Plant Soil (2015) 394:165–175
DOI 10.1007/s11104-015-2530-5

REGULAR ARTICLE

Organic acids metabolism in roots of grapevine rootstocks under severe iron deficiency

José Ignacio Covarrubias · Adamo Domenico Rombolà

Received: 25 March 2015 / Accepted: 19 May 2015 / Published online: 26 May 2015

Abstract
Background and aims In many important viticultural areas of the Mediterranean basin, plants often face prolonged periods of scarce iron (Fe) availability in the soil. The objective of the present work was to perform a comparative analysis of physiological and biochemical responses of *Vitis* genotypes to severe Fe deficiency.
Methods Three grapevine rootstocks differing in susceptibility to Fe chlorosis were grown with and without Fe in the nutrient solution.
Results 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), however, it accumulated high levels of citric acid. By contrast, rootstock 110 Richter, tolerant to Fe chlorosis, maintained an active metabolism of organic acids, but citric acid accumulation was lower than in 101-14. Similarly to 101-14, rootstock SO4 showed a strong decrease in PEPC and MDH activities. Nevertheless it maintained moderate citric acid levels in the roots, mimicking the response by 110 Richter.
Conclusions Root PEPC and MDH activities can be used as tools for screening Fe chlorosis tolerance. Conversely, organic acids accumulation in roots may not be a reliable indicator of Fe chlorosis tolerance, particularly under conditions of severe Fe deficiency, because of their probable exudation by roots. Our results show that drawing sound conclusions from screening programs involving Fe deficiency tolerance requires short as well as long-term assessment of responses to Fe deprivation.

Keywords Iron chlorosis · Phosphoenolpyruvate carboxylase · Organic acids · Enzyme activity · Grapevine genotypes

Abbreviations

BSA	Bovine serum albumin
CoA	Coenzyme A
CS	Citrate synthase
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
FW	Fresh weight
MDH	Malate dehydrogenase
NADP+-IDH	Isocitrate dehydrogenase
PEPC	Phosphoenolpyruvate carboxylase
TCA	Tricarboxylic acid

Introduction

Iron (Fe) is the fourth most abundant element in the Earth's crust. It is an essential microelement in all plants

Responsible Editor: Yong Chao Liang.

J. I. Covarrubias
Facultad de Ciencias Agronómicas, Universidad de Chile, Av. Santa Rosa, 11315 Santiago, Chile

A. D. Rombolà (✉)
Department of Agricultural Sciences, University of Bologna, Viale G. Fanin 44, 40127 Bologna, Italy
e-mail: adamo.rombola@unibo.it

including woody crops with relatively low Fe requirement (50–100 mg Fe kg^{-1} dry weight) (Tagliavini and Rombolà 2001). However, its deficiency represents an important nutritional disorder in susceptible fruit tree crops grown in alkaline soils with high levels of calcium carbonates and bicarbonates (>5 mM) (Nikolic et al. 2000). Several fruit tree crops (e.g. grapevine, avocado, *Citrus,* kiwifruit, peach, pear, *Vaccinium spp*.) grown under such soil conditions are known to develop symptoms of Fe deficiency. Typical Fe deficiency involves the interveinal yellowing of apical leaves (Fe chlorosis) accompanied by a reduction in the growth rate of shoots and roots (Rombolà and Tagliavini 2006). Among the aforementioned fruit crops, grapevine (*Vitis vinifera* L.) is a representative crop of great economic importance worldwide grown mostly on rootstocks of American *Vitis spp*. due to sanitary threats. However, in established vineyards with calcareous soils, Fe deficiency is one of the main nutritional disorders in grafted grapevines. The iron deficiency results in reduced yield (Bavaresco et al. 2003) and changes in berry composition (Bavaresco et al. 2010). To overcome this problem, Fe deficiency tolerant rootstocks have long been used, whereas other sustainable strategies for improving grapevine Fe nutrition, such as intercropping with soil Fe-solubilizing graminaceous species (Bavaresco et al. 2010; Covarrubias et al. 2014) and applying Fe-containing animal blood-based fertilizers, have recently been proposed as alternatives to expensive and environmentally risky synthetic Fe chelates (Yunta et al. 2013; López-Rayo et al. 2014).

It has been well documented that plant species display a high variation in susceptibility to Fe deficiency (Tagliavini and Rombolà 2001). Such differences are mostly due to the plant's ability to solubilize Fe in the rhizosphere for absorption and subsequent transport to the aerial organs. Studies dealing with annual species (De Nisi and Zocchi 2000; López-Millán et al. 2000; Jelali et al. 2010) and fruit tree crops (Nikolic et al. 2000; Rombolà et al. 2002; Donnini et al. 2009; Covarrubias and Rombolà 2013) have characterized the principal mechanisms of both Fe acquisition and the physiological responses to Fe deficiency. For instance, dicotyledonous and non-graminaceous monocotyledonous plants (Strategy I plants for Fe absorption) take up Fe from the soil as Fe^{+2}, which in sub-alkaline and alkaline soils is oxidized to Fe^{+3}, causing low levels of available Fe for plant uptake (Römheld and Marschner 1986; Kim and Guerinot 2007). However, under these conditions the Fe deficiency-tolerant species can reduce the pH in the rhizosphere by extruding protons through the root plasma-membrane ATPase enzyme activity, thus increasing the solubility of Fe^{3+} (Kim and Guerinot 2007). Other Fe deficiency responses generally exerted by Fe deficiency-tolerant Strategy I plants involve increases in root ferric chelate reductase (FCR) activity that directly reduces the Fe^{3+} located in the rhizosphere to the more soluble Fe^{2+}. In addition, phenolic compounds and organic acids are frequently reported to be the main components of root exudates in response to Fe deficiency in Strategy I plants (Cesco et al. 2010). Therefore, the importance of the reducing and complexing properties of phenolic compounds is widely accepted (Cesco et al. 2010).

