

# Nitrogen nutrition influences some biochemical responses to iron deficiency in tolerant and sensitive genotypes of *Vitis*

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**Abstract** The effects of nitrogen source on iron deficiency responses were investigated in two *Vitis* genotypes, one tolerant to limestone chlorosis Cabernet Sauvignon (*Vitis vinifera* cv.) and the other susceptible Gloire de Montpellier (*Vitis riparia* cv.). Plants were grown with or without Fe(III)-EDTA, and with  $\text{NO}_3^-$  alone or a mixture of  $\text{NO}_3^-$  and  $\text{NH}_4^+$ . Changes in pH of the nutrient solution and root ferric chelate reductase (FC-R) activity were monitored over one week. We carried out quantitative metabolic profiling ( $^1\text{H-NMR}$ ) and determined the activity of enzymes involved in organic acid metabolism in root tips. In iron free-solutions, with  $\text{NO}_3^-$  as the sole nitrogen source, the typical Fe-deficiency response reactions as acidification of the growth medium and enhanced FC-R activity in the roots were observed only in the tolerant genotype. Under the same nutritional conditions, organic

acid accumulation (mainly citrate and malate) was found for both genotypes. In the presence of  $\text{NH}_4^+$ , the sensitive genotype displayed some decrease in pH of the growth medium and an increase in FC-R activity. For both genotypes, the presence of  $\text{NH}_4^+$  ions decreased significantly the organic acid content of roots. Both *Vitis* genotypes were able to take up  $\text{NH}_4^+$  from the nutrient solution, regardless of their sensitivity to iron deficiency. The presence of N- $\text{NH}_4^+$  modified typical Fe stress responses in tolerant and sensitive *Vitis* genotypes.

**Keywords** Ammonium · Ferric chelate reductase · Grapevine · Organic acids · Rhizosphere acidification · Rootstocks

## Abbreviations

2OG	2-oxoglutarate
BPDS	bathophenanthrolinedisulfonic acid disodium salt hydrate
BSA	bovine serum albumin
CoA	coenzyme A
DEA	diethanolamine
DTNB	5-5'-dithio-bis-2-nitrobenzoic acid
DTT	DL-dithiothreitol
EDTA	ethylenediaminetetracetic acid
ERETIC	electronic reference to access in vivo concentrations
FW	fresh weight
GOGAT	glutamate synthetase

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GS	glutamine synthetase
MDH	malate dehydrogenase
MES	2-( <i>N</i> -morpholino)ethanesulfonic acid
NMR	nuclear magnetic resonance
PEP	phosphoenolpyruvate
PEPC	phosphoenolpyruvate carboxylase
PMSF	phenylmethylsulfonyl fluoride
PVPP	polyvinylpyrrolidone
TCA	tricarboxylic acid
TEA	triethylamine
TSP	(trimethyl) propionic-2,3,3,3- <i>d</i> 4 acid sodium salt

## Introduction

Plants differ in susceptibility to iron (Fe) deficiency. Several perennial crops, including grapevine, are particularly susceptible when grown on calcareous and alkaline soils. The development of chlorosis symptoms in orchards and vineyards may severely reduce fruit yield and quality (Álvarez-Fernández et al. 2006). Among many parameters, the form of N present in the soil was shown to impact Fe nutrition and the development of chlorotic symptoms (Korcak 1987; Lucena 2000; Marschner 1995).

The adaptive response of so-called Strategy I plants, including grapevine, involves morphological and physiological changes in the roots (Andaluz et al. 2002; Bavaresco et al. 1991; Römheld 1987; Rombolà et al. 2002). These typical responses include proton extrusion, release of reducing or chelating substances, and increase in membrane-bound ferric chelate reductase activity (FC-R) in the roots, to reduce Fe<sup>III</sup> before its uptake via an Fe (II) transporter (Briat and Lobréaux 1997). Proton extrusion and root Fe<sup>III</sup> reducing capacities have been demonstrated for several *Vitis* genotypes, and shown to be related to their adaptation to Fe deficiency (Bavaresco et al. 1991; Brancadoro et al. 1995; Mengel and Malissiovas 1982; Nikolic et al. 2000; Varanini and Maggioni 1982).

Proton extrusion has been attributed to activation of the root plasma membrane H<sup>+</sup>-ATPase, based on the activity and steady-state levels of the

enzyme throughout the root (Dell'Orto et al. 2000b) and the co-localization of intense immunolabeling of the H<sup>+</sup>-ATPase protein and proton extrusion in the subapical root zones of Fe-deficient plants (Schmidt 2003). Acidification of the rhizosphere facilitates the mobilization of sparingly soluble ferric iron. Proton extrusion has been shown to be associated with an increase in Fe<sup>III</sup> reduction (Toulon et al. 1992), although the induction of root FC-R activity can be uncoupled from the acidification (Yi and Guerinot 1996). FC-R is highly sensitive to pH and is inhibited at high pH (Kosegarten et al. 2004; Nikolic et al. 2000; Susin et al. 1996).

Several metabolic changes have also been described in Fe-deficient roots, including the accumulation of organic acids, shifts in the redox state of the cytoplasm, and increases in the activities of phosphoenolpyruvate carboxylase (PEPC) and several enzymes of the Krebs cycle and of the glycolytic pathway (Agnolon et al. 2001; López-Millán et al. 2000a; McCluskey et al. 2004; Rombolà et al. 2002). Citrate, which accumulates in large amounts in roots under Fe deficiency, is thought to be beneficial for Fe nutrition, and has been linked to Fe transport, proton extrusion and the capacity to produce reducing power in the form of NADPH (Bienfait 1996; Brown and Tiffin 1965; Landsberg 1981). Organic acid accumulation has been reported as part of the grapevine response to Fe deficiency and the presence of bicarbonates (Ollat et al. 2003).

