Scientia Horticulturae 199 (2016) 1–8

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Contrasting physiological responses to iron deficiency in Cabernet Sauvignon grapevines grafted on two rootstocks

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ARTICLE INFO

Article history:
Received 26 June 2015
Received in revised form 1 December 2015
Accepted 11 December 2015
Available online 28 December 2015

Keywords:
Iron chlorosis symptoms
Net photosynthesis
Organic acids
Enzyme activity
Grapevine genotypes

ABSTRACT

The present work aimed to compare the physiological responses to Fe deficiency in *Vitis vinifera* L. cv. Cabernet Sauvignon grafted on two *Vitis* rootstocks with different Fe chlorosis susceptibility: 110 Richter (moderately tolerant) and 3309-C (susceptible). Plants were grown in hydroponic solutions with 50 μM of Fe-EDTA or without Fe. Our data showed that, in a first step (18 days after treatment, DAT), Fe deficiency reduced net photosynthesis in leaves from both variety/rootstock combinations, without modifying leaf chlorophyll concentration. Moreover, Fe deficiency increased the activity of PEPC and $NADP^+$-IDH enzymes and the concentration of malic and total organic acids in roots of both rootstocks. During such period, no differences related to Fe level were recorded in the main shoot length and biomass. After a longer exposure to Fe deficiency (32 DAT), the activity of PEPC and organic acid-linked enzymes in roots of both genotypes remained unaltered. However, at 32 DAT, plants grafted on 110 Richter reacted to Fe deficiency by reducing the main shoot length and biomass, whereas such reduction was not observed in plants grafted on 3309-C. In addition, at 32 DAT Fe deficiency reduced the net photosynthesis as well as the chlorophyll concentration in plants grafted on 3309-C, without modifying these parameters in 110 Richter plants. In conclusion, our results revealed significant differences between the two variety/rootstock combinations in severity of Fe deficiency symptoms and physiological responses.

1. Introduction

Grapevine is a crop of high economic importance worldwide, mainly grown on rootstocks because of sanitary threats. Cabernet Sauvignon is the most widespread red grape variety. Such genotype is characterized by a high adaptability to different soils and climates and is tolerant to iron (Fe)-chlorosis (Jiménez et al., 2007; Ollat et al., 2003). However, by grafting on pure *Vitis* species or hybrids, Cabernet Sauvignon may display different Fe deficiency-induced responses (Bavaresco et al., 1994; Covarrubias et al., 2014). Iron chlorosis is a major concern in grafted vines cultivated in calcareous soils. The susceptibility to Fe chlorosis in grapevine is highly variable for different genotypes. *Vitis vinifera* and *Vitis berlandieri* species are highly tolerant and tolerant to Fe chlorosis, respectively, *Vitis riparia* is considered as highly susceptible and *Vitis rupestris* slightly susceptible to Fe chlorosis (Brancadoro et al., 1995; Covarrubias and Rombolà, 2015; Tagliavini and Rombolà, 2001).

Investigations conducted on several plant species (Covarrubias and Rombolà, 2013; Covarrubias and Rombolà, 2015; De Nisi and Zocchi, 2000; Donnini et al., 2009; Jelali et al., 2010; López-Millán et al., 2000; Nikolic et al., 2000; Rombolà et al., 2002) have studied root mechanisms of Fe uptake. Grapevine behaves as dicotyledonous and non-graminaceous monocotyledonous species regarding the root Fe uptake strategy. Consequently, grapevine absorbs Fe from the soil as Fe^{2+}, a form scarcely present in subalkaline and alkaline soils where Fe^{3+} prevails, causing low levels of available Fe for plant uptake (Kim and Guerinot, 2007; Römheld and Marschner, 1986). The induction of Fe chelate reductase (FCR) activity as well as the proton release into the rhizophere, represent the main mechanisms of the tolerant grapevine rootstocks to overcame Fe deficiency (Bavaresco et al., 2003; Covarrubias and Rombolà, 2013, 2015; Jiménez et al., 2007; Ollat et al., 2003). In addition, it has been demonstrated that Fe deficiency tolerant grapevines submitted to Fe depletion may increase the activity of enzymes related to the organic acids biosynthesis in roots. Some root enzymes that increase their activity under Fe deficiency are phosphoenolpyruvate carboxylase (EC 4.1.1.31), malate dehydrogenase (EC 1.1.1.37), citrate synthase (EC 4.1.3.7), and isocitrate dehydrogenase (EC

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http://dx.doi.org/10.1016/j.scienta.2015.12.013
0304-4238/