Experiments conducted with *Vitis spp.* under controlled conditions (hydroponics) (Brancadoro et al. 1995; Ollat et al. 2003; Jimenez et al. 2007; Covarrubias and Rombolà 2013) have suggested that some genotypes, especially *Vitis vinifera* and *Vitis berlandieri* hybrids, are more responsive to Fe deficiency because they trigger physiological responses related to reductions in rhizosphere pH and the synthesis/accumulation of organic acids in roots. On the other hand, experiments with model plants have shown that the main organic acids of Fe deficiency tolerant genotypes subjected to Fe depletion are citrate and malate, and to a lesser extent, succinate, quinate, *cis*-aconitate, fumarate, 2-oxoglutarate, oxalate and ascorbate (Brancadoro et al. 1995; López-Millán et al. 2000, 2009; Rombolà et al. 2002; Ollat et al. 2003; Jimenez et al. 2007; Covarrubias and Rombolà 2013). However, an increase in tartrate has been reported in some grapevine Fe deficiency-tolerant genotypes (e.g. 140 Ruggeri rootstock and Cabernet Sauvignon) exposed to Fe depletion (Ollat et al. 2003; Covarrubias and Rombolà 2013). Such organic acids accumulation in roots originates from the increased activities of organic acids synthetizing enzymes, and/or the reduction in the activity of enzymes that degrade/convert them (Covarrubias and Rombolà 2013). For instance, in Cabernet Sauvignon (Ollat et al. 2003; Jimenez et al. 2007) and 140 Ruggeri (Covarrubias and Rombolà 2013), an increase in the activity of the key enzyme PEPC occurs in roots as a response to Fe deficiency. Similar patterns have been observed in the Fe deficiency-tolerant genotypes of *Cucumis sativus* L. (De Nisi and Zocchi 2000), *Pisum sativum* (Jelali et al. 2010), *Beta vulgaris* L. (López-Millán et al. 2000), *Actinidia deliciosa*

(Rombolà et al. 2002), *Pyrus communis* (Donnini et al. 2009). Moreover, in *Cucumis sativus* L., the absence of Fe in the nutrient solution induced the expression of *Cspepc1* transcripts in roots, corresponding to an increase in the enzyme activity (De Nisi et al. 2010). In addition, an increase in the tricarboxylic acid cycle (TCA) related enzymes such as citrate synthase (CS), malate dehydrogenase (MDH) and isocitrate dehydrogenase ($NADP^+$-IDH) has been observed in the roots of Fe deficiency tolerant-genotypes of several species subjected to Fe depletion. Citrate synthase, an enzyme located exclusively in the mitochondria, catalyzes the formation of citrate from oxalacetate and acetyl coenzyme A. The increase in this enzyme in roots as a response to Fe deficiency has been reported in some model plants and in grapevines genotypes, e.g. *Vitis riparia* Gloire de Montpellier (Jimenez et al. 2007) and 140 Ruggeri rootstock (Covarrubias and Rombolà 2013), whereas in *Pisum sativum* this effect was observed in leaves as well as roots (Jelali et al. 2010). Malate dehydrogenase in the cytosol promotes the formation of malate but catalyzes its degradation in the mitochondria, favoring the formation of oxaloacetate. NADP-dependent isocitrate dehydrogenase produces 2-oxoglutarate through the oxidative decarboxylation of isocitrate (Foyer et al. 2011). This enzyme is located in several cell compartments, mainly in the cytosol and mitochondria. Similar to PEPC and CS, MDH and $NADP^+$-IDH have been reported as enzymes responding to Fe deficiency in the root tissues of *Beta vulgaris* L. (López-Millán et al. 2000), *Pisum sativum* (Jelali et al. 2010), *Lycopersicon esculentum* L. (López-Millán et al. 2009), *Vitis riparia* Gloire de Montpellier (Jimenez et al. 2007) and 140 Ruggeri grapevine rootstocks (Covarrubias and Rombolà 2013).

Under field conditions, grapevines frequently encounter prolonged periods of Fe scarcity. Only Fe deficiency-tolerant genotypes are able to overcome this constraint, thereby avoiding the detrimental effects on vegetative and reproductive growth. Most studies into Fe deficiency tolerant genotypes examined the short-term (2 weeks) biochemical response mechanisms to Fe-shortage. Not much is known as to how these species respond to an extended period of Fe deficiency. The main objective of the present work was to compare physiological and biochemical response mechanisms to a severe Fe-deficiency in *Vitis* genotypes with varying degrees of tolerance to Fe chlorosis. The study was conducted with three rootstocks varying in susceptibility to Fe chlorosis and subjected to two levels of Fe in nutrient solution.

Materials and methods

Plant material, growth conditions and treatments

Micropropagated plants of rootstocks 101-14 (*Vitis riparia* x *Vitis rupestris*), 110R (*Vitis berlandieri* x *Vitis rupestris*) and SO4 (*Vitis berlandieri* x *Vitis riparia*) were acclimated in peat for 1 month and pruned to maintain one main shoot on each plant. The plants (6 per container, a total of 36) were transferred to a greenhouse in 10 L plastic containers covered with aluminum foil and filled with 8 L of a half Hoagland nutrient solution, which was continuously aerated. 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.

The three grapevine genotypes were grown with Fe (+Fe; 10 μmol/L of Fe-EDDHA) and without Fe (−Fe). The composition of the half Hoagland nutrient solution was: 2.5 mM KNO_3; 1 mM $MgSO_4$; 1 mM KH_2PO_4; 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 nutrient solution was renewed twice a week, the pH was monitored daily at 9:00 am and adjusted to 6.0 with HCl 0.1 M. The experiment was concluded 32 days after imposing the treatments when apical leaves of Fe deficient plants displayed extremely severe yellowing.

