

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

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2 acidification, rootstocks.

3

4 **Abstract**

5 The effects of nitrogen source on iron deficiency responses were investigated in two *Vitis*
6 genotypes, one tolerant to limestone chlorosis "Cabernet Sauvignon" (*Vitis vinifera* cv.) and
7 the other susceptible "Gloire de Montpellier" (*Vitis riparia* cv.). Plants were grown with or
8 without Fe(III)-EDTA, and with NO₃⁻ alone or a mixture of NO₃⁻ and NH₄⁺. Changes in pH of
9 the nutrient solution and root ferric chelate reductase (FC-R) activity were monitored over one
10 week. We carried out quantitative metabolic profiling (¹H-NMR) and determined the activity
11 of enzymes involved in organic acid metabolism in root tips. In iron free-solutions, with NO₃⁻
12 as the sole nitrogen source, the typical Fe-deficiency response reactions as acidification of the
13 growth medium and enhanced FC-R activity in the roots were observed only in the tolerant
14 genotype. Under the same nutritional conditions, organic acid accumulation (mainly citrate
15 and malate) was found for both genotypes. In the presence of NH₄⁺, the sensitive genotype
16 displayed some decrease in pH of the growth medium and an increase in FC-R activity. For
17 both genotypes, the presence of NH₄⁺ ions decreased significantly the organic acid content of
18 roots. Both *Vitis* genotypes were able to take up NH₄⁺ from the nutrient solution, regardless of
19 their sensitivity to iron deficiency. The presence of N-NH₄⁺ modified typical Fe stress
20 responses in tolerant and sensitive *Vitis* genotypes.

21

22 *Abbreviations:* 2-OG, 2-oxoglutarate; BPDS, bathophenanthrolinedisulfonic acid disodium
23 salt hydrate; BSA, bovine serum albumin; CoA, coenzyme A; DEA, diethanolamine; DTNB,
24 5-5'-dithio-bis-2-nitrobenzoic acid; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetracetic
25 acid; ERETIC, electronic reference to access *in vivo* concentrations; FW, fresh weight;

1 GOGAT, glutamate synthetase; GS, glutamine synthetase; MDH, malate dehydrogenase;
2 MES, 2-(N-morpholino)ethanesulfonic acid; NMR, nuclear magnetic resonance; PEP,
3 phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PMSF,
4 phenylmethylsulfonyl fluoride; PVPP, polyvinylpolypyrrolidone, TCA, tricarboxylic acid;
5 TEA, triethylamine; TSP, (trimethyl) propionic-2,3,3,3-*d*4 acid sodium salt

6

7

8 **Introduction**

9 Plants differ in susceptibility to iron deficiency. Several perennial crops, including grapevine,
10 are particularly susceptible when grown on calcareous and alkaline soils. The development of
11 chlorotic symptoms in orchards and vineyards may severely reduce fruit yield and quality
12 (Álvarez-Fernández *et al.*, 2006). Among many parameters, the form of N present in the soil
13 was shown to impact iron nutrition and the development of chlorotic symptoms (Korcak,
14 1987; Lucena, 2000; Marschner, 1995).

15

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

1 Proton extrusion has been attributed to activation of the root plasma membrane H⁺-ATPase,
2 based on the activity and steady-state levels of the enzyme throughout the root (Dell'Orto *et*
3 *al.*, 2000b) and the co-localization of intense immunolabeling of the H⁺-ATPase protein and
4 proton extrusion in the subapical root zones of Fe-deficient plants (Schmidt, 2003).
5 Acidification of the rhizosphere facilitates the mobilization of sparingly soluble Fe. Proton
6 excretion has been shown to be associated with an increase in Fe^{III} reduction (Toulon *et al.*,
7 1992), although the induction of root FC-R activity can be uncoupled from the acidification
8 (Yi and Guerinot, 1996). FC-R is highly sensitive to pH and is inhibited at high pH
9 (Kosegarten *et al.*, 2004a; Nikolic *et al.*, 2000; Susin *et al.*, 1996).

10

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

21

22 Most plants can make use of either ammonium or nitrate ions. The uptake of these two forms
23 of nitrogen (N) is controlled by genotype, plant development and physiological status, and
24 also by soil properties such as texture, structure, water content and pH (Lea and Morot-
25 Gaudry, 2001; Loulakakis and Roubelakis-Angelakis, 2001). Ammonium uptake results in

1 strong acidification of the rhizosphere, due to the excretion of protons via the H^+ -ATPase. In
2 contrast, nitrate uptake is associated with the proton consumptions via $2H^+/NO_3^-$ symport,
3 leading to an increase in the pH of the outer solution (Mengel and Kirkby, 2001). Ammonium
4 assimilation requires carbon skeletons in the form of keto acids, mainly tricarboxylic acid
5 cycle intermediates. Nitrate triggers a shift from starch biosynthesis to organic acid
6 production (Foyer *et al.*, 2003). The activity of key enzymes, such as PEPC, is modified by
7 the form of nitrogen in the growth medium (Pasqualini *et al.*, 2001). Little is known about the
8 *Vitis* genotypes with respect to their different assimilation capacity for the forms of nitrogen.
9 The expression of genes encoding various enzymes involved in ammonium assimilation, such
10 as glutamate synthase, glutamine synthetase and glutamate dehydrogenase has been identified
11 in grapevine roots (Loulakakis and Roubelakis-Angelakis, 2001). Nitrate reductase activity
12 has also been detected in roots and varied according to the seasonal root growth pattern
13 (Hunter and Ruffner, 1997).