1.1.1.42) (Covarrubias and Rombolà, 2013, 2015; Jiménez et al., 2007; Ollat et al., 2003). Such responses are frequently associated to an increase in organic acids concentration in roots (Covarrubias and Rombolà, 2013, 2015; Jiménez et al., 2007; Ollat et al., 2003). Citric acid, which accumulates in high amounts in Fe-deficient roots, is closely involved in plant Fe homeostasis, since it is linked to Fe transport in xylem vessels, proton efflux through the root plasma membrane and the capacity to produce reducing power as NADPH (see Jiménez et al., 2007 and references therein). Other organic acids whose concentration in roots increase under Fe deficiency and bicarbonate in the nutrient solution are malate (Covarrubias and Rombolà, 2013; Covarrubias et al., 2014; Jiménez et al., 2007; Ollat et al., 2003) and tartrate (Covarrubias and Rombolà, 2013; Ollat et al., 2003). Phosphoenolpyruvate carboxylase (PEPC) catalyzes the carboxylation of phosphoenolpyruvateto oxaloacetate (Chollet et al., 1996). Oxalacetate could be subsequently reduced to malate via cytosolic malate dehydrogenase (MDH), and malate could then be transported to the mitochondria and converted to citrate by citrate synthase (CS) (López-Millán et al., 2000). The NADP-dependent isocitrate dehydrogenase (NADP$^+$-IDH) ηασ α κεΨ ρολε ινττηε 2-oxoglutarate production by the oxidative decarboxylation of isocitrate (Foyer et al., 2011), and has been reported as an enzyme responding to Fe deficiency in root tissues of *Beta vulgaris* L. (López-Millán et al., 2000), *Pisum sativum* (Jelali et al., 2010) and *Lycopersicon esculentus* L. (López-Millán et al., 2009).

It has been observed that the organic acids metabolism under Fe deficiency is differently induced among grapevine genotypes (Brancadoro et al., 1995; Covarrubias and Rombolà, 2013, 2015; Dell'Orto et al., 2000; Jiménez et al., 2007; Ksouri et al., 2006; Rombolà and Tagliavini, 2006) depending on the level of Fe deficiency tolerance exhibited by the different genotypes in the field. In such context, the Fe chlorosis tolerant genotype 140 Ruggeri submitted to Fe depletion for 17 days significantly reacted by increasing the activity of PEPC, CS and NADP$^+$-IDH, and the concentration of citrate, and tartrate in roots (Covarrubias and Rombolà, 2013). A similar effect was observed in Cabernet Sauvignon (*V. vinifera*), where Fe deficiency increased the activity of PEPC and the concentration of malate, citrate and succinate in roots, whereas the Fe chlorosis susceptible genotype Gloire de Montpellier (*V. riparia*) differentially triggered such response mechanisms up to a different extent (Jiménez et al., 2007). The large amount of scientific evidences recorded in several plant species has appointed organic acids accumulation in roots and the activity of related enzymes, in particular PEPC as biochemical markers for Fe deficiency status and tolerance degree to Fe deficiency in plants (Jiménez et al., 2007; Ollat et al., 2003; Rombolà et al., 2002; Rombolà and Tagliavini, 2006). However, a recent experiment conducted on three grapevine genotypes subjected to Fe deficiency for a longer period (32 days), Covarrubias and Rombolà (2015) observed that the rootstock 101-14, susceptible to Fe chlorosis, responded to severe Fe deficiency by reducing the root activity of phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH) and accumulating high levels of citric acid, whereas the rootstock 110 Richter, tolerant to Fe chlorosis, showed a lower citric acid accumulation (Covarrubias and Rombolà, 2015). Such evidences suggest that organic acids accumulation in roots might not be a Fe chlorosis reliable indicator, particularly under conditions of severe Fe deficiency (Covarrubias and Rombolà, 2015). The contrasting physiological behaviors exhibited by grapevine genotypes submitted to short and long length of Fe depletion, suggest that only some genotypes are able to modulate their strategy to cope with Fe deficiency according to its duration and severity.

Indeed, information concerning the behavior of grapevine genotypes under prolonged Fe deficiency are scarce. The present work is aimed at comparing physiological responses to Fe deficiency in *V. vinifera* L. cv. Cabernet Sauvignon grafted on two *Vitis* genotypes with different Fe chlorosis tolerance: the Fe chlorosis moderately tolerant 110 Richter (*V. berlandieri* × *V. rupestris*) and the Fe chlorosis susceptible 3309-C (*V. riparia* × *V. rupestris*).

2. Materials and methods

2.1. Plant material, growth conditions and treatments

The experiment was carried out at the Experimental Station of the Facultad de Ciencias Agronómicas, Universidad de Chile (Santiago, Chile). One year grapevine cuttings of the rootstocks 110 Richter (*V. berlandieri* × *V. rupestris*) and 3309-C (*V. riparia* × *V. rupestris*) grafted with Cabernet Sauvignon were placed in a greenhouse, acclimated in peat for 2 months and pruned to maintain one main 10–15 cm shoot per plant. The plants were transferred to 10 L plastic containers covered with aluminum foil and filled with 8 L of a half Hoagland nutrient solution continuously aerated. The composition of the half Hoagland nutrient solution was: 2.5 mM KNO_3; 2 mM MgSO4; 1 mM KH_2PO4; 2.5 mM $Ca(NO_3)_2$; 4.6 μM $MnCl_2$; 23.2 μM H_3BO_3; 0.06 μM Na_2MoO_4; 0.4 μM $ZnSO_4$; 0.19 μM $CuSO_4$ (Covarrubias and Rombolà, 2013). The two genotypes were grown with 50 μM of Fe-EDTA (+Fe) or without Fe (−Fe) in groups of 5 plants for each container. The nutrient solution was renewed twice a week, the pH was adjusted to 5.5 (HCl 0.1 M) to maintain Fe-EDTA stability and monitored daily at 9:00. The plants were grown with natural photoperiod (16 h of light and 8 h of darkness) in a greenhouse wherein the temperature was 25–30 °C with 70–75% relative humidity. After 18 and 32 days from treatments imposition (DAT = days after treatment imposition), groups of 5 plants of each treatment were divided into roots, main shoot and leaves to determine dry weight and the following analyses were performed on fresh root samples. The experiment was concluded after 32 days, when apical leaves of at least one variety/rootstock −Fe combination displayed Fe deficiency symptoms (SPAD value value lower than 10).

2.2. Plant growth and leaf chlorophyll content

The main shoot length was measured twice a week, simultaneously to the nutrient solution renewal. During the experiment, leaf chlorophyll content on each plant was periodically monitored on five points of the first completely expanded leaf with the portable chlorophyll meter SPAD MINOLTA 502 (Konica Minolta, Inc., Osaka, Japan).

2.3. Enzyme assays

At the end of the experiment, about 100 mg of root tips (20–30 mm long) were collected from each plant, rinsed in deionized water, weighed, deep-frozen in liquid nitrogen, and kept at −80 °C for enzyme activity analysis. The root extraction was performed as described by Jiménez et al. (2007).

Phosphoenolpyruvate carboxylase was determined by coupling its activity to malate dehydrogenase-catalyzed NADH oxidation (Vance et al., 1983). Malate dehydrogenase activity was determined by monitoring the increase in absorbance at 340 nm due to the enzymatic reduction of NAD$^+$ (Smith, 1974). Citrate synthase activity was assayed by monitoring the reduction of acetyl coenzyme A to coenzyme A with DTNB at 412 nm (Srere, 1967). Isocitrate dehydrogenase activity was assayed as described by Goldberg and Ellis (1974), by monitoring the reduction of NADP$^+$ at 340 nm.

2.4. Determination of organic acids in roots

The organic acid content was determined according to Neumann (2006). Frozen samples of root tips collected at the end of the

experiment were submerged in a pre-cooled (4 °C) mortar with liquid nitrogen. For extraction and deproteinization, 5% H_3PO_4 was utilized. Organic acids were quantified as described by Neumann (2006) by high-performance liquid chromatography (HPLC) with 250 × 4 mm LiChrospher 5 μm RP-18 column (Supelco Inc., PA 16823-0048, USA). The elution buffer was 18 mM KH_2PO_4, pH 2.1 adjusted with H_3PO_4. Chromatograms were run for 40 min using a detection wavelength of 210 nm. During the analysis, four organic acids have been identified and quantified: tartrate, citrate, malate and ascorbate.

2.5. Measurement of leaf gas exchange

Measurement of leaf CO_2 exchange was performed on each plant at 18 and 32 DAT, using an infrared gas analyser (IRGA, LCi—ADC, UK). Measurements were performed on the first completely expanded leaf from the middle third of the shoot. Leaves were illuminated providing a photosynthetic photon flux density of around 500 μmol m^{-2} s^{-1}. Net photosynthesis (A_n) was measured when foliar CO_2 uptake was steady. During the experiment, gas-exchange measurements including CO_2 assimilation rate and stomatal conductance (g_s) were done in the morning between 9:00 am and 12:00 pm.

2.6. Statistics

Data were analyzed by a two-way analysis of variance with SAS software (SAS Institute, Cary, NC). A factorial experimental design with two factors (Genotype and Iron) and two levels of each factor were employed. If *F*-test revealed a significant interaction between factors, statistical comparisons among the 4 possible treatments (2 Genotype × 2 Fe levels) were performed. In these cases, the standard error of the interaction means (SEM) was calculated and the treatments were considered as significantly different when the difference between data were greater than 2× SEM. In the absence of significant interaction between factors, the statistical comparison was performed by the *F*-test ($P \leq 0.05$) between the levels of each independent factor. We adopted such methodological approach to clearly address the main objective of the factorial experiment (Rombolà et al., 2002; Covarrubias and Rombolà, 2013, 2015).

3. Results

3.1. Plant growth, leaf chlorophyll content and pH of the nutrient solution

Main shoot length was significantly influenced by genotype and Fe availability (Fig. 1). From the beginning of the experiment until 18 DAT, plants grafted on 3309-C rootstock reached a higher main shoot as compared with plants grafted on 110 Richter, regardless of Fe level (Fig. 1). Later, at 32 DAT, data showed an interaction between factors; Fe deficiency induced a significant reduction in the length of the main shoot in plants grafted on 110 Richter, whereas such reduction was not observed when grafted on 3309-C genotype (Fig. 1).

At 18 DAT, plants grafted on 3309-C rootstock reached a higher main shoot and leaf dry biomass as compared with plants grafted on 110 Richter, regardless of Fe level (Table 1). As for root biomass, at 18 DAT an interaction between genotype and Fe level occurred (Table 1). Plants grafted on 110 Richter grown in −Fe conditions resulted in a higher root biomass as compared with control plants, whereas in 3309-C root biomass did not change in response to Fe level (Table 1). At the end of the experiment (32 DAT), Fe deficiency reduced the leaf biomass of plants grafted on both rootstocks (Table 1).

Treatments did not modify the leaf chlorophyll content (expressed by SPAD value) at 4 and 18 DAT, whereas at 32 DAT, Fe deficiency reduced the SPAD value in plants grafted on 3309-C, but not on 110 Richter (Fig. 2).

Plants grafted on 110 Richter were more effective in decreasing the nutrient solution pH, under Fe depletion, compared to those grafted on 3309-C (Fig. 3).

3.2. Measurement of leaf gas exchange

Iron deficiency reduced the net photosynthesis in leaves from both rootstocks already at 18 DAT, whereas at 32 DAT an interaction Fe × Genotype was observed (Table 2). Iron deficiency, at 32 DAT, decreased net photosynthesis in leaves of plants grafted on 3309-C, whereas in plants grafted on 110 Richter Fe deficiency did not affect leaf gas exchange (Table 2). During the experiment, nor the genotype neither the Fe level influenced leaf stomatal conductance.

3.3. Enzyme activities in root extracts

At 18 DAT, Fe deficiency increased the root activity of PEPC and $NADP^+$-IDH enzymes in both genotypes (Table 3). In addition, and irrespective of Fe level, the rootstock 3309-C showed a higher PEPC activity in roots compared to the genotype 110 Richter (Table 3).

On the other hand, at 32 DAT, the treatments did not modify the activity of PEPC and organic acid linked enzymes in roots (Table 4).

3.4. Organic acids concentration in roots

During the experiment, the major organic acids present in root extracts were tartaric, followed by citric, malic and ascorbic (Tables 5 and 6). At 18 DAT, differences due to both genotype and Fe level were registered on malic acid, as well as total organic acids concentrations in roots (Table 5). Upon low Fe levels, a higher malic and total organic concentration in root tip extracts occurred in plants grafted on both 3309-C and 110 Richter genotypes. In the former, such changes were higher than in the later (Table 5). Later, at 32 DAT, plants grafted on 3309-C accumulated more tartaric acid in root tips, regardless of Fe level (Table 6).

4. Discussion

Plants of Cabernet Sauvignon grafted on 3309-C and 110 Richter rootstocks were grown under Fe depletion for 32 days. After 18 days under deficiency conditions, a clear effect of the rootstock on the leaf chlorophyll content and growth of the grafted variety was recorded. During such period, Fe-deficient plants grafted on 3309-C presented the typical interveinal leaf yellowing (Fig. 2), though maintaining the same shoot length of control plants (Fig. 1). Conversely, leaf chlorophyll concentration of plants grafted on 110 Richter was not affected by Fe deficiency (Fig. 2). Interestingly, even though plants on 110 Richter presented a reduced shoot length after 18 DAT, a change in the carbon allocation was triggered, inducing a higher root biomass in the Fe-deficient plants (Fig. 1; Table 1). It is not possible to establish whether this is an indirect response resulting by Fe deficiency on the grafted variety, or the result of root specific responses, such as the Fe deficiency induced signaling for ABA synthesis intermediates (Zhao et al., 2014). Several investigations show inter- and intra-specific differences in plants' tolerance to Fe deficiency, particularly on plant genotypes grown in hydroponics, reporting differences in Fe deficiency symptoms such as interveinal yellowing in young leaves and shoot growth reduction. In the Fe deficiency tolerant cv Cabernet Sauvignon, a short (7-days) Fe deficiency period reduced leaf chlorophyll content and the shoot fresh weight, whereas the Fe chlorosis susceptible *V. riparia* Gloire de Montpellier decreased leaf chlorophyll content but not shoot

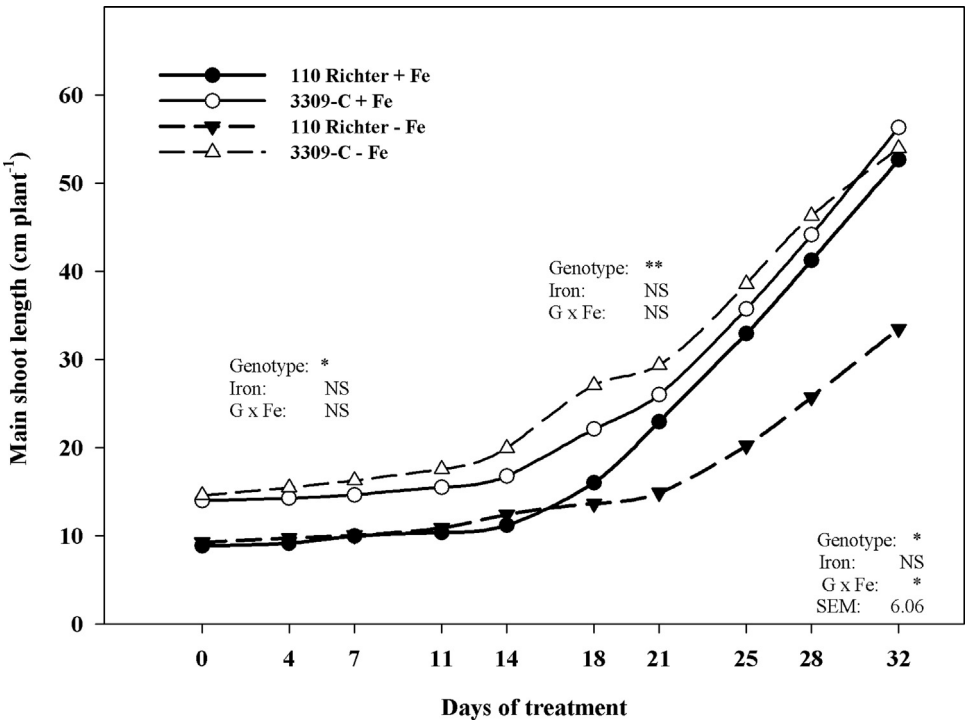

Fig. 1. Shoot length (cm plant^{-1}) of Cabernet Sauvignon grafted on two grapevine genotypes (110R; 3309-C) grown in a nutrient solution containing 0 μM and 50 μM of Fe-EDTA for 32 days.

Table 1
Dry biomass yield of different organ (g DW plant^{-1}) determined at 18 and 32 DAT for Cabernet Sauvignon plants grafted on two grapevine genotypes (110R; 3309-C) grown in a nutrient solution containing 0 μM and 50 μM of Fe-EDTA. Data are means ± SE of five replicates.

Genotype	Iron	Roots		Main shoot		Leaves	
(G)	(Fe)	18 days	32 days	18 days	32 days	18 days	32 days
110 Richter	+Fe	1.4 ± 0.4	3.1 ± 0.7	0.12 ± 0.03	1.5 ± 0.4	0.38 ± 0.07	1.8 ± 0.3
110 Richter	−Fe	2.6 ± 0.4	1.7 ± 0.5	0.32 ± 0.06	0.8 ± 0.2	0.74 ± 0.09	1.1 ± 0.2
3309-C	+Fe	2.3 ± 0.2	1.8 ± 0.3	0.54 ± 0.20	1.4 ± 0.3	1.11 ± 0.28	2.0 ± 0.2
3309-C	−Fe	1.7 ± 0.2	2.3 ± 0.2	0.64 ± 0.22	1.4 ± 0.3	0.94 ± 0.19	1.7 ± 0.2
Statistics							
G treatment		NS	NS	*	NS	*	NS
Fe treatment		NS	NS	NS	NS	NS	*
G × Fe interaction		*	NS	NS	NS	NS	NS
SEM[a]		0.31					

Abbreviation and symbols: NS, * = not significant and significant at $p \leq 0.05$, respectively.

[a] SEM: standard error of the interaction means. Within each column, the treatments are considered as significantly different when the difference between values is higher than 2 × SEM.

Table 2
Net photosynthesis, A_n (μmol CO_2 m^{-2} s^{-1}) and stomatal conductance, g_s (mol H_2O m^{-2} s^{-1}), measured in the first mature leaf of the main shoot. Data were recorded at 18 and 32 DAT for Cabernet Sauvignon plants grafted on two grapevine genotypes (110R; 3309-C) grown in a nutrient solution containing 0 μM and 50 μM of Fe-EDTA. Data are means ± SE of five replicates.

Genotype	Iron	Data recorded at 18 days		Data recorded at 32 days	
(G)	(Fe)	A_n	g_s	A_n	g_s
110 Richter	+Fe	3.5 ± 0.4	0.08 ± 0.009	1.6 ± 0.1	0.07 ± 0.009
110 Richter	−Fe	2.6 ± 0.4	0.07 ± 0.006	1.8 ± 0.3	0.11 ± 0.025
3309-C	+Fe	3.6 ± 0.3	0.07 ± 0.004	1.9 ± 0.2	0.08 ± 0.012
3309-C	−Fe	2.4 ± 0.5	0.06 ± 0.004	0.9 ± 0.2	0.06 ± 0.009
Statistics					
G treatment		NS	NS		NS
Fe treatment		*	NS		NS
G × Fe interaction		NS	NS	*	NS
SEM[a]				0.18	

Abbreviation and symbols: NS, * = not significant and significant at $p \leq 0.05$, respectively.

[a] SEM: standard error of the interaction means. Within each column, the treatments are considered as significantly different when the difference between values is higher than 2 × SEM.

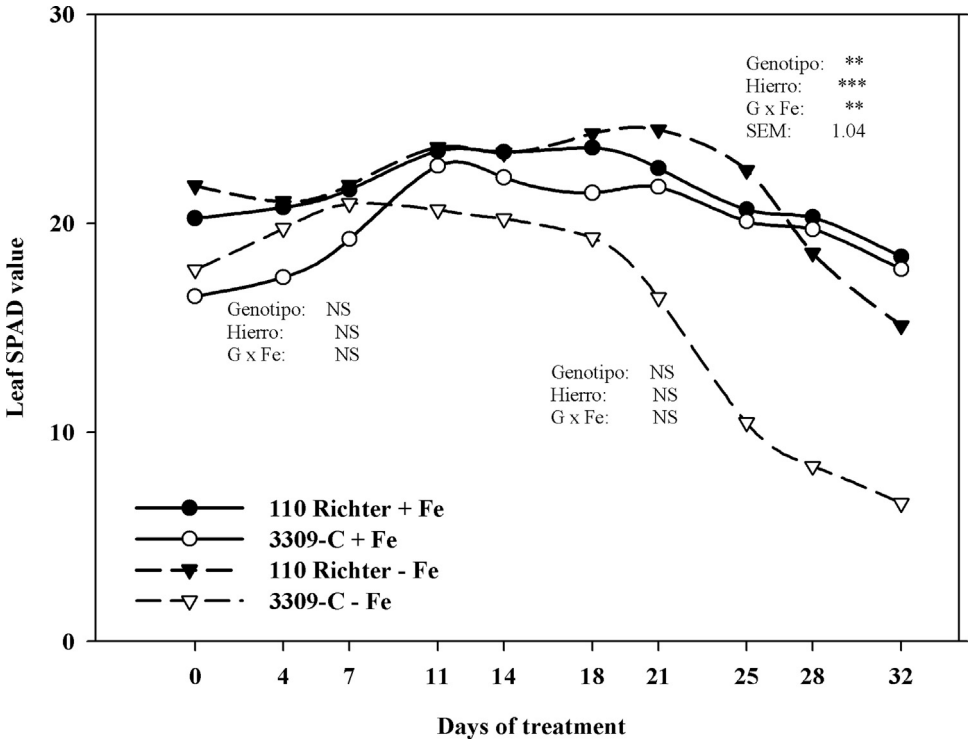

Fig. 2. SPAD index determined in the first expanded leaf along the 32 days of the experiment in Cabernet Sauvignon grafted on two grapevine genotypes (110R; 3309-C) grown in a nutrient solution containing 0 μM and 50 μM of Fe-EDTA for 32 days.

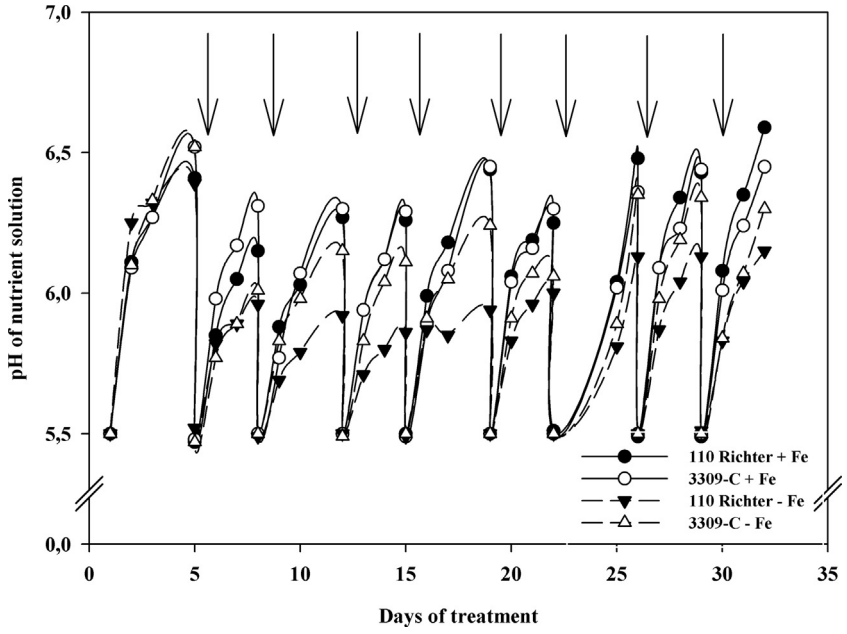

Fig. 3. Changes in the pH of the nutrient solution (10-L containers with 5 plants in each) for Cabernet Sauvignon plants grafted on two grapevine genotypes (110R; 3309-C) grown in a nutrient solution containing 0 μM and 50 μM of Fe-EDTA for 32 days. The nutrient solution was renewed twice a week during the experiment (arrow).

biomass (Jiménez et al., 2007). When cultivated in a calcareous soil, plants of cv Syrah grafted on rootstock SO4, moderately tolerant to Fe chlorosis (Covarrubias and Rombolà, 2015; Tagliavini and Rombolà, 2001), exhibited a severe shoot growth inhibition starting early in the vegetative season (Römheld, 2000). In a pot experiment specifically focused on the appearance of Fe deficiency symptoms, plants of *V. riparia* 1G (highly susceptible to Fe deficiency) and SO4 (moderately tolerant to Fe deficiency) showed severe shoot growth depression, by 50% and higher, before yellowing started or without leaf yellowing in *V. riparia* 1G and SO4, respectively (Gruber and Kosegarten, 2002). Other than affecting chlorophyll synthesis (Terry and Abadia, 1986), Fe deficiency is well known to affect photosynthesis by impairing other components of the photosynthetic machinery (Morales et al., 2000). In fact, contrarily to the Cabernet Sauvignon/3309-C graft combination, suffering a marked decrease in leaf photosynthetic activity as a result of a prolonged (32 DAT) Fe deficiency, plants of Cabernet Sauvignon/110 Richter maintained high levels of photosynthetic capacity regardless of Fe

Table 3
Activities (nmol mg^{-1} root min^{-1}) of PEPC, MDH, CS, $NADP^+$-IDH measured in root extracts determined at 18 DAT for Cabernet Sauvignon plants grafted two grapevine genotypes (110R; 3309-C) grown in a nutrient solution containing 0 μM and 50 μM of Fe-EDTA for 32 days. Data are means ± SE of five replicates.

Genotype (G)	Iron (Fe)	PEPC	MDH	CS	$NADP^+$-IDH
110 Richter	+Fe	2.4 ± 0.52	49.7 ± 9.1	0.42 ± 0.04	2.31 ± 0.17
110 Richter	−Fe	3.6 ± 0.62	49.4 ± 6.1	0.43 ± 0.07	2.96 ± 0.33
3309-C	+Fe	2.9 ± 0.30	43.9 ± 2.7	0.46 ± 0.04	2.55 ± 0.13
3309-C	−Fe	5.8 ± 0.18	59.1 ± 7.8	0.40 ± 0.02	2.71 ± 0.10
Statistics					
G treatment		*	NS	NS	NS
Fe treatment		**	NS	NS	*
G × Fe interaction		NS	NS	NS	NS

Abbreviation and symbols: NS, *, ** = not significant and significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.

Table 4
Activities (nmol mg^{-1} root min^{-1}) of PEPC, MDH, CS, $NADP^+$-IDH measured in root extracts determined at 32 DAT for Cabernet Sauvignon plants grafted on two grapevine genotypes (110R; 3309-C) grown in a nutrient solution containing 0 μM and 50 μM of Fe-EDTA for 32 days. Data are means ± SE of five replicates.

Genotype (G)	Iron (Fe)	PEPC	MDH	CS	$NADP^+$-IDH
110 Richter	+Fe	3.38 ± 0.16	35.9 ± 1.3	0.38 ± 0.03	2.13 ± 0.06
110 Richter	−Fe	3.90 ± 0.54	37.6 ± 8.5	0.32 ± 0.08	2.10 ± 0.24
3309-C	+Fe	3.58 ± 0.52	47.0 ± 6.0	0.42 ± 0.04	2.38 ± 0.20
3309-C	−Fe	3.68 ± 0.46	39.6 ± 2.1	0.38 ± 0.04	1.90 ± 0.19
Statistics					
G treatment		NS	NS	NS	NS
Fe treatment		NS	NS	NS	NS
G × Fe interaction		NS	NS	NS	NS

Abbreviation and symbols: NS = not significant.

Table 5
Organic acid concentrations (mg g^{-1} FW) in root tip extracts determined at 18 DAT for Cabernet Sauvignon plants grafted on two grapevine genotypes (110R; 3309-C) grown in a nutrient solution containing 0 μM and 50 μM of Fe-EDTA for 32 days. Data are means ± SE of five replicates.

Genotype (G)	Iron (Fe)	Citric	Tartaric	Malic	Ascorbic	Total
110 Richter	+Fe	0.56 ± 0.05	2.0 ± 0.11	0.32 ± 0.05	0.08 ± 0.020	2.8 ± 0.3
110 Richter	−Fe	0.52 ± 0.13	2.2 ± 0.07	0.53 ± 0.12	0.08 ± 0.003	3.3 ± 0.2
3309-C	+Fe	0.69 ± 0.13	2.3 ± 0.09	0.63 ± 0.14	0.09 ± 0.010	3.4 ± 0.4
3309-C	−Fe	1.07 ± 0.33	2.3 ± 0.11	1.50 ± 0.30	0.09 ± 0.006	4.7 ± 0.2
Statistics						
G treatment		NS	NS	**	NS	**
Fe treatment		NS	NS	*	NS	**
G × Fe interaction		NS	NS	NS	NS	NS

Abbreviation and symbols: NS, *, ** = not significant and significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.

Table 6
Organic acid concentrations (mg g^{-1} FW) in root tip extracts determined at 32 DAT for Cabernet Sauvignon plants grafted on two grapevine genotypes (110R; 3309-C) grown in a nutrient solution containing 0 μM and 50 μM of Fe-EDTA for 32 days. Data are means ± SE of five replicates.

Genotype (G)	Iron (Fe)	Citric	Tartaric	Malic	Ascorbic	Total
110 Richter	+Fe	0.58 ± 0.15	1.6 ± 0.11	0.24 ± 0.05	0.05 ± 0.007	2.4 ± 0.3
110 Richter	−Fe	0.42 ± 0.07	1.7 ± 0.07	0.30 ± 0.13	0.05 ± 0.007	2.3 ± 0.3
3309-C	+Fe	0.62 ± 0.04	2.1 ± 0.09	0.42 ± 0.08	0.06 ± 0.005	2.9 ± 0.1
3309-C	−Fe	0.77 ± 0.25	1.9 ± 0.15	0.42 ± 0.10	0.05 ± 0.006	2.8 ± 0.6
Statistics						
G treatment		NS	**	NS	NS	NS
Fe treatment		NS	NS	NS	NS	NS
G × Fe interaction		NS	NS	NS	NS	NS

Abbreviation and symbols: NS, ** = not significant and significant at $p \leq 0.01$, respectively.

status (Table 2). In an investigation conducted in grapevines cv Aurora, Fe deficiency reduced the CO_2 assimilation rate in leaves, increasing the transpiration rate (Bavaresco and Poni, 2003). In our study Fe deficiency did not change leaf and stomatal conductance (Table 2) and transpiration rate (data not reported) in none of the two graft combinations. Similarly, previous reports in *B. vulgaris* plants imposed to Fe deficiency, showed that net photosynthesis per leaf area drastically decreased, without a significant effect of Fe regime on leaf transpiration rates and stomatal conductance (Rombolà et al., 2005). Clearly, therefore, the observed reduction of

net photosynthesis in the 3309-C grafted plants is not coupled to a CO_2 limitation of the process but, likely, to a reduced capacity for energy capture as revealed by the concomitant reduction of chlorophyll concentration in this plants. As a result, the instantaneous water use efficiency of Cabernet Sauvignon/3309-C combination is severely reduced. Altogether, the shoot and leaf growth reduction displayed by Cabernet Sauvignon/110 Richter combination may be a convenient strategy to overcome Fe deficiency since it results in the maintenance of high leaf chlorophyll concentration and, consequently, the photosynthesis activity per leaf area. Therefore, it is unlikely that any reduction of the main shoot length in the former genotype was the result of damage; on the contrary, the growth reallocation probably resulted from a physiological response.

In the present work, 6 days after the beginning of treatment, a lower pH was recorded in the nutrient solution of plants grown under Fe deficiency, as compared with the nutrient solution of control plants (Fig. 3). Under Fe depletion plants grafted on 110 Richter decreased the pH of the nutrient solution more effectively than those grafted on 3309-C. The effect was more pronounced between 15 and 20 DAT (Fig. 3). The capability to decrease the pH of the nutrient solution exerted by these genotypes is in line with their different tolerance levels to Fe chlorosis and in agreement with what has been reported in literature (Tagliavini and Rombolà, 2001). In the study of Brancadoro et al. (1995), the Fe deficiency susceptible *V. riparia* did not decrease the pH of the nutrient solution when grown in the absence of Fe, whereas the Fe deficiency tolerant *V. vinifera* (cv Cabernet Sauvignon and Pinot blanc) and *V. berlandieri* showed to be efficient in triggering such response mechanism. In agreement with such findings, the rootstock 3309-C originated from *V. riparia* showed a low capability to decrease the pH in the rhizosphere, as already found for other hybrids from *V. riparia*. Conversely, 110 Richter is an hybrid originated from the Fe chlorosis tolerant *V. berlandieri* and the Fe chlorosis slightly susceptible *V. rupestris*. Other hybrids from *V. berlandieri* and *V. rupestris*, such as the rootstock 140 Ruggeri, have been described as efficient in reducing the pH of the nutrient solution under Fe deficiency conditions (Covarrubias and Rombolà, 2013; Ksouri et al., 2005). Along with the effect of the nutrient solution pH, the genotypes showed a significant reaction to Fe deficiency increasing the concentration of malate and total organic acids in root tips at 18 DAT (Table 5) and the activity of PEPC and $NADP^+$-IDH enzymes (Table 3), involved in the synthesis of organic acids, without significant differences between them. However, is important to highlight that 3309-C grafted plants accumulated a higher organic acids concentration in roots as compared with 110 Richter grafted plants, independently of the Fe level. In a previous experiment conducted in hydroponic conditions, Covarrubias and Rombolà (2015) reported a strong accumulation of citric acid and, to a relatively lower extent, malic acid in roots of 101-14 vines submitted to a severe Fe deficiency. The rootstock 101-14 employed in that experiment, exhibited a similar behavior to plants grafted on 3309-C assessed in the present experiment. As previously proposed (Covarrubias and Rombolà, 2015) the accumulation of organic acids in roots of Fe chlorosis susceptible genotypes under Fe deficiency may be the result of a decreased organic acids exudation rate. This could be a common trait of rootstocks 101-14 and 3309-C, sharing the same genetic origin (*V. riparia* × *V. rupestris).* Conversely, the lower accumulation of malic and total organic acids recorded in roots of 110 Richter (Table 5) suggests a higher capability of this rootstock to exudate into the rhizosphere or load in the xylem the organic acids. Noteworthy, in *Medicago ciliaris*, the Fe deficiency tolerant genotype TN11.11 was more efficient in reducing the pH in the Fe depleted nutrient solution, by increasing the root H^+-ATPase activity and exudation of citric and malic acids to the rhizosphere, as compared with the susceptible genotype TN8.7 (M'sehli et al., 2008). In addition, the Fe deficiency tolerant genotype showed a higher root biomass when it was subjected to Fe deficiency (M'sehli et al., 2008). Therefore, the higher capability of 110 Richter rootstock to decrease the pH of the nutrient solution under Fe deficiency may be also related to the higher root biomass and surface for H^+ and organic acids extrusion.

Consequently, under the experimental conditions employed in this experiment, is possible to conclude that in a first step under Fe depletion in the nutrient solution, both grapevine variety/rootstock combinations react to Fe deficiency increasing the synthesis and accumulation of organic acids in roots. In a subsequent step, Fe-depleted genotypes diminish the synthesis and/or accumulation of organic acids in root tips, and begin to show Fe deficiency symptoms in leaves and shoots, at different degree according to their Fe chlorosis tolerance degree. During the latter Fe deficiency stage, the rootstock 110 Richter (Fe chlorosis moderately tolerant) induces in the grafted variety a reduction in shoot growth rate, maintaining the leaf chlorophyll content and photosynthesis rate in leaves, whereas 3309-C (Fe chlorosis susceptible) induces a reduction in leaf chlorophyll content and photosyhthesis, without reducing the shoot growth rate in the grafted variety. Plants grafted on 110 Richter exhibited a relatively higher ability to decrease the nutrient solution pH under Fe depletion.

Acknowledgment

This study has been supported by the Comisión Nacional de Investigación Científica y Tecnológica (CONICYT) of Chile (project "Desarrollo del Área de Nutrición Vegetal en el Departamento de Producción Agrícola de la Facultad de Ciencias Agronómicas de la Universidad de Chile" – PAI – No. 7912010003).

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