Plant growth and leaf chlorophyll content

Leaf chlorophyll content was periodically monitored during the experiment on five points of the first completely expanded leaf with the portable chlorophyll meter SPAD MINOLTA 502 (Konica Minolta, Inc., Osaka, Japan). At time 0, the leaf SPAD value was 14.2 in 101.14, 14.3 in 110 Richter and 14.2 in SO4. On day 32, plants were divided into roots, main shoot and leaves to determine dry weight and the following analyses were performed on the fresh root samples.

Enzyme assays and protein concentration in roots

At the end of the experiment, root tip (20–30 mm long) samples (100 mg FW) 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 activities of phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MDH), citrate synthase (CS), and isocitrate dehydrogenase ($NADP^+$-IDH) were determined. The root extraction was performed as described by Jimenez 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 by monitoring the reduction of $NADP^+$ at 340 nm as described by Goldberg and Ellis (1974). Protein concentration was determined by the Bradford method, using bovine serum albumin (BSA) as the standard (Bradford 1976). Data obtained from the enzyme assays were referred to protein concentration of roots (nmol mg^{-1} protein min^{-1}).

Determination of kinetic properties of PEPC

Kinetic analysis was performed by varying each time the HCO_3^- concentration, buffering the pH at 8.1 (Vance et al. 1983). The substrate dependence of PEPC on HCO_3^- concentration was characterized by determining the PEPC activity with different concentrations of HCO_3^- in 9 points in a range from 0 to 10 mM. Decarbonated water was used for the determination of HCO_3^- kinetics. V_{max} and *Km* values were calculated using Eadie-Hofstee plots.

Organic acids concentration in roots

The organic acid concentrations were 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. After liquid nitrogen evaporation, the tissue was homogenized with a pestle. For extraction and deproteinization, 5 % H_3PO_4 was utilized. Organic acids were quantified as described by Neumann (2006) using high-performance liquid chromatography (HPLC) with a 250×4 mm LiChrospher 5 μm RP-18 column (Supelco Inc., PA 16823-0048 USA). High-performance liquid chromatography elution buffer was 18 mmol/L 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, two organic acids were identified and quantified (citrate and malate).

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 three levels for genotype and two levels of iron was used. If the F-test revealed a significant interaction between factors, then statistical comparisons were performed among the 6 possible treatments (3 genotypes x 2 Fe levels). 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 was greater than 2 x 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 this methodological approach to address the main objective of the factorial experiment more clearly (Covarrubias and Rombolà 2013; Rombolà et al. 2002).

Results

Plant growth and chlorophyll content

Shoot length of vines was influenced by Fe and genotype (Table 1). Until 21 days from treatments imposition, all genotypes displayed differences in shoot length independently of the Fe level. In the presence of Fe, 101-14 showed a higher shoot length than the other rootstocks (Table 1). Starting from 14 days after treatment imposition, Fe deficiency significantly decreased shoot length irrespective of the genotype (Table 1). At the end of the experiment, data showed an interaction between factors. Iron deficiency decreased shoot length by 80 % in the 101-14 rootstock, 71 % in SO4 and 51 % in 110 Richter.

Following the imposition of treatments, Fe deficiency decreased leaf chlorophyll content regardless of genotype until 14 days (Table 2). Thereafter, an interaction between Fe level and genotype was detected (Table 2). At the end of the experiment, Fe deficiency decreased

Table 1 Time course of main shoot length (cm plant^{-1}) determined during the experiment for three grapevine genotypes (110 Richter; SO4; 101-14) grown in a nutrient solution containing 0 μM and 10 μM of Fe-EDDHA. Data are means±SE of six replicates

Genotype	Iron	Time course of main shoot length (cm plant^{-1}) Days of treatments						
(G)	(Fe)	7	11	14	18	21	26	29
110 Richter	+Fe	7.9±0.4	9.5±0.9	11.3±1.6	15.0±3.0	18.4±4.4	24.5±6.5	29.4±7.7
110 Richter	− Fe	7.8±0.2	8.7±0.2	9.6±0.5	11.1±0.8	12.0±1.0	13.6±1.3	14.3±1.5
SO4	+Fe	6.9±0.4	8.2±0.5	10.0±0.8	13.8±1.8	18.4±3.6	30.6±8.1	38.1±10.8
SO4	− Fe	6.8±0.4	8.2±0.7	9.2±0.7	9.9±1.0	10.2±1.1	10.7±1.2	10.9±1.2
101-14	+Fe	9.8±0.6	12.5±1.1	15.9±1.5	27.9±6.2	32.2±3.2	54.8±4.1	67.5±4.8
101-14	− Fe	9.9±0.5	11.2±0.4	11.9±0.5	12.7±0.6	13.2±0.5	13.5±0.6	13.8±0.5
Statistics								
G treatment		***	***	***	*	**		
Fe treatment		*NS*	*NS*	*	**	***		
G x Fe interaction		*NS*	*NS*	*NS*	*NS*	*NS*	*	**
SEM [b]							4.63	5.80

[a] Abbreviation and symbols: *NS, *, **, ****=not significant and significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ levels, respectively

[b] *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 x SEM

the chlorophyll content by 99.6 % in 101-14.92 % in SO4 and 72 % in 110 Richter.