14

15 Nitrogen is taken up almost exclusively as nitrate by the roots of plants growing in calcareous
16 soil, in which ammonium is rapidly nitrified (Mengel, 1994). The high nitrate levels of
17 calcareous soils are thought to favor the development of iron chlorosis (Korcak, 1987;
18 Tagliavini and Rombolà, 2001). Several studies have demonstrated that nitrate can induce Fe
19 deficiency chlorosis in plants (Aktas and Van Egmond, 1979; Kosegarten *et al.*, 1998; Mengel
20 *et al.*, 1994; Smolders *et al.*, 1997). The primary cause of Fe deficiency in NO_3^- -fed plants is
21 the high root apoplastic pH as a consequence of the removal of protons during H^+/NO_3^-
22 cotransport, which impairs Fe uptake by the roots, most probably by inhibiting Fe^{III} reduction
23 (Kosegarten *et al.*, 2004b; Nikolic and Römheld, 2003). Aktas and Van Egmond, (1979)
24 studied the effect of nitrogen fertilization on soybean genotypes with different susceptibilities
25 to iron chlorosis. They showed that increasing the amount of nitrate supplied to plants

1 growing in calcareous soils worsened the symptoms of chlorosis in Fe-inefficient cultivars,
2 but increased the growth of Fe-efficient cultivars. They suggested that the Fe-inefficient
3 cultivar was unable to balance the large increase in pH resulting from very active NO_3^-
4 uptake.

5

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

9

10

11 **Materials and methods**

12

13 *Plant material*

14 Micropropagated plants of the iron chlorosis tolerant genotype *Vitis vinifera* cv "Cabernet
15 Sauvignon" (CS) (Brancadoro *et al.*, 1995; Dell'Orto *et al.*, 2000a; Tagliavini and Rombolà,
16 2001) and the iron chlorosis susceptible genotype *Vitis riparia* cv "Gloire de Montpellier"
17 (RG) (Bavaresco *et al.*, 1991) were acclimated in perlite for three weeks. The plants were then
18 transferred to 10 L plastic containers filled with 8 L of a continuously aerated nutrient
19 solution, with 20 plants per container. The growth chamber was programmed for a 16 h
20 photoperiod ($300 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) at 25°C and 8 h of darkness at 20°C, with 75%
21 relative humidity. The two genotypes were grown separately throughout the experiment. The
22 nutrient solutions contained either nitrate as the only nitrogen source [NO_3] or both
23 ammonium and nitrate [NH_4/NO_3]. The macronutrient composition of the [NO_3] solution was
24 2.5 mM 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 KH_2PO_4 and that of the
25 [NH_4/NO_3] solution was 1 mM NH_4NO_3 , 1 mM KNO_3 , 2.5 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.87 mM

1 MgSO₄·7H₂O, 0.5 mM K₂SO₄, 1 mM KH₂PO₄. Nitrogen concentrations have been set
2 according to Rodriguez-Lovelle *et al.*, (2002) in order to maintain ion equilibrium in both
3 conditions. The two solutions had identical micronutrient compositions: 9.1 μM MnCl₂·4H₂O,
4 46.3 μM H₃BO₃, 2.4 μM ZnSO₄·H₂O, 0.5 μM CuSO₄, 0.013 μM (NH₄)₆Mo₇O₂₄·4H₂O. Iron
5 was supplied in the form of 90 μM Fe(III)-EDTA (Sigma). After four days, two thirds of the
6 plants for each genotype and nitrogen source were transferred to iron-free solutions [-Fe]. The
7 rest of the plants were maintained in the solution containing 90 μM Fe(III)-EDTA [+Fe].
8 There were therefore four different nutrient solutions for each genotype: [+FeNH₄/NO₃], [-
9 FeNH₄/NO₃], [+FeNO₃], [-FeNO₃]. The day on which plants were transferred to iron-free
10 solution was counted as day 0 of the experiment. The plants were grown under these
11 conditions for one week. The pH of the nutrient solutions was adjusted to 6 at day 0. The pH
12 changes of the medium by roots was monitored daily.

