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REGULAR ARTICLE

Physiological and biochemical responses of the iron chlorosis tolerant grapevine rootstock 140 Ruggeri to iron deficiency and bicarbonate

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Abstract
Background and aims Iron (Fe) deficiency chlorosis associated with high levels of soil bicarbonate is one of the main nutritional disorders observed in sensitive grapevine genotypes. The aim of the experiment was to assess both the independent and combined effects of Fe and bicarbonate nutrition in grapevine.
Methods Plants of the Fe chlorosis tolerant 140 Ruggeri rootstock were grown with and without Fe(III)-EDTA and bicarbonate in the nutrient solution. SPAD index, plant growth, root enzyme (PEPC, MDH, CS, $NADP^+$ −IDH) activities, kinetic properties of root PEPC, organic acid concentrations in roots and xylem sap and xylem sap pH were determined. A factorial statistical design with two factors (Fe and BIC) and two levels of each factor was adopted: +Fe and −Fe, and +BIC and −BIC.
Results This rootstock strongly reacted to Fe deficiency by activating several response mechanisms at different physiological levels. The presence of bicarbonate in the nutrient solution changed the activity of PEPC and TCA related enzymes (CS, $NADP^+$-IDH) and the accumulation/translocation of organic acids in roots of Fe-deprived plants. Moreover, this genotype increased root biomass and root malic acid concentration in response to high bicarbonate levels in the substrate. Bicarbonate also enhanced leaf chlorophyll content.
Conclusions Along with a clear independent effect on Fe nutrition, our data support a modulating role of bicarbonate on Fe deficiency response mechanisms at root level.

Keywords Iron · Bicarbonate · Grapevine · Enzyme activity · Organic acids

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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

In many important wine regions of the Mediterranean basin, characterized by soils with high concentration of active lime and alkaline pH, iron (Fe) chlorosis is one of the main nutritional deficiencies in grapevine (Tagliavini and Rombolà 2001). This nutritional disorder can cause a dramatic reduction in vineyard longevity and productivity, root and shoot growth and losses in grape yield and quality (Bavaresco et al. 2003). However, mild Fe chlorosis can enhance wine quality due to improvements in grape quality attributes such as soluble solids, pH, anthocyanins and resveratrol (Bavaresco et al. 2005). Grapevine belongs to Strategy I plants, and therefore under Fe deficiency it is able to increase Fe reductase activity and net release of protons and organic compounds in roots (eg. organic acids, phenolics, etc.), lowering the pH and increasing the solubility of Fe(III) (Brancadoro et al. 1995; Ksouri et al. 2006; Jiménez et al. 2007). The control of Fe chlorosis with Fe chelates is a widespread agronomical practice in vineyards, but it involves high costs and potential environmental and health risks (Rombolà and Tagliavini 2006; Tagliavini and Rombolà 2001). Such constraints strongly warrant the need for adopting alternative strategies to manage Fe nutrition according to soil and plant parameters. In this context, the use of tolerant rootstocks may represent an economical and efficient method for preventing Fe chlorosis. It is well known that the susceptibility level to Fe chlorosis in grapevine rootstocks is highly variable in function of the genotype. For instance, 140 Ruggeri has been classified as highly tolerant rootstock (Ksouri et al. 2006; Tagliavini and Rombolà 2001). Rootstock 140 Ruggeri is an interspecific hybrid between *Vitis berlandieri x Vitis rupestris*. It inherited a high tolerance to lime soil from *Vitis berlandieri* and tolerance to drought stress from *Vitis rupestris* (Eynard and Dalmasso 1990). Ksouri et al. (2006) found that the high tolerance of 140 Ruggeri to Fe chlorosis is in part due to a high root Fe(III)-reductase activity and its ability to release phenolic compounds in the medium. Currently this rootstock is largely employed in south Mediterranean and North Africa viticulture areas, characterized by lime soils and dry environmental conditions.