Most plants can make use of either ammonium or nitrate ions. The uptake of these two forms of nitrogen (N) is controlled by genotype, plant development and physiological status, and also by soil properties such as texture, structure, water content and pH (Lea and Morot-Gaudry 2001; Loulakakis and Roubelakis-Angelakis 2001). Ammonium uptake results in strong acidification of the rhizosphere, due to the excretion of protons via the H<sup>+</sup>-ATPase. In contrast, nitrate uptake is associated with the proton consumptions via 2H<sup>+</sup>/NO<sub>3</sub><sup>-</sup> symport, leading to an increase in the pH of the outer solution (Mengel and Kirkby 2001). Ammonium assimilation requires carbon skeletons in the form of keto acids, mainly tricarboxylic acid cycle intermediates. Nitrate

triggers a shift from starch biosynthesis to organic acid production (Foyer et al. 2003). The activity of key enzymes, such as PEPC, is modified by the form of nitrogen in the growth medium (Pasqualini et al. 2001). Little is known about the *Vitis* genotypes with respect to their different assimilation capacity for the forms of nitrogen. The expression of genes encoding various enzymes involved in ammonium assimilation, such as glutamate synthase, glutamine synthetase and glutamate dehydrogenase has been identified in grapevine roots (Loulakakis and Roubelakis-Angelakis 2001). Nitrate reductase activity has also been detected in roots and varied according to the seasonal root growth pattern (Hunter and Ruffner 1997).

Nitrogen is taken up almost exclusively as nitrate by the roots of plants growing in calcareous soil, in which ammonium is rapidly nitrified (Mengel 1994). The high nitrate levels of calcareous soils are thought to favor the development of Fe chlorosis (Korcak 1987; Tagliavini and Rombolà 2001). Several studies have demonstrated that nitrate can induce Fe-deficiency chlorosis in plants (Aktas and Van Egmond 1979; Kosegarten et al. 1998; Mengel et al. 1994; Smolders et al. 1997). The primary cause of Fe deficiency in  $\text{NO}_3^-$ -fed plants is the high root apoplastic pH as a consequence of the removal of protons during  $\text{H}^+/\text{NO}_3^-$  cotransport, which impairs Fe uptake by the roots, most probably by inhibiting  $\text{Fe}^{\text{III}}$  reduction (Kosegarten et al. 2004; Nikolic and Römheld 2003). Aktas and Van Egmond (1979) studied the effect of nitrogen fertilization on *Glycine max* L. genotypes with different susceptibilities to Fe chlorosis. They showed that increasing the amount of nitrate supplied to plants growing in calcareous soils worsened the symptoms of chlorosis in Fe-inefficient cultivars, but increased the growth of Fe-efficient cultivars. They also suggested that the Fe-inefficient cultivar was unable to balance the large increase in rhizosphere pH resulting from very active  $\text{NO}_3^-$  uptake.

The aim of this work was to study the combined effects of Fe and the form of nitrogen on some Strategy I responses and root metabolism of two *Vitis* cultivars, one considered tolerant and the other susceptible to Fe deficiency.

## Materials and methods

### Plant material

Micropropagated plants of the Fe chlorosis tolerant genotype *Vitis vinifera* cv. Cabernet Sauvignon (CS) (Brancadoro et al. 1995; Dell'Orto et al. 2000a; Tagliavini and Rombolà 2001) and the Fe chlorosis-susceptible genotype *Vitis riparia* cv. Gloire de Montpellier (RG) (Brancadoro et al. 1995; Nikolic et al. 2000) were acclimated in perlite for 3 weeks. The plants were then transferred to 10-l plastic containers filled with 8-l of a continuously aerated nutrient solution, with 20 plants per container. The growth chamber was programmed for a 16 h photoperiod ( $300 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) at 25°C and 8 h of darkness at 20°C, with 75% relative humidity. The two genotypes were grown separately throughout the experiment. The nutrient solutions contained either nitrate as the only nitrogen source [ $\text{NO}_3^-$ ] or both ammonium and nitrate [ $\text{NH}_4^+/\text{NO}_3^-$ ]. The macronutrient composition of the half-strength Hoagland solution [ $\text{NO}_3^-$ ] was 2.5 mM  $\text{KNO}_3$ , 2.5 mM  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 1 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1 mM  $\text{KH}_2\text{PO}_4$  and that of the [ $\text{NH}_4^+/\text{NO}_3^-$ ] solution was 1 mM  $\text{NH}_4\text{NO}_3$ , 1 mM  $\text{KNO}_3$ , 2.5 mM  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 0.87 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 mM  $\text{K}_2\text{SO}_4$ , 1 mM  $\text{KH}_2\text{PO}_4$ . Nitrogen concentrations have been set according to Rodriguez-Lovelle and Gaudillère (2002), in order to maintain ion equilibrium in both conditions. The two solutions had identical micronutrient compositions: 9.1  $\mu\text{M}$   $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 46.3  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ , 2.4  $\mu\text{M}$   $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$ , 0.5  $\mu\text{M}$   $\text{CuSO}_4$ , 0.013  $\mu\text{M}$   $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ . Iron was supplied in the form of 90  $\mu\text{M}$  Fe(III)-EDTA. After 4 days, two thirds of the plants for each genotype and nitrogen source were transferred to Fe-free solutions [-Fe]. The rest of the plants were maintained in the solution containing 90  $\mu\text{M}$  Fe(III)-EDTA [+Fe]. There were therefore four different nutrient solutions for each genotype: [+Fe $\text{NH}_4^+/\text{NO}_3^-$ ], [-Fe $\text{NH}_4^+/\text{NO}_3^-$ ], [+Fe $\text{NO}_3^-$ ], [-Fe $\text{NO}_3^-$ ]. The day on which plants were transferred to Fe-free solution was counted as day 0 of the experiment. The plants were grown under these conditions for one week. The pH of the nutrient solutions was adjusted to 6

at day 0. The pH changes of the medium by roots was monitored daily.