Interactions between genotype and Fe level were also observed for organ biomass (Table 3). Iron deficiency

Table 2 Time course of chlorophyll content (SPAD value) determined during the experiment in the first expanded apical leaf for three grapevine genotypes (110 Richter; SO4; 101-14) grown in a nutrient solution containing 0 μM and 10 μM of Fe-EDDHA. Data are means±SE of six replicates

Genotype	Iron	Time course of leaf chlorophyll content (SPAD value) Days of treatments						
(G)	(Fe)	7	11	14	18	21	26	29
110 Richter	+Fe	15.4±0.9	17.6±1.0	18.0±0.9	19.1±1.1	21.2±0.8	22.4±1.1	21.9±1.1
110 Richter	− Fe	14.4±1.0	15.1±0.9	14.6±1.5	11.6±1.7	7.6±1.2	6.5±2.9	6.1±2.8
SO4	+Fe	18.8±1.1	20.1±1.8	20.1±1.4	22.0±1.6	22.7±1.7	22.6±2.2	21.3±2.0
SO4	− Fe	12.9±1.3	15.1±1.3	11.2±0.9	4.5±0.8	3.0±0.7	1.8±0.8	1.7±0.8
101-14	+Fe	16.2±1.0	19.5±1.3	20.4±1.3	23.9±0.5	24.8±0.6	25.1±0.7	26.3±0.9
101-14	− Fe	13.1±0.7	14.1±0.9	13.3±1.0	6.5±0.6	0.9±0.5	0.4±0.2	0.2±0.0
Statistics								
G treatment		*NS*	*NS*	*NS*				
Fe treatment		**	**	***				
G x Fe interaction		*NS*	*NS*	*NS*	***	***	*	**
SEM [b]					1.14	1.02	1.60	1.55

[a] Abbreviation and symbols: *NS, *, **, ****=not significant and significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ levels, respectively

[b] *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 x *SEM*

Table 3 Organ biomass (g DW plant^{-1}) determined at the end of experiment for three grapevine genotypes (110 Richter; SO4; 101-14) grown in a nutrient solution containing 0 μM and 10 μM of Fe-EDDHA. Data are means±SE of six replicates

Genotype	Iron	Organ biomass (g DW plant^{-1})			
(G)	(Fe)	Roots	Shoot	Leaves	Total
110 Richter	+Fe	0.44±0.07	0.30±0.11	0.45±0.13	1.19±0.30
110 Richter	− Fe	0.31±0.03	0.09±0.02	0.21±0.04	0.61±0.07
SO4	+Fe	0.50±0.14	0.65±0.23	0.92±0.29	2.07±0.65
SO4	− Fe	0.34±0.06	0.16±0.03	0.30±0.02	0.80±0.11
101-14	+Fe	0.98±0.10	1.78±0.23	2.03±0.23	4.79±0.55
101-14	− Fe	0.37±0.04	0.20±0.03	0.46±0.05	1.03±0.06
Statistics					
G treatment					
Fe treatment					
G x Fe interaction		**	***	**	**
SEM [c]		0.08	0.14	0.16	0.37

[a] Abbreviation and symbols: **, ***=significant at $p \leq 0.01$ and $p \leq 0.001$ levels, respectively

[b] *SEM* standard error of the interaction means. Within each column, the treatments are considered as significantly different when the difference between values ise higher than 2 x *SEM*

decreased the biomass of roots, shoots, leaves and total weight of the plants. The highest decrease occurred in the 101-14 genotype and the lowest in 110 Richter (Table 3). For the SO4 rootstock, the effect of Fe deficiency on dry biomass was intermediate (Table 3). At the end of the experiment, Fe deprivation reduced the total biomass by 78 % in 101-14, 62 % in SO4 and 48 % in 110 Richter (Table 3).

Enzyme activities and protein concentration in root extracts

At the end of the experiment, the activities of PEPC and enzymes linked to the organic acid metabolism were determined in the root tip extracts (100 mg FW). In the roots of the 101-14 and SO4 genotypes, Fe deficiency decreased the PEPC activity by 68 % and 81 %, respectively (Table 4), whereas Fe deficiency did not change PEPC activity in 110 Richter plants (Table 4). Iron deficiency induced a decrease in the root activity of MDH in 101-14 (36 %) and SO4 (46 %) (Table 4), whereas no differences were recorded for 110 Richter (Table 4). The root activity of $NADP^+$-IDH differed among rootstocks, regardless of Fe level (Table 4). Citrate synthase activity and protein concentration in roots were not influenced by the treatments (Table 4).

Determination of kinetic properties of PEPC

The saturation kinetics curves of PEPC were established by adding different concentrations of bicarbonate to the buffer assay in a range of 0 to 10 mM. In 101-14 and SO4 roots, Fe deficiency decreased the V_{max} of PEPC activity by 46 % and 62 %, respectively, compared to Fe-sufficient plants (Table 5). In contrast, Fe deficiency did not modify the V_{max}in roots of 110 Richter (Table 5). *Km* was not altered by the treatments (Table 5).

Organic acids concentration in roots

At the end of the experiment, the major organic acids present in the root extracts were malic and citric (Table 6). Significant interactions between Fe level and genotype were recorded. Iron deficiency increased citric acid concentration in the roots of all three genotypes. The highest increase in citric acid was recorded in the 101-14 genotype (27-fold) (Table 6). In the roots of 101-14, Fe-deficiency induced an increase in malic acid concentration by 54 %. By contrast, in 110 Richter and SO4 Fe deficiency decreased the concentration of malic acid by 35 % and 27 %, respectively (Table 6). Iron deficiency enhanced total organic acids concentration in

Table 4 Activities (nmol mg^{-1} protein min^{-1}) of PEPC, MDH, CS, $NADP^+$-IDH and protein concentration (mg g^{-1} FW) measured in root tip extracts for three grapevine genotypes (110 Richter; SO4; 101-14) grown in a nutrient solution containing 0 μM and 10 μM of Fe-EDDHA. Data are means±SE of six replicates