Bicarbonate is one of the main factors causing Fe chlorosis in Strategy I plants (Mengel et al. 1984). In calcareous soils, bicarbonate concentrations can reach values up to 15 mM (Boxma 1972). Nevertheless, the mechanisms involved in bicarbonate-induced Fe chlorosis are still not clear. The morphological and physiological responses demonstrated that woody cuttings of 140 Ruggeri rootstock (not grafted) can withstand high concentrations of bicarbonate in the soil (10 mM), showing only slight/moderate decreases in leaf chlorophyll and plant biomass (Ksouri et al. 2005). In several studies bicarbonate or calcium (Ca) carbonate has been routinely included in the nutrient solution for exacerbating expected Fe deficiency symptoms and stimulating response mechanisms (Lopez-Millán et al. 2000, 2009; Rombolà et al. 2005). In a study carried out on grapevine rootstocks, Nikolic et al. (2000) concluded that bicarbonate-induced Fe chlorosis was caused by an inhibition of ^{59}Fe uptake and translocation due to inhibited Fe(III) reduction by root cells, with these processes being less inhibited in chlorosis-resistant rootstocks. Mengel et al. (1994) hypothesized that bicarbonate-induced chlorosis was caused by a transport of bicarbonate into the steel, leading to an alkalinization of the xylem sap and, in turn, of the leaf apoplast. Several authors have tested Mengel hypothesis by measuring the effects of bicarbonate on the xylem sap pH. There are contrasting results showing that bicarbonate does not cause a physiologically relevant increase of the xylem sap/leaf apoplastic fluid pH in several plant species, including grapevine (see Nikolic and Roemheld 2007 and references therein). However, using a novel xylem pH probe enabling *in situ* measurements, Wegner and Zimmermann (2004) clearly demonstrated that bicarbonate induced an alkalinization of the xylem sap in intact maize seedlings, supporting Mengel hypothesis. Bicarbonate, at extremely high concentrations (20–30 mM), could induce Fe chlorosis by inhibiting the expression of the genes encoding ferric reductase, Fe transporters and H^+-ATPase (Lucena et al. 2007). Some authors (Donnini et al. 2009; Jelali et al. 2011; Rombolà 1998) added bicarbonate and Fe to the nutrient solution in order to mimic field conditions where Fe deficiency is caused by the low availability of Fe in the soil induced by the presence of bicarbonate. Results obtained in nutrient culture experiments (Donnini et al. 2009; Jelali et al. 2010; Rombolà 1998) indicate that Fe chlorosis tolerant genotypes grown in Fe supplied nutrient solutions with bicarbonate (+Fe +bicarbonate; low Fe availability) are generally less affected than plants subjected to Fe-free nutrient solutions (−Fe - bicarbonate; absolute Fe deficiency), whereas in

Fe chlorosis sensitive genotypes the effect of absolute and low Fe availability are often the same. In the field, it has been observed that several woody crops can tolerate lime soils, growing and producing normally without the need for adopting any specific agronomic techniques to amend Fe chlorosis.

This work is aimed at studying the physiological and biochemical response mechanisms of grapevine to Fe and bicarbonate in the substrate as independent and combined factors. For this purpose, a nutrient solution experiment with two levels of Fe and two levels of bicarbonate has been conducted with the Fe chlorosis tolerant rootstock 140 Ruggeri. Such experimental approach allowed to identify the effects of each factor and their interaction.

Materials and methods

Plant material, growth conditions and treatments

The experiment was carried out at the Experimental Station of the Agriculture Faculty (Cadriano, Bologna). Micropropagated plants of the Fe chlorosis tolerant genotype cv 140 Ruggeri (*Vitis berlandieri x Vitis rupestris*) were acclimated in peat for 3 weeks and pruned maintaining one main shoot per plant. The plants (36) were transferred to 35 l plastic containers (9 plants for each container) covered with aluminum and filled with 30 l of a half Hoagland nutrient solution continuously aerated. The growth chamber was programmed for a 16 h photoperiod (150–200 μmol photons $m^{-2}s^{-1}$ at leaf level) at 25–30 °C and 8 h of darkness at 25 °C, with 70–75 % relative humidity. Plants were treated with two levels of Fe-EDTA (0 and 50 μM) and two levels of bicarbonate (0 and 5 mM) in the solution. The bicarbonate concentration was lower than levels often employed in hydroponic experiments. The treatments established were: 1) +Fe +BIC; 2) +Fe −BIC; 3) −Fe +BIC; 4) −Fe −BIC. The potassium supplied in the treatments with $KHCO_3$ was balanced with K_2SO_4 in the treatments without bicarbonate. The composition of the half Hoagland nutrient solution was: 2.5 mM KNO_3; 2 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 $CuSO_4$. The nutrient solution was renewed twice a week and the pH was adjusted to 7.4 and 6.0 in the containers with and without bicarbonate, respectively, after every renewal with HCl 0.1 M. Also, the pH was monitored daily. The experiment was concluded when apical leaves of Fe deficient plants displayed severe yellowing.

Leaf chlorophyll content and plant growth

Leaf chlorophyll content was monitored periodically during the experiment on five different areas of the first expanded leaf with a portable chlorophyll meter (SPAD MINOLTA 502, Osaka, Japan). When apical leaves of −Fe plants showed severe Fe chlorosis symptoms (SPAD index value of around 5), plants were divided into roots, main shoot, lateral shoots and leaves for dry weight (DW) determination, total leaf area and subsequent analysis.

Enzyme assays and protein concentration in roots

At the end of the experiment, root (20–30 mm long) samples 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 activity of phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MDH), citrate synthase (CS), and isocitrate dehydrogenase ($NADP^+$-IDH) in root extracts were determined. The extraction of enzymes 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 CoA to CoA with DTNB at 412 nm (Srere 1967). Isocitrate dehydrogenase was assayed as described by Goldberg and Ellis (1974), by monitoring the reduction of $NADP^+$ at 340 nm. Protein concentration was determined by the Bradford method, using BSA as standard (Bradford 1976). Data obtained in enzyme assays were referred to protein concentration of roots.

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 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 acid concentrations 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 evaporation, the tissue was ground and homogenized with a pestle. For extraction and deproteinization, 0.5 M H_3PO_4 was utilized. Organic acids were quantified as described by Neumann (2006) by HPLC with 250×4 mm LiChrospher 5 μm RP-18 column (Supelco Inc., PA 16823-0048 USA). HPLC 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 (citrate, tartrate, malate and ascorbate).

Xylem sap collection and analysis

Xylem sap was collected from each plant at the end of the experiment applying a pressure of 5 bar to the root system using a Schölander pressure chamber, as described by Rombolà et al. (2002). Approximately 500–1000 μl of xylem sap per plant were collected with microcapillary tubes in 2 ml Eppendorf. The pH of the xylem sap samples was determined with a microelectrode (Hamilton AG, CH-7402 Bonaduz, Switzerland), and the samples were subsequently frozen at −20 °C for organic acid determinations. Organic acids were quantified by HPLC as described above.

Statistics

Data were analyzed by a two-way analysis of variance with SAS software (SAS Institute, Cary, NC). A factorial statistical design with two factors (Fe and BIC) and two levels of each factor was adopted: +Fe and −Fe, and +BIC and −BIC. When a significant interaction between factors was obtained by the F-test, the statistical comparison between the 4 possible treatments (2 BIC levels × 2 Fe levels) has been 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 was higher than 2 × SEM. When the interaction between factors was not significant, the statistical comparison has been performed by the F-test ($P \leq 0.05$) between the levels of each independent factor. We adopted such methodological approach in order to clearly pursue the main objective of the factorial experiment (Rombolà et al. 2002).

Results

Plants response to Fe deficiency

At the end of the experiment, higher SPAD index values were recorded in the first expanded leaf of + Fe (moderate leaf chlorosis) treatment, as compared with −Fe (severe leaf chlorosis) treatment (Table 1). The presence of bicarbonate in the nutrient solution increased leaf chlorophyll content in the two levels of Fe assessed (Table 1). Iron deficiency decreased root biomass independently of bicarbonate level, whereas bicarbonate nutrition induced an increase in root biomass at the two levels of Fe (Table 1). Total leaf area and biomass yield of the main shoot, lateral shoots, leaves, and total biomass did not show significant differences due to the two factors investigated (Table 1).

Since six days from treatments imposition, a lower pH was recorded in −Fe as compared to +Fe nutrient solution. This effect resulted more pronounced in the presence of bicarbonate. In +BIC treatment, the largest difference (by 0.6 units) has been recorded at 8 days from treatments imposition (Fig. 1), whereas in −BIC treatment, the largest difference has been recorded at 9 days (by 0.3 units) (Fig. 1).

Root enzyme activities

At the end of the experiment, the activity of PEPC and some enzymes related to Krebs cycle were determined in root extracts. Iron deficiency induced an increase in PEPC activity (Table 2), whereas bicarbonate decreased the activity of this enzyme (Table 2). Malate dehydrogenase activity did not change in response to Fe level (Table 2), whereas bicarbonate

Table 1 Biomass yield of the different organs (g DW plant^{-1}), total leaf area (cm^2 plant^{-1}) and SPAD index determined at the end of the experiment in 140 Ruggeri plants grown with two levels of bicarbonate (+BIC and −BIC) under Fe-sufficient (+Fe) and Fe-deficient (−Fe) conditions. Data are means of nine plants (replicates). Since the BIC × Fe interaction was not significant for any parameter, the F-test ($P<0.05$) between the levels of each independent factor was used

Bicarbonate (BIC)	Iron (Fe)	Plant biomass (g DW plant^{-1})					Leaf area	SPAD index
		Roots	Main Shoot	Lateral shoots	Leaves	Total plant		
−BIC	+Fe	1.7±0.18	0.7±0.10	0.4±0.11	1.1±0.10	4.0±0.34	193.7±19.8	8.5±1.1
−BIC	−Fe	1.5±0.08	0.8±0.07	0.2±0.05	1.1±0.07	3.6±0.18	183.9±15.8	2.8±0.6
+BIC	+Fe	2.7±0.17	0.7±0.08	0.2±0.05	1.0±0.10	4.6±0.32	192.6±23.1	10.5±1.0
+BIC	−Fe	2.3±0.17	0.6±0.04	0.2±0.05	1.0±0.06	4.1±0.26	173.6±15.3	6.5±0.9
Statistics								
BIC treatment		***	NS	NS	NS	NS	NS	**
Fe treatment		*	NS	NS	NS	NS	NS	***
BIC × Fe interaction		NS	NS	NS	NS	NS	NS	NS

Data are mean ± SE of nine replicates

NS *, **, *** not significant and significant at $p\leq0.05$, $p\leq0.01$, and $p\leq0.001$ levels, respectively

supply reduced the activity of MDH (Table 2). Citrate synthase and NADP$^+$−IDH data showed an interaction between both factors. Iron deficiency increased the activity of these enzymes in bicarbonate fed-plants and the opposite effects were recorded in −BIC plants (Table 2).

The saturation kinetics curves of PEPC were studied by adding different concentrations of bicarbonate -in a range of 0 to 10 mM to the buffer assay. The assays showed that bicarbonate concentration in the buffer assay increased the activity of PEPC in all treatments, up to a concentration of approximately 5 mM (data not shown). With concentrations of bicarbonate between 5 mM and 10 mM, the activity of the enzyme did not change significantly (data not shown). Iron deficiency increased the rate of PEPC activity, reaching a higher V_{max} than that obtained with Fe-sufficient plant extracts (Table 3). In contrast, bicarbonate did not modify the V_{max} (Table 3). The substrate-affinity (*Km*) was not altered by treatments (Table 3).

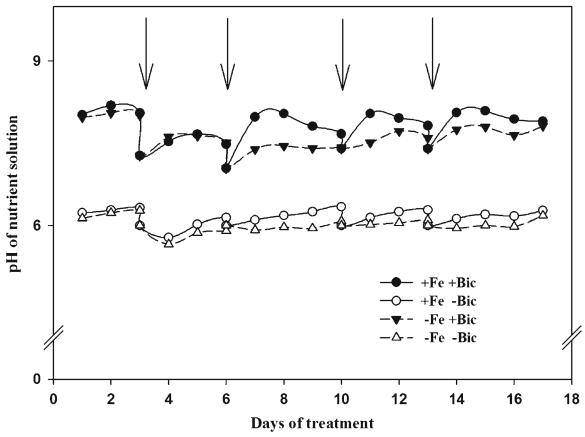

Fig. 1 Changes in the pH of the nutrient solution (35-L containers with 9 plants in each) for two levels of bicarbonate (+BIC and −BIC) under Fe-sufficient (+Fe) and Fe-deficient (−Fe) conditions. The nutrient solution was renewed twice a week during the experiment (*arrow down*)

Organic acid concentrations in root tissue and xylem sap

At the end of the experiment, the major organic acids investigated in root extracts were tartaric, followed by malic, citric and ascorbic acids (Table 4). Significant interactions between Fe and bicarbonate were found. Iron-deficiency increased citric acid concentration in roots (Table 4). However, the effect of Fe was less pronounced in bicarbonate-fed plants. Iron shortage enhanced the concentration of tartaric acid only in −BIC plants (73 %), whereas no differences were detected in plants submitted to bicarbonate nutrition (Table 4). Limiting Fe did not alter malic acid level, while bicarbonate enhanced malic acid concentration in the roots (Table 4). In −BIC plants, root ascorbic acid concentration was lowered by Fe deficiency, whereas Fe status did not affect ascorbic acid levels in +BIC roots (Table 4). Iron

Table 2 Activities (nmol mg^{-1} protein min^{-1}) of PEPC, MDH, CS, $NADP^+$–IDH and protein concentration (mg g^{-1} FW) measured in root extracts of 140 Ruggeri plants grown with two levels of bicarbonate (+BIC and −BIC) under Fe-sufficient (+Fe) and Fe-deficient (−Fe) conditions. Data are means of nine plants (replicates). When the BIC × Fe interaction was not significant, the F-test ($P \leq 0.05$) between the levels of each independent factor was used. When the BIC × Fe interaction was significant, the statistical comparison between the 4 possible treatments (2 BIC levels × 2 Fe levels) was performed. The treatments were considered as significantly different when the difference between data was higher than 2 × SEM

Bicarbonate (BIC)	Iron (Fe)	PEPC	MDH	CS	$NADP^+$-IDH	Protein
−BIC	+Fe	30.2±2.0	283.5±30.8	10.8±1.8	28.8±3.5	31.4±3.2
−BIC	−Fe	40.1±5.2	228.9±43.2	6.0±1.1	19.2±2.6	28.8±3.1
+BIC	+Fe	14.1±1.4	185.6±20.2	5.5±0.1	13.2±1.2	35.8±2.0
+BIC	−Fe	26.4±4.1	190.5±19.1	9.4±1.4	18.3±1.8	24.6±2.4
Statistics						
BIC treatment		**	*			NS
Fe treatment		**	NS			*
BIC × Fe interaction		NS	NS	*	*	NS
SEM [a]				0.37	0.72	

Data are mean ± SE of nine replicates

NS *, ** not significant and significant at $p \leq 0.05$ and $p \leq 0.01$ levels, respectively

[a] *SEM* standard error of the interaction means

deficiency enhanced by 2-fold total root organic acids concentration in −BIC plants (Table 4), whereas under bicarbonate nutrition, similar levels were recorded in +Fe and −Fe plants (Table 4).

In xylem sap, the predominant organic acid was malic acid, followed by citric and ascorbic acids (Table 5). Iron deficiency increased the concentration of citric acid without affecting the pH of the xylem sap (Table 5). Bicarbonate decreased citric acid concentration and increased xylem sap pH (Table 5). Malic, ascorbic and total organic acids concentration did not change in response to Fe and bicarbonate levels (Table 5).

Table 3 Kinetic parameters *Km* (mM of $NaHCO_3^-$) and V_{max} (nmol mg^{-1} protein min^{-1}) of phosphoenolpyruvate carboxylase activity (PEPC) in root extracts of 140 Ruggeri plants roots grown with two levels of bicarbonate (+BIC and −BIC) under Fe-sufficient (+Fe) and Fe-deficient (−Fe) conditions. Data are means of three plants (replicates). Since the BIC × Fe interaction was not significant for any parameter, the F-test ($P \leq 0.05$) between the levels of each independent factor was used

Bicarbonate (BIC)	Iron (Fe)	*Km*	V_{max}
−BIC	+Fe	0.07±0.01	25.0±3.3
−BIC	−Fe	0.10±0.04	42.4±3.2
+BIC	+Fe	0.08±0.03	12.8±1.6
+BIC	−Fe	0.12±0.04	38.8±7.3
Statistics			
BIC treatment		NS	NS
Fe treatment		NS	**
BIC × Fe interaction		NS	NS

Data are mean ± SE of three replicates

NS ** not significant and significant at $p \leq 0.01$ level

Discussion

Under Fe deficiency conditions (−Fe), the Fe chlorosis tolerant rootstock 140 Ruggeri showed a marked decrease in leaf chlorophyll content concomitant with a reduction in root biomass (Table 1). Such symptoms, as well as a reduction of shoot growth, have been previously described in grapevine and other woody species (Bavaresco et al. 2003; Jiménez et al. 2007; Rombolà and Tagliavini 2006).

The presence of bicarbonate in the nutrient solution (5 mM) increased the SPAD index of apical leaves and root biomass (Table 1). A stimulation of root biomass induced by bicarbonate has been reported in *Medicago ciliaris* (M'sehli et al. 2009), in which the presence of bicarbonate increased root DW in a line more tolerant to Fe chlorosis. In a pot experiment, similar results were observed with pear and quince genotypes grown in soils with different $CaCO_3$ content (Tagliavini et al.

Table 4 Organic acid concentrations (mg g^{-1} FW) in root extracts of 140 Ruggeri plants grown with two levels of bicarbonate (+BIC and −BIC) under Fe-sufficient (+Fe) and Fe-deficient (−Fe) conditions. Data are means of nine plants (replicates). When the BIC × Fe interaction was not significant, the F-test ($P \leq 0.05$) between the levels of each independent factor was used. When the BIC × Fe interaction was significant, the statistical comparison between the 4 possible treatments (2 BIC levels × 2 Fe levels) was performed. The treatments were considered as significantly different when the difference between data was higher than 2 × SEM

Bicarbonate (BIC)	Iron (Fe)	Citrate	Tartrate	Malate	Ascorbate	Total
−BIC	+Fe	0.22±0.01	0.93±0.03	0.38±0.03	0.038±0.005	1.6±0.1
−BIC	−Fe	0.91±0.12	1.61±0.06	0.46±0.05	0.018±0.003	3.0±0.2
+BIC	+Fe	0.37±0.01	1.37±0.06	0.56±0.04	0.031±0.001	2.3±0.1
+BIC	−Fe	0.73±0.08	1.25±0.11	0.72±0.17	0.031±0.005	2.7±0.4
Statistics						
BIC treatment				*		
Fe treatment				NS		
BIC × Fe interaction		*	***	NS	*	*
SEM [a]		0.065	0.072		0.004	0.21

Data are mean ± SE of nine replicates

NS *, *** not significant and significant at $p \leq 0.05$ and $p \leq 0.001$ levels, respectively

[a] *SEM* standard error of the interaction means

1993). In *Parietaria diffusa*, bicarbonate supply induced a shorter root system, with the appearance of structures similar to "proteoid roots", that provide an enhanced surface of contact between plant and soil (Donnini et al. 2012). The presence of such structures in Fe-sufficient plants grown with bicarbonate but not in Fe-deficient plants suggests that this should not be a specific response to Fe deficiency but to a more general condition of low nutrient availability (Donnini et al. 2012).

In the present work, since 6 days from treatments imposition, a lower pH was recorded in −Fe as compared to +Fe nutrient solution. This effect resulted more pronounced in the presence of bicarbonate (Fig. 1). These results are in line with those of Ksouri et al. (2005) and demonstrate the capacity of this Fe chlorosis tolerant genotype to decrease the pH of the medium under Fe deficiency conditions. In grapevine, the Fe chlorosis tolerant genotype Cabernet Sauvignon (*Vitis vinifera*) decreased the pH more effectively than

Table 5 pH and organic acid concentrations (μM) in xylem sap of 140 Ruggeri plants grown with two levels of bicarbonate (+BIC and −BIC) under Fe-sufficient (+Fe) and Fe-deficient (−Fe) conditions. Data are means of nine plants (replicates). Since the BIC × Fe interaction was not significant for any parameter, the F-test ($P \leq 0.05$) between the levels of each independent factor was used

Bicarbonate (BIC)	Iron (Fe)	pH	Citrate	Malate	Ascorbate	Total
−BIC	+Fe	6.5±0.41	86.3±8.3	322.7±14.1	20.7±1.8	429.7±22.4
−BIC	−Fe	6.0±0.04	127.9±13.7	270.2±45.7	16.5±2.3	414.6±58.5
+BIC	+Fe	6.7±0.13	69.5±7.2	297.9±29.3	17.3±4.0	384.7±39.8
+BIC	−Fe	6.8±0.14	86.9±11.4	330.7±43.7	18.9±2.8	436.6±53.7
Statistics						
BIC treatment		*	*	NS	NS	NS
Fe treatment		NS	**	NS	NS	NS
BIC × Fe interaction		NS	NS	NS	NS	NS

Data are mean ± SE of nine replicates

NS *, ** not significant and significant at $p \leq 0.05$ and $p \leq 0.01$ levels, respectively

the Fe chlorosis susceptible genotype Riparia Gloire de Montpellier (Jiménez et al. 2007).

The rootstock 140 Ruggeri reacted to Fe deficiency by increasing the activity of PEPC in root extracts. Iron deficiency increased PEPC activity in root extracts of the Fe chlorosis tolerant genotype Cabernet Sauvignon by approximately 3-fold, whereas the Fe chlorosis susceptible genotype Gloire de Montpellier increased PEPC activity by approximately 2.5-fold (Jiménez et al. 2007). According to preliminary results reported by Ollat et al. (2003), in vitro PEPC activity was also stimulated in roots of grapevine genotypes grown with low Fe concentration (0.5 μM) in the nutrient solution. Similar results have been observed in *Cucumis sativus* L. (de Nisi and Zocchi 2000), *Pisum sativum* (Jelali et al. 2010), *Beta vulgaris* L. (López-Millán et al. 2000), *Actinida deliciosa* (Rombolà et al. 2002), *Pyrus communis* (Donnini et al. 2009) submitted to Fe deficiency. The increase in PEPC activity in Fe-deficient roots was associated with a higher concentration of some organic acids (mainly citrate, discussed later), as observed in grapevine by Ollat et al. (2003) and Jiménez et al. (2007). These results support the significance of this enzyme for organic acid accumulation, a typical response of Strategy I plants to Fe deficiency (Abadía et al. 2002; López-Millán et al. 2000; Rombolà et al. 2002).

The enhancement induced by Fe deficiency on PEPC V_{max} without changes in *Km* (Table 3) suggests a possible increase in PEPC concentration in roots, as discussed for *Cucumis sativus* L. by De Nisi and Zocchi (2000). In *Beta vulgaris* L., the amount of PEPC protein determined by immuno-blotting was, on a protein basis, 35-fold larger in Fe-deficient than in Fe-sufficient root tips (Andaluz et al. 2002). In *Medicago truncatula*, transcript levels of PEPC (MtPEPC1-TC129218), assessed by semi-quantitative RT-PCR, showed a slightly increase in Fe-deficient roots, indicating that adaptation to Fe deficiency in roots of this species is in part mediated by a transcriptional regulation (Andaluz et al. 2009). In the grapevine genotype 140 Ruggeri, information regarding protein concentration and transcript levels in Fe-deficient roots are necessary to assess the regulation of this enzyme.

The presence of bicarbonate decreased the activity of PEPC and MDH regardless of Fe, whereas the effect of bicarbonate on the activity of CS and $NADP^+$-IDH depended on the Fe status of plants (Table 2). A decrease in PEPC activity induced by bicarbonate was also observed in *Pisum sativum* (Jelali et al. 2010) and in *Parietaria diffusa* (Donnini et al. 2012). Several hypotheses have been proposed to explain this effect. This enzyme is negatively regulated by L-malate (Chollet et al. 1996). López-Millán et al. (2000) proposed that malic acid concentrations of 0.5 mM could have an inhibitory effect on PEPC activity, and in this experiment bicarbonate increased malic acid concentration in roots reaching 0.64 $mg g^{-1}$ FW, 52 % higher than in roots grown without bicarbonate. Recently, Donnini et al. (2012) suggested that in the calcicole species *Parietaria diffusa*, the presence of bicarbonate in the substrate changes significantly the responses of plant to Fe deficiency. The PEP produced by glycolysis would be in part channeled into the shikimate pathway and converted into phenolics rather than assimilated by PEPC (Donnini et al. 2012). Data showed a clear reduction on PEPC regardless of Fe level indicating an independent effect of bicarbonate on the enzyme activity. Therefore, an adverse effect of bicarbonate on PEPC activity has been observed in various species including those tolerant to calcareous soils. In such conditions the lower bicarbonate fixation inside roots cells could lead to an increased in ion accumulation in the cytoplasm compartments. Data of our experiment showed that bicarbonate in the nutrient solution slightly increased xylem sap pH (Table 5). Such result suggests that bicarbonate was presumably loaded into the xylem and transported to the shoots and leaves (Mengel et al. 1994; Wegner and Zimmermann 2004). Moreover, it has been reported that increases in the pH of xylem sap can increase the concentration of CO_2 dissolved compounds (H_2CO_3, HCO_3^- and CO_3^{-2}) (Levy et al. 1999). The increase in SPAD value induced by bicarbonate observed in this experiment suggests that this ion may slow down leaf chlorophyll degradation as occurs in green vegetables subjected to high CO_2 atmosphere in postharvest treatments (Eason et al. 2007). The possibility that xylem sap CO_2 represents a source of carbon for leaf Rubisco has been previously proposed (Stringer and Kimmerer 1993). The effect of bicarbonate on the SPAD index could also be the consequence of an improved plant Fe status.

The activities of the enzymes related to organic acid biosynthesis in roots indicate that response mechanisms to Fe deficiency and bicarbonate are complex and the accumulation of organic acids may change according to these factors or to their interaction. The presence of bicarbonate in the nutrient solution slowed

down the activity of MDH regardless to Fe level. In contrast, bicarbonate decreased the activity of CS and $NADP^+$-IDH only under Fe-sufficiency conditions. The slowdown of CS and $NADP^+$-IDH caused by bicarbonate in +Fe plants, resulted in an accumulation of malate in roots, in spite of the PEPC reduction induced by bicarbonate. Under Fe deficiency conditions the presence of bicarbonate increased the activity of CS in roots. This result indicates that under the concomitant occurrence of Fe deficiency and bicarbonate in the nutrient solution, this genotype primarily reacts to Fe deficiency increasing the synthesis of citric acid by CS. Iron deficient roots of 140 Ruggeri grown in the presence of bicarbonate showed also a higher $NADP^+$–IDH activity (Table 2). Both enzymes (CS and $NADP^+$–IDH) have been reported as enzymes responding to Fe deficiency in root tissues of *Beta vulgaris* L. (López-Millán et al. 2000), *Pisum sativum* (Jelali et al. 2010), *Lycopersicon esculentus* L. (López-Millán et al. 2009). The effect of Fe deficiency on enzyme activities related to organic acid biosynthesis recorded in the grapevine rootstock 140 Ruggeri was different than that found in *Cucumis sativus* L. (Vigani et al. 2009) and *Beta vulgaris* (López-Millán et al. 2000). Several factors such as species, Fe status, preculture and experimental conditions, measurements on cell organelles or tissue extracts, organic acids concentration and location, etc. may contribute to explain this discrepancy.

The addition of bicarbonate in the nutrient solution induced an increase in malate concentration in roots (Table 4) without enhancing its concentration in xylem sap (Table 5). In the grapevine tolerant genotype Cabernet Sauvignon, the presence of bicarbonate in the nutrient solution increased malate concentration by 4.5 and 4.1-fold in +Fe and −Fe plants, respectively (Ollat et al. 2003). However, for the Fe chlorosis susceptible grapevine genotype Riparia Gloire de Montpellier, bicarbonate increased malate concentration by only 1.2-fold, independently of the Fe level (Ollat et al. 2003). In plants of *Pisum sativum*, the bicarbonate supply to +Fe treatment increased malate concentration at root level (Jelali et al. 2010); this effect was more pronounced in the tolerant than in the susceptible cultivar. In *Parietaria diffusa*, Donnini et al. (2012) showed that bicarbonate increased the concentration and release of malic acid by roots.

We hypothesize that root malic acid accumulation induced by bicarbonate is partially due to an inhibition exerted by bicarbonate on the root activity of MDH, as previously observed in *Pisum sativum* by Jelali et al. (2010). Inside the mitochondrion, this enzyme catalyzes the malate oxidation into oxaloacetate. We may speculate that if additional oxaloacetate is required by the increased CS activity in −Fe+ BIC plants, this compound would be imported from cytoplasm through di–tricarboxylate carriers (Vigani et al. 2009). Since no mitochondrial purification was performed, it is not possible to attribute such modification exclusively to mitochondrial MDH.

The interactions found between Fe and BIC factors indicate that bicarbonate level modulates the concentrations of tartrate, citrate and ascorbate in the roots of 140 Ruggeri rootstock as a response to Fe deficiency (Table 4). Iron deficiency increased citrate concentration in roots (Table 4) for both levels of bicarbonate in the nutrient solution, and in xylem sap (Table 5). This is a common response in many plant species and genotypes with a medium-high level of tolerance to iron chlorosis, and several authors suggested that citrate concentrations in roots could be used as a biochemical marker of Fe chlorosis tolerance level (Ollat et al. 2003; Rombolà et al. 2002). However, the differences in root citrate concentration between Fe levels recorded in plants grown in +BIC are lower than in −BIC conditions (Table 4). A similar effect of bicarbonate on citrate concentration between Fe levels has been reported by Ollat et al (2003) for the grapevine tolerant genotype Cabernet Sauvignon. However, opposite results have been observed for the susceptible genotype Riparia Gloire de Montpellier (Ollat et al. 2003), indicating that the effect of bicarbonate on the citrate root accumulation as a response to Fe deficiency is dependent on the genotype. In our experiment, Fe deficiency increased the concentration of tartaric acid and decreased ascorbic acid concentration in roots grown without bicarbonate (Table 4). In bicarbonate-fed plants, Fe deficiency did not modify the concentration of tartaric and ascorbic acids in roots. Tartaric acid was the major organic acid found in roots of 140 Ruggeri (Table 4). This organic acid is specific to *Vitis* species (Ruffner 1982), and is one of the main acids in berries. In the present experiment, Fe deficiency increased the concentration of tartaric acid in plants grown without bicarbonate (Table 4). An increase of tartaric acid as a response to Fe deficiency has been observed in the Fe chlorosis tolerant genotype Cabernet Sauvignon, whereas reverse effects were found in the Fe chlorosis sensitive genotype 101-14 and

Gloire de Montpellier (Ollat et al. 2003), suggesting that the increase of tartrate concentration in roots is a typical response of tolerant genotypes. In bicarbonate-fed plants, Fe deficiency did not modify the concentration of tartaric acid in roots. Ascorbic acid has been reported as a synthesis precursor of tartaric acid (DeBolt et al. 2007), therefore it is possible that the lower concentration of ascorbic acid recorded in −Fe-BIC roots as compared to +Fe-BIC, is due the conversion of ascorbate into tartrate through the tartaric acid synthesis pathway.

In conclusion, the present work shows physiological and biochemical mechanisms adopted by the Fe chlorosis tolerant rootstock 140 Ruggeri to Fe deficiency. The methodological approach adopted in this experiment allowed to demonstrating that the presence of bicarbonate in the nutrient solution modulates response mechanisms to Fe deficiency by shifting the activities of TCA related enzymes (CS and $NADP^+$-IDH) and the accumulation/translocation of organic acids in roots. In the absence of bicarbonate, Fe deficiency decreases the activity of CS and $NADP^+$-IDH increasing organic acid concentrations, particularly citrate and tartrate. In contrast, in bicarbonate-fed plants, Fe deficiency increases the activity of CS and $NADP^+$-IDH, without changing tartrate level and increasing citrate accumulation. Regardless of Fe level, bicarbonate depresses PEPC and MDH activities, favoring the accumulation of malic acid. Moreover, this genotype increased root biomass in response to high bicarbonate levels in the substrate. In addition, bicarbonate *per se* enhanced the leaf chlorophyll content. Further investigations are needed to clarify the metabolic fate of bicarbonate in the leaf apoplast and the possible implications of graft combinations.

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