#### In vivo root Fe(III)-EDTA reduction by intact plants

The root FC-R (EC 1.16.1.7) activity of whole plants was determined by monitoring the formation of the Fe(II)-disulfonic acid BPDS<sub>3</sub> complex from Fe(III)-EDTA (Bienfait et al. 1983). Individual plants were transferred to 50-ml plastic beakers (covered with black tape to exclude light) containing 46 ml of 300 μM BPDS (Sigma), 10 mM MES, pH 6.0, as described by Gogorcena et al. (2000). Measurements were made in the growth chamber, under illumination (300 μmol photons m<sup>-2</sup> s<sup>-1</sup>). The buffer solution was continually aerated by means of plastic tubing. Once the plants were placed in the beaker, 1 ml of Fe(III)-EDTA was added from a stock solution, to give a final concentration of 500 μM. After keeping the final solution for one hour under light exclusion, a 1 ml aliquot was removed from each beaker to measure the absorbance at 535 nm. An extinction coefficient of 22.14 mM<sup>-1</sup> cm<sup>-1</sup> was used to calculate the concentration of the Fe(II)-BPDS<sub>3</sub> complex.

#### Determination of plant parameters and root sample collection

After FC-R determination, the plants were used to measure the length of the stem and the fresh weights of the stems and roots. The chlorophyll content of the leaves was determined, using a SPAD 502 chlorophyll meter (Minolta Co., Osaka Japan). Root tip samples (20–30 mm long) were taken from each plant assessed for FC-R activity, rinsed in deionized water, weighed, deep-frozen in liquid nitrogen, and kept at –80°C for metabolic profiling and enzyme activity determination.

#### Metabolic profiles obtained by <sup>1</sup>H-NMR spectroscopy on root tips

Root tips were freeze-dried, weighed and crushed. Polar compounds were extracted by aqueous ethanol at 80°C, in three incubation steps, each lasting 20 min (step 1: 0.75 ml 80%

ethanol; steps 2 and 3: 0.75 ml 50% ethanol) and then centrifuged for 10 min at 4,800g. Slurries were pooled. The ethanol was allowed to evaporate and the dry extracts were solubilized in 1 ml 200 mM oxalate buffer to maintain the pH of the extracts at 4.0. To improve spectrum resolution and to eliminate paramagnetic ions the extracts were further purified on 200 mg of Chelex 100 resin (BioRad, Marnes-la-Coquette) in oxalate buffer (pH = 4.0). The resin was rinsed three times with 1 ml double-distilled water. The pH of each extract was checked after this step. The extracts were lyophilized, solubilized in 500 μl D<sub>2</sub>O and lyophilized again to eliminate residual water. The dried extracts were stored in a dry atmosphere until <sup>1</sup>H-NMR analysis.

Dried root extracts were solubilized in 500 μl D<sub>2</sub>O, followed by the addition of the sodium salt of (trimethyl) propionic-2,3,3,3-*d*4 acid (TSP) in D<sub>2</sub>O to a final concentration of 0.01%, for chemical shift calibration. The mixture was transferred to an NMR tube and <sup>1</sup>H-NMR spectra were recorded as previously described (Moing et al. 2004) at 500.162 MHz and 300 K on a Bruker Avance spectrometer (Wissenbourg, France), using a 5-mm dual probe. We acquired 64 scans of 64 K data points with a spectral width of 6,000 Hz and an acquisition time of 2.73 s. The recycle delay was 15 s. The ERETIC method was used to determine absolute concentrations of metabolites (Akoka et al. 1999), using calibration curves for C1-H(α + β) glucose.

#### Enzyme extraction

The enzymes assayed included 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 1.1.1.42).

Extracts for enzyme assays were prepared by grinding the root material in liquid nitrogen in a mortar with 50% PVPP (w/w FW) and 1 ml of extraction buffer containing 400 mM Tricine (pH 7.6), 5 mM MgSO<sub>4</sub>, 0.25 mM EDTA, 10% (v/v) glycerol, 0.5% (w/v) BSA, 5 mM NaHCO<sub>3</sub>, 2 mM PMSF, 10 mM sodium ascorbate and 1% (v/v) Triton. The slurry was filtered through glass wool and the filtrate was desalted on a Sephadex G-25

column (equilibrated with extraction buffer). The desalted extracts were centrifuged at 1,400g for 2 min (4°C) and used for enzyme activity assays directly, or after a short period of storage (less than 2 h) in liquid N<sub>2</sub>.

#### Enzyme assays

Phosphoenolpyruvate carboxylase was determined by coupling its activity to malate dehydrogenase-catalyzed NADH oxidation (Vance et al. 1983) with 25 µl of extract in 1 ml of 5 mM NaHCO<sub>3</sub>, 0.2 mM NADH, 2 mM DTT, 3 units ml<sup>-1</sup> MDH (Sigma), 2.2 mM PEP, 2.5 mM MgSO<sub>4</sub>, 0.25 mM EDTA and 100 mM tricine, pH 8.1. Nicotinamide adenine dinucleotide (NADH) consumption was determined spectrophotometrically by monitoring the decrease in absorbance at 340 nm, at 25°C. Malate dehydrogenase activity was determined by monitoring the increase in absorbance at 340 nm, due to the enzymatic reduction of NAD<sup>+</sup> at 25°C (Smith 1974). The reaction was carried out with 5 µl of extract in 1 ml of 3 mM NAD<sup>+</sup>, 29 mM malate, 5 mM MgCl<sub>2</sub> and 100 mM DEA buffer, pH 9.2. Citrate synthase activity was assayed by monitoring the reduction of acetyl CoA to CoA with DTNB at 412 nm and 25°C (Srere 1967). The reaction mixture contained 25 µl of extract in 1 ml of 0.4 mM acetyl CoA, 0.1 mM DTNB, 0.5 mM oxalacetate and 200 mM Tris-HCl, pH 8.1. Isocitrate dehydrogenase was assayed spectrophotometrically, as described by Goldberg and Ellis (1974), by monitoring the reduction of NADP<sup>+</sup> at 340 nm and 37°C. The reaction was carried out with 25 µl of extract in 1 ml of 3.9 mM MgCl<sub>2</sub>, 0.42 mM NADP<sup>+</sup>, 6.7 mM sodium isocitrate and 100 mM TEA buffer, pH 7.3.

