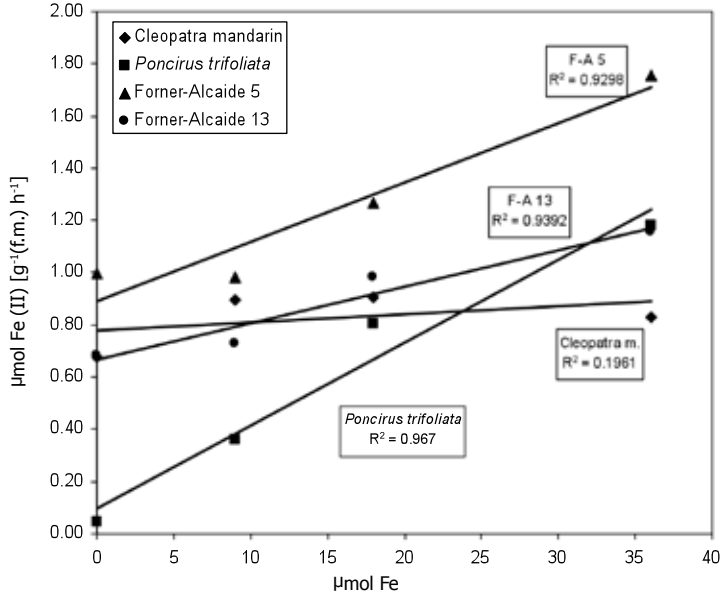

Fig. 2. Root ferric chelate reductase activities (µmol Fe (II)•g^{-1} (f.m.)•h^{-1}) of different rootstocks grown 60 days with four different Fe concentrations.

Discussion

F-A 5 and F-A 13 are two citrus rootstocks, both Cleopatra mandarin x *P. trifoliata* hybrids, developed at the Centro de Citricultura y Producción Vegetal at IVIA (Moncada, Spain) and currently commercialized in Spain. The productivity of trees on F-A 5 and F-A 13 is higher than trees on Carrizo citrange, but their tree size is also smaller (16). Thus, these two hybrids have high production efficiency per unit of canopy volume. Both hybrids also are resistant to CTV and tolerant to salinity (18), and their horticultural performance in experimental plots has been very good (17). Now, we have tested these rootstocks for susceptibility to iron chlorosis.

A number of authors have classified iron tolerance of citrus rootstocks in terms of growth and chlorosis parameters of shoots (5, 21, 34). Iron deficiency tolerance is primarily determined by the rootstock, but the scion cultivar also exerts an effect. Cleopatra mandarin is considered a tolerant genotype to iron chlorosis whereas *P. trifoliata* is very susceptible to iron chlorosis (7). Table 1 shows how zero iron treatments affected leaf weight. Differences were found among the different genotypes included in the experiment: Cleopatra mandarin has larger entire leaves than *P. trifoliata*, F-A 5 and F-A 13 which all have smaller trifoliate leaves. Pestana et al. (30) describe an increase in root biomass in Troyer and *C. taiwanica* (Tan. and Shim.) orange plants grown in the absence of Fe. This response has already been described for other species (25, 26, 36); however, no root morphological changes were found in response to low Fe in peach (31). Here, root weight increased with increasing iron in the treatments. Some authors used the root:shoot ratio to assess the distribution of photoassimilates between shoots and roots (26), and an effect of Fe on root:shoot ratio in some young citrus rootstocks has been described (30). Pestana et al. (30) connected the constant root:shoot ratio of Swingle citrumelo with its high susceptibility to iron chlorosis, but in our experiment all rootstocks had a constant root:shoot ratio so it was impossible for us to correlate the root:shoot ratio to iron chlorosis susceptibility.

F-A 5 and Cleopatra mandarin had a higher total dry weight than the other two rootstocks (Table 1). It is clear, however, that plants grown with little or no iron were smaller and produced less dry matter than those grown with high levels of Fe. This is especially true in *P. trifoliata* where the reduction in growth was about 53% in the absence of Fe. The evaluation of growth parameters may not be sufficient to evaluate iron-chlorosis tolerance in citrus rootstocks (30).

Percent dry weight of iron in chlorotic leaves, is frequently greater than in green leaves (1, 27). This was called the "chlorosis paradox" (33) and results from either the inactivation of iron in leaves or from an inhibition of leaf growth due to iron chlorosis (27, 28). This phenomenon did not occur in young hydroponically grown citrus seedlings (12). Chouliaras et al. (13) found that the absence of iron in the nutrient solution reduced catalase activity in leaves of citrus plants when enzyme activity was used as an indicator of iron chlorosis in orange cultivars. This is in agreement with our results in that 0 μM Fe decreased catalase activity in all the rootstocks except F-A 13; however, the increase of catalase activity is not significantly correlated with the iron concentrations (Table 2).

Chouliaras et al. (13) also found that catalase and peroxidase activity were significantly higher in leaves of two cultivars grafted on *C. aurantium* than when they were grafted on Swingle citrumelo so *C. aurantium* was more tolerant to iron chlorosis than Swingle citrumelo. These results disagree with those obtained in our experiment, because catalase activity in Cleopatra mandarin, an iron-chlorosis-tolerant rootstock, did not differ from the susceptible *P. trifoliata* at any given level of Fe tested.

Iron catalyzes chlorophyll biosynthesis (11) and is believed to affect leaf chlorophyll content. F-A 5 and F-A 13 maintained significantly higher chlorophyll contents than Cleopatra mandarin and *P. trifoliata* (Fig. 1). Sudahono et al. (34) reported that the values of chlorophyll may give information not only

about the degree of chlorosis but also about the different behavior of genotypes to iron chlorosis. The chlorophyll data suggest that *P. trifoliata* was the most sensitive of all the rootstocks in agreement with the results published by other authors (7, 21) and the data also suggest that F-A 5 and F-A 13 are tolerant to iron deficiency.

Determination of FCR activity has been widely used as a screening technique for selecting iron chlorosis-tolerant genotypes (12, 20, 23). Increases in FCR activity when iron was omitted from the nutrient solution have been found in other woody plants under different experimental conditions (12, 14). Several reports have indicated that in other fruit tree species, iron deficiency alone does not always produce increases in FCR activity (19, 31, 35). Tagliavini et al. (35) did not find any FCR induction in iron-deficient rootstocks. There have been other preliminary reports that the addition of iron to the nutrient solution of Fe-deficient trees may cause an enhancement of root FCR activity (4, 15).

Under our working conditions, an increase in root FCR activity was observed with increasing iron concentration in the nutrient solution, except in Cleopatra mandarin, in which no differences were found among the four iron treatments applied (Figure 2). F-A 5, F-A 13 and *P. trifoliata* displayed increased FCR activity with increasing iron concentration in the medium. FCR activity of *P. trifoliata* was the lowest for the 0 and 9 μM Fe treatments.

In conclusion, the results indicated that the rootstock F-A 5 showed high FCR activity in roots, high chlorophyll content in leaves and iron content in roots, and also high total dry weight. F-A 5 is an iron-chlorosis-tolerant rootstock like Cleopatra mandarin, but as it does not have the important agronomic drawbacks associated with Cleopatra mandarin, it is a valuable rootstock for soils affected by this abiotic problem.

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