Evaluation of sustainable management techniques for preventing iron chlorosis in the grapevine

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Abstract

Background and Aims: The control of iron (Fe) chlorosis by synthetic Fe chelates is costly and their application can have adverse environmental impacts. We investigated the effectiveness of alternative vineyard strategies to prevent Fe chlorosis in grapevines.

Methods and Results: An experiment was conducted over two consecutive seasons on *Vitis vinifera* L. cv. Cabernet Sauvignon grafted on the Fe-chlorosis susceptible *Vitis riparia* grown in pots filled with calcareous soil. Intercropping with *Festuca rubra* enhanced leaf chlorophyll index and reduced the root activity of phosphoenolpyruvate carboxylase enzyme, a physiological marker of Fe deficiency. This response was similar to that of supplying Fe-ethylenediamine-N,N′-bis(2-hydroxyphenyl)acetic acid to soil. Application of ammonium with 3,4-dimethylpyrazole phosphate (a nitrification inhibitor) increased leaf chlorophyll index and stomatal length, and induced root biochemical responses similar to those with Fe-ethylenediamine-N,N′-bis(2-hydroxyphenylaceticacid) application. Leaf-applied Fe-ethylenediaminetetraacetic acid induced a high root citric acid concentration, suggesting a limited translocation of Fe from leaves to roots. Intercropping with *Festuca rubra* decreased the leaf fluorescence-derived parameters in the first year and increased the leaf stomata conductance in the second year of the experiment.

Conclusions: The results demonstrate the potential for preventing grapevine Fe chlorosis more sustainably through managing ammonium nutrition and adopting intercropping with Fe-efficient grasses.

Significance of the Study: The data provide evidence of the effectiveness and physiological responses of agronomic strategies, alternative to synthetic Fe chelates, for preventing Fe deficiency in the grapevine.

Keywords: *ammonium, intercropping, leaf structural property, organic acid, photosynthetic apparatus*

Introduction

Iron (Fe) deficiency chlorosis is one of the main nutritional deficiencies of woody plants, including grapevine (Tagliavini and Rombolà 2001). It can dramatically reduce orchard longevity, and lower yield and fruit quality (Rombolà and Tagliavini 2006). Grapevine is a Strategy I plant that when exposed to Fe deficiency is able to increase Fe reductase activity and enhance net excretion of protons and root organic compounds, such as organic acids and phenols, lowering the pH and increasing the solubility of Fe(III) (Brancadoro et al. 1995, Dell'Orto et al. 2000, Jiménez et al. 2007, Covarrubias and Rombolà 2013). Under controlled conditions, tolerant grapevine genotypes have enhanced root Fe uptake mechanisms under Fe limiting conditions, such as an increased Fe-chelate reductase activity and a higher extrusion of protons into the rhizosphere (Brancadoro et al. 1995, Dell'Orto et al. 2000, Rombolà and Tagliavini 2006, Jiménez et al. 2007). In addition, tolerant grapevine genotypes may increase the activity of phosphoenolpyruvate carboxylase (PEPC) enzyme and the concentration of organic acids in roots (particularly citric acid) in response to Fe deficiency (Rombolà et al. 2002, Ollat et al. 2003, Rombolà and Tagliavini 2006, Jiménez et al. 2007, Covarrubias and Rombolà 2013).

In fruit trees, Fe deficiency impairs aspects of leaf physiology, and Fe deficiency-mediated reduction in net photosynthesis has been reported for many plant species (Terry and Abadía 1986, Abadía 1992, Morales et al. 2000, Larbi et al. 2006). In *Pyrus communis*, reduced photosynthesis in Fe chlorotic leaves has been attributed to reduced efficiency of Photosystem II (PSII) because of the closure of PSII reaction centres and reduced intrinsic PSII efficiency (Morales et al. 2000). Moreover, the efficiency of light absorption and Rubisco carboxylation in leaves was down-regulated in response to Fe deficiency (Larbi et al. 2006). Scanning electron microscopy (SEM) studies have observed leaf structural changes associated with Fe deficiency chlorosis in pear and peach (Fernández et al. 2008b), supporting the role of Fe nutrition on leaf structure and cellular organisation. In the grapevine cultivar Aurora grafted on SO4 rootstock, Fe deficiency reduced leaf and whole canopy photosynthesis, grape yield, and total dry matter production (Bavaresco and Poni 2003). In plants of cv. Pinot Blanc grafted on the Fe-chlorosis susceptible 3309 C rootstock, cultivated in a calcareous soil, Fe deficiency strongly reduced the leaf chlorophyll content and shoot length (Bavaresco et al. 2003). Information, however, concerning the effect of Fe deficiency on components of leaf photosynthesis and leaf structural properties in grapevine is limited.

The control of Fe chlorosis with Fe chelates is an effective and widespread agronomical practice in vineyards and orchards (Rombolà and Tagliavini 2006). Nevertheless, Fe chelates are expensive, require repeated application and, because of their

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high stability and solubility, increase the risk of leaching metals and chelating agents into deep soil layers and the water table (Rombolà and Tagliavini 2006). These environmental consequences have stimulated the development of sustainable management techniques for preventing Fe chlorosis in several Fe deficiency susceptible crops. It has been reported that intercropping with graminaceous species can prevent Fe chlorosis in kiwifruit and citrus (Rombolà et al. 2003, Ammari and Rombolà 2010). Graminaceous species are able to release high affinity Fe(III) chelating compounds (phytosiderophores) into the rhizosphere, solubilising Fe and to take it up by roots as intact Fe-phytosiderophore complexes (Ma et al. 2003, Cesco et al. 2006, Cesco and Rombolà 2007, Ueno et al. 2007). Recent studies have shown that the presence of graminaceous species, when grown alongside non-graminaceous species, may stimulate the expression of AhFRO1 and AhIRT1 genes encoding proteins responsible for Fe absorption, and such changes have been mainly attributed to soil Fe level, cultivation conditions and phenology (Ding et al. 2009, 2010).