#### Statistical analysis

Each set of data were analyzed by a two-way (Fe × N) analysis of variance (MANOVA) with SPSS software version 12.0 (SPSS, Inc., Chicago, USA). Analyses were performed on raw data or Ln-transformed data, to maximize variance homogeneity. If a significant result was obtained for the *F*-test, means were compared using the Newman and Keuls test ( $p \leq 0.05$ ).

## Results

### Plant growth and appearance of leaf chlorosis symptoms

After one week of Fe deprivation, leaf chlorophyll content was significantly reduced in both genotypes, and plants displayed initial symptoms of Fe chlorosis (Table 1). At this stage, CS plants displayed significantly higher rates of root and shoot growth under [NH<sub>4</sub>/NO<sub>3</sub>] treatment than under [NO<sub>3</sub>] treatment. The shoot growth of CS plants was reduced by Fe deficiency if NO<sub>3</sub><sup>-</sup> was used as the sole nitrogen source. The root and shoot growth was unaffected by any treatment in RG plants.

### Nutrient solution acidification

When CS plants were grown without Fe, the pH of the nutrient solutions quickly decreased (Fig. 1). The pH reached 3.5 after 5 days for [NH<sub>4</sub>/NO<sub>3</sub>] and after 7 days for [NO<sub>3</sub>]. In the presence of Fe, the pH of the [NH<sub>4</sub>/NO<sub>3</sub>] solution decreased slightly, whereas the [NO<sub>3</sub>] treatment had the opposite effect. For RG plants, the pH of the Fe-free [NH<sub>4</sub>/NO<sub>3</sub>] solution decreased to 4.9 by 7 days. In all other treatments, the pH remained close to 6.

### Root FC-R (ferric chelate reductase) activity

Root FC-R activities of CS and RG plants were measured after 3, 5 and 7 days (Fig. 2).

For CS plants, the FC-R activity of [+Fe] plants remained low and steady during the growing period whereas that of [-Fe] plants increased significantly by days 5 and 7, under both nitrogen treatments. The Fe effect was significant ( $p < 0.05$ ) on day 5 and highly significant ( $p < 0.01$ ) on day 7. At this stage, the presence of NH<sub>4</sub><sup>+</sup> in the nutrient solution significantly decreased FC-R activity and the interaction with Fe treatments was highly significant. For RG plants, the elimination of Fe from the growth medium had a significant positive effect on FC-R activity ( $p < 0.05$ ) on day 7 only. The highest levels of activity were recorded for the [-FeNH<sub>4</sub>/NO<sub>3</sub>] treatment at this time point. FC-R activity

**Table 1** Plant growth parameters and SPAD values of the second fully developed leaf from apex for two grapevine genotypes, one chlorosis tolerant (CS) and the other chlorosis susceptible (RG), grown during 7 days with 0  $\mu\text{M}$

Fe(III)-EDTA [-Fe] or 90  $\mu\text{M}$  Fe(III)-EDTA [+Fe], and with  $\text{NO}_3^-$  as the only source of nitrogen [ $\text{NO}_3^-$ ] or with a mixed  $\text{NH}_4^+/\text{NO}_3^-$  supply [ $\text{NH}_4/\text{NO}_3^-$ ]

Genotype	N source	Fe treatment	Root fresh weight (g)	Shoot fresh weight (g)	SPAD 2nd leaf	
CS	[ $\text{NO}_3^-$ ]	+Fe	0.91 $\pm$ 0.10	1.58 $\pm$ 0.10	28.6 $\pm$ 0.9	
		-Fe	0.63 $\pm$ 0.10	0.87 $\pm$ 0.13	21.1 $\pm$ 0.7	
	[ $\text{NH}_4/\text{NO}_3^-$ ]	+Fe	1.06 $\pm$ 0.15	1.61 $\pm$ 0.18	29.5 $\pm$ 0.9	
		-Fe	1.21 $\pm$ 0.22	1.60 $\pm$ 0.18	23.3 $\pm$ 0.2	
	<i>Significance</i> <sup>a</sup>					
	N source			*	*	NS
Fe treatment			NS	*	**	
N source $\times$ Fe treatment			NS	*	NS	
RG	[ $\text{NO}_3^-$ ]	+Fe	0.51 $\pm$ 0.07	0.97 $\pm$ 0.07	21.5 $\pm$ 1.1	
		-Fe	0.49 $\pm$ 0.18	0.88 $\pm$ 0.27	18.5 $\pm$ 1.3	
	[ $\text{NH}_4/\text{NO}_3^-$ ]	+Fe	0.57 $\pm$ 0.08	0.93 $\pm$ 0.12	25.1 $\pm$ 0.9	
		-Fe	0.43 $\pm$ 0.05	0.77 $\pm$ 0.13	19.1 $\pm$ 0.1	
	<i>Significance</i> <sup>a</sup>					
	N source			NS	NS	NS
Fe treatment			NS	NS	*	
N source $\times$ Fe treatment			NS	NS	NS	

Data are mean  $\pm$  SE of three replicates

<sup>a</sup> MANOVA analysis (Fe treatment  $\times$  N source) was performed for a linear model, on raw data. Significance: \*\*  $p < 0.01$ ; \*  $p < 0.05$ ; NS, not significant

for this treatment was two times higher than for the corresponding [+Fe] treatment. However, the interaction between nitrogen and Fe treatments was not significant.

#### <sup>1</sup>H-NMR metabolic profiling of root tips

<sup>1</sup>H-NMR metabolic profiles of root tip extracts were obtained after 6 days of Fe deficiency. We identified and quantified 17 compounds in root tip extracts, including two sugars, six organic acids and eight amino acids. The major compounds identified in root tip extracts were organic acids, primarily malate and citrate (Table 2). Glutamine and glutamate, which could not be quantified separately, were the main amino acids identified in the root tips (Table 3). Data for the rest of compounds are not shown.