Genotype	Iron	Enzyme activities (nmol mg^{-1} protein min^{-1})				Protein concentration
(G)	(Fe)	PEPC	MDH	CS	$NADP^+$-IDH	(mg g^{-1} FW)
110 Richter	+Fe	3.0±0.6	252.5±32.2	5.7±1.0	0.5±0.2	37.0±4.0
110 Richter	− Fe	3.5±0.4	283.5±15.0	6.6±0.3	0.9±0.1	37.5±1.7
SO4	+Fe	9.8±2.4	373.2±70.5	7.1±1.3	1.3±0.2	35.1±2.4
SO4	− Fe	1.9±0.4	199.7±18.0	6.7±0.3	2.1±0.2	40.8±3.2
101-14	+Fe	10.6±1.4	367.4±40.0	7.3±0.5	3.6±0.5	43.2±2.9
101-14	− Fe	3.4±1.0	233.7±36.9	7.1±0.8	4.4±0.8	36.2±3.0
Statistics						
G treatment				*NS*	***	*NS*
Fe treatment				*NS*	*NS*	*NS*
G x Fe interaction		*	**	*NS*	*NS*	*NS*
SEM [c]		1.15	37.5			

[a] Abbreviation and symbols: *NS, *, **, ***=not significant and significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ levels, respectively

[b] *SEM* standard error of the interaction means. Within each column, the treatments are considered as significantly different when the difference between values ise higher than 2 x *SEM*

Table 5 Kinetic parameters *Km* (mM of $NaHCO_3^-$) and V_{max} (nmol mg^{-1} protein min^{-1}) of phosphoenolpyruvate carboxylase activity (PEPC) in extracts for three grapevine genotypes (110 Richter; SO4; 101-14) grown in a nutrient solution containing 0 μM and 10 μM of Fe-EDDHA. Data are means±SE of three replicates

Genotype (G)	Iron (Fe)	*Km* (mM of $NaHCO_3^-$)	V_{max} (nmol mg^{-1} protein min^{-1})
110 Richter	+Fe	0.05±0.02	3.5±0.3
110 Richter	− Fe	0.04±0.02	3.7±0.2
SO4	+Fe	0.04±0.01	8.7±2.2
SO4	− Fe	0.05±0.02	3.3±0.4
101-14	+Fe	0.03±0.01	8.1±0.8
101-14	− Fe	0.02±0.01	4.4±0.3
Statistics			
G treatment		NS	
Fe treatment		NS	
G x Fe interaction		NS	*
SEM [b]			0.98

[a] Abbreviation and symbols: *NS, *=not significant and significant at $p \leq 0.05$ level

[b] *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 x *SEM*

Table 6 Organic acids in root tissue (mg g^{-1} FW) determined at the end of experiment for three grapevine genotypes (110 Richter; SO4; 101-14) grown in a nutrient solution containing 0 μM and 10 μM of Fe-EDDHA. Data are means±SE of six replicates

Genotype (G)	Iron (Fe)	Citrate (mg g^{-1} FW)	Malate (mg g^{-1} FW)	Total (mg g^{-1} FW)
110 Richter	+Fe	0.07±0.03	0.60±0.09	0.67±0.11
110 Richter	− Fe	0.35±0.01	0.39±0.06	0.74±0.05
SO4	+Fe	0.21±0.03	0.60±0.10	0.81±0.11
SO4	− Fe	0.47±0.08	0.44±0.17	0.91±0.16
101-14	+Fe	0.03±0.01	0.61±0.07	0.64±0.08
101-14	− Fe	0.81±0.06	0.94±0.14	1.75±0.16
Statistics				
G treatment				
Fe treatment				
G x Fe interaction		***	*	***
SEM [c]		0.045	0.110	0.114

[a] Abbreviation and symbols: *, ***=significant at $p \leq 0.05$ and $p \leq 0.001$ levels, respectively

[b] *SEM* standard error of the interaction means. Within each column, the treatments are considered as significantly different when the difference between values ise higher than 2 x *SEM*

roots of 101-14 2.7-fold. Similar values were recorded in 110 Richter and SO4 rootstocks (Table 6).

Discussion

Data on shoot length and leaf chlorophyll content (SPAD value) clearly indicated that a 14-day period of Fe-depletion did not discriminate among these genotypes (Tables 1 and 2). However, when plants were subjected to a prolonged period of Fe deficiency (at the end of the experiment), the lowest reductions in shoot length and SPAD value respect to the control occurred in the 110 Richter rootstock. In contrast, the highest reduction in these parameters compared to the control plants was exhibited by 101-14. Intermediate values were recorded in the SO4 rootstock. Although the prolonged Fe deprivation period resulted in severe Fe deficiency symptoms even in the Fe chlorosis-tolerant genotype (110 Richter), possibly due to our experimental conditions (young, small, micropropagated plants), data regarding shoot length and leaf chlorophyll content suggested a higher tolerance to a severe Fe deficiency by 110 Richter than by the 101-14 and SO4 rootstocks. The degree of Fe chlorosis severity shown by genotypes is consistent with grapevine tolerance levels to Fe chlorosis reported in the literature (Tagliavini and Rombolà 2001), and this stems from the fact that these species are of hybrid origin (Tagliavini and Rombolà 2001; Ollat et al. 2003; Jimenez et al. 2007; Covarrubias and Rombolà 2013). The tolerance level of the originating species may also explain the intermediate Fe chlorosis symptoms exhibited by SO4, a hybrid from *Vitis berlandieri x Vitis riparia*.

The biomass data indicated a significantly higher growth rate in Fe-sufficient 101-14 than in the other genotypes grown in the presence of Fe, and this effect exacerbated the differences between+Fe and -Fe for the 101-14 rootstock (Table 3). The higher Fe deficiency tolerance of SO4 and 110 Richter was probably related to their slower growth rate and, for this reason they required less Fe, withstanding Fe deprivation for a longer period.

