Plant and Soil **241:** 49–56, 2002.

A comparison of ferric-chelate reductase and chlorophyll and growth ratios as indices of selection of quince, pear and olive genotypes under iron deficiency stress

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Received 19 October 2000. Accepted in revised form 3 September 2001

Key words: iron chlorosis, iron stress, nutrient solution, olive, pear, quince

Abstract

Quince (*Cydonia oblonga* Mill.), pear (*Pyrus communis* L.) and olive (*Olea europaea* L.) genotypes were evaluated for their tolerance to iron deficiency stress by growing young plants in three types of aerated nutrient solutions: (1) with iron, (2) without iron or (3) low in iron and with 10 m*M* bicarbonate. Plants were obtained either from rooted softwood cuttings or from germination of seeds. The degree of tolerance was evaluated with several indices: (1) the chlorophyll content, (2) the root Fe^{3+} reducing capacity and (3) the whole plant relative growth. Fifteen hours before Fe^{3+} reducing capacity determination, iron was applied to the roots of plants with iron-stress, since this method resulted in increasing the reductase activity. All quince and pear genotypes increased the root Fe^{3+} reducing capacity when grown in the treatments for iron-stress, in relation to control plants of the same genotypes. In olive cultivars, the Fe^{3+} reducing capacity was lower in the iron-stress treatments than in the control one. Studying the relationship between relative growth and chlorophyll content for each genotype under iron-stress, in relation to both indices in control plants, a classification of species and genotypes was established. According to that, most olive cultivars and some pear rootstocks and cultivars appear more iron-efficient than quince rootstocks. Our study shows that in some woody species, determining root Fe^{3+} reducing capacity is not the best method to establish tolerance to iron deficiency stress.

Introduction

Quince (*Cydonia oblonga* Mill.) and pear (Pyrus communis L.) fruit trees show iron chlorosis when grown on calcareous soils, especially when pear varieties are grafted on quince rootstocks (Tagliavini et al., 1995). Olive (*Olea europaea* L.) trees have been grown on calcareous soils of the mediterranean area without apparent problems of chlorosis, however in new fertigated olive plantations in calcareous soils, iron chlorosis is present in some cultivars, and differences in tolerance among cultivars have been shown (Cordeiro et al., 1995).

Many plant species develop physiological responses under iron-deficiency which increase iron uptake. These responses, for dicots and non-graminaceous monocots (Strategy I plants), include release of hydrogen ions and reductants from roots and increasing Fe^{3+} reduction at the root surface (Bienfait, 1988; Jolley et al., 1996; Römheld and Marschner, 1986).

To screen plants for resistance to iron chlorosis, several methods can be used: some of them are based on screening for leaf iron-deficiency chlorosis on calcareous soils in field trials or in nutrient solutions in growth chambers (Zaiter et al., 1986, 1987); others use the physiological responses induced by iron-deficiency (Ellsworth et al., 1997, 1998; Jolley et al., 1992). Good relationship was found between Fe^{3+} reduction capacity (RC) and the resistance to iron-deficiency in the field for some herbaceous dicot plants, as soybean (Jolley et al., 1992) and dry bean

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genotypes (Ellsworth et al., 1997). Induced H^+ ions release under iron-deficiency has also given good relationship with resistance to iron-deficiency in subclover (Wei et al., 1997) and in soybean, but Fe^{3+} reduction provided better screening ability in soybean (Ellsworth et al., 1998).

In woody plants, increase in Fe^{3+} reduction capacity under iron stress has been found in apple (Ao et al., 1985), in peach rootstocks or cultivars (De la Guardia et al., 1995; Egilla et al., 1994; Romera et al., 1991), in grapevine (Bavaresco et al., 1991; Brancadoro et al., 1995), in kiwi (Vizzotto et al., 1997; 1999) and in two quince rootstocks (Cinelli, 1995). However, no increase in ferric reductase under iron-stress has been found either in mango (Shenker et al., 1991) or in several quince and pear rootstocks (Tagliavini et al., 1995).

Cordeiro et al. (1995) studied the differences in tolerance to iron-deficiency of several olive cultivars following the time course of leaf chlorosis and chlorophyll, shoot elongation and shoot dry matter accumulation. However, no physiological responses to iron-deficiency, as RC of roots, were studied neither in that work nor in other publications about olive.

In some studies with herbaceous dicots, stimulation of ferric reductase activity was reported when a small amount of iron was supplied to iron-deficient plants a few hours before ferric reductase activity determination (Grusak et al., 1990; Romera et al., 1996). In some early work with iron-deficient plants, no increase in ferric reductase activity was found, and this may be related to using a less appropriate method.