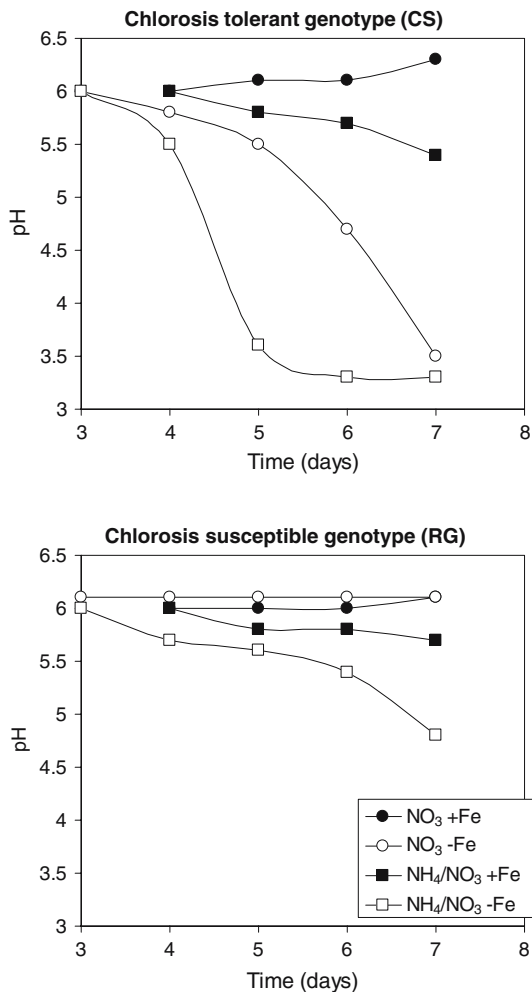
MANOVA analysis of organic acid content showed that Fe treatment significantly increased the concentration of root organic acids other than fumarate for both genotypes (Table 2). Citrate predominated in [-Fe] CS roots, whereas malate was the main organic acid in RG roots. Nitrogen source had a significant effect on malate, citrate

and succinate concentrations in CS roots, and on malate and fumarate in RG roots. The presence of  $\text{NH}_4^+$  decreased organic acid concentration in the roots of both genotypes. The concentration of amino acids in the roots was influenced by the form of N, but not by Fe status (Table 3). With the exception of alanine in RG plants, the presence of  $\text{NH}_4^+$  in the nutrient solution increased significantly amino acid levels.

#### Enzyme activities in root extracts

The activities of some enzymes involved in organic acid metabolism were measured in the root tips of the plants from all treatments after 6 days of Fe starvation (Table 4).

Fe deficiency induced significant increase in PEPC activity in both genotypes. In RG plants, MDH and citrate synthase activities were stimulated by Fe deficiency. Fe status did not affect these activities in CS plants. Regardless of the genotype, there was no nitrogen effect on these enzymatic activities. In RG plants, Fe deficiency significantly decreased NADP<sup>+</sup>-IDH activity in  $\text{NH}_4^+$ -fed plants whereas it had an opposite effect



**Fig. 1** Changes in the pH of the medium (8-l containers with 20 plants in each) for two grapevine genotypes, one chlorosis tolerant (CS) and the other chlorosis susceptible (RG), during the first week of growth in a nutrient solution containing 0  $\mu\text{M}$  Fe(III)-EDTA [-Fe] or 90  $\mu\text{M}$  Fe(III)-EDTA [+Fe] and with  $\text{NO}_3^-$  as the only source of nitrogen [ $\text{NO}_3^-$ ] or a mixed  $\text{NH}_4^+/\text{NO}_3^-$  supply [ $\text{NH}_4^+/\text{NO}_3^-$ ]. The nutrient solution was not replenished during this monitoring

in  $\text{NO}_3^-$ -treated plants. No significant effect on NADP<sup>+</sup>-IDH activity was demonstrated for CS plants.

## Discussion

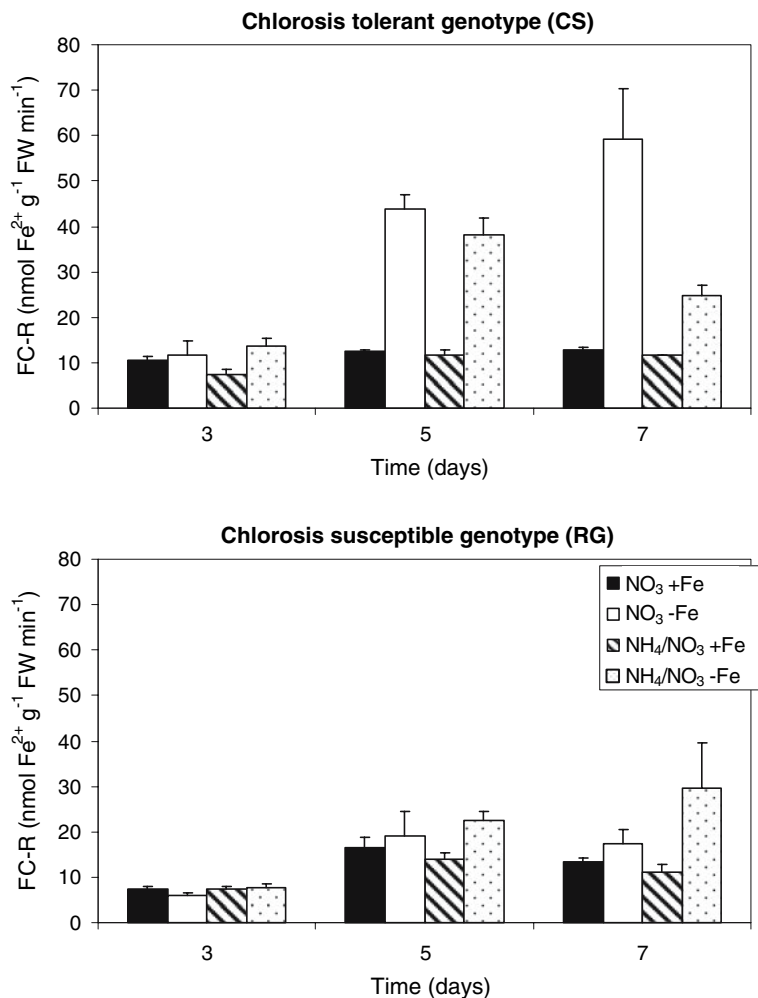
After one week of Fe starvation, the chlorophyll content of the leaves, as indicated by SPAD readings, had significantly decreased for both

genotypes. The shoot growth of the chlorosis tolerant genotype CS plants was significantly decreased by the lack of Fe, whereas root growth was not. Similar increase in root-to-shoot ratio has been previously reported for grapevine (Gruber and Kosegarten 2002) and peach (Shi et al. 1993) under conditions of Fe deficiency. Indeed, in grapevine, the shoots appear to be more sensitive to Fe deficiency regarding dry matter increment than the roots. However, some species, such as *Beta vulgaris* L., display the opposite pattern (Rombolà et al. 2005). Shoot and root growth of chlorosis-susceptible genotype did not respond to Fe deficiency nor to nitrogen source. The low growth rate registered for this genotype may partly explain this lack of response.