An increase in root PEPC and MDH activity induced by Fe deficiency has been reported for several species including grapevine (Covarrubias and Rombolà 2013; Covarrubias et al. 2014), and is considered one of the main responses to Fe deficiency in root tissues (López-Millán et al. 2000; Zocchi 2006; Rombolà and Tagliavini 2006). Moreover, PEPC activityin roots has been proposed as a biochemical marker for Fe deficiency status in Fe chlorosis tolerant species (Rombolà et al. 2002; Ollat et al. 2003; Rombolà and Tagliavini 2006; Jimenez et al. 2007). The enzyme PEPC catalyzes the production of oxaloacetate (a C4 organic acid) from phosphoenolpyruvate (C3) and bicarbonate. Oxaloacetate generated by PEPC catabolism in the cytosol compartment is converted to malate by MDH (Lance and Rustin 1984). This process is an important component of organic acid synthesis and the pH-stat mechanism inside the cell. The interactions recorded in root PEPC and MDH activities indicated genotype differences in the responses to prolonged Fe deficiency. Severe Fe deficiency did not modify the activity of PEPC and MDH in the 110 Richter rootstock, whereas in 101-14 and SO4 Fe deficiency decreased the activity of these enzymes (Table 4). In addition, Fe deficiency did not modify the V_{max} of PEPC in the 110 Richter roots, whereas the 101-14 and SO4 rootstocks, subjected to Fe depletion, showed a decrease in V_{max} compared to Fe sufficient plants (Table 5). Contrasting results were reported for the Fe chlorosis-tolerant grapevine rootstock 140 Ruggeri, in which enhancement by Fe deficiency on PEPC V_{max} without changes in *Km* suggested a possible increase in the PEPC concentration in roots (Covarrubias and Rombolà 2013). In grapevine plants subjected to a short period of Fe depletion (7 days), a 2.9-fold increase in root PEPC activity was recorded in the Fe chlorosis-tolerant genotype Cabernet Sauvignon, whereas a lower increase (2.2-fold) was observed in the sensitive cv Gloire de Montpellier (Jimenez et al. 2007). The lower activity of PEPC and MDH recorded in the 101-14 and SO4 rootstocks subjected to severe Fe starvation conditions may reflect the scarce availability of substrate in roots of plants with a strong reduction in photosynthetic activity (see SPAD values in Table 2) and the general metabolic reprogramming associated with protein turnover caused by Fe deficiency. In *Cucumis sativus* roots subjected to Fe deficiency, Donnini et al. (2010) reported an increase in the glycolytic flux, in the anaerobic metabolism and in enzymes linked to the protein turnover, and observed a decrease in the amount of enzymes linked to the biosynthesis of complex carbohydrates of the cell wall. In *Medicago trunculata* roots, Fe deficiency induced an accumulation of proteins related to nitrogen recycling and protein catabolism, and an increase in glycolysis, tricarboxylic

acid (TCA) cycle, and stress-related processes (Rodríguez-Celma et al. 2011). The activity of PEPC and MDH recorded in the Fe chlorosis-tolerant 110 Richter rootstock (Table 4) indicated the ability of this genotype to maintain for a longer period a root metabolism still able to cope with low photosynthesis as well as a slower protein turnover, which is also reflected by PEPC V_{max} values (Table 5). Likewise, a decrease in root Fe-reducing capacity -in part dependent on FCR- as a response to a prolonged Fe-deficiency (50 days) has been observed in Fe chlorosis susceptible rootstocks, but not in Fe chlorosis-tolerant rootstocks of quince and pear species (Tagliavini et al. 1995). The PEPC and MDH activity data obtained in our experiment indicated differences in the root metabolism between the three grapevine genotypes subjected to a severe Fe deficiency, which could be used as biochemical indicators for screening Fe deficiency-tolerance levels.

Isocitrate dehydrogenase and CS activities in roots were not affected by Fe deficiency, whereas $NADP^+$-IDH showed differences among genotypes (Table 4). NADP-dependent isocitrate dehydrogenase is part of the TCA cycle, and produces 2-oxoglutarate by the oxidative decarboxylation of isocitrate (Foyer et al. 2011). This enzyme is located in several cell compartments, mainly in the cytosol and mitochondria. Isocitrate dehydrogenase has been reported as an enzyme responding to Fe deficiency in the root tissues of *Beta vulgaris* L. (López-Millán et al. 2000), *Pisum sativum* (Jelali et al. 2010), *Lycopersicon esculentum* L. (López-Millán et al. 2009). However, in our experimental conditions this root response mechanism to Fe deficiency was not observed for the three grapevine genotypes. Some authors have suggested that $NADP^+$-IDH (cytosolic and mitochondrial) is directly involved in the production of 2-oxoglutarate for N-assimilation and glutamate synthesis (GS-GOGAT cycle) (see Foyer et al. 2011, and references therein). In addition, cytosolic $NADP^+$-IDH plays a role in the cycling, redistribution, and export of amino acids during leaf senescence (Masclaux et al. 2000). The higher root $NADP^+$-IDH activity recorded in 101-14 suggested it to possesses a different metabolism in roots, probably associated with N nutrition. In the sensitive grapevine cv Gloire de Montpellier, Jimenez et al. (2007) reported an increase in root $NADP^+$-IDH in Fe deficient plants fed with nitrate-N in the nutrient solution. However, contrasting results were observed in plants grown in the presence of both ammonium-N and nitrate-N in the nutrient solution. By contrast, the presence of Fe and the nitrogen species in the nutrient solution did not modify the activity of root $NADP^+$-IDH in the tolerant grapevine genotype Cabernet Sauvignon. These authors suggested that this enzyme determined the production of reducing power required by FCR (see Jimenez et al. 2007 and references therein). Such evidence suggests that in a Fe deficiency-sensitive genotype, the activity of $NADP^+$-IDH in roots changes according to the Fe level and nitrogen form (NH_4^+ or NO_3^-) in the nutrient solution.

