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 NO_3^- alone or a mixture of NO_3^- and 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}H-NMR) and determined the activity of enzymes involved in organic acid metabolism in root tips. In iron free-solutions, with 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 $NH4^+$, 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 NH_4^+ ions decreased significantly the organic acid content of roots. Both *Vitis* genotypes were able to take up NH_4^+ from the nutrient solution, regardless of their sensitivity to iron deficiency. The presence of N-NH_4^+ modified typical Fe stress responses in tolerant and sensitive *Vitis* genotypes.

*Abbreviations***:** 2-OG, 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; GS, glutamine synthetase; MDH, malate dehydrogenase; MES, 2-(N-morpholino)ethanesulfonic acid; NMR, nuclear magnetic resonance; PEP, phospho*enol*pyruvate; PEPC, phospho*enol*pyruvate carboxylase; PMSF, phenylmethylsulfonyl fluoride; PVPP, polyvinylpolypyrrolidone, TCA, tricarboxylic acid; TEA, triethylamine; TSP, (trimethyl) propionic-2,3,3,3-*d4* acid sodium salt

Introduction

Plants differ in susceptibility to iron deficiency. Several perennial crops, including grapevine, are particularly susceptible when grown on calcareous and alkaline soils. The development of chlorotic 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 iron 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; Brancadoro *et al.*, 1995; 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^{III} before its uptake via an iron transporter (Briat and Lobréaux, 1997). Proton extrusion and root Fe^{III} reducing capacities have been demonstrated for several *Vitis* genotypes, and shown to be related to their adaptation to iron deficiency (Brancadoro *et al.*, 1995; Mengel and Malissiovas, 1982; Nikolic and Kastori, 2000; Varanini and Maggioni, 1982).

Proton extrusion has been attributed to activation of the root plasma membrane H^+-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^+-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 Fe. Proton excretion has been shown to be associated with an increase in Fe^{III} 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*., 2004a; 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 phospho*enol*pyruvate 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 iron deficiency, is thought to be beneficial for Fe nutrition, and has been linked to iron 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 iron 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^+-ATPase. In contrast, nitrate uptake is associated with the proton consumptions via $2H^+/NO_3^-$ 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 iron 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 NO_3^--fed plants is the high root apoplastic pH as a consequence of the removal of protons during H^+/NO_3^- cotransport, which impairs Fe uptake by the roots, most probably by inhibiting Fe^{III} reduction (Kosegarten *et al.*, 2004b; Nikolic and Römheld, 2003). Aktas and Van Egmond, (1979) studied the effect of nitrogen fertilization on soybean genotypes with different susceptibilities to iron 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 suggested that the Fe-inefficient cultivar was unable to balance the large increase in pH resulting from very active NO_3^- uptake.

The aim of this work was to study the combined effects of iron 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 iron deficiency.

Materials and methods

Plant material

Micropropagated plants of the iron chlorosis tolerant genotype *Vitis vinifera* cv "Cabernet Sauvignon" (CS) (Brancadoro *et al.*, 1995; Dell'Orto *et al.*, 2000a; Tagliavini and Rombolà, 2001) and the iron chlorosis susceptible genotype *Vitis riparia* cv "Gloire de Montpellier" (RG) (Bavaresco *et al.*, 1991) were acclimated in perlite for three 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 µmol photons m^{-2} 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 [NO_3] or both ammonium and nitrate [NH_4/NO_3]. The macronutrient composition of the [NO_3] solution was 2.5 m*M* KNO_3, 2.5 m*M* $Ca(NO_3)_2.4H_2O$, 1 m*M* $MgSO_4.7H_2O$, 1 m*M* KH_2PO_4 and that of the [NH_4/NO_3] solution was 1 m*M* NH_4NO_3, 1 m*M* KNO_3, 2.5 m*M* $Ca(NO_3)_2.4H_2O$, 0.87 m*M*