In well-aerated agricultural soils, average annual ammonium (NH_4^+) concentration is frequently 10–1000 times lower than that of nitrate (NO_3^-), rarely exceeding 50 µmol/L (Marschner 1995). Despite this low concentration in soils, NH_4^+ can be taken up by plant roots at a high rate. Indeed, NH_4^+ uptake is of major importance for N nutrition under numerous circumstances (von Wirén et al. 2001). In controlled experiments, it has been demonstrated that the presence of NH_4^+ in the substrate can counteract the negative effect of NO_3^- for Fe uptake by roots (Jiménez et al. 2007). Nitrate absorption is normally mediated by two-proton co-transport, which can increase the pH in the rhizosphere or neutralise the protons released by roots to increase Fe solubility in the soil or in the apoplast (Nikolic and Römheld 2002, Kosegarten et al. 2004). Consequently, the presence of NO_3^- in the soil contributes to induce Fe-deficiency chlorosis in plants cultivated in lime soils (Mengel et al. 1994). In contrast, NH_4^+ uptake induces an acidification of the rhizosphere because of the excretion of protons via the H^+-ATPase, favouring the reduction of Fe(III). In order to improve the effectiveness of NH_4^+ for alleviating Fe chlorosis in plants, it may be crucial to develop effective strategies to maintain the concentration of NH_4^+ in the soil at a medium-to-low level by slowing down the oxidation of NH_4^+ to NO_3^- (nitrification). In this context, the employment of nitrification inhibitors applied to the soil may optimise N and Fe nutrition simultaneously. Some authors have reported the effectiveness of 3,4-dimethylpyrazole phosphate (DMPP) as a nitrification inhibitor (Zerulla et al. 2001, Irigoyen et al. 2003). The utilisation of DMPP at 0.5–1.5 kg/ha can be sufficient to achieve optimal inhibition of nitrification in the substrate, reducing significantly NO_3^- leaching and N_2O emission, and improving crop yields (Zerulla et al. 2001). The effectiveness of DMPP may be drastically reduced in soils with extremely high temperature (>30°C), and this should be taken into account in warm climates (Irigoyen et al. 2003).

The maintenance of a limited concentration of ammonium in the rhizosphere and intercropping with Fe-efficient grasses have partially controlled Fe chlorosis in fruit crops. These management techniques, however, have not been investigated in the grapevine, and their effect on physiological and biochemical mechanisms in roots and leaves are unknown. This study evaluated the effectiveness of several alternative treatments for preventing Fe chlorosis in the grapevine. The effect of the treatments on the metabolism of root organic acids, leaf photosynthesis, and leaf structure and morphology was also evaluated.

Materials and methods

Plant material, growth conditions and treatments

The experiment was undertaken over two consecutive seasons (2010–2011) at the Experimental Station of the Agriculture Faculty of Bologna University. In summer 2010, grapevines *Vitis vinifera* L. cv. Cabernet Sauvignon clone ISV-F-V5 grafted on the Fe-chlorosis susceptible genotype *Vitis riparia* Mchx. cv. Gloire de Montpellier were acclimated under a black shade net (30% of shading) for 3 weeks and pruned to one main 10–20 cm long shoot per plant. Plants were transferred to 33 L plastic pots (one plant per pot) filled with a calcareous soil (Table 1) and placed under a structure covered with transparent polyethylene film (thickness 0.16 mm). The pots were covered with light reflecting aluminium film (Cuki Cofresco S.p.a., Turin, Italy) to maintain the soil temperature below 30°C.

The treatments tested were: (i) control (bare soil); (ii) soil-applied Fe-ethylenediamine-N,N′-bis(2-hydroxyphenyl)acetic acid (Fe-EDDHA) chelate; (iii) leaf-applied Fe-ethylenediaminetetraacetic acid (Fe-EDTA) chelate; (iv) intercropping with *Festuca rubra* (graminaceous species); (v) soil-applied NH_4^+; and (vi) soil-applied NH_4^+ + nitrification inhibitor. The experimental design was completely randomised, and each treatment was applied to seven plants. The nitrification inhibition was maintained during the experiment with DMPP. To determine the effectiveness of the nitrification inhibitor, additional pots (three replicates) without plants were treated with NO_3^-, NH_4^+ and NH_4^+ + DMPP.

Iron chelates were applied according to the leaf Soil Plant Analysis Development (SPAD) index to maintain an intensive green colour of leaves (SPAD index >25). A dose of 100 mL of 6% Fe-EDDHA solution (1 g Fe/L) was applied to pots three and two times, respectively, during the 2010 and 2011 seasons. The solution of Fe-EDTA (2 mmol/L pH 6.0, without surfactant) was sprayed on the canopy until complete leaf wetting at around 7:00 am. The solution was applied nine and six times during the 2010 and 2011 seasons, respectively. The graminaceous species *Festuca rubra* was sown in pots 1 day after transplanting at a density of 20 000 seeds/m^2. Ammonium-fertilised vines received 100 mL of $(NH_4)_2SO_4$ (1 g N/L) or 100 mL of $(NH_4)_2SO_4$ (1 g N/L) + DMPP (1% of the N amount supplied) solutions applied weekly during the season until the vine had received 1.5 g N.

The same amount of N supplied in the pots treated with N-NH_4^+ was also applied as N-NO_3^- to the other treatments (control, Fe-EDDHA, Fe-EDTA and intercropping), as a solution of $Ca(NO_3)_2$ (1 g N/L). In intercropped pots, an additional supply of N and water (+20% compared with the dose applied to the other treatments) was added. Each pot for all treatments was irrigated daily with one 2 L/h out-line microdrip emitter (Netafim Ltd, Tel Aviv, Israel) maintaining a constant level of soil moisture, close to field capacity (40% saturation). Weeds were manually removed, and pest and disease protection was regularly carried out. In 2011, an additional supply of potassium (2 g of K as K_2SO_4) was added to the intercropped pots to overcome visible potassium deficiency symptoms in the grapevines leaves. In February 2011, vines were pruned to one spur with three buds per vine.

Leaf chlorophyll content and plant growth

Leaf chlorophyll content was periodically measured during the experiment on five points of the first completely expanded leaf with a Minolta SPAD 502 portable greenness meter (Konica Minolta, Inc., Osaka, Japan). In 2010, after leaf abscission, the dry mass of shoots, leaves and pruning was measured. At the

Table 1. Chemical and physical properties of the soil utilised in the experiment.