At the end of the experiment, significant interactions were observed between Fe level and genotype for citric and malic acids root concentrations. Iron deficiency increased the citric acid concentration in roots of the three genotypes, with the concentration being highest in 101-14 (27-fold) followed by 110 Richter and SO4 (5-fold and 2-fold respectively) (Table 6). Severe Fe deficiency increased the concentration of malic acid in the roots of 101-14 rootstock, whereas for 110 Richter and SO4 the malic acid concentration did not change. The heavy accumulation of citric acid and, to a relatively lesser extent, malic acid recorded in roots of 101-14 -Fe vines contrasted with the low activity of PEPC and MDH. What may have caused such phenomenon is not known. Some studies have shown that L-malate inhibits PEPC (Wong and Davies 1973; Chollet et al. 1996; López-Millán et al. 2000). In addition, citric acid is known to inhibit the PEPC activity (Wong and Davies 1973). Accordingly, it is possible that the high accumulation of organic acids in roots as a consequence of prolonged Fe deficiency in the 101-14 genotype contributed to deceleration of PEPC and MDH activity due to an inhibitory effect caused by these acids. In the 110 Richter rootstock, the moderate increases in citric acid concentration (Table 6) and the PEPC and MDH activity (Table 4) in the roots of Fe-deficient plants indicated that the organic acids metabolism was still active after a prolonged Fe-shortage. The finding that the root concentrations of organic anions (citrate and malate) are negatively correlated with the tolerance to Fe chlorosis is in contrast to the study by Brancadoro et al. (1995). The physiological explanation of this paradoxical phenomenon, that a tolerant genotype shows a lower intensity of one of the Strategy I root responses to Fe deficiency than the susceptible one, is unknown. The organic acids efflux, which could be lower in the Fe chlorosis-susceptible genotype, must be determined. It is possible that the lower accumulation of organic acids in the roots of Fe deficiency-tolerant genotype may be the result of

the increased anion exudation rate. A different behavior was observed in SO4. Similarly to 101-14, lower V_{max} of PEPC and MDH activity were recorded in SO4 Fe-deficient plants respect to the control plants. This is a clear indication of a deceleration of these organic acids-related enzymes. Conversely, the root concentration of organic acids was moderate under Fe deficiency conditions, suggesting that when SO4 was subjected to a severe Fe-deficiency, it behaved like 110 Richter in certain tolerance responses to Fe chlorosis (organic acids accumulation in roots), whereas other responses were similar to 101-14 (slowing down the PEPC and MDH activities in roots). These physiological observations are in line with the intermediate level of Fe deficiency symptoms reflected in leaf chlorophyll content and plant biomass production compared with 110 Richter and 101-14. Additional physiological responses to a severe Fe deficiency, related to the reduction capacity of roots and exudation/translocation of organic compounds in different genotypes, may explain the diverse Fe chlorosis tolerance of these grapevine rootstocks.

Conclusions

Our data showed that root PEPC and MDH could serve as tools for screening Fe chlorosis tolerance among genotypes. However, the high levels of organic acid accumulation recorded in the 101-14 and SO4 genotypes after a severe exposure to Fe deficiency suggested exercising caution in their adoption as screening parameters. Based on our results we suggest that screening programs assess the degree of Fe deficiency tolerance of genotypes by taking the short as well as long-term response mechanisms to Fe deprivation into consideration.

Acknowledgments The authors gratefully acknowledge the Comisión Nacional de Investigación Científica y Tecnológica (CONICYT) of Chile and the Erasmus Mundus External Cooperation Window for Chile (Lot 17)-European Union Community for Doctoral Scholarships to José Ignacio Covarrubias.

References

Bavaresco LE, Giachino E, Pezzutto S (2003) Grapevine rootstock effects on lime-induced chlorosis, nutrient uptake, and source-sink relationships. J Plant Nutr 26:1451–1465

Bavaresco L, van Zeller MI, Civardi S, Gatti M, Ferrari F (2010) Effects of traditional and new methods on overcoming lime-induced chlorosis of grapevine. Am J Enol Vitic 61:186–190

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Brancadoro L, Rabotti G, Scienza A, Zocchi G (1995) Mechanisms of Fe-efficiency in roots of *Vitis spp*. in response to iron deficiency stress. Plant Soil 171:229–234

Cesco S, Neumann G, Tomasi N, Pinton R, Weisskopf L (2010) Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition. Plant Soil 329:1–25

Chollet R, Vidal J, O'Leary MH (1996) Phosphoenolpyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants. Annu Rev Plant Physiol Plant Mol Biol 47:273–298

Covarrubias JI, Rombolà AD (2013) Physiological and biochemical responses of the iron chlorosis tolerant grapevine rootstock 140 Ruggeri to iron deficiency and bicarbonate. Plant Soil 370:305–315

Covarrubias JI, Pisi A, Rombolà AD (2014) Evaluation of sustainable management techniques for preventing iron chlorosis in the grapevine. Aust J Grape Wine Res 20:149–159

De Nisi P, Zocchi G (2000) Phosphoenolpyruvate carboxylase in cucumber (*Cucumis sativus* L.) roots under iron deficiency: activity and kinetic characterization. J Exp Bot 51(352): 1903–1909

De Nisi P, Vigani G, Zocchi G (2010) Modulation of iron responsive gene expression and enzymatic activities in response to changes of the iron nutritional status in *Cucumis sativus* L. Available from Nature Precedings. doi:10.1038/npre.2010.4658.1