$MgSO_4.7H_2O$, 0.5 m*M* K_2SO_4, 1 m*M* KH_2PO_4. Nitrogen concentrations have been set according to Rodriguez-Lovelle *et al.*, (2002) in order to maintain ion equilibrium in both conditions. The two solutions had identical micronutrient compositions: 9.1 µ*M* $MnCl_2.4H_2O$, 46.3 µ*M* H_3BO_3, 2.4 µ*M* $ZnSO_4.H_2O$, 0.5 µ*M* $CuSO_4$, 0.013 µ*M* $(NH_4)\ 6Mo_7O_{24}.4H_2O$. Iron was supplied in the form of 90 µ*M* Fe(III)-EDTA (Sigma). After four days, two thirds of the plants for each genotype and nitrogen source were transferred to iron-free solutions [-Fe]. The rest of the plants were maintained in the solution containing 90 µ*M* Fe(III)-EDTA [+Fe]. There were therefore four different nutrient solutions for each genotype: [+$FeNH_4/NO_3$], [-$FeNH_4/NO_3$], [+$FeNO_3$], [-$FeNO_3$]. The day on which plants were transferred to iron-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)-bathophenanthrolinedisulfonic acid ($BPDS_3$) 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 µ*M* BPDS (Sigma), 10 m*M* MES, pH 6.0, as described by Gogorcena *et al*. (2000). Measurements were made in the growth chamber, under illumination (300 µmol photons m^{-2} s^{-1}). 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 (Sigma) was added from a stock solution, to give a final concentration of 500 µ*M*. After one hour, a 1 mL aliquot was removed from each beaker to measure the

absorbance at 535 nm under exclusion of light. An extinction coefficient of 22.14 mM^{-1} cm^{-1} was used to calculate the concentration of the Fe(II)-$BPDS_3$ 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 to 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 ^{1}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 4800 g. Slurries were pooled. The ethanol was allowed to evaporate and the dry extracts were solubilized in 1 mL 200 m*M* 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_2O and lyophilized again to eliminate residual water. The dried extracts were stored in a dry atmosphere until ^{1}H-NMR analysis.

Dried root extracts were solubilized in 500 µL D_2O, followed by the addition of the sodium salt of (trimethyl) propionic-2,3,3,3-*d4* acid (TSP) in D_2O to a final concentration of 0.01%, for chemical shift calibration. The mixture was transferred to an NMR tube and ^{1}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 6000 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 phospho*enol*pyruvate 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 m*M* Tricine (pH 7.6), 5 m*M* $MgSO_4$, 0.25 m*M* EDTA, 10% (v/v) glycerol, 0.5% (w/v) BSA, 5 m*M* $NaHCO_3$, 2 m*M* PMSF, 10 m*M* 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 1400 g for 2 min (4ºC) and used for enzyme activity assays directly, or after a short period of storage (less than 2 h) in liquid N_2.

Enzyme assays

Phospho*enol*pyruvate 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 m*M* $NaHCO_3$, 0.2 m*M* NADH, 2 m*M* DTT, 3 units mL^{-1} MDH (Sigma), 2.2 m*M* PEP, 2.5 m*M* $MgSO_4$, 0.25 m*M* EDTA and 100 m*M* tricine, pH 8.1. Nicotinamide adenine dinucleotide (NADH) consumption was determined spectrophotometrically by monitoring the decrease in absorbance at 340 nm, at 25ºC. Malate dehydrogenase activity was determined by monitoring the increase in absorbance at 340 nm, due to the enzymatic reduction of NAD^+ at 25°C (Smith, 1974). The reaction was carried out with 5 μL of extract in 1 mL of 3 m*M* NAD^+, 29 m*M* malate, 5 m*M* $MgCl_2$ and 100 m*M* 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 25ºC (Srere, 1967). The reaction mixture contained 25 μL of extract in 1 mL of 0.4 m*M* acetyl CoA, 0.1 m*M* DTNB, 0.5 m*M* oxalacetate and 200 m*M* Tris-HCl, pH 8.1. Isocitrate dehydrogenase was assayed spectrophotometrically, as described by Goldberg and Ellis (1974), by monitoring the reduction of $NADP^+$ at 340 nm and 37ºC. The reaction was carried out with 25 μL of extract in 1 mL of 3.9 m*M* $MgCl_2$, 0.42 m*M* $NADP^+$, 6.7 m*M* sodium isocitrate and 100 m*M* TEA buffer, pH 7.3.