13

14

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

16 The root FC-R (EC 1.16.1.7) activity of whole plants was determined by monitoring the
17 formation of the Fe(II)-bathophenanthrolinedisulfonic acid (BPDS₃) complex from Fe(III)-
18 EDTA (Bienfait *et al.*, 1983). Individual plants were transferred to 50 mL plastic beakers
19 (covered with black tape to exclude light) containing 46 mL of 300 μM BPDS (Sigma), 10
20 mM MES, pH 6.0, as described by Gogorcena *et al.* (2000). Measurements were made in the
21 growth chamber, under illumination (300 μmol photons m⁻² s⁻¹). The buffer solution was
22 continually aerated by means of plastic tubing. Once the plants were placed in the beaker, 1
23 mL of Fe(III)-EDTA (Sigma) was added from a stock solution, to give a final concentration
24 of 500 μM. After one hour, a 1 mL aliquot was removed from each beaker to measure the

1 absorbance at 535 nm under exclusion of light. An extinction coefficient of $22.14 \text{ mM}^{-1} \text{ cm}^{-1}$
2 was used to calculate the concentration of the Fe(II)-BPDS₃ complex.

3

4 *Determination of plant parameters and root sample collection*

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

11

12 *Metabolic profiles obtained by ¹H-NMR spectroscopy on root tips*

13 Root tips were freeze-dried, weighed and crushed. Polar compounds were extracted by
14 aqueous ethanol at 80°C , in three incubation steps, each lasting 20 min (step 1: 0.75 mL 80%
15 ethanol; steps 2 and 3: 0.75 mL 50% ethanol) and then centrifuged for 10 min at 4800 g.
16 Slurries were pooled. The ethanol was allowed to evaporate and the dry extracts were
17 solubilized in 1 mL 200 mM oxalate buffer to maintain the pH of the extracts at 4.0. To
18 improve spectrum resolution and to eliminate paramagnetic ions the extracts were further
19 purified on 200 mg of Chelex 100 resin (BioRad, Marnes-la-Coquette) in oxalate buffer (pH =
20 4.0). The resin was rinsed three times with 1 mL double-distilled water. The pH of each
21 extract was checked after this step. The extracts were lyophilized, solubilized in 500 μL D₂O
22 and lyophilized again to eliminate residual water. The dried extracts were stored in a dry
23 atmosphere until ¹H-NMR analysis.

24

1 Dried root extracts were solubilized in 500 μ L D₂O, followed by the addition of the sodium
2 salt of (trimethyl) propionic-2,3,3,3-*d*4 acid (TSP) in D₂O to a final concentration of 0.01%,
3 for chemical shift calibration. The mixture was transferred to an NMR tube and ¹H-NMR
4 spectra were recorded as previously described (Moing *et al.*, 2004) at 500.162 MHz and 300
5 K on a Bruker Avance spectrometer (Wissenbourg, France), using a 5-mm dual probe. We
6 acquired 64 scans of 64 K data points with a spectral width of 6000 Hz and an acquisition
7 time of 2.73 s. The recycle delay was 15 s. The ERETIC method was used to determine
8 absolute concentrations of metabolites (Akoka *et al.*, 1999), using calibration curves for C1-
9 H(α + β) glucose.

10

11 *Enzyme extraction*

12 The enzymes assayed included phosphoenolpyruvate carboxylase (EC 4.1.1.31), malate
13 dehydrogenase (EC 1.1.1.37), citrate synthase (EC 4.1.3.7), and isocitrate dehydrogenase (EC
14 1.1.1.42).

15

16 Extracts for enzyme assays were prepared by grinding the root material in liquid nitrogen in a
17 mortar with 50% PVPP (w/w FW) and 1 mL of extraction buffer containing 400 mM Tricine
18 (pH 7.6), 5 mM MgSO₄, 0.25 mM EDTA, 10% (v/v) glycerol, 0.5% (w/v) BSA, 5 mM
19 NaHCO₃, 2 mM PMSF, 10 mM sodium ascorbate and 1% (v/v) Triton. The slurry was filtered
20 through glass wool and the filtrate was desalted on a Sephadex G-25 column (equilibrated
21 with extraction buffer). The desalted extracts were centrifuged at 1400 g for 2 min (4°C) and
22 used for enzyme activity assays directly, or after a short period of storage (less than 2 h) in
23 liquid N₂.

24

1 *Enzyme assays*

2 Phosphoenolpyruvate carboxylase was determined by coupling its activity to malate
3 dehydrogenase-catalyzed NADH oxidation (Vance *et al.*, 1983) with 25 μL of extract in 1 mL
4 of 5 mM NaHCO_3 , 0.2 mM NADH, 2 mM DTT, 3 units mL^{-1} MDH (Sigma), 2.2 mM PEP,
5 2.5 mM MgSO_4 , 0.25 mM EDTA and 100 mM tricine, pH 8.1. Nicotinamide adenine
6 dinucleotide (NADH) consumption was determined spectrophotometrically by monitoring the
7 decrease in absorbance at 340 nm, at 25°C. Malate dehydrogenase activity was determined by
8 monitoring the increase in absorbance at 340 nm, due to the enzymatic reduction of NAD^+ at
9 25°C (Smith, 1974). The reaction was carried out with 5 μL of extract in 1 mL of 3 mM
10 NAD^+ , 29 mM malate, 5 mM MgCl_2 and 100 mM DEA buffer, pH 9.2. Citrate synthase
11 activity was assayed by monitoring the reduction of acetyl CoA to CoA with DTNB at 412
12 nm and 25°C (Srere, 1967). The reaction mixture contained 25 μL of extract in 1 mL of 0.4
13 mM acetyl CoA, 0.1 mM DTNB, 0.5 mM oxalacetate and 200 mM Tris-HCl, pH 8.1.
14 Isocitrate dehydrogenase was assayed spectrophotometrically, as described by Goldberg and
15 Ellis (1974), by monitoring the reduction of NADP^+ at 340 nm and 37°C. The reaction was
16 carried out with 25 μL of extract in 1 mL of 3.9 mM MgCl_2 , 0.42 mM NADP^+ , 6.7 mM
17 sodium isocitrate and 100 mM TEA buffer, pH 7.3.

18

19 *Statistical analysis*

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

24

25

1 **Results**

2

3 *Plant growth and appearance of leaf chlorosis symptoms*

4 After one week of Fe deprivation, leaf chlorophyll content was significantly reduced in both
5 genotypes, and plants displayed initial symptoms of Fe chlorosis (Table 1). At this stage, CS
6 plants displayed significantly higher rates of root and shoot growth under [NH₄/NO₃]
7 treatment than under [NO₃] treatment. The shoot growth of CS plants was reduced by iron
8 deficiency if NO₃⁻ was used as the sole nitrogen source. The root and shoot growth was
9 unaffected by any treatment in RG plants.

10

11 *Nutrient solution acidification*

12 When CS plants were grown without iron, the pH of the nutrient solutions quickly decreased
13 (Figure 1). The pH reached 3.5 after 5 days for [NH₄/NO₃] and after 7 days for [NO₃]. In the
14 presence of iron, the pH of the [NH₄/NO₃] solution decreased slightly, whereas the [NO₃]
15 treatment had the opposite effect. For RG plants, the pH of the iron-free [NH₄/NO₃] solution
16 decreased to 4.9 by 7 days (Figure 1). In all other treatments, the pH remained close to 6.

17

18 *Root FC-R (ferric chelate reductase) activity*

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

20

21 For CS plants (Figure 2), the FC-R activity of [+Fe] plants remained low and steady during
22 the growing period whereas that of [-Fe] plants increased significantly by days 5 and 7, under
23 both nitrogen treatments. The iron effect was significant ($p < 0.05$) on day 5 and highly
24 significant ($p < 0.01$) on day 7. At this stage, the presence of NH₄⁺ in the nutrient solution
25 significantly decreased FC-R activity and the interaction with iron treatments was highly

1 significant. For RG plants (Figure 2), the elimination of iron from the growth medium had a
2 significant positive effect on FC-R activity ($p < 0.05$) on day 7 only. The highest levels of
3 activity were recorded for the $[-\text{FeNH}_4/\text{NO}_3]$ treatment at this time point. FC-R activity for
4 this treatment was two times higher than for the corresponding $[\text{+Fe}]$ treatment. However, the
5 interaction between nitrogen and iron treatments was not significant.

6

7 *¹H-NMR metabolic profiling of root tips*

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

14

15 MANOVA analysis of organic acid content showed that iron treatment significantly increased
16 the concentration of root organic acids other than fumarate for both genotypes (Table 2).
17 Citrate predominated in $[-\text{Fe}]$ CS roots, whereas malate was the main organic acid in RG
18 roots. Nitrogen source had a significant effect on malate, citrate and succinate concentrations
19 in CS roots, and on malate and fumarate in RG roots. The presence of NH_4^+ decreased
20 organic acid concentration in the roots of both genotypes. The concentration of amino acids in
21 the roots was influenced by the form of N, but not by Fe status (Table 3). With the exception
22 of alanine in RG plants, the presence of NH_4^+ in the nutrient solution increased significantly
23 amino acid levels.

24

25 *Enzyme activities in root extracts*

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

3
4 Iron deficiency induced significant increase in PEPC activity in both genotypes. In RG plants,
5 MDH and citrate synthase activities were stimulated by Fe deficiency. Fe status did not affect
6 these activities in CS plants. Regardless of the genotype, there was no nitrogen effect on these
7 enzymatic activities. In RG plants, iron deficiency significantly decreased NADP⁺-IDH
8 activity in NH₄⁺-fed plants whereas it had an opposite effect in NO₃⁻-treated plants. No
9 significant effect on NADP⁺-IDH activity was demonstrated for CS plants.

10

11 **Discussion**

12

13 After one week of iron starvation, the chlorophyll content of the leaves, as indicated by SPAD
14 readings, had significantly decreased for both genotypes. The shoot growth of the chlorosis
15 tolerant genotype "Cabernet Sauvignon" plants was significantly decreased by the lack of
16 iron, whereas root growth was not. Similar increase in root-to-shoot ratio has been previously
17 reported for grapevine (Grüber and Kosegarten, 2002) and peach (Shi *et al.*, 1993) under
18 conditions of iron deficiency. Indeed, in grapevine, the shoots appear to be more sensitive to
19 iron deficiency regarding dry matter increment than the roots. However, some species, such as
20 sugar beet, display the opposite pattern (Rombolà *et al.*, 2005). Shoot and root growth of
21 chlorosis susceptible genotype did not respond to iron deficiency nor to nitrogen source. The
22 low growth rate registered for this genotype may partly explain this lack of response.

23

24 Acidification of the rhizosphere is known to be part of the mechanism by which some
25 dicotyledonous plants respond to Fe starvation. This acidification has been attributed to

1 activation of the root plasma membrane H^+ -ATPase (Dell'Orto *et al.*, 2000b). Ammonium
2 uptake also results in strong rhizosphere acidification whereas NO_3^- uptake increases the pH
3 of the outer solution (Mengel and Kirkby, 2001). Under our growing conditions, the pH of the
4 medium depended on both mechanisms: response to iron deficiency and nitrogen uptake. The
5 chlorosis tolerant genotype plants displayed high capacity of acidification when grown
6 without iron, regardless of the nitrogen source, indicating a marked response to iron
7 deficiency. The pH decreased more rapidly if NH_4^+ was present in the nutrient solution. A
8 slight decrease in pH was also observed when plants were supplied with iron, indicating that
9 active NH_4^+ uptake occurred. For the chlorosis susceptible genotype "Riparia Gloire de
10 Montpellier" plants fed with NH_4^+ , the pH reached 4.9 after seven days of iron deficiency,
11 whereas no decrease was observed for plants supplied with NO_3^- . The changes in pH
12 depended primarily on nitrogen source for this genotype.

13
14 "Cabernet Sauvignon" is known to be tolerant to iron deficiency, as other *Vitis vinifera*
15 genotypes. As previously reported (Brancadoro *et al.*, 1995), it displays typical Strategy I
16 responses. "Riparia Gloire de Montpellier" is considered to be susceptible (Bavaresco *et al.*,
17 1991). However, when supplied with NH_4^+ , this genotype was also able to acidify the growth
18 medium and to increase FC-R activity. Kosegarten *et al.* (2004) demonstrated close
19 relationships between the form of nitrogen in the nutrient solution, root apoplast pH and FC-R
20 activity in *Helianthus annuus*. Based on these relationships, the stimulation of FC-R activity
21 observed for this susceptible genotype may be linked to the decrease in pH associated with
22 NH_4^+ uptake. The known differences in susceptibility to iron deficiency of the two *Vitis*
23 genotypes studied here may therefore be related to their capacity to induce FC-R, and may
24 also depend on H^+ -ATPase activity in roots and the regulation of apoplastic pH, as suggested
25 by Nikolic *et al.* (2003) and Kosegarten *et al.* (2004). The transcriptional regulation of a

1 specific isoform of H⁺-ATPase in response to Fe deficiency was recently demonstrated in
2 cucumber (Santi *et al.*, 2005). However as root apoplastic pH was not determined in our work
3 and using MES could have affected FC-R response to *in vivo* apoplastic pH, further work is
4 required to investigate this relationship in different *Vitis* genotypes.

5

6 Although grapevine rootstocks are known to display different responses to N fertilization
7 under field and pot trial conditions (Keller *et al.*, 2001; Zerihun and Treeby, 2002), their
8 capacity to assimilate different forms of N forms has been little investigated. This capacity to
9 assimilate different forms of nitrogen depends on ammonia detoxification capacity, which
10 itself depends directly on the availability of keto acids (Loulakakis and Roubelakis-Angelakis,
11 2001). Our data suggest that both *Vitis* genotypes can take up NH₄⁺ from the nutrient solution.
12 Significantly higher growth rate of "Cabernet Sauvignon" plants supplied with NH₄⁺ and the
13 slight acidification of the [+FeNH₄/NO₃] nutrient solution observed with this genotype
14 suggest that it presents a good capacity to assimilate this form of nitrogen.

15

16 Among the 17 metabolites quantified simultaneously in the roots with 1H-RMN analysis,
17 organic acid accumulated in larger amount in response to iron deficiency, confirming previous
18 results obtained with other methodologies for grapevine (Ollat *et al.*, 2003), other woody
19 species (Rombolà *et al.*, 2002; Sun *et al.*, 1987) and herbaceous plants (Abadía *et al.*, 2002).
20 Similar increase in malate concentrations was found in both genotypes. The chlorosis tolerant
21 genotype "Cabernet Sauvignon" was characterized by a very high citrate concentration in the
22 root tips and a ratio of citrate concentration under -Fe conditions to citrate concentration
23 under +Fe conditions varying from 4 to 7. These data suggest that citrate could be used as
24 biochemical marker of iron chlorosis tolerance in some species, as suggested by Ollat *et al.*
25 (2003) and Rombolà *et al.* (2002).

1
2 For both genotypes, the changes in root organic acid composition under iron deficiency
3 conditions were associated with an increase in maximal PEPC activity. This difference was
4 highly significant for the chlorosis tolerant one. Root tip citrate content and PEPC activity
5 measured *in vitro* were significantly related (citrate content = $4.3 e^{1.03PEPC_{activity}}$, $r^2 = 0.74$, $n =$
6 8 , $p < 0.01$). This stimulation of PEPC activity under iron deficiency conditions appears to be
7 a general feature (Landsberg, 1986; López-Millán *et al.*, 2000b; Nisi and Zocchi, 2000;
8 Andaluz *et al.*, 2002; Rombolà *et al.*, 2005) consistent with the increase in CO₂ fixation in the
9 roots and the increase in organic acid concentration. In the absence of iron, the chlorosis
10 susceptible genotype "Riparia Gloire de Montpellier" presented significantly higher MDH and
11 citrate synthase activities. The stimulation of citrate synthase activity under iron deficiency
12 conditions was not reported previously by Rombolà *et al.* (2002) and McCluskey *et al.*
13 (2004).

14
15 These results suggest that PEPC activity may be one of the limiting steps for citrate
16 accumulation. Other activities, such as MDH and citrate synthase activities, are probably in
17 excess of the normal cellular requirements (Delhaize *et al.*, 2003). Other factors, such as
18 reducing power and compartmentalization of substrates and products, may control the
19 activities of these enzymes *in vivo*. Export to the external medium and to the xylem sap must
20 also be taken into account.

21
22 Organic acid and amino acid contents were significantly affected by nitrogen source. The
23 effect on citrate content was significant for "Cabernet Sauvignon" only, whereas the effect on
24 malate content was significant for both genotypes. Citrate and malate contents were lower in
25 the presence of NH₄⁺ than in its absence. Amino acid content was also significantly higher in

1 the presence of NH_4^+ . Cabernet Sauvignon roots contained very high concentrations of
2 glutamate and glutamine when NH_4^+ was added to the nutrient solution.

3

4 Amino acid biosynthesis requires the allocation of assimilated carbon — 2-oxoglutarate (2-
5 OG) in particular — for ammonia incorporation, and the production of C skeletons by the
6 TCA cycle for amino acid synthesis downstream from the GS/GOGAT pathway (Von Wiren
7 *et al.*, 2001). Ammonium assimilation may direct more organic acids to the GS/GOGAT
8 pathway, resulting in lower levels of accumulation in the roots (Pasqualini *et al.*, 2001) and an
9 increase in carbon flow through the TCA cycle. In our study, the lower organic acid
10 concentration in the root tips of NH_4^+ -fed plants probably resulted from the use of these
11 compounds to produce 2-OG for NH_4^+ assimilation. This was particularly clear for "Cabernet
12 Sauvignon", in which citrate concentrations were half those in the presence of NH_4^+ , and
13 glutamine + glutamate concentrations were six times higher in the same treatments .

14

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

24

1 Our results show that nitrogen uptake and metabolism interfere with iron metabolism in *Vitis*
2 roots in a complex manner. Both *Vitis* genotypes were able to take up NH_4^+ . When fed with
3 NH_4^+ , both genotypes displayed active growth and higher amino acid concentrations in roots.
4 The chlorosis tolerant *Vitis vinifera* genotype "Cabernet Sauvignon" showed medium
5 acidification, the induction of root FC-R activity and citrate accumulation in the roots when
6 subjected to iron deficiency. The acidification of the nutrient solution resulting from the
7 ammonium uptake may, at least partly, account for the unexpected stimulation of FC-R
8 activity in the *Vitis riparia* genotype "Gloire de Montpellier", which is known to be chlorosis
9 susceptible. Our results confirm that citrate is a biochemical marker of iron deficiency and
10 that PEPC activity limits the accumulation of this compound. However, the assimilation of
11 NH_4^+ also interfered with organic acid accumulation in roots, particularly in "Cabernet
12 Sauvignon" plants, limiting citrate accumulation. Ammonium in the medium changes the
13 typical biochemical responses to iron deficiency for both tolerant and susceptible cultivars,
14 and this cation should therefore be avoided in screening methods based on nutrient solution
15 experiments.

16

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25

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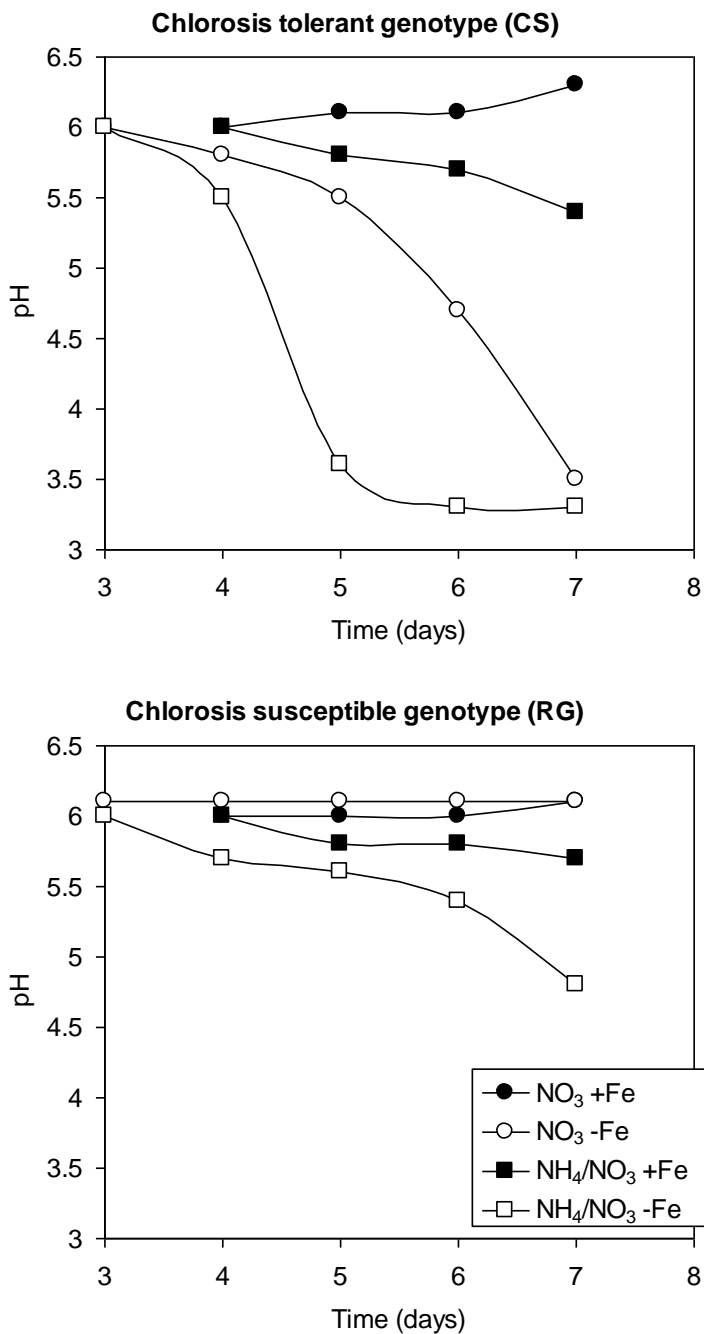


Figure 1. Changes in the pH of the medium (8 L containers with 20 plants in each) for two grapevine genotypes, one chlorosis tolerant (CS) and the other chlorosis susceptible (RG), during the first week of growth in a nutrient solution containing 0 μM Fe(III)-EDTA [-Fe] or 90 μM Fe(III)-EDTA [+Fe] and 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.

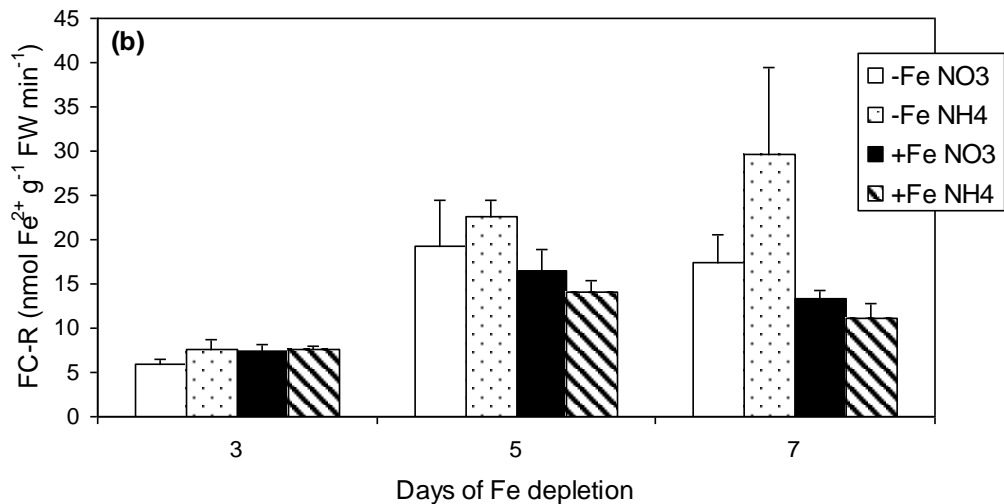
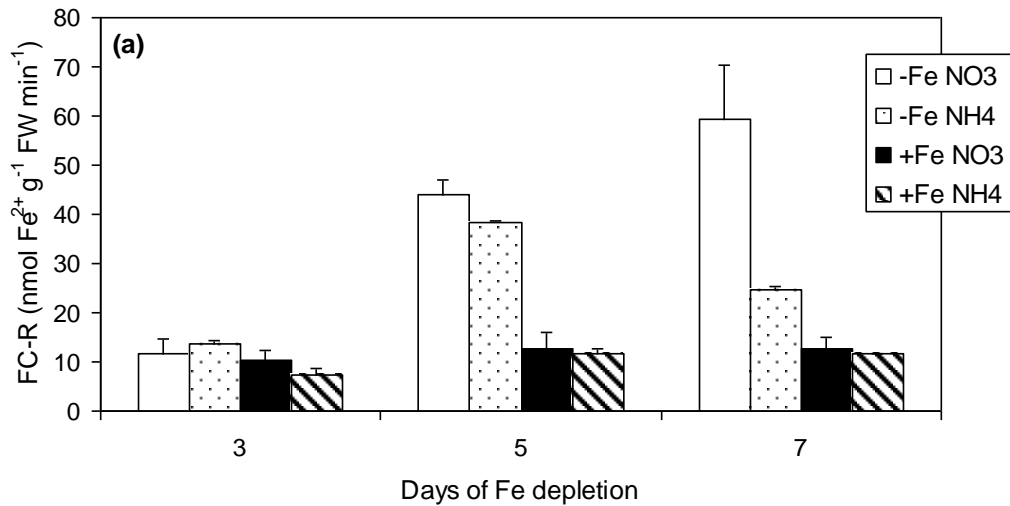


Figure 2. Time course of root ferric chelate reductase (FC-R, $\text{nmol Fe}^{2+} \text{g}^{-1} \text{FW min}^{-1}$) activity for whole plants of two grapevine genotypes, one chlorosis tolerant (CS) and the other chlorosis susceptible (RG), plants growing in nutrient solution containing 0 mM Fe(III)-EDTA [-Fe] or 90 mM Fe(III)-EDTA [+Fe], and with NO_3^- as the only source of nitrogen [NO_3^-] or a mixed $\text{NH}_4^+/\text{NO}_3^-$ supply [NH_4/NO_3]. Values were obtained after 3, 5, 7 days of Fe depletion. Data are means \pm 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 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 with a mixed $\text{NH}_4^+/\text{NO}_3^-$ supply [NH_4/NO_3]. Data are mean \pm 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 \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	
	[NH_4/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^a</i>					
	N source			*	*	NS
	Fe treatment			NS	*	**
	N source x Fe treatment			NS	*	NS
RG	[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	
	[NH_4/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^a</i>					
	N source			NS	NS	NS
	Fe treatment			NS	NS	*
	N source x Fe treatment			NS	NS	NS

^aMANOVA 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 ¹H-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 NO₃⁻ as the only source of nitrogen [NO₃] or with a mixed NH₄⁺/NO₃⁻ supply [NH₄/NO₃]. 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	
	<i>Significance^a</i>						
	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	
	<i>Significance^a</i>						
	N source			**	NS	NS	*
	Fe treatment			**	*	**	NS
	N source x Fe treatment			NS	NS	NS	NS

^aMANOVA 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 ¹H-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 NO₃⁻ as the only source of nitrogen [NO₃] or with a mixed NH₄⁺/NO₃⁻ supply [NH₄/NO₃]. 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	
	<i>Significance^a</i>					
	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	
	<i>Significance^a</i>					
	N source			NS	*	**
	Fe treatment			NS	NS	NS
	N source x Fe treatment			NS	NS	NS

^aMANOVA 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 ($\text{nmol mg}^{-1} \text{FW min}^{-1}$) of phosphoenolpyruvate 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 μM Fe(III)-EDTA [-Fe] or 90 μM Fe(III)-EDTA [+Fe], with NO₃⁻ as the only source of nitrogen [NO₃] or with a mixed NH₄⁺/ NO₃⁻ supply [NH₄/NO₃]. Activities were measured after 6 days of culture. Data are means \pm SE of three replicates.

Genotype	N source	Fe treatment	PEPC	MDH	Citrate synthase	NADP ⁺ -IDH	
CS	[NO ₃]	+ Fe	0.81 \pm 0.18	28.10 \pm 2.73	0.82 \pm 0.24	1.40 \pm 0.23	
		- Fe	2.26 \pm 0.72	27.99 \pm 3.30	0.88 \pm 0.14	1.44 \pm 0.26	
	[NH ₄ /NO ₃]	+ Fe	0.76 \pm 0.22	24.54 \pm 3.33	0.90 \pm 0.19	3.44 \pm 1.55	
		- Fe	2.33 \pm 1.29	26.01 \pm 1.89	1.08 \pm 0.21	1.24 \pm 0.20	
	<i>Significance^a</i>						
	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 \pm 0.25	23.46 \pm 5.26	0.51 \pm 0.09	0.67 \pm 0.11	
		- Fe	1.37 \pm 0.50	32.34 \pm 5.53	0.94 \pm 0.14	1.37 \pm 0.03	
	[NH ₄ /NO ₃]	+ Fe	0.88 \pm 0.26	29.55 \pm 3.65	0.72 \pm 0.11	2.79 \pm 0.35	
		- Fe	1.83 \pm 0.69	36.11 \pm 4.09	0.74 \pm 0.21	0.92 \pm 0.34	
	<i>Significance^a</i>						
	N source			NS	NS	NS	**
	Fe treatment			*	*	*	**
	N source x Fe treatment			NS	NS	*	**

^aMANOVA 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.