Donnini S, Castagna A, Ranieri A, Zocchi G (2009) Differential responses in pear and quince genotypes induced by Fe deficiency and bicarbonate. J Plant Physiol 166:1181–1193

Donnini S, Prinsi B, Negri AS, Vigani G, Espen L, Zocchi G (2010) Proteomic characterization of iron deficiency responses in *Cucumis sativus* L. roots. BMC Plant Biol 10:268

Foyer CH, Noctor G, Hodges M (2011) Respiration and nitrogen assimilation: targeting mitochondria-associated metabolism as a means to enhance nitrogen use efficiency. J Exp Bot 62(4):1467–1482

Goldberg DM, Ellis G (1974) Isocitrate dehydrogenase. In: Bergmeyer HU (ed) Methods of enzymatic analysis. VerlagChemie/Academic Press, New York, pp 183–189

Jelali N, Wissal M, Dell'Orto M, Abdellya C, Gharsalli M, Zocchi G (2010) Changes of metabolic responses to direct and induced Fe deficiency of two *Pisum sativum* cultivars. Environ Exp Bot 68:238–246

Jimenez S, Gogorcena Y, Hévin C, Rombolà AD, Ollat N (2007) Nitrogen nutrition influences some biochemical responses to iron deficiency in tolerant and sensitive genotypes of *Vitis*. Plant Soil 290:343–355

Kim SA, Guerinot ML (2007) Mining iron: iron uptake and transport in plants. FEBS Lett 581:2273–2280

Lance C, Rustin P (1984) The central role of malate in plant metabolism. Physiol Veg 22(5):625–641

López-Millán AF, Morales F, Andaluz S, Gogorcena Y, Abadía A, De Las Rivas J, Abadía J (2000) Responses of sugar beet roots to iron deficiency. Changes in carbon assimilation and oxygen use. Plant Physiol 124:885–897

López-Millán AF, Morales F, Gogorcena Y, Abadía A, Abadía J (2009) Metabolic responses in iron deficient tomato plants. J Plant Physiol 166:375–384

López-Rayo S, Di Foggia M, Bombai G, Yunta F, Rodrigues-Moreira E, Filippini G, Pisi A, Rombolà AD (2014) Blood-derived compounds can efficiently prevent iron deficiency in grapevine. Aust J Grape Wine Res 21:135–142

Masclaux C, Valadier MH, Brugière N, Morot-Gaudry JF, Hirel B (2000) Characterization of the sink/source transition in tobacco (*Nicotiana tabacum* L.) shoots in relation to nitrogen management and leaf senescence. Planta 211:510–518

Neumann G (2006) Root exudates and organic composition of plant roots. In: Luster J, Finlay R (eds) Handbook of methods used in rhizosphere research. Swiss Federal Research Institute WSL, Birmensdorf, 536 p

Nikolic M, Römheld V, Merkt N (2000) Effect of bicarbonate on uptake and translocation of ^{59}Fe in two grapevine rootstocks differing in their resistance to Fe deficiency chlorosis. Vitis 39(4):145–149

Ollat N, Laborde B, Neveux M, Diakou-Verdin P, Renaud C, Moing A (2003) Organic acid metabolism in roots of various grapevine (*Vitis*) rootstocks submitted to iron deficiency and bicarbonate nutrition. J Plant Nutr 26(10&11): 2165–2176

Rodríguez-Celma J, Lattanzio G, Grusak MA, Abadía A, Abadía J, López-Millán AF (2011) Root responses of *Medicago truncatula* plants grown in two different iron deficiency conditions: changes in root protein profile and riboflavin biosynthesis. J Proteome Res 10:2590–2601

Rombolà AD, Tagliavini M (2006) Iron nutrition of fruit tree crops. In: Abadía J, Barton L (eds) Iron nutrition in plants and rhizospheric microorganisms. Springer, Berlin, pp 61–83

Rombolà AD, Brüggemann W, López-Millán AF, Tagliavini M, Abadía J, Marangoni B, Moog PR (2002) Biochemical responses to iron deficiency in kiwifruit (*Actinidia deliciosa*). Tree Physiol 22:869–875

Römheld V, Marschner H (1986) Mobilitation of iron in the rhizosphere of different plant species. Adv Plant Nutr 2: 123–218

Smith F (1974) Malate dehydrogenase. In: Bergmeyer HU (ed) Methods of enzymatic analysis. VerlagChemie/Academic Press, New York, pp 163–175

Srere PA (1967) Citrate synthase. In: Colowick SP, Kaplan NO (eds) Methods in enzymology. Academic, New York, pp 3–11

Tagliavini M, Rombolà AD (2001) Iron deficiency and chlorosis in orchard and vineyard ecosystems. Eur J Agron 15:71–92

Tagliavini M, Rombolà AD, Marangoni B (1995) Response to Fe-deficiency stress of pear and quince genotypes. J Plant Nutr 18(11):2465–2482

Vance CP, Stade S, Maxwell CA (1983) Alfalfa root nodule carbon dioxide fixation. I: association with nitrogen fixation and incorporation into amino acids. Plant Physiol 72:469–473

Wong KF, Davies DD (1973) Regulation of phosphoenolpyruvate carboxylase of *Zea mays* by metabolites. Biochem J 131: 451–458

Yunta F, Di Foggia M, Bellido-Díaz V, Morales-Calderón M, Tessarin P, López-Rayo S, Tinti A, Kovács K, Klencsár Z, Fodor F, Rombolà AD (2013) Blood meal-based compound. Good choice as iron fertilizer for organic farming. J Agric Food Chem 61:3995–4003

Zocchi G (2006) Metabolic changes in iron-stressed dicotyledonous plants. In: Abadía J, Barton L (eds) Iron nutrition in plants and rhizospheric microorganisms. Springer, Berlin, pp 359–370