Statistical analysis

Each set of data were analyzed by a two-way (Fe x 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_4/NO_3] treatment than under [NO_3] treatment. The shoot growth of CS plants was reduced by iron deficiency if NO_3^- 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 iron, the pH of the nutrient solutions quickly decreased (Figure 1). The pH reached 3.5 after 5 days for [NH_4/NO_3] and after 7 days for [NO_3]. In the presence of iron, the pH of the [NH_4/NO_3] solution decreased slightly, whereas the [NO_3] treatment had the opposite effect. For RG plants, the pH of the iron-free [NH_4/NO_3] solution decreased to 4.9 by 7 days (Figure 1). 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 (Figure 2).

For CS plants (Figure 2), 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 iron 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_4^+ in the nutrient solution significantly decreased FC-R activity and the interaction with iron treatments was highly

significant. For RG plants (Figure 2), the elimination of iron 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 [-$FeNH_4/NO_3$] treatment at this time point. FC-R activity for this treatment was two times higher than for the corresponding [+Fe] treatment. However, the interaction between nitrogen and iron treatments was not significant.

^{1}H-NMR metabolic profiling of root tips

^{1}H-NMR metabolic profiles of root tip extracts were obtained after 6 days of iron 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 iron 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 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 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 form all treatments after six days of iron starvation (Table 4).

Iron 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, iron deficiency significantly decreased $NADP^+$-IDH activity in NH_4^+-fed plants whereas it had an opposite effect in NO_3^--treated plants. No significant effect on $NADP^+$-IDH activity was demonstrated for CS plants.

Discussion

After one week of iron 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 "Cabernet Sauvignon" plants was significantly decreased by the lack of iron, whereas root growth was not. Similar increase in root-to-shoot ratio has been previously reported for grapevine (Grüber and Kosegarten, 2002) and peach (Shi *et al.*, 1993) under conditions of iron deficiency. Indeed, in grapevine, the shoots appear to be more sensitive to iron deficiency regarding dry matter increment than the roots. However, some species, such as sugar beet, display the opposite pattern (Rombolà *et al.*, 2005). Shoot and root growth of chlorosis susceptible genotype did not respond to iron 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 H^+-ATPase (Dell'Orto *et al*., 2000b). Ammonium uptake also results in strong rhizosphere acidification whereas 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 iron deficiency and nitrogen uptake. The chlorosis tolerant genotype plants displayed high capacity of acidification when grown without iron, regardless of the nitrogen source, indicating a marked response to iron deficiency. The pH decreased more rapidly if NH_4^+ was present in the nutrient solution. A slight decrease in pH was also observed when plants were supplied with iron, indicating that active NH_4^+ uptake occurred. For the chlorosis susceptible genotype "Riparia Gloire de Montpellier" plants fed with NH_4^+, the pH reached 4.9 after seven days of iron deficiency, whereas no decrease was observed for plants supplied with NO_3^-. The changes in pH depended primarily on nitrogen source for this genotype.

"Cabernet Sauvignon" is known to be tolerant to iron deficiency, as other *Vitis vinifera* genotypes. As previously reported (Brancadoro *et al*., 1995), it displays typical Strategy I responses. "Riparia Gloire de Montpellier" is considered to be susceptible (Bavaresco *et al*., 1991). However, when supplied with NH_4^+, this genotype was also able to acidify the growth medium and to increase FC-R activity. Kosegarten *et al*. (2004) demonstrated close relationships between the form of nitrogen in the nutrient solution, root apoplast pH and FC-R activity in *Helianthus annus*. Based on these relationships, the stimulation of FC-R activity observed for this susceptible genotype may be linked to the decrease in pH associated with NH_4^+ uptake. The known differences in susceptibility to iron deficiency of the two *Vitis* genotypes studied here may therefore be related to their capacity to induce FC-R, and may also depend on H^+-ATPase activity in roots and the regulation of apoplastic pH, as suggested by Nikolic *et al.* (2003) and Kosegarten *et al.* (2004). The transcriptional regulation of a

specific isoform of H^+-ATPase in response to Fe deficiency was recently demonstrated in cucumber (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.

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. This capacity to assimilate different forms of nitrogen depends on ammonia detoxification capacity, which itself depends directly on the availability of keto acids (Loulakakis and Roubelakis-Angelakis, 2001). Our data suggest that both *Vitis* genotypes can take up NH_4^+ from the nutrient solution. Significantly higher growth rate of "Cabernet Sauvignon" plants supplied with NH_4^+ and the slight acidification of the [+$FeNH_4/NO_3$] nutrient solution observed with this genotype suggest that it presents a good capacity to assimilate this form of nitrogen.

Among the 17 metabolites quantified simultaneously in the roots with 1H-RMN analysis, organic acid accumulated in larger amount in response to iron deficiency, confirming previous results obtained with other methodologies for grapevine (Ollat *et al.*, 2003), other woody species (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 "Cabernet Sauvignon" 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 iron 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 iron 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 $^{1.03PEPCactivity}$, $r^2 = 0.74$, n = 8, $p < 0.01$). This stimulation of PEPC activity under iron deficiency conditions appears to be a general feature (Landsberg, 1986; López-Millán *et al*., 2000b; Nisi and Zocchi, 2000; Andaluz *et al*., 2002; Rombolà *et al*., 2005) consistent with the increase in CO_2 fixation in the roots and the increase in organic acid concentration. In the absence of iron, the chlorosis susceptible genotype "Riparia Gloire de Montpellier" presented significantly higher MDH and citrate synthase activities. The stimulation of citrate synthase activity under iron deficiency conditions was not reported previously by Rombolà *et al*. (2002) and McCluskey *et al.* (2004).

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 "Cabernet Sauvignon" only, whereas the effect on malate content was significant for both genotypes. Citrate and malate contents were lower in the presence of NH_4^+ than in its absence. Amino acid content was also significantly higher in

the presence of NH_4^+. Cabernet Sauvignon roots contained very high concentrations of glutamate and glutamine when 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 Wiren *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 NH_4^+-fed plants probably resulted from the use of these compounds to produce 2-OG for NH_4^+ assimilation. This was particularly clear for "Cabernet Sauvignon", in which citrate concentrations were half those in the presence of NH_4^+, and glutamine + glutamate concentrations were six times higher in the same treatments .

The interaction between Fe treatment and N source was highly significant for $NADP^+$-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, which is required for ammonium assimilation in the GS/GOGAT cycle (Suárez *et al.*, 2002). In our study, the interaction identified for $NADP^+$-IDH activity may be the expression of these two roles.

Our results show that nitrogen uptake and metabolism interfere with iron metabolism in *Vitis* roots in a complex manner. Both *Vitis* genotypes were able to take up NH_4^+. When fed with $NH4^+$, both genotypes displayed active growth and higher amino acid concentrations in roots. The chlorosis tolerant *Vitis vinifera* genotype "Cabernet Sauvignon"showed medium acidification, the induction of root FC-R activity and citrate accumulation in the roots when subjected to iron 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 *Vitis riparia* genotype "Gloire de Montpellier", which is known to be chlorosis susceptible. Our results confirm that citrate is a biochemical marker of iron deficiency and that PEPC activity limits the accumulation of this compound. However, the assimilation of NH_4^+ also interfered with organic acid accumulation in roots, particularly in "Cabernet Sauvignon" plants, limiting citrate accumulation. Ammonium in the medium changes the typical biochemical responses to iron 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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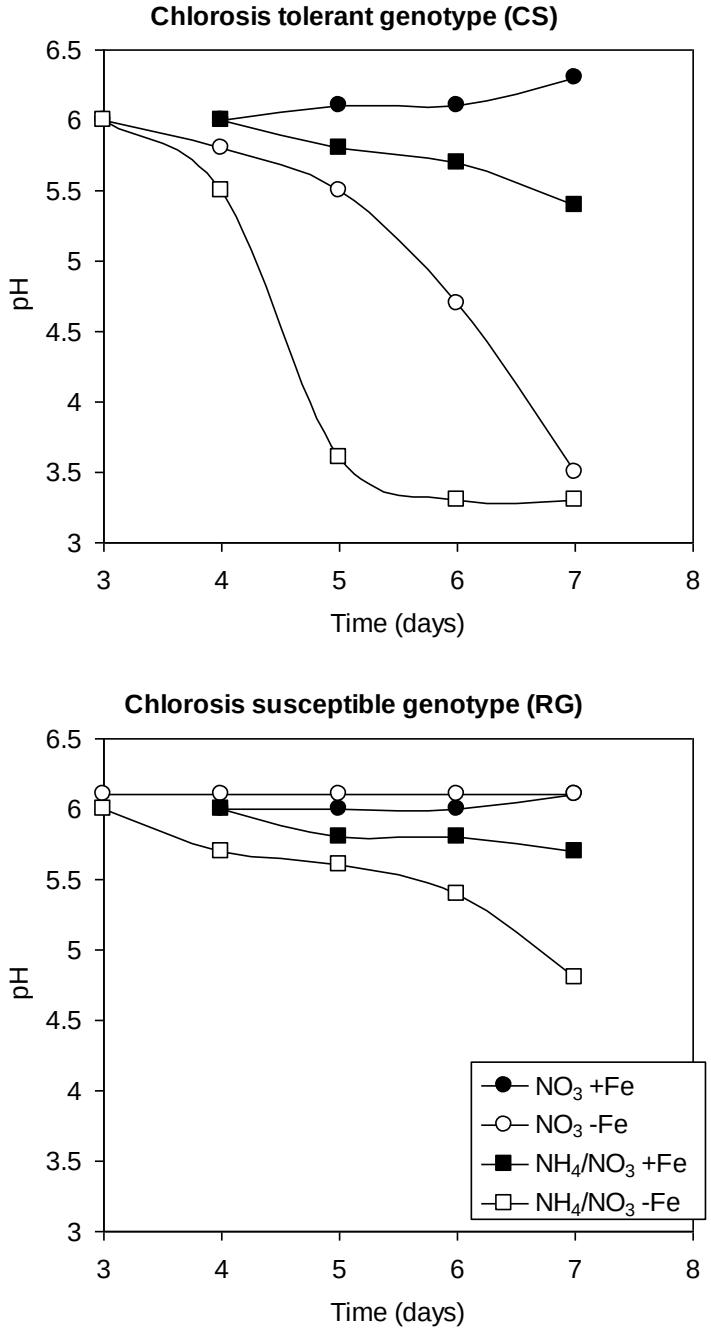

Figure 1. Changes in the pH of the medium (8 L containers with 20 plants in each) for two grapevine genotypes, one chlorosis tolerant (CS) and the other chlorosis susceptible (RG), during the first week of growth in a nutrient solution containing 0 μM Fe(III)-EDTA [-Fe] or 90 μM Fe(III)-EDTA [+Fe] and with NO_3^- as the only source of nitrogen [NO_3] or a mixed NH_4^+/ NO_3^- supply [NH_4/NO_3]. The nutrient solution was not replenished during this monitoring.

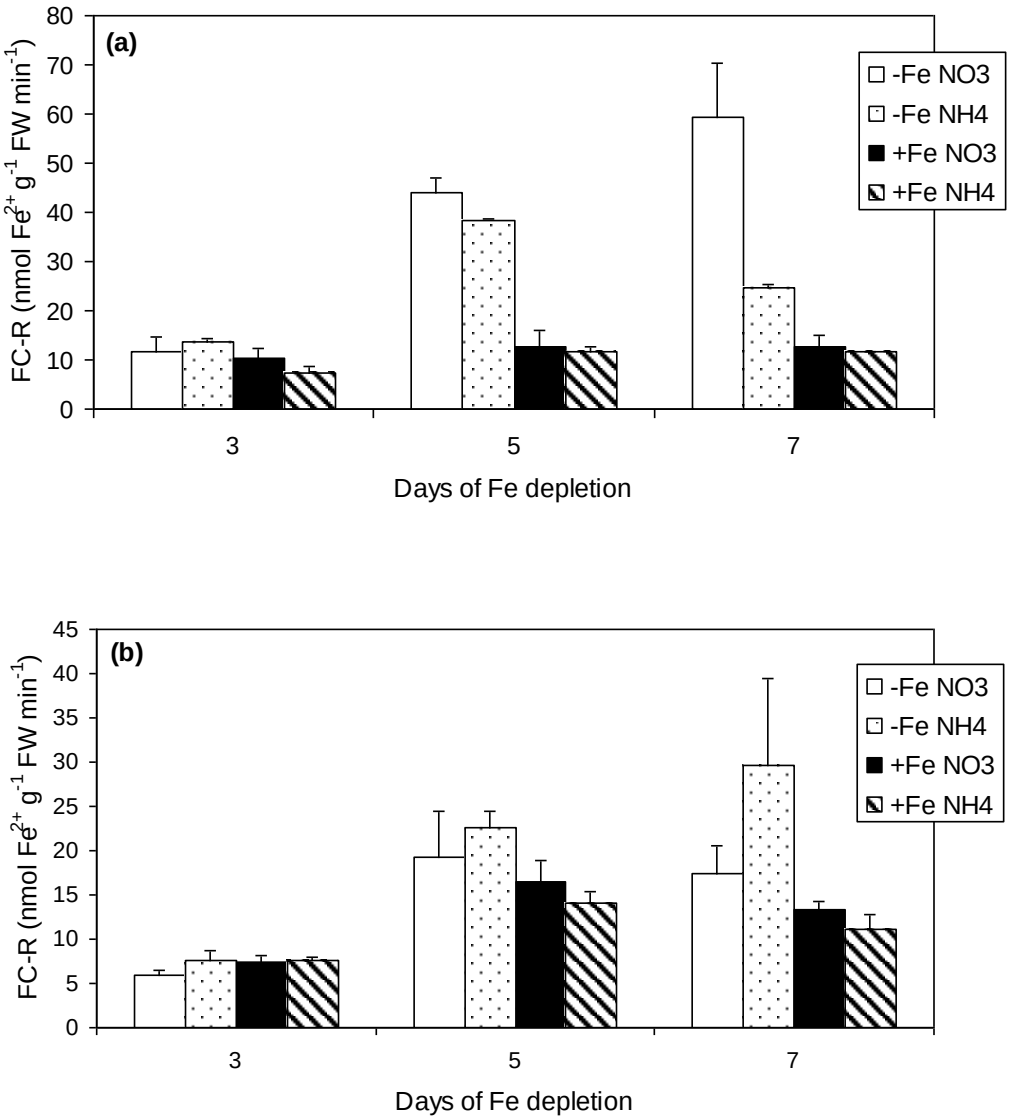

Figure 2. Time course of root ferric chelate reductase (FC-R, nmol Fe^{2+} g^{-1} FW min^{-1}) activity for whole plants of two grapevine genotypes, one chlorosis tolerant (CS) and the other chlorosis susceptible (RG), plants growing in nutrient solution containing 0 m*M* Fe(III)-EDTA [-Fe] or 90 m*M* Fe(III)-EDTA [+Fe], and with NO_3^- as the only source of nitrogen [NO_3] or a mixed NH_4^+/ NO_3^- supply [NH_4/NO_3]. Values were obtained after 3, 5, 7 days of Fe depletion. Data are means ± SE of three replicates.

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 μ*M* Fe(III)-EDTA [-Fe] or 90 μ*M* Fe(III)-EDTA [+Fe], and with NO_3^- as the only source of nitrogen [NO_3] or with a mixed NH_4^+/NO_3^- supply [NH_4/NO_3]. Data are mean ± SE of three replicates.

Genotype	N source	Fe treatment	Root fresh weight (g)	Shoot fresh weight (g)	SPAD 2nd leaf
CS	[NO_3]	+ Fe	0.91 ± 0.10	1.58 ± 0.10	28.6 ± 0.9
		- Fe	0.63 ± 0.10	0.87 ± 0.13	21.1 ± 0.7
	[NH_4/NO_3]	+ Fe	1.06 ± 0.15	1.61 ± 0.18	29.5 ± 0.9
		- Fe	1.21 ± 0.22	1.60 ± 0.18	23.3 ± 0.2
	Significance[a]				
	N source		*	*	NS
	Fe treatment		NS	*	**
	N source x Fe treatment		NS	*	NS
RG	[NO_3]	+ Fe	0.51 ± 0.07	0.97 ± 0.07	21.5 ± 1.1
		- Fe	0.49 ± 0.18	0.88 ± 0.27	18.5 ± 1.3
	[NH_4/NO_3]	+ Fe	0.57 ± 0.08	0.93 ± 0.12	25.1 ± 0.9
		- Fe	0.43 ± 0.05	0.77 ± 0.13	19.1 ± 0.1
	Significance[a]				
	N source		NS	NS	NS
	Fe treatment		NS	NS	*
	N source x Fe treatment		NS	NS	NS

[a]MANOVA analysis (Fe treatment x N source) was performed for a linear model, on raw data. Significance: ** $p<0.01$; * $p<0.05$; NS, not significant.

Table 2. Concentration of the main organic acids (mg g^{-1} DW), determined by ^{1}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 μ*M* Fe(III)-EDTA [-Fe] or 90 μ*M* Fe(III)-EDTA [+Fe], with NO_3^- as the only source of nitrogen [NO_3] or with a mixed NH_4^+/NO_3^- supply [NH_4/NO_3]. Data are means ± SE of three replicates.

Genotype	N source	Fe treatment	Malate	Citrate	Succinate	Fumarate
CS	[NO_3]	+ 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_4/NO_3]	+ 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
	Significance[a]					
	N source		*	*	*	NS
	Fe treatment		*	*	**	NS
	N source x Fe treatment		NS	NS	NS	NS
RG	[NO_3]	+ 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_4/NO_3]	+ 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
	Significance[a]					
	N source		**	NS	NS	*
	Fe treatment		**	*	**	NS
	N source x Fe treatment		NS	NS	NS	NS

[a]MANOVA analysis (Fe treatment x 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^{-1} DW), determined by ^{1}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 μ*M* Fe(III)-EDTA [-Fe] or 90 μ*M* Fe(III)-EDTA [+Fe], with NO_3^- as the only source of nitrogen [NO_3] or with a mixed NH_4^+/NO_3^- supply [NH_4/NO_3]. Data are means ± SE of three replicates.

Genotype	N source	Fe treatment	Alanine	Histidine	Glutamine + Glutamate
CS	[NO_3]	+ 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_4/NO_3]	+ 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
	Significance[a]				
	N source		*	**	**
	Fe treatment		NS	NS	NS
	N source x Fe treatment		NS	NS	NS
RG	[NO_3]	+ 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_4/NO_3]	+ 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
	Significance[a]				
	N source		NS	*	**
	Fe treatment		NS	NS	NS
	N source x Fe treatment		NS	NS	NS

[a]MANOVA analysis (Fe treatment x N source) was performed for a linear model, on raw data. Significance: ** $p<0.01$; * $p<0.05$; NS, not significant.

Table 4. Activities (nmol mg^{-1} FW min^{-1}) of phospho*enol*pyruvate carboxylase (PEPC), malate dehydrogenase (MDH), citrate synthase, and isocitrate dehydrogenase $NADP^+$-dependent ($NADP^+$-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 μ*M* Fe(III)-EDTA [-Fe] or 90 μ*M* Fe(III)-EDTA [+Fe], with NO_3^- as the only source of nitrogen [NO_3] or with a mixed NH_4^+/ NO_3^- supply [NH_4/NO_3]. Activities were measured after 6 days of culture. Data are means ± SE of three replicates.

Genotype	N source	Fe treatment	PEPC	MDH	Citrate synthase	$NADP^+$-IDH
CS	[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
	[NH_4/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
	Significance[a]					
	N source		NS	NS	NS	NS
	Fe treatment		**	NS	NS	NS
	N source x Fe treatment		NS	NS	NS	NS
RG	[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
	[NH_4/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
	Significance[a]					
	N source		NS	NS	NS	**
	Fe treatment		*	*	*	**
	N source x Fe treatment		NS	NS	*	**

[a]MANOVA analysis (Fe treatment x N source) was performed for a linear model, on raw data. Significance: ** $p<0.01$; * $p<0.05$; NS, not significant.