Acidification of the rhizosphere is known to be part of the mechanism by which some dicotyledonous plants respond to Fe starvation. This acidification has been attributed to activation of the root plasma membrane  $\text{H}^+$ -ATPase (Dell'Orto et al. 2000b). Ammonium uptake also results in strong rhizosphere acidification whereas  $\text{NO}_3^-$  uptake increases the pH of the outer solution (Mengel and Kirkby 2001). Under our growing conditions, the pH of the medium depended on both mechanisms: response to Fe deficiency and nitrogen uptake. The chlorosis-tolerant genotype plants displayed high capacity of acidification when grown without Fe, regardless of the nitrogen source, indicating a marked response to Fe deficiency. The pH decreased more rapidly if  $\text{NH}_4^+$  was present in the nutrient solution. A slight decrease in pH was also observed when plants were supplied with Fe, indicating that active  $\text{NH}_4^+$  uptake occurred. For the chlorosis-susceptible genotype Riparia Gloire de Montpellier plants fed with  $\text{NH}_4^+$ , the pH reached 4.9 after 7 days of Fe deficiency, whereas no decrease was observed for plants supplied with  $\text{NO}_3^-$ . The changes in pH depended primarily on nitrogen source for this genotype.

As previously demonstrated (Brancadoro et al. 1995), medium acidification and root  $\text{Fe}^{\text{III}}$  reducing capacities for the two *Vitis* genotypes studied here are related to their adaptation to Fe deficiency. However, when supplied with  $\text{NH}_4^+$  under Fe-deficiency conditions, both genotypes displayed unexpected responses. The root FC-R

**Fig. 2** Time course of root ferric chelate reductase (FC-R, nmol  $\text{Fe}^{2+} \text{g}^{-1} \text{FW min}^{-1}$ ) activity for whole plants of two grapevine genotypes, one chlorosis tolerant (CS) and the other chlorosis susceptible (RG), plants growing in nutrient solution containing 0  $\mu\text{M}$   $\text{Fe(III)-EDTA}$  [-Fe] or 90  $\mu\text{M}$   $\text{Fe(III)-EDTA}$  [+Fe], and with  $\text{NO}_3^-$  as the only source of nitrogen [ $\text{NO}_3$ ] or a mixed  $\text{NH}_4^+/\text{NO}_3^-$  supply [ $\text{NH}_4/\text{NO}_3$ ]. Values were obtained after 3, 5, 7 days of Fe depletion. Data are means  $\pm$  SE of three replicates



activity of the chlorosis-tolerant genotype was significantly reduced. On the opposite the susceptible one was able to acidify the nutrient solution and to increase FC-R activity. Kosegarten et al. (2004) and Nikolic and Römheld (2003) demonstrated close relationships between the form of nitrogen in the nutrient solution, root apoplast pH and FC-R activity in *Helianthus annuus* L. Based on these relationships, the stimulation of FC-R activity observed for the susceptible genotype may be linked to the decrease in pH associated with  $\text{NH}_4^+$  uptake. Nevertheless, our results for the chlorosis tolerant genotype are not in agreement with the expected effect of various nitrogen forms on  $\text{Fe}^{\text{III}}$  reduction. The very low pH of the nutrient solution associated to  $\text{NH}_4^+$  uptake may have increased the solubility of sparingly soluble

Fe compounds in the root apoplast resulting in lower FC-R activity under these conditions (Gogorcena et al. 2001).

The known differences in susceptibility to Fe deficiency of the two *Vitis* genotypes studied here may therefore be related to their capacity to induce FC-R, and may also depend on the regulation of apoplastic pH, as suggested by Kosegarten et al. (2004). The transcriptional regulation of a specific isoform of  $\text{H}^+$ -ATPase in response to Fe deficiency was recently demonstrated in *Cucumis sativus* L. (Santi et al. 2005). However, as root apoplastic pH was not determined in our work and using MES could have affected FC-R response to in vivo apoplastic pH, further work is required to investigate this relationship in different *Vitis* genotypes.



**Table 2** Concentration of the main organic acids (mg g<sup>-1</sup> DW), determined by <sup>1</sup>H-NMR spectrophotometry, in root tip extracts from two grapevine genotypes, one chlorosis tolerant (CS) and the other chlorosis susceptible (RG), after 6 days of growth in nutrient solution containing 0 μM Fe(III)-EDTA [-Fe] or 90 μM Fe(III)-EDTA [+Fe], with NO<sub>3</sub><sup>-</sup> as the only source of nitrogen [NO<sub>3</sub>] or with a mixed NH<sub>4</sub><sup>+</sup>/NO<sub>3</sub><sup>-</sup> supply [NH<sub>4</sub>/NO<sub>3</sub>]

Genotype	N source	Fe treatment	Malate	Citrate	Succinate	Fumarate	
CS	[NO <sub>3</sub> ]	+Fe	16.8 ± 6.0	15.5 ± 2.3	0.6 ± 0.2	0.15 ± 0.04	
		-Fe	43.9 ± 13.7	61.1 ± 17.8	1.4 ± 0.7	0.19 ± 0.08	
	[NH <sub>4</sub> /NO <sub>3</sub> ]	+Fe	8.3 ± 2.6	4.8 ± 1.0	0.3 ± 0.1	0.11 ± 0.02	
		-Fe	15.5 ± 20.1	32.5 ± 15.9	0.6 ± 0.8	0.11 ± 0.14	
	<i>Significance</i> <sup>a</sup>						
	N source			*	*	*	NS
	Fe treatment			*	*	**	NS
N source × Fe treatment			NS	NS	NS	NS	
RG	[NO <sub>3</sub> ]	+Fe	16.6 ± 3.4	10.6 ± 6.0	0.5 ± 0.1	0.22 ± 0.07	
		-Fe	44.2 ± 5.7	25.9 ± 5.8	1.9 ± 0.3	0.33 ± 0.07	
	[NH <sub>4</sub> /NO <sub>3</sub> ]	+Fe	11.9 ± 6.1	7.1 ± 5.2	0.4 ± 0.2	0.17 ± 0.07	
		-Fe	23.7 ± 9.2	26.9 ± 15.4	1.3 ± 0.4	0.17 ± 0.08	
	<i>Significance</i> <sup>a</sup>						
	N source			**	NS	NS	*
	Fe treatment			**	*	**	NS
N source × Fe treatment			NS	NS	NS	NS	

Data are means ± SE of three replicates

<sup>a</sup> MANOVA analysis (Fe treatment × N source) was performed for a linear model, on raw data. Significance: \*\*  $p < 0.01$ ; \*  $p < 0.05$ ; NS, not significant

**Table 3** Concentration of the main amino acids (mg g<sup>-1</sup> DW), determined by <sup>1</sup>H-NMR spectrophotometry, in root tip extracts from two grapevine genotypes, one chlorosis tolerant (CS) and the other chlorosis susceptible (RG), after 6 days of growth in nutrient solution containing 0 μM Fe(III)-EDTA [-Fe] or 90 μM Fe(III)-EDTA [+Fe], with NO<sub>3</sub><sup>-</sup> as the only source of nitrogen [NO<sub>3</sub>] or with a mixed NH<sub>4</sub><sup>+</sup>/NO<sub>3</sub><sup>-</sup> supply [NH<sub>4</sub>/NO<sub>3</sub>]

Genotype	N source	Fe treatment	Alanine	Histidine	Glutamine + Glutamate	
CS	[NO <sub>3</sub> ]	+Fe	0.30 ± 0.08	0.29 ± 0.13	3.0 ± 1.0	
		-Fe	0.20 ± 0.08	0.08 ± 0.02	1.7 ± 0.3	
	[NH <sub>4</sub> /NO <sub>3</sub> ]	+Fe	0.37 ± 0.08	0.43 ± 0.39	12.8 ± 4.9	
		-Fe	0.61 ± 0.24	0.59 ± 0.30	14.6 ± 6.4	
	<i>Significance</i> <sup>a</sup>					
	N source			*	**	**
	Fe treatment			NS	NS	NS
N source × Fe treatment			NS	NS	NS	
RG	[NO <sub>3</sub> ]	+Fe	0.12 ± 0.05	0.12 ± 0.01	2.2 ± 0.5	
		-Fe	0.25 ± 0.09	0.10 ± 0.06	1.8 ± 0.6	
	[NH <sub>4</sub> /NO <sub>3</sub> ]	+Fe	0.19 ± 0.06	0.19 ± 0.04	6.3 ± 2.2	
		-Fe	1.43 ± 1.09	0.18 ± 0.05	6.4 ± 3.3	
	<i>Significance</i> <sup>a</sup>					
	N source			NS	*	**
	Fe treatment			NS	NS	NS
N source × Fe treatment			NS	NS	NS	

Data are means ± SE of three replicates

<sup>a</sup> MANOVA analysis (Fe treatment × N source) was performed for a linear model, on raw data. Significance: \*\*  $p < 0.01$ ; \*  $p < 0.05$ ; NS, not significant

Among the 17 metabolites quantified simultaneously in the roots with <sup>1</sup>H-NMR analysis, organic acid accumulated in larger amount in

response to Fe deficiency, confirming previous results obtained with other methodologies for grapevine (Ollat et al. 2003), other woody species

**Table 4** Activities ( $\text{nmol mg}^{-1} \text{FW min}^{-1}$ ) of phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MDH), citrate synthase, and isocitrate dehydrogenase NADP<sup>+</sup>-dependent (NADP<sup>+</sup>-IDH) measured under optimal conditions, in root tip extracts of two grapevine genotypes, one chlorosis tolerant (CS) and the

other chlorosis susceptible (RG), grown in nutrient solutions containing 0  $\mu\text{M}$  Fe(III)-EDTA [-Fe] or 90  $\mu\text{M}$  Fe(III)-EDTA [+Fe], with  $\text{NO}_3^-$  as the only source of nitrogen [ $\text{NO}_3^-$ ] or with a mixed  $\text{NH}_4^+/\text{NO}_3^-$  supply [ $\text{NH}_4^+/\text{NO}_3^-$ ]

Genotype	N source	Fe treatment	PEPC	MDH	Citrate synthase	NADP <sup>+</sup> -IDH
CS	[ $\text{NO}_3^-$ ]	+Fe	0.81 ± 0.18	28.10 ± 2.73	0.82 ± 0.24	1.40 ± 0.23
		-Fe	2.26 ± 0.72	27.99 ± 3.30	0.88 ± 0.14	1.44 ± 0.26
	[ $\text{NH}_4^+/\text{NO}_3^-$ ]	+Fe	0.76 ± 0.22	24.54 ± 3.33	0.90 ± 0.19	3.44 ± 1.55
		-Fe	2.33 ± 1.29	26.01 ± 1.89	1.08 ± 0.21	1.24 ± 0.20
	<i>Significance</i> <sup>a</sup>					
			NS	NS	NS	NS
			**	NS	NS	NS
			NS	NS	NS	NS
RG	[ $\text{NO}_3^-$ ]	+Fe	0.55 ± 0.25	23.46 ± 5.26	0.51 ± 0.09	0.67 ± 0.11
		-Fe	1.37 ± 0.50	32.34 ± 5.53	0.94 ± 0.14	1.37 ± 0.03
	[ $\text{NH}_4^+/\text{NO}_3^-$ ]	+Fe	0.88 ± 0.26	29.55 ± 3.65	0.72 ± 0.11	2.79 ± 0.35
		-Fe	1.83 ± 0.69	36.11 ± 4.09	0.74 ± 0.21	0.92 ± 0.34
	<i>Significance</i> <sup>a</sup>					
			NS	NS	NS	**
			*	*	*	**
			NS	NS	*	**

Activities were measured after 6 days of culture. Data are means ± SE of three replicates

<sup>a</sup> MANOVA analysis (Fe treatment × N source) was performed for a linear model, on raw data. Significance: \*\*  $p < 0.01$ ; \*  $p < 0.05$ ; NS, not significant

(Rombolà et al. 2002; Sun et al. 1987) and herbaceous plants (Abadía et al. 2002). Similar increase in malate concentrations was found in both genotypes. The chlorosis-tolerant genotype CS was characterized by a very high citrate concentration in the root tips and a ratio of citrate concentration under -Fe conditions to citrate concentration under +Fe conditions varying from 4 to 7. These data suggest that citrate could be used as biochemical marker of Fe chlorosis tolerance in some species, as suggested by Ollat et al. (2003) and Rombolà et al. (2002).

For both genotypes, the changes in root organic acid composition under Fe-deficiency conditions were associated with an increase in maximal PEPC activity. This difference was highly significant for the chlorosis tolerant one. Root tip citrate content and PEPC activity measured in vitro were significantly related (citrate content =  $4.3 e^{1.03 \text{PEPC activity}}$ ,  $r^2 = 0.74$ ,  $n = 8$ ,  $p < 0.01$ ). This stimulation of PEPC activity under Fe-deficiency conditions appears to be a general feature (Andaluz et al. 2002; Landsberg 1986; López-Millán et al. 2000b; Nisi and Zocchi

2000; Rombolà et al. 2005) consistent with the increase in  $\text{CO}_2$  fixation in the roots and the increase in organic acid concentration. In the absence of Fe, the chlorosis-susceptible genotype Riparia Gloire de Montpellier presented significantly higher MDH and citrate synthase activities. The stimulation of citrate synthase activity under Fe-deficiency conditions was not reported previously by McCluskey et al. (2004) and Rombolà et al. (2002).

These results suggest that PEPC activity may be one of the limiting steps for citrate accumulation. Other activities, such as MDH and citrate synthase activities, are probably in excess of the normal cellular requirements (Delhaize et al. 2003). Other factors, such as reducing power and compartmentalization of substrates and products, may control the activities of these enzymes in vivo. Export to the external medium and to the xylem sap must also be taken into account.

Organic acid and amino acid contents were significantly affected by nitrogen source. The effect on citrate content was significant for CS only, whereas the effect on malate content was

significant for both genotypes. Citrate and malate contents were lower in the presence of  $\text{NH}_4^+$  than in its absence. Amino acid content was also significantly higher in the presence of  $\text{NH}_4^+$ . The roots of CS plants contained six times higher concentrations of glutamate and glutamine when  $\text{NH}_4^+$  was added to the nutrient solution.

Amino acid biosynthesis requires the allocation of assimilated carbon—2-oxoglutarate (2-OG) in particular—for ammonia incorporation, and the production of C skeletons by the TCA cycle for amino acid synthesis downstream from the GS/GOGAT pathway (von Wirén et al. 2001). Ammonium assimilation may direct more organic acids to the GS/GOGAT pathway, resulting in lower levels of accumulation in the roots (Pasqualini et al. 2001) and an increase in carbon flow through the TCA cycle. In our study, the lower organic acid concentration in the root tips of  $\text{NH}_4^+$ -fed plants probably resulted from the use of these compounds to produce 2-OG for  $\text{NH}_4^+$  assimilation.

Although grapevine rootstocks are known to display different responses to N fertilization under field and pot trial conditions (Keller et al. 2001; Zerihun and Treeby 2002), their capacity to assimilate different forms of N forms has been little investigated. Our data suggest that both *Vitis* genotypes can take up  $\text{NH}_4^+$  from the nutrient solution. Significantly higher growth rate of CS plants supplied with  $\text{NH}_4^+$ , the slight acidification of the  $[\text{+FeNH}_4/\text{NO}_3]$  nutrient solution observed with this genotype, and high root amino acid concentration recorded for both genotypes may illustrate the ability to assimilate this form of nitrogen.

The interaction between Fe treatment and N source was highly significant for NADP<sup>+</sup>-IDH activity in Riparia Gloire de Montpellier. This enzyme has several roles in cells, depending on the particular isoform considered and its subcellular distribution (Hodges et al. 2003). This enzyme is determinant for production of the reducing power (Hodges et al. 2003; López-Millán et al. 2000b; McCluskey et al. 2004) required for FC-R activity, as previously suggested by Bienfait (1996). It is also thought to be involved in the production of 2-OG (Suárez et al. 2002). In our study, the interaction identified for NADP<sup>+</sup>-IDH activity may be the expression of these two roles.

Our results show that nitrogen uptake and metabolism interfere with Fe metabolism in *Vitis* roots in a complex manner. The chlorosis-tolerant genotype *Vitis vinifera* cv. CS showed medium acidification, the induction of root FC-R activity and citrate accumulation in the roots when subjected to Fe deficiency. The acidification of the nutrient solution resulting from the ammonium uptake may, at least partly, account for the unexpected stimulation of FC-R activity in the genotype *Vitis riparia* cv. Gloire de Montpellier, which is known to be chlorosis susceptible. Our results confirm that citrate is a biochemical marker of Fe deficiency and that PEPC activity limits the accumulation of this compound. However, the assimilation of  $\text{NH}_4^+$  also interfered with organic acid accumulation in roots, particularly in CS plants, limiting citrate accumulation. Ammonium in the medium changes some typical biochemical responses to Fe deficiency for both tolerant and susceptible cultivars, and this cation should therefore be avoided in screening methods based on nutrient solution experiments.

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