Fe-EDDHA Alleviates Chlorosis in ‘Concord’ Grapevines Grown at High pH

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Abstract. ‘Concord’ grapevines (*Vitis labruscana* Bailey) can readily develop iron deficiency-induced leaf chlorosis when grown on calcareous or high pH soils. Iron (Fe) chelates are often applied to the soil to remedy chlorosis but can vary in their stability and effectiveness at high pH. We transplanted own-rooted 1-year-old ‘Concord’ grapevines into a peat-based medium adjusted to pH 7.5 and fertigated them with 0, 0.5, 1.0, 2.0, or 4 mg·L⁻¹ Fe from Fe-EDDHA [ferric ethylenediamine di (*o*-hydroxyphenylacetic) acid] to determine the effectiveness of this Fe chelate for alleviating Fe deficiency-induced chlorosis at high pH. Vines were sampled midseason for iron, chlorophyll, CO₂ assimilation, and photosystem II quantum efficiency (PSII) and at the end of the season for leaf area, dry weight, and cane length. We found that leaf total Fe concentration was similar across all treatments, but active Fe (extracted with 0.1 N HCl) concentration increased as the rate of Fe-EDDHA increased. Chlorophyll concentration increased curvilinearly as applied Fe increased and was highly correlated with active Fe concentration. CO₂ assimilation, stomatal conductance, and PSII were very low without any supplemental Fe and increased rapidly in response to Fe application. Total leaf area, foliar dry weight, and cane length all increased as Fe application increased to 1 mg·L⁻¹ Fe, but above this rate, a further increase in Fe did not significantly increase growth. Our results demonstrate that Fe-EDDHA is very effective in alleviating Fe deficiency-induced leaf chlorosis in ‘Concord’ grapevines grown at high pH, which provides a foundation for continuing research related to the optimum rate and timing of application of Fe-EDDHA in ‘Concord’ vineyards on calcareous soils. Compared with total Fe, leaf “active Fe” better indicates the actual Fe status of ‘Concord’ vines.

‘Concord’ grapevines (*Vitis labruscana* Bailey) are native to the northeastern United States (Schofield, 1988) and are tolerant of the acidic soils that characterize the region. As soil pH increases and iron (Fe) becomes less available, own-rooted ‘Concord’ grapevines can readily develop Fe deficiency-induced leaf chlorosis. In ‘Concord’ vineyards where calcareous or high pH soils predominate such as in Washington State, leaf chlorosis can be a limiting factor in production (Davenport and Stevens, 2006).

Iron is a key nutrient in a number of metabolic pathways. The major symptom of Fe deficiency is chlorosis as a result of the requirement for Fe in protochlorophyllide synthesis (Totter et al., 2003). Fe is essential in reactions that regulate CO₂ assimilation and antioxidant protection, and we have shown that Fe-deficient source leaves have

lower levels of nonstructural carbohydrates and are source-limited (Chen et al., 2004; Smith and Cheng, 2005). In addition, organic acid metabolism is influenced by Fe status (Abadia et al., 2002).

When Fe deficiency-induced chlorosis occurs, chlorotic leaves can often have same or even higher total Fe levels than healthy green leaves, which has been referred to as the “chlorosis paradox” (Bavaresco et al., 1999; Römhelt, 2000). Thus, leaf total Fe concentration is not a good indicator of plant Fe status; instead, “active Fe,” extracted with dilute acids or ferrous Fe chelates, correlates better with chlorophyll concentration and leaf function (Chen et al., 2004; Gezgin and Er, 2001; Römhelt, 2000).

There are several means for alleviating Fe chlorosis in grapevines. Grafting scions to high pH tolerant or “Fe-efficient” rootstocks can be effective in preventing chlorosis (Brancadoro et al., 1995). However, ‘Concord’ vines are typically grown own-rooted to lower establishment cost. Other methods to control Fe deficiency include: 1) foliar Fe sprays, 2) lowering soil pH, and 3) supplementing the soil with Fe chelates (Tagliavini and Rombolà, 2001). Foliar Fe sprays can be effective but only provide a temporary solution; although lowering soil pH is the best means to overcome Fe deficiency, it is not always feasible. Ferric chelates applied to the soil can often alleviate chlorosis, but they

vary in their stability and effectiveness at high pH. For instance, EDDHA [ethylenediamine di (*o*-hydroxyphenylacetic) acid] can form a very stable ferric chelate from pH 4 to 9, whereas Fe-EDTA (ethylenediaminetetraacetic acid) is stable only at low pH and loses its ability to chelate Fe³⁺ above pH 6.5 (Norvell, 1991).

Fe-EDDHA has been used to alleviate Fe-chlorosis in a variety of crops, yet only a few reports address the use of Fe-EDDHA for correcting Fe-deficiency in *Vitis* spp. (Gruber and Kosegarten, 2002; Tagliavini and Rombolà, 2001). In addition, most of the published work with Fe nutrition in grapevines is concerned primarily with *V. vinifera*, and little to no information exists regarding the effectiveness of Fe-EDDHA for Fe correction in *V. labruscana*. Compared with *V. vinifera*, *V. labruscana* has a lower optimum pH (Bates et al., 2002), and the use of Fe-EDDHA to alleviate lime-induced chlorosis in the high pH-sensitive (Fe-inefficient) *V. labruscana* has not been characterized. The objective of this work was to determine if Fe-EDDHA can effectively alleviate chlorosis in ‘Concord’ grapevines grown at high pH, which will provide a foundation for further research related to the optimum rate and timing of application of Fe-EDDHA in ‘Concord’ vineyards with calcareous soils.