Parameter	Unit	Value	Extractant/method
pH	–	8.39	Water/Potentiometric
Total carbonates ($CaCO_3$)	%	78	Hydrochloric acid/De Astis method
Active lime ($CaCO_3$)	%	19.2	Ammonium oxalate (Drouineau 1942)
Organic matter	%	0.54	Walkley-Black 1919 (Soltner 1988)
Total nitrogen (N)	‰	0.39	Kjeldahl method
Assimilable phosphorous (P)	mg/kg	3	Olsen (Olsen and Sommers 1982)
Exchangeable potassium (K)	mg/kg	195	Barium chloride (Hendershot and Duquette 1986)
Exchangeable sodium (Na)	mg/kg	186	Barium chloride (Hendershot and Duquette 1986)
Exchangeable calcium (Ca)	mg/kg	2611	Barium chloride (Hendershot and Duquette 1986)
Exchangeable magnesium (Mg)	mg/kg	47	Barium chloride (Hendershot and Duquette 1986)
Assimilable iron (Fe)	mg/kg	2.68	DTPA (Soltanpour and Schwab 1977)
Assimilable manganese (Mn)	mg/kg	1	DTPA (Soltanpour and Schwab 1977)
Assimilable zinc (Zn)	mg/kg	0.51	DTPA (Soltanpour and Schwab 1977)
Assimilable copper (Cu)	mg/kg	2.2	DTPA (Soltanpour and Schwab 1977)
Assimilable boron (B)	mg/kg	0.29	Calcium chloride (Bingham 1982)
Relation C/N	–	8.03	
Cation exchange capacity (CEC)	meg/100 g	14.72	Barium chloride (Hendershot and Duquette 1986)
Soil texture			
Sand	%	24	
Lime	%	53	Gee and Bauder 1986
Clay	%	23	

DTPA, diethylenetriaminepentaacetic acid.

end of the experiment (July 2011), plants were divided into roots, main shoot and leaves for dry mass determination.

Nitrate and ammonium concentration in the soil

At the end of the experiment, soil samples were collected from each pot at a depth of 15 cm. Nitrate- and ammonium-N fractions were extracted with a 2-mol/L solution of KCl (Mulvaney 1966). Samples were centrifuged, and the supernatant solution was collected and stored at −20°C until analysis. Nitrate- and ammonium-N concentration in the soil extracts was determined by ultraviolet-spectrophotometric auto-analyser (Auto Analyzer AA-3; Bran + Luebbe, Norderstadt, Germany). Both NH_4^+ and NO_3^- absorbance were converted to concentration with reference to a calibration curve prepared from standard solutions.

Concentration of organic acids and PEPC activity in roots

At the end of the experiment, root tip (20–30 mm long) samples were collected from each plant, rinsed in deionised water, weighed, deep-frozen in liquid nitrogen and stored at −80°C for analysis. The activity of PEPC, and the concentration of the proteins and the main organic acids were determined in root extracts. The root extract for PEPC activity analysis was prepared according to Jiménez et al. (2007), and PEPC activity was determined by coupling its activity to malate dehydrogenase-catalysed nicotinamide adenine dinucleotide hydrate oxidation (Vance et al. 1983). Protein concentration was determined by the Bradford method using bovine serum albumin as standard (Bradford 1976). Data obtained in the enzyme assays were expressed as nmol/(mg protein·min). The concentration of organic acids was determined according to Neumann (2006). Samples of frozen root tips were submerged in a precooled (4°C) mortar with liquid nitrogen. After evaporation, the tissue was ground and homogenised with a pestle. A 5% H_3PO_4 solution was utilised for extraction and deproteinisation. Organic acids were quantified by high-performance liquid chromatography with 250 × 4 mm LiChrospher 5 µm RP-18 column (Supelco, Inc., Bellefonte, PA, USA). High-performance liquid chromatography elution buffer was 18 mmol/L KH_2PO_4, pH 2.1 adjusted with H_3PO_4. Chromatograms were run for 40 min, and the detection wavelength was 210 nm. During the analysis, the three organic acids, citrate, malate and ascorbate, were identified and quantified.

Measurement of leaf gas exchange and chlorophyll fluorescence

Simultaneous measurement of leaf CO_2 gas exchange and chlorophyll fluorescence in 2010 and 2011 was determined on three plants per treatment using an infrared gas analyser (LI-COR 6400 IRGA with an integrated 6400-40 leaf chamber fluorometer, Li-Cor, Inc., Lincoln, NE, USA). During the experiment, measures were performed on the first mature leaf inserted in the middle third of the shoot. Leaves were illuminated by the LI-COR 6400 LED light source providing a photosynthetic photon flux density of around 1000 µmol/(m^2·s). Net photosynthesis (A_n) was measured when foliar CO_2 uptake was steady. During the experiment, gas-exchange measurements including CO_2 assimilation rate and stomatal conductance (g_s) were made in the morning between 9:00 am and 12:00 pm. The level of CO_2 was fixed at 380 mg/L within the leaf chamber. Chlorophyll fluorescence was simultaneously recorded with gas-exchange measurements. Fluorescence parameters were set for light-adapted leaves. Saturation pulses of approximately 8000 µmol photons/(m^2·s) with a 0.8-s duration were applied in order to saturate the PSII reaction centres to estimate the maximum fluorescence (Fm′). Additionally, the 'dark pulse'

routine was performed in order to estimate the yield of fluorescence in absence of an actinic (photosynthetic) light (Fo′). Comparison of these values with the steady-state yield of fluorescence in the light (Fs) has been used for determining the efficiency of photochemical quenching. The fluorescence parameters for light-adapted leaves were calculated using the equations proposed by Genty et al. (1989) and reviewed by Maxwell and Jhonson (2000). The efficiency of PSII photochemistry was calculated as:

$$\Phi_{PSII} = (Fm' - Fs)/Fm' \quad (1)$$

and the linear electron transport rate (ETR) as:

$$ETR = \Phi_{PSII} \times PFD \times 0.5 \quad (2)$$

Photon flux density (PFD) is absorbed light [µmol photon/(m^2·s)] (measured using an integrating sphere), and 0.5 is the factor that accounts for the partitioning of energy between PSII and Photosystem I. It was also calculated as the efficiency of excitation energy capture by open PSII reaction centres (Fv′/Fm′), and the expression was given by the equation:

$$Fv'/Fm' = (Fm' - Fo')/Fm' \quad (3)$$

Scanning electron microscopy

At the end of the experiment, one mature leaf per treatment from the middle third of the plant was collected for SEM. Small samples (2–3 mm long) from leaf intervenial areas, petioles and main veins were cut and fixed for 2 h at 5°C in 0.1 mol/L phosphate buffer (pH 7.2) containing 5% (w/v) glutaraldehyde (Pisi and Filippini 1994). After washing in the buffer, the tissue pieces were dehydrated at an increasing concentration of aqueous ethanol (10, 20, 30, 50, 75 and 95%) for 15 min at 5°C and in 100% ethanol at room temperature for 5 min. Then samples were dried with a critical point drier unit Emitech K850 (Emitech Ltd, Ashford, England) mounted on aluminum stubs with silver glue and coated with gold-palladium film using an ion sputtering unit Emitech K500 (Emitech Ltd). Samples were observed with a Philips 515 SEM scanning electron microscope (Philips, Eindhoven, The Netherlands) at 10 kV, and pictures were taken with a Nikon 5400 Coolpix digital camera (Nikon, Tokyo, Japan).

Statistics

Comparison of means and analysis of variance between treatments were done using SAS software (SAS Institute, Cary, NC, USA). Analyses were performed on raw data to maximise variance homogeneity. If significant differences were found with *F*-tests, means were compared using the Newman and Keuls test ($P \leq 0.05$).

Results

Leaf chlorophyll content and plant growth

Fifteen days after the onset of the treatments in 2010, plants started displaying differences in the leaf greenness according to the treatments (Figure 1) without any change to leaf area or the dry mass of the shoots (data not reported). Application of Fe-EDDHA increased leaf SPAD value during the season (Figure 1). Until 21 days after the onset of treatments, intercropped vines exhibited the lowest leaf SPAD index (Figure 1). At days 28 and 35, statistical differences were recorded only between the intercropping and Fe-EDDHA treatments (Figure 1). At the end of the 2010 season (63 and 70 days

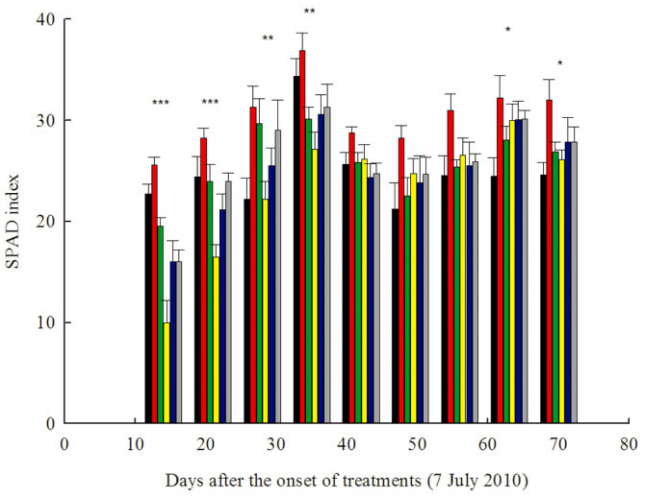

Figure 1. Effect of soil-applied iron (Fe)-ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid (■), leaf-applied Fe-ethylenediaminetetraacetic acid (■), intercropping (■), ammonium (■) and ammonium + 3,4-dimethylpyrazole phosphate (■), and the control (■) as treatments for the prevention of iron chlorosis in the grapevine on the time course of chlorophyll content [Soil Plant Analysis Development (SPAD) index] determined during the 2010 season in the first expanded apical leaf. Data are means of seven replicates. *Significant at $P \leq 0.05$, **Significant at $P \leq 0.01$, ***Significant at $P \leq 0.001$ level.

after the onset of treatments), control vines exhibited a greenness intensity significantly lower than that of vines treated with Fe-EDDHA, whereas vines treated with Fe-EDTA, NH_4^+ and NH_4^+ + DMPP, and intercropping exhibited intermediate leaf SPAD values, not statistically different to either that of the control or Fe-EDDHA treatments (Figure 1).

During the second season (2011), plants treated with Fe-EDDHA showed the highest leaf green intensity, always significantly higher than that of the control and of the other treatments on most measurement dates (Figure 2). Plants treated with Fe-EDTA, NH_4^+ (with and without DMPP) and intercropped with *Festuca rubra* displayed intermediate values, not always significantly different to that of the control or Fe-EDDHA treatments (Figure 2). In particular, starting from day 29, the SPAD value of the NH_4^+ treatment alone was not significantly different to that of the control (Figure 2).

Data collected at the end of the experiment showed that Fe-EDTA application significantly increased the leaf area and mass as compared with that of the control (1.5-fold for both parameters), whereas intercropping substantially decreased the leaf area of vines (Table 2). Other strategies to prevent Fe chlorosis did not modify the leaf area (Table 2). Intercropping decreased the dry mass of roots (1.6-fold), shoots (2-fold), leaves (1.9-fold) and plant (1.7-fold). Application of Fe-EDDHA, NH_4^+ and NH_4^+ + DMPP did not affect leaf area and dry mass of organs compared with that of control vines (Table 2).

*Nitrate- and ammonium-*N *concentration in the soil*

At the end of the experiment, significant differences in the soil N-NO_3^- concentration were recorded, whereas N-NH_4^+ concentration did not differ between treatments (Table 3). Soil collected from intercropping pots showed the largest decrease (3.9-fold) in N-NO_3^- concentration as compared with that of the control, followed by Fe-EDTA (2.4-fold) and NH_4^+ + DMPP (1.9-fold) (Table 3). In pots without plants, fertilisation with NH_4^+ + DMPP significantly increased N-NH_4^+ concentration in

Table 2. Impact of treatments for the prevention of iron chlorosis in the grapevine on leaf area and mass of organs after 2 years.

Treatment	Leaf area (m^2)	Mass of organs (g dry mass/plant)				
		Roots	Trunk	Shoots	Leaves	Total
Control	0.18 b	24.5 ab	15.4	10.7 ab	12.8 b	63.5 ab
Fe-EDDHA (soil-applied)	0.23 ab	26.8 ab	15.9	14.3 a	16.6 ab	73.5 ab
Fe-EDTA (leaf-applied)	0.27 a	31.3 a	16.7	15.8 a	19.1 a	82.9 a
Intercropping	0.11 c	15.0 c	11.0	5.1 b	6.8 c	37.9 c
Ammonium	0.19 b	22.1 b	13.4	10.1 ab	12.9 b	58.6 b
Ammonium + DMPP	0.18 b	23.3 ab	16.4	9.5 ab	12.4 b	61.6 ab
Significance	**	*	n.s.	*	**	*

*Significant at $P \leq 0.01$, **significant at $P \leq 0.001$ level. Means followed by the same letter in each column were not significantly different according to the Student-Newman-Keuls test. Data are means of seven replicates. DMPP, 3,4-dimethylpyrazole phosphate; EDDHA, ethylenediamine-N,N′-bis(2-hydroxyphenyl)acetic acid; EDTA, ethylenediaminetetraacetic acid; Fe, iron; n.s., not significant.

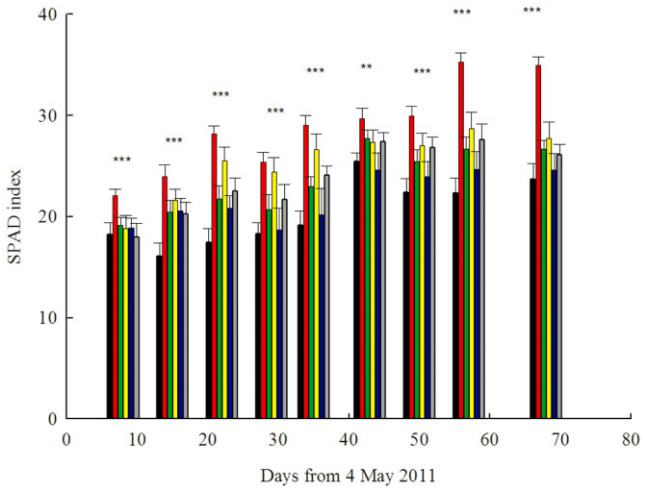

Figure 2. Effect of soil-applied iron (Fe)-ethylenediamine-N,N'-bis(2-hydroxyphenylaceticacid (■), leaf-applied Fe-ethylenediaminetetraacetic acid (■), intercropping (■), ammonium (■) and ammonium + 3,4-dimethylpyrazole phosphate (■), and the control (■) for the prevention of iron chlorosis in the grapevine on the time course of chlorophyll content [Soil Plant Analysis Development (SPAD) index] determined during the 2011 season in the first expanded apical leaf. Data are means of seven replicates. **Significant at $P \leq 0.01$, ***Significant at $P \leq 0.001$ level.

the soil as compared with the application of NH_4^+ alone (data not reported). Consequently, supply of DMPP effectively inhibited nitrification processes.

Concentration of organic acids and PEPC activity in roots

The activity of PEPC was determined in root extracts. At the end of the experiment, roots from control plants showed PEPC activity higher than that of the other treatments (Table 4). Moreover, the major organic acid present in root extracts was malic acid, followed by citric acid and ascorbic acid (Table 4). Application of Fe-EDDHA and NH_4^+ + DMPP decreased citric acid concentration in the roots compared with that of the control (Table 5). None of the treatments affected the concentration of malic acid or ascorbic acid in the roots (Table 4).

Leaf gas exchange and chlorophyll fluorescence

During the experiment, leaf gas exchange and fluorescence parameters were measured in the first mature leaf. In 2010,

Table 3. Nitrate and ammonium concentration in soil samples collected from pots after 2 years.

Treatment	Nitrate	Ammonium (mg/kg)
Control	6.6 a	0.055
Fe-EDDHA (soil-applied)	5.2 ab	0.055
Fe-EDTA (leaf-applied)	2.8 bc	0.003
Intercropping	1.7 c	0.038
Ammonium	4.6 abc	0.003
Ammonium + DMPP	3.5 bc	0.003
Significance	*	n.s.

*Significant at $P \leq 0.01$ level. Means followed by the same letter in each column were not significantly different according to the Student-Newman-Keuls test. Data are means of seven replicates. DMPP, 3,4-dimethylpyrazole phosphate; EDDHA, ethylenediamine-N,N′-bis(2-hydroxyphenyl)acetic acid; EDTA, ethylenediaminetetraacetic acid; Fe, iron; n.s., not significant.

treatments did not modify net photosynthesis or stomatal conductance in leaves, whereas intercropping decreased Fv′/Fm′, ΦPSII and ETR values as compared with those of the control (Table 5). In 2011, intercropping increased leaf net photosynthesis as compared with that of the vines treated with Fe-EDTA, with the other treatments showing intermediate values (Table 6). In addition, intercropping with *Festuca rubra* increased leaf stomatal conductance as compared with that of the control vines. Treatments did not influence fluorescence parameters (Table 6).

Scanning electron microscopy

Leaf stomatal density of plants treated with NH_4^+ was lower than that of the other treatments (data not reported). The leaf stomatal length of vines treated with NH_4^+ + DMPP (Figure 3c) and intercropped with *Festuca rubra* (Figure 3b) was enhanced compared with that of the control (Figure 3a). Although the treatments affected internal leaf structure, leaf thickness was not altered (data not reported). The images of the leaf transversal sections show that vascular bundles and palisade parenchyma cells were better organised and defined in leaves of vines treated with Fe-EDDHA (Figure 3e) as compared with those of Fe-EDTA-treated leaves (Figure 3f). Leaves treated with Fe-EDDHA (Figure 3e) and Fe-EDTA (Figure 3f) had a more porous spongy parenchyma, with larger intracellular spaces

Table 4. Effect of treatments for the prevention of iron chlorosis in the grapevine on the activity of phosphoenolpyruvate carboxylase and the concentration of organic acids in an extract of roots sampled after 2 years.

Treatment	PEPC activity [nmol/(mg protein · min)]	Organic acid concentration (mg/g FM)			
		Citric acid	Malic acid	Ascorbic acid	Total organic acids
Control	27.3 a	0.60ab	2.4 ab	0.05 ab	3.1 ab
Fe-EDDHA (soil-applied)	16.4 b	0.39 c	2.1 ab	0.05 ab	2.5 b
Fe-EDTA (leaf-applied)	17.4 b	0.69 a	2.7 a	0.06 ab	3.5 a
Intercropping	11.9 b	0.49 bc	1.7 b	0.07 a	2.3 b
Ammonium	16.5 b	0.46 bc	1.9 b	0.03 b	2.4 b
Ammonium + DMPP	12.0 b	0.41 c	2.2 ab	0.03 b	2.6 b
Significance	**	**	**	*	**

*Significant at $P \leq 0.05$, **significant at $P \leq 0.01$. Means followed by the same letter in each column were not significantly different according to the Student-Newman-Keuls test. Data are means of seven replicates. DMPP, 3,4-dimethylpyrazole phosphate; EDDHA, ethylenediamine-N,N′-bis(2-hydroxyphenyl)acetic acid; EDTA, ethylenediaminetetraacetic acid; Fe, iron; FM, fresh mass; PEPC, phosphoenolpyruvate carboxylase.

Table 5. Effect of treatments for the prevention of iron chlorosis in the grapevine on leaf gas exchange and chlorophyll fluoresencemeasured in the first three mature leaves from the middle third shoot in the 2010 season.

Treatments	A_n [μmol CO_2/(m^2·s)]	g_s [mol H_2O/(m^2·s)]	Fv′/Fm′ (relative units)	ΦPSII (relative units)	ETR (relative units)
Control	7.7	0.24	0.47 a	0.19 ab	79.1 ab
Fe-EDDHA (soil-applied)	6.8	0.27	0.47 a	0.19 ab	79.0 ab
Fe-EDTA (leaf-applied)	8.0	0.29	0.43 a	0.21 a	89.0 a
Intercropping	5.8	0.28	0.34 b	0.13 c	54.2 c
Ammonium	7.8	0.25	0.43 a	0.18 ab	78.3 ab
Ammonium + DMPP	6.5	0.27	0.42 a	0.15 bc	64.1 bc
Significance	n.s.	n.s.	***	**	*

*Significant at $P \leq 0.05$, **significant at $P \leq 0.01$, ***significant at $P \leq 0.001$ level. Means followed by the same letter in each column were not significantly different according to the Student-Newman-Keuls test. ΦPSII, efficiency of Photosystem II photochemistry; A_n, net photosynthesis; DMPP, 3,4-dimethylpyrazole phosphate; EDDHA, ethylenediamine-N,N′-bis(2-hydroxyphenyl)acetic acid; EDTA, ethylenediaminetetraacetic acid; ETR, linear electron transport rate; Fe, iron; Fv′/Fm′, efficiency of excitation capture by open Photosystem II reaction centres; g_s, stomatal conductance; n.s., not significant.

Table 6. Effect of treatments for the prevention of iron chlorosis in the grapevine on leaf gas exchange and chlorophyll fluorescence measured on the first three mature leaves from the middle third shoot in the 2011 season.

Treatments	A_n [μmol CO_2/(m^2·s)]	g_s [mol H_2O/(m^2·s)]	Fv′/Fm′ (relative units)	ΦPSII (relative units)	ETR (relative units)
Control	7.9 ab	0.10 b	0.53	0.20	86.9
Fe-EDDHA (soil-applied)	7.8 ab	0.09 b	0.51	0.24	99.9
Fe-EDTA (leaf-applied)	7.0 b	0.10 b	0.51	0.20	86.7
Intercropping	11.2 a	0.19 a	0.53	0.27	114.2
Ammonium	8.2 ab	0.14 b	0.56	0.22	94.8
Ammonium + DMPP	8.3 ab	0.13 b	0.49	0.23	95.7
Significance	*	**	n.s.	n.s.	n.s.

*Significant at $P \leq 0.05$; **significant at $P \leq 0.001$ level. Means followed by the same letter in each column were not significantly different according to the Student-Newman-Keuls test. ΦPSII, efficiency of Photosystem II photochemistry; A_n, net photosynthesis; ETR, linear electron transport rate; DMPP, 3,4-dimethylpyrazole phosphate; EDDHA, ethylenediamine-N,N′-bis(2-hydroxyphenyl)acetic acid; EDTA, ethylenediaminetetraacetic acid; Fe, iron; Fv′/Fm′, efficiency of excitation capture by open Photosystem II reaction centres; g_s, stomatal conductance; n.s., not significant.

compared with that of the other treatments (Figure 3d,g,h,i). The epidermal cells were similar in size and shape in the control (Figure 3d) and in the Fe-EDDHA treatment (Figure 3e), while more heterogeneous in Fe-EDTA, intercropping, NH_4^+ and NH_4^+ + DMPP treatments (Figure 3f,g,h,i).

Treatment with Fe-EDDHA (Figure 3e) and Fe-EDTA (Figure 3f) led to thick and homogeneous epidermal cell walls similar to that of the control (Figure 3d), whereas intercropped- and NH_4^+-fed plants (Figure 3g,h,i) had thinner, discontinuous and apparently heterogeneous epidermal cell walls. The

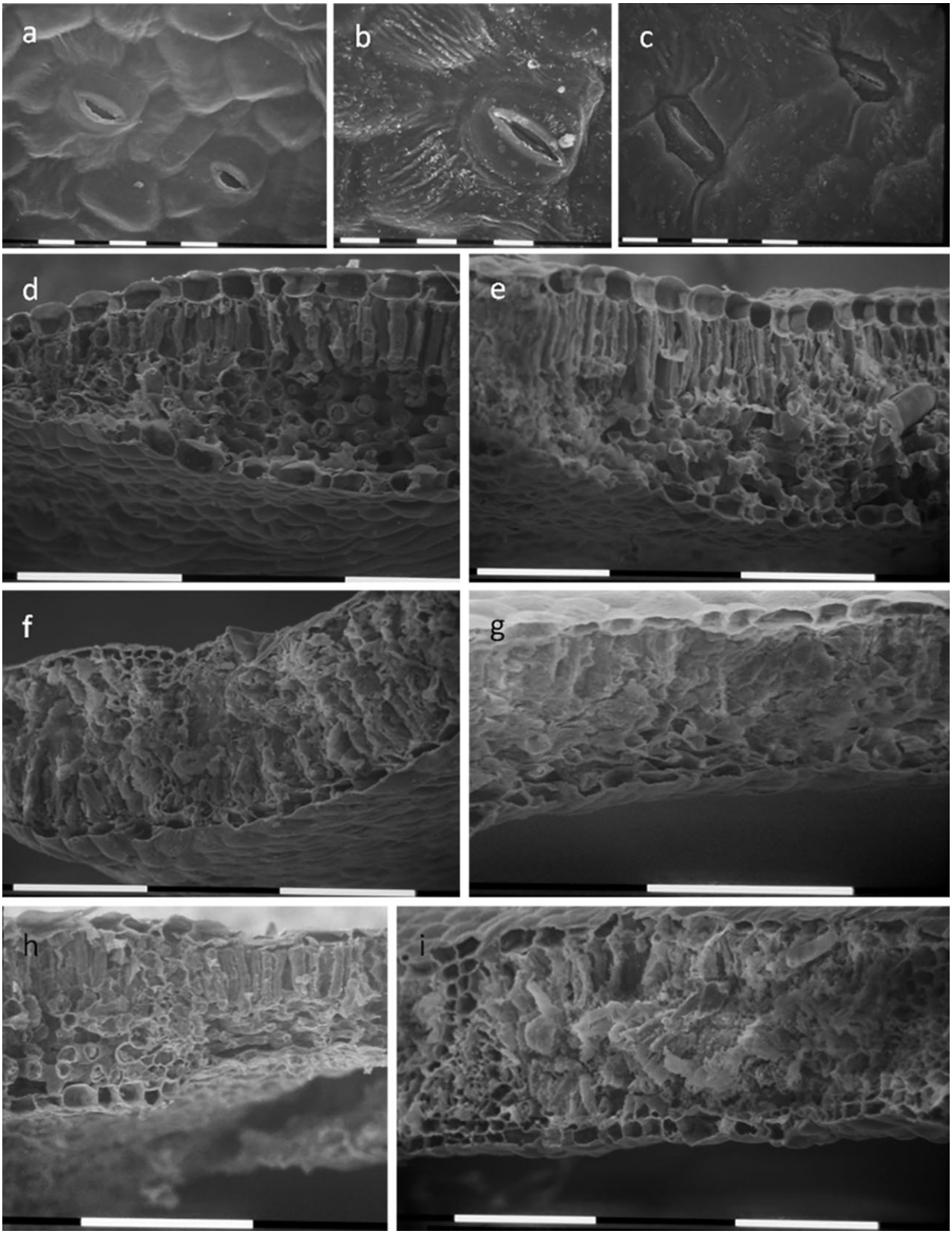

Figure 3. Scanning electron micrographs (SEM) of stomata in abaxial grapevine leaf surfaces: (a) in control vines; (b) in vines intercropping with *Festuca rubra*; and (c) treated with ammonium + 3,4-dimethylpyrazole phosphate (DMPP). SEM micrographs of grapevine leaf transversal sections: (d) epidermal cells in control plants; (e) vascular bundles and palisade parenchyma cells in vines treated with soil-applied Fe-ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid and (f) with leaf-applied Fe-ethylenediaminetetraacetic acid; (g) epidermal cells in vines intercropping with *Festuca rubra*; (h) treated with ammonium; and (i) with ammonium + DMPP. Bar = 10 μm (from a to c), bar = 0.1 mm (from d to i).

morphology of the abaxial and adaxial leaf surface was slightly modified by the treatments. Vines treated with Fe-EDDHA (Figure 3e), Fe-EDTA (Figure 3f) and intercropping (Figure 3g) appeared to have more epicuticular waxes as compared with that of the control (Figure 3d), NH_4^+ (Figure 3h) and NH_4^+ + DMPP (Figure 3i) treatments as indicated by a smoother, glazed-like surface.

Discussion

During the first season, the leaf greenness index of plants fertilised with NH_4^+ (alone or with DMPP) was intermediate between that of control and Fe-EDDHA-treated vines (Figure 1); however, NH_4^+ supply did not lead to statistically measurable changes. In the following season (2011), the application of NH_4^+ alone did not induce a significant increase in greenness intensity, whereas the addition of DMPP to NH_4^+ supply resulted in an increased SPAD index (Figure 2).

Several studies conducted with the grapevine have established a good correlation between the leaf greenness intensity assessed by the SPAD index and the leaf chlorophyll content (Petrie et al. 2000, Porro et al. 2001, Steele et al. 2008) suggesting that the index is a reliable indicator of leaf chlorophyll concentration. In addition, in a long-term trial conducted in the grapevine by Porro et al. (2001), SPAD values were significantly correlated with the concentration of leaf N and P. A study conducted on cv. Pinot Blanc grafted on Kober 5BB showed that the concentration of leaf chlorophyll and Fe expressed on fresh mass and area basis, as well as the concentration per individual leaf, was significantly related (Bavaresco et al. 1999). Taken together, this suggests that the SPAD index is a reliable indicator of Fe nutrition in plants. Beneficial effects of NH_4^+ on Fe (and other nutrients) uptake that countered the adverse effects of NO_3^-, were reported by Houdusse et al. (2007); however, these effects were associated with a reduction in plant growth as compared with that of plants fed with mixed N forms (NO_3^-, NH_4^+ and urea) (Houdusse et al. 2007). This suggests that the maintenance of a limited NH_4^+ concentration along with other N forms in the soil may be effective for improving Fe nutrition in plants.

The initial leaf yellowing of intercropped vines probably because of the competition for Fe (and other nutrients) exercised by *Festuca rubra*, which occurred in the first 21 days of the 2010 season, progressively disappeared over time, and values intermediate between that of the control and of the Fe-EDDHA treatment were recorded at the end of the season (Figure 1). During 2011, intercropping increased the leaf chlorophyll content indicating that this soil management system improved Fe nutrition (Figure 2). The presence of graminaceous species, however, strongly reduced leaf area and plant mass in intercropped vines (Table 2). Similar (Inal et al. 2007) but also contrasting (Zuo et al. 2000) results have been reported for peanut (*Arachis hypogaea* L.) intercropped with maize, suggesting that the effect of intercropping on the growth of a co-cultivated crop may vary according to the experimental conditions (water and nutrient availability and sowing density of the cover crop). In a pot experiment conducted in citrumelo 'Swingle', intercropping with graminaceous species did not reduce dry mass of co-cultivated plants (Ammari and Rombolà 2010). In grapevine, Bavaresco et al. (2010) did not observe a significant reduction of shoot length in potted vines intercropped with *Festuca ovina* after 4 years of cultivation on calcareous soil. A possible cause of growth reduction in intercropped plants observed in our experiment could be the strong competition for mineral elements and water uptake exerted by the graminaceous species, in spite of the additional (20%) water and N supplied. Such a hypothesis is supported by the lower concentration of N-NO_3^- recorded in the soil covered with the intercropping system (Table 3). In addition, restriction of root growth caused by space limitation in pots would exacerbate competition for these essential sources between species. In studies performed in the grapevine, shoot growth was severely inhibited despite continuous water and nitrogen supply, suggesting possible allelopathic effects (Lopes et al. 2004). It is important to note that intercropping can be an effective tool for controlling the vigour and with potential benefits on grape composition across a range of climates and soils.

In our experiment, Fe deficiency increased the activity of PEPC in roots (Table 4). The enzyme PEPC catalyses the incorporation of bicarbonate into a C_3 organic acid, phosphoenolpyruvate, generating oxalacetate, which is converted to malate by malate dehydrogenase (Lance and Rustin 1984). This process is an important component of the pH-stat mechanism inside the cell (Davies 1973). An increase in root PEPC activity induced by Fe deficiency was reported for several model plants grown in a hydroponic system, and PEPC was proposed as a biochemical marker for Fe deficiency status in tolerant species (Rombolà et al. 2002, Rombolà and Tagliavini 2006, Jiménez et al. 2007). In grapevines grown hydroponically, an increase in PEPC activity in roots as a response to Fe deficiency was reported for the Fe-chlorosis tolerant genotype Cabernet Sauvignon (*Vitis vinifera*) and, at a lower level, for the Fe-chlorosis susceptible Gloire de Montpellier (*Vitis riparia*) (Jiménez et al. 2007). Moreover, the Fe-chlorosis tolerant rootstock 140 Ruggeri subjected to Fe deficiency significantly increased the activity of the root PEPC enzyme (Covarrubias and Rombolà 2013). To the best of our knowledge, however, the activity of this enzyme has not been reported in experiments where grapevines are grown in soil. Our data indicate that the Fe-chlorosis susceptible *Vitis riparia* rootstock is able to activate tolerance mechanisms to Fe deficiency in soil-grown grapevines. The activity of root PEPC in co-cultivated and NH_4^+-fed plants was similar to that recorded in plants supplied with Fe chelates, suggesting that both treatments improved Fe nutrition. An increases in the concentration of organic acids in roots of Fe-deficient plants is fairly ubiquitous and occurs both in Strategy I and Strategy II plant species (Abadía et al. 2002). The major organic acid present in root extracts of *Vitis riparia* rootstock was malate, followed by citrate and ascorbate (Table 4). Citric acid has an important role in Fe absorption and xylem transport of Fe in plants, and citrate metabolism may be associated with proton extrusion and with Fe(III) reduction activity (Ollat et al. 2003). Our data showed a lower concentration of citrate in roots of vines treated with Fe-EDDHA and NH_4^+ + DMPP (Table 4), supporting the effectiveness of these treatments for prevention of Fe deficiency. Surprisingly, foliar application of Fe-EDTA did not reduce the concentration of citric acid in roots, and Fe-EDTA-treated vines exhibited a behaviour similar to that of Fe-deficient plants (Table 4). This may be due to a reduced translocation of Fe applied as Fe-EDTA from leaves to roots, inducing an increase in the citric acid concentration in roots as a response to Fe deficiency. In *Prunus persica* L., Fernández et al. (2008a) reported a limited Fe translocation from treated to untreated areas within the same leaf, indicating a low Fe mobility into the tissues. Limited phloem translocation from shoots to roots may be also exacerbated by graft incompatibility between different genotypes because of a lower continuity of functional phloem between the two internodes (Espen et al. 2005). In the grapevine, incompatibility

between commercial cultivars and rootstock has been reported by Kocsis and Bakonyi (1994).

In the first season, intercropping decreased the fluorescence-derived parameters (Fv′/Fm′, ΦPSII and ETR) measured in light-adapted leaves (Table 5). A reduction in the photosynthesis rate in Fe-deficient chlorotic leaves was attributed to a decrease in PSII efficiency because of closure of PSII reaction centres and a decrease of the intrinsic PSII efficiency (Morales et al. 2000). Leaf Fv′/Fm′ represents the efficiency of light harvesting by open reaction centres of PSII (Genty et al. 1989). The lower leaf SPAD index recorded in intercropped vines (Figure 1) may partially explain the lower light harvesting efficiency registered. In addition, ΦPSII measures the proportion of light absorbed by chlorophyll associated with PSII used in photochemistry, while ETR is an indicator of overall photosynthetic capacity (Maxwell and Jhonson 2000). Therefore, data collected in the first season indicate that the establishment of intercropping induced photochemical changes in vines consistent with a temporary deficiency of Fe (and other nutrients), decreasing leaf photosynthetic capacity independently of stomatal conductance (g_s). Such results indicate that in the case of adopting intercropping management, the possibility of transient nutrient deficiencies should be taken into account. Interestingly, in the second season, intercropping increased g_s and ETR compared with that of the control (Table 6). The higher leaf stomatal conductance found in intercropped vines can be attributed to the enhanced leaf stomatal length recorded in the leaf surface of intercropped vines (Figure 3b). Similar results were reported by Fernández et al. (2008b), who noticed that Fe deficiency appeared to reduce the average size of stomatal pores in pear and peach species. Data concerning leaf g_s and stomatal length obtained in this study suggest that intercropping improved Fe nutrition in vines. In our experiment, leaf stomatal density was not significantly affected by Fe supply (Fe-EDDHA and Fe-EDTA) and by intercropping, as also reported by Fernández et al. (2008b), whereas, NH_4^+ supply decreased stomatal density probably because of a direct effect of NH_4^+ independent of Fe nutrition. Our data show that vascular bundle and palisade parenchyma cells were not well organised and defined in leaves of vines treated with Fe-EDTA (Figure 3f), whereas soil-applied Fe-EDDHA (Figure 3e) slightly improved structural and morphological leaf parameters as compared with those of the control (Figure 3d). In addition, Fe application induced a more porous leaf spongy parenchyma with larger empty intracellular spaces. Our results showed that the different management techniques aimed to prevent Fe deficiency in the grapevine can alter leaf morphology. Similar results were reported by Fernández et al. (2008b) for peach and pear, whereas contrasting results were reported by Maldonado-Torres et al. (2006) in *Citrus aurantifolia*, perhaps indicating that besides Fe nutrition, multiple factors, such as environmental conditions, water soil content and plant species, can modulate effects on leaf structure and morphology.

Leaves from intercropped vines appeared to have more epicuticular waxes, similar to those in Fe-chelate-treated vines. Lipidic components are required for adequate leaf growth, and their synthesis may be affected by Fe deficiency (Fernández et al. 2008b). Leaf transpiration rate (water loss) through the cuticle may be higher in Fe-deficient than in Fe-sufficient leaves, as observed in peach and pear by Fernández et al. (2008b), suggesting that cuticular properties are important for the leaf water status of plants. Data concerning fluorescence parameters (Table 6), however, revealed that intercropping decreased the water use efficiency of vines, calculated by A_n/g_s as described by Salazar-Parra et al. (2012), presumably because it enhanced water loss through leaf stomata.

Conclusions

Intercropping with *Festuca rubra* increased leaf SPAD index, stomata length and amount of leaf epicuticular waxes in grapevines. In addition, intercropping reduced the activity of PEPC in grapevine roots, as did the Fe-chelate supply treatments, suggesting that this was effective for prevention of Fe chlorosis. The employment of this management technique will require properly selected and managed grasses according to crop and environmental parameters. Application of NH_4^+ with a nitrification inhibitor can prevent Fe deficiency, increase leaf chlorophyll content and leaf stomata length. Moreover, NH_4^+ + DMPP induces root biochemical responses (PEPC activity and the concentration of organic acids) that are similar to those for Fe-EDDHA-treated vines, suggesting that it is effective for preventing Fe chlorosis in vineyards located in calcareous soils. In contrast, without the addition of nitrification inhibitors, the effectiveness of NH_4^+ application for prevention of Fe chlorosis decreased significantly. Leaf chlorophyll concentration was increased more effectively by Fe-EDDHA treatment than Fe-EDTA, improving leaf vascular bundle and parenchyma cell organisation and inducing more homogeneous leaf epidermal cells. Iron-EDTA induces a high concentration of root citric acid, suggesting a limited translocation of Fe, applied as Fe-EDTA, from leaves to roots. Intercropping decreased the leaf fluorescence-derived parameters in the first year and increased the leaf stomata conductance in the second year of the experiment. Future studies should concentrate on identifying Fe-efficient graminaceous species with low water and nutrient requirements and the use of natural nitrification inhibitors.

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