The objectives of this work were: (1) to study the effect of applying iron (1 or 100 μM Fe) to the nutrient solution of iron-deficient quince seedlings the fifteen hours prior to measurement on their root Fe^{3+} reduction capacity; we next used the proven method for all subsequent determinations (2) to screen quince, pear and olive genotypes for tolerance to iron-deficiency stress by growing the plants in nutrient solutions with different degree of iron-deficiency. In the screening method, several indices were used: chlorosis development, relative growth and root Fe^{3+} reducing capacity.

Material and methods

Plant material

Plants of quince and pear rootstocks and olive cultivars were obtained from softwood cuttings rooted under mist. The quince rootstocks were: EM-A and EM-C selected at East Malling (UK); Sydo selected by Institut National de la Recherche Agronomique (France); Adams selected in Belgium; and Provence from France. The pear rootstocks were selections from OHxF (Old Home × Farmingdale): OHF 333, F-69 and F-87 selected in US (Brooks, 1984). The rootstocks original material was obtained from commercial nurseries.

The olive cultivars were: Picual, Picudo and Loaime (from a farm at Granada, Spain), and Arbequina, Nevadillo negro, Hojiblanca and Megaritiki (from the Experimental Farm of Alameda del Obispo at Córdoba, Spain). Also, quince and pear seedlings were obtained after germination of seeds of commercial cultivars: Córdoba, Gamboa and Wranja (quince); Conference, Mantecosa and Blanquilla (pear). The seeds were maintained in wet perlite in cold (5 °C) for 2 or 3 months to break dormancy.

Plant culture and treatments

Plants were transferred from the rooting or germination medium to continuously aerated nutrient solution with 4 plants per four L plastic container. The composition of the basic nutrient solution (BNS) was the same as in previous experiments (Romera et al., 1991). For quince and pear plants, the boron and calcium concentrations were increased to 25 μM (as H_3BO_3) and to 2 mM (as $CaCl_2$), since B and Ca deficiencies appeared in the shoot apices at the lower B and Ca concentrations of BNS.

After three or four weeks of preculture in BNS and 10 μM FeEDDHA, plants were selected to start the different experiments. At this time, the fresh weight of each plant was recorded.

In each set of experiments, three treatments were established: control (BNS with 20 μM FeEDDHA, pH 6.0), Bic (BNS with 3 μM FeEDDHA, 10 mM $NaHCO_3$ and 0.5 g L^{-1} $CaCO_3$, pH 7.5), and zero-iron (BNS, pH 6.0). Four plants per treatment were growing in the same container. The nutrient solutions were renewed every week and the experiments were maintained during three or four weeks for quince and pear, and five weeks for olive.

The experiments were conducted in a growth chamber at 24 °C during the day and 20 °C at night, 14 h photoperiod, relative humidity 60–80% and a photosynthetic irradiance of 200μmol quanta m^{-2} s^{-1} provided by fluorescent tubes (Sylvania cool white VHO).

Table 1. Root ferric reducing capacity (RC) of seedlings of quince cultivars grown in nutrient solution with different treatments for iron deficiency and different conditions for RC determination

Cultivar	Treatment	Fe addition[a]	Reducing capacity	
		μM	nmol Fe^{2+} g^{-1}FW h^{-1}	% of control
Córdoba	Control	0	250 ± 40	100
	–Fe	0	1150 ± 120	460
	–Fe	1	2080 ± 230	832
Gamboa	Control	0	79 ± 10	100
	Bic[b]	0	65 ± 10	82
	Bic[b,c]	100	285 ± 50	360
Wranja	Control	0	300 ± 30	100
	Bic[b]	0	135 ± 30	45
	Bic[b,c]	100	580 ± 80	193

[a] Iron (FeEDDHA) was applied to nutrient solution to reach 1 or 100 μM, 15 h before RC determination.
[b] Treatment with 10 m*M* bicarbonate.
[c] The solution for measuring RC was buffered with MES to pH 5.0.

Determinations

The levels of chlorophyll in the youngest expanded leaves were weekly recorded by taking SPAD (Soil Plant Analysis Development) readings with a SPAD-502 Chlorophyll Meter (Minolta Camera Co., Ltd, Japan) (see Wood et al., 1992). A close relationship between chlorophyll content and SPAD reading was obtained previously (Cordeiro et al., 1995). At the end of the experiments, the Fe^{3+} reducing capacity of the roots was determined with intact plants, as follows. After washing, the roots were mantained in a 0.5 m*M* $CaSO_4$ solution for 30 min, thereafter the roots of each plant were submerged in 200 ml of a reduction assay solution containing BNS without micronutrients, 3×10^{-4} M Ferrozine (3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine) and 10^{-4} M FeEDTA (ferric ethylene diamine-tetraacetate). The solution was buffered at pH 5.0 with 1 m*M* MES (2-(N-Morpholino) ethane sulfonic acid), except for some plants (see Table 1). After 3 h of Fe^{3+} reduction in light, while protecting the roots from light, the absorbance was determined at 562 nm.

The total plant fresh weight and the root fresh weight (after drying the roots with filter paper) were determined at the end of each experiment. Some experiments were repeated and for simplification only the most significant results are presented. At least four plant replicates were used for each treatment in each experiment.

Results

Chlorophyll index

SPAD readings (chorophyll index) taken at the end of the treatment period (Figure 1) showed that leaves of olive plants had higher SPAD readings than leaves of quince or pear plants, regardless of treatment, probably because they are thicker. Green leaves of control plants had SPAD readings of about 60 for olive and between 30 and 35 for quince and pear. Iron deficiency had a big influence on this index decreasing it for all three species studied. The zero-iron treatment produced the lowest chlorophyll index for almost all genotypes. The effect was especially marked in quince and pear plants in which leaves were almost yellow due to low chlorophyll contents (SPAD reading less than 10). In the bicarbonate treatment, the SPAD readings, relative to the control, were higher for olive (between 34 and 76%), and pear (between 38 and 92%), than for quince plants (between 18 and 39%).

Root Fe^{3+} reducing capacity

In a preliminary experiment, the effect of applying to the roots a small amount of iron (1 μM) to zero-iron plants and higher quantities (100 μM) to bicarbonate treated plants, a few hours (15 h) before reducing capacity (RC) determination on RC was studied. In addition, the effect of buffering the reduction assay

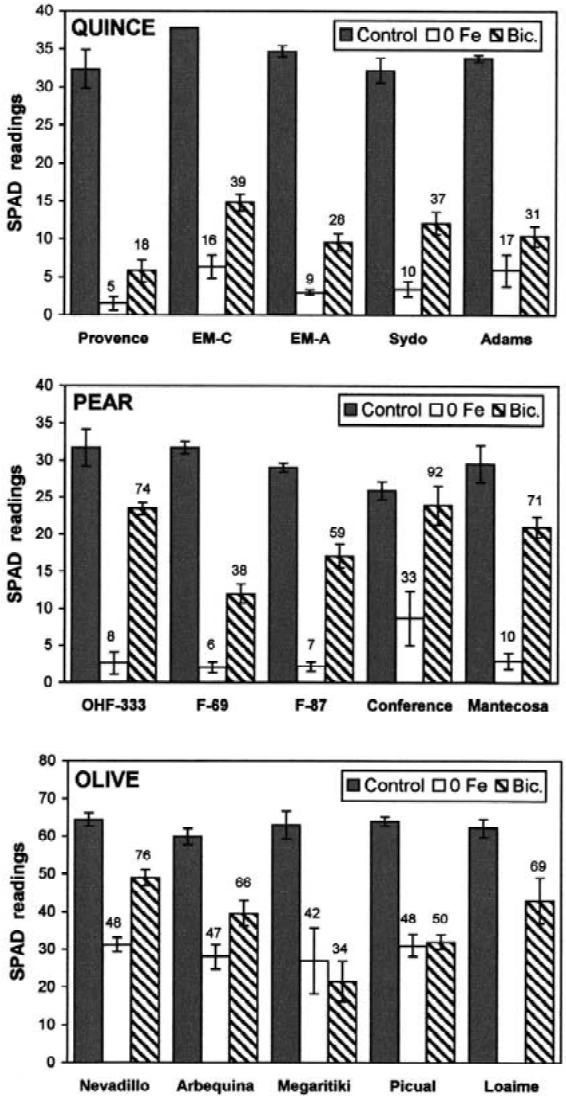

Figure 1. SPAD readings of the youngest leaves of five quince rootstocks, three pear rootstocks, two pear cultivars and five olive cultivars in each of three treatments at the end of experiments. Mean±SE (n=4). For each genotype, the ratio of treated – control plants is given as percentage over the corresponding bar. Note a different y-axis scale for olive compared to quince and pear.

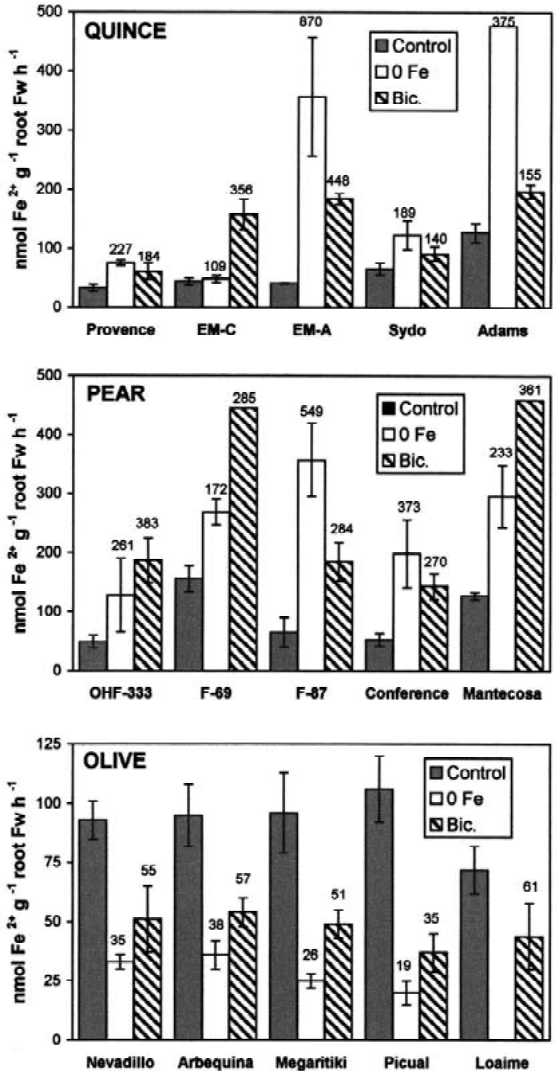

Figure 2. Ferric-chelate reducing capacity (RC) of roots of five quince rootstocks, three pear rootstocks, two pear cultivars and five olive cultivars in each of three treatments at the end of experiments. Mean±SE (n=4). For each genotype, the ratio of treated – control plants is given as percentage over the corresponding bar. Note a different y-axis scale for olive compared to quince and pear.

solution at pH 5.0 with MES for the plants previously treated with bicarbonate was studied. The results showed that previous application of iron had a big effect on increasing RC; cultivar Córdoba in (−Fe) increased RC to 832% of the control (Table 1). The cultivars Gamboa and Wranja did not show RC increase when they did not receive iron addition previous to RC determination (RC was 82 and 45% of the control); however, they showed RC increase to 360 and 193%, respectively, of their controls, with previous iron application and measuring RC with MES (Table 1). After these results, all the following RC determinations were done with prior iron application (1 μM for zero-iron and 100 μM for Bic treatments) and in a solution buffered at pH 5.0 with MES.

The five quince rootstocks and the five pear genotypes presented in Figure 2 showed increase in RC when grown in iron deficiency conditions, in relation to the control plants grown with sufficient iron. The ratio of RC of iron stressed plants (zero-iron or Bic)

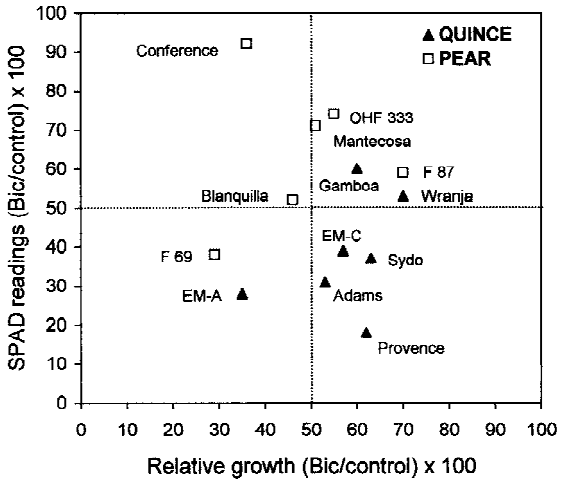

Figure 3. Relationship between chlorophyll content, as SPAD readings, and relative growth of quince and pear plants in bicarbonate treatment. Both indices are expressed as percentage of control.

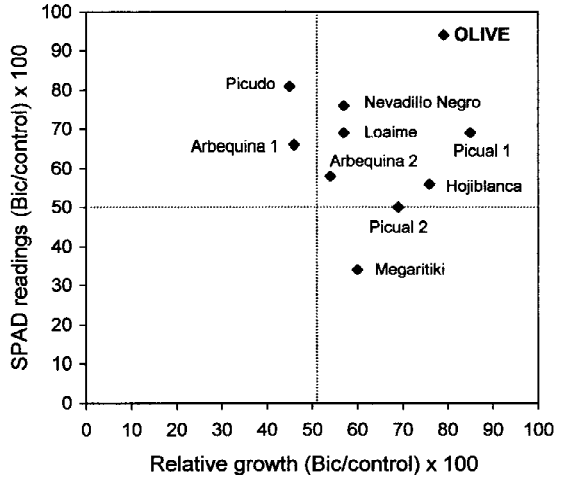

Figure 4. Relationship between chlorophyll content, as SPAD readings, and relative growth of olive plants in bicarbonate treatment. Both indices are expressed as percentage of control.

to iron sufficient plants (control) showed large differences among genotypes and treatments. Some genotypes (EM-A, Adams and F-87) showed higher RC in zero-iron, and others (EM-C, F-69 and Mantecosa) had higher RC in Bic treatment.

In olive plants, results were quite different (Figure 2). The RC under iron stress (zero-iron or Bic treatments) was lower than in control plants for all cultivars tested.

Whole plant relative growth

The total plant increase in fresh weight was determined by subtracting from the final weight (Wf) the initial weight (Wi). The relative growth was obtained dividing the increase (Wf–Wi) by the initial weight (Wi). The ratio of relative growth of iron-stressed plants (zero-iron or bicarbonate) to control plants was obtained for all the genotypes studied. The ratios obtained with the zero-iron treatment for each genotype were similar to those obtained with the bicarbonate treatment (data not shown). Thus, for simplicity only the ratios of relative growth of the bicarbonate treatment – control plants are presented in relation to the ratios of SPAD readings of bicarbonate treatment – control plants for each genotype (Figures 3 and 4).

The ratios of relative growth for quince and pear genotypes were between 29 and 70%; and for olive cultivars were between 45 and 85%. For two olive cultivars, two determinations of the same cultivar are presented in Figure 4 (Picual and Arbequina) to give information about the variability of the method.

Discussion

The results show that the protocol followed to measure RC greatly affects the values of RC obtained (Table 1) and pose questions about earlier studies in which no increase in RC was found under iron-deficiency conditions in mango (Shenker et al., 1991) or in several quince and pear rootstocks (Tagliavini et al., 1995). In the present work, following previous iron application and using MES for pH buffering, iron-stressed quince and pear plants showed increases in RC for all the cultivars studied. However, iron-stressed olive plants did not show such an increase. These surprising results of lower reductase activity in iron-stressed olive plants relative to controls can be questioned since the RC measurements were performed after 5 weeks of iron-deficiency and we might have missed a potential increase in RC, that could have occurred earlier in the iron-deficiency treatment, but we did not find increase in another experiments with shorter periods of iron-stress. The 5 weeks period was needed in order to get enough growth in a species of relative slow growth.

Moreover, no decrease in the pH of the nutrient solution in which the zero-iron plants were grown was observed for all the species or cultivars studied, which is one of the physiological responses of Strategy I plants.

According to a classification for iron-efficiency based in the physiological responses induced by iron-deficiency, as the increase in RC (Bienfait, 1988; Römheld and Marschner, 1986), we could say that the quince and pear genotypes studied are iron-efficient and the olive cultivars are iron-inefficient. However, this is not in agreement with leaf chlorosis developed for these genotypes in the field and with other classification indices used in this work.

In another species, chlorosis susceptibility relates closely to the magnitude and timing of iron reduction, as in soybean (Jolley et al., 1992) and dry bean cultivars (Ellsworth et al., 1997). Peach genotypes and peach rootstocks also showed good correlation between iron root reduction and chlorosis development observed in the field (De la Guardia et al., 1995; Romera et al., 1991). However since time course of iron reduction shows oscillations, one time measurement of iron reduction is a questionable practice for estimating iron-efficiency, and better results were obtained when the sum of seven daily iron reduction measurements were used (Ellsworth et al., 1997; Jolley et al., 1992). Wei et al. (1997) question if increased iron reduction capacity is essential for plant iron-efficiency in calcareous soils, since they found a significant difference in H^+ release between two extreme cultivars of subclover, but not differences in root iron reduction capacity were observed between the resistant and the susceptible cultivar.

Development of chlorosis in growth chambers is seldom similar to field grown plants. Thus, SPAD reading data in growth chamber should be related to field estimates of iron-efficiency. If we apply the method of studying the relation between the relative growth and the SPAD reading to the same species and cultivars, different results appear. Following this method, an iron-efficient cultivar is one that under iron-stress decreases little its chlorophyll level and its relative growth, in relation to the control plants. In the presentation of Figures 3 and 4, the most iron-efficient cultivars would be the ones localized more closed to the upper and right corner, and the most iron-inefficient ones would be represented at positions lower and toward the left side. There are differences among species and genotypes. The quince rootstocks are the most iron-inefficient, and the quince cultivars (Gamboa and Wranja) are more iron-efficient than the rootstocks. In pears, OHF 333 and Mantecosa are more iron-efficient than F-69, and in olive cultivars, Megaritiki is the most iron-inefficient one.

As we can see the responses of the three species studied are different depending on the index considered. Quince and pear plants showed increased RC and olive did not show that (Figure 2), while olive showed as good or better response, in relative growth and SPAD index, than quince and pear plants (Figures 3 and 4). The results in Figures 3 and 4 are in agreement with the response of plants of the same cultivar grown in pots with calcareous soils (unpublished data) and with observations of plants of these species growing in calcareous fields of Southern Spain. Quince develop high chlorosis, pear on quince rootstocks also shows chlorosis, but not on its own roots, and olive chlorosis is very unusual especially in old plantations. Thus, the method of studying the increase in RC when plants grow under iron-stress does not appear a good method for classifying genotypes of these species for establishing their degree of tolerance.

The question of how the olive overcomes the iron-stress in calcareous soil without increasing the RC of their roots is open. In Figure 2, the olive control plants (growing with adequate iron) show a relatively high basic RC (among 72 and 106 nmol Fe^{2+} g^{-1} root Fw h^{-1}) in relation to the control plants of quince and pear, most of its genotypes show RC below 100 nmol Fe^{2+} g^{-1} root Fw h^{-1}. This basic RC must be the expresion of the standard reductase, a constitutive mechanism present in the plasma membrane, which activity is not affected by the iron nutritional status and it has a low capacity to reduce synthetic ferric-chelates (Marschner and Römheld, 1994). Probably the basic RC must be enough to get the iron that the plant needs, considering the slow growth of this species. Another reason could be the existence of non specific mechanisms for enhaced mobilization and uptake of iron in the rhizosphere, as the root induced pH decreases because preferencial cation uptake. Striking differences can be observed between cultivars and along an individual root (Marschner and Römheld, 1983).

The method based on relative growth and chlorophyll content followed in this and in a previous work (De la Guardia et al.,1995) can be useful to compare species or cultivars for their responses to iron-deficiency stress over short time periods. However, the need to have enough plants of each genotype in order to select the uniform ones to start the treatments and to reduce the high variability of results that appears in some of the indices studied must be considered. Duplication of experiments also must be considered before classifying one cultivar. As we can see in Figure 4, duplicated studies of Arbequina gave very close

results, but in Picual, the duplicated study done with plants that were one month older gave divergent ones.

One interesting result is that seedlings from commercial quince cultivars, Gamboa and Wranja in Figure 3, gave better response than the established rootstocks of this species: EM-C, EM-A, Sydo, Adams and Provence. This opens the way to do selection in both cultivars to obtain rootstocks with better performance to iron-stress.

In conclusion, our study shows that in some woody species, there is not clear relationship between root ferric RC and other indices, as chlorophyll content and relative growth, when plants are grown under iron-stress. Similarly, there is no relationship of RC with the observations of plants that grow in calcareous soils. Most of quince genotypes studied had poor performance growing in iron-stress conditions. However they had high or very high root ferric RC. Probably in this species there are other problems with iron transport to the shoot or with iron reduction in the leaves. The situation is different for the olive genotypes; they had better performance in iron-stress conditions, although they did not increase their root ferric RC. Another nonspecific mechanism must cooperate for acquisition of enough iron from calcareous soils in this plant species.

Acknowledgements

This work was supported by the Comision Interministerial de Ciencia y Tecnología (Projects AGF96-1081 and OLI96-2183) and Junta de Andalucía (Research Group AGR 115). The autors thank Dr. F.J. Romera his commments to text. The technical assistance of A. Sánchez Palenzuela and N. Ruíz Baena is greatfully acknowledged.

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