Materials and Methods

Own-rooted 1-year-old ‘Concord’ grapevines were transplanted on 5 May 2005 into 19.8-L plastic pots containing an 80% peat : 20% perlite (v/v) growth medium (Conrad Fafard Inc., Agawam, Mass). Substrate was adjusted to pH 7.5 with 16.3 kg·m⁻³ CaCO₃ (Grade R2; Mississippi Lime, St. Genevieve, Mo.), and vines were grown outdoors from 17 May 2005 to 29 Sept. 2005. At budbreak, extra shoots were removed and two shoots were allowed to grow on each plant.

Twice weekly, each plant was supplied with 1 L of a complete nutrient solution, including micronutrients and 0, 0.5, 1.0, 2.0, or 4.0 mg·L⁻¹ Fe from Fe-EDDHA (Dissolvevine; GreenCare Fertilizers, Chicago). Nutrients were blended as a water-soluble fertilizer based on Hoagland’s No. 2 solution by GreenCare Fertilizers and applied at a rate of (mg·L⁻¹) 210 N, 31 P, 235 K, 160 Ca, 49 Mg, 0.50 Mn, 0.05 Zn, 0.02 Cu, 0.01 Mo, and 0.49 B. Micronutrient cations were applied as sulfate salts, and solution was supplemented with 80 mg·L⁻¹ K from KCl. There were five replicates (three plants per replicate) for each treatment in a completely randomized design. Approximately 9 weeks after planting, one recently mature leaf from each shoot on one plant per rep was selected for midday CO₂ assimilation and photosystem II (PSII) quantum efficiency measurements. Selected leaves were then harvested for total Fe, active Fe, and chlorophyll analysis.

Gas exchange was measured with a CIRAS-1 system (PP Systems, Herts, U.K.) at ambient CO₂ (360 μmol·mol⁻¹) at noon

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under a photon flux density of $1540 \pm 68 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and a leaf temperature of $29.8 \pm 0.8^\circ\text{C}$. Chlorophyll fluorescence was measured with a pulse-modulated fluorometer (FMS2; Hansatech Instruments, Norfolk, U.K.) under the same environmental conditions, and PSII quantum efficiency (proportion of light absorbed by PSII used in photochemistry) was calculated as [maximum fluorescence (F_m) – steady-state fluorescence (F_s)]/ F_m (Genty et al., 1989).

After gas exchange and PSII measurements, chlorophyll was extracted from two 1.2-cm² leaf discs according to (Arnon, 1949). Leaves were then washed in 0.1 N HCl, rinsed in deionized H₂O, and dried at 70°C for 7 d. Dried samples were ground through a 0.5-mm screen using a Cyclotec sample mill.

Active Fe was extracted by shaking 50 mg dried leaf tissue in 1.0 mL of 0.1 N HCl for 24 h. The solution was centrifuged at 10,000 g for 5 min, and the supernatant was passed through a 0.45- μm polyvinylidene fluoride (PVDF) syringe filter. A 500- μL aliquot was removed and mixed with 250 μL of 500 mM ascorbic acid and 250 μL of 1 mM bathophenanthroline (BP). The Fe(II)-BP chromophore was developed by raising the pH to 4.5 with 500 μL of 2.5 M NaOAc-pH 4.5 (Diehl et al., [1980]; 100 mL NaOAc was decontaminated by adding 2 mL 500 mM AsA, 4 mL of 1 mM BP, and 10 mL 1-octanol and shaking and allowing the layers to separate. The alcohol layer was removed and additional 1-octanol was added and removed until there was no visible color.) Samples were mixed vigorously and allowed to sit for 5 min before the addition of 400 μL of 1-octanol. Solution was mixed again and the Fe(II)-BP complex was moved into the immiscible, 1-octanol phase after settling for 15 min. Samples were centrifuged at 1000 g_n for 5 min to promote phase separation. Three hundred microliters of the upper phase (containing Fe(II)-BP in 1-octanol) was transferred to a chemical resistant ultramicrocuvette (BrandTech, Essex, Conn.), capped, and absorbance measured at 535 nm against an appropriate blank. Fe concentration was calculated from a standard curve using Fe atomic absorption standard solution (Aldrich Chemical, Milwaukee, Wisc.) in 0.1 N HCl and extracting along with samples.

Total Fe was determined in a similar manner. Fifty milligrams of dried leaf tissue was digested in HNO₃ and 30% H₂O₂ according to Jones (1991). Clear digests were brought up to 3.5 mL with 0.1 N HCl and passed through a 0.45- μm PVDF syringe filter. Fe was quantified by mixing a 500- μL aliquot of the digest with 3.0 mL of 0.1 N HCl, 2.0 mL of 500 mM ascorbic acid (fresh), and 2 mL of 1 mM BP. After mixing over a 5-min period, we added 5 mL of 5 M NaOAc-pH 4.5 to develop the chromophore. Solutions were mixed and the Fe(II)-BP complex was moved into the upper phase with the addition of 1 mL of 1-octanol. Samples were allowed to sit overnight for maximum color formation, remixed, and centrifuged at 1000 g_n for 5

min. We removed 600 μL of the alcohol layer and quantified Fe as described for active Fe. We also spiked samples with 2 μg Fe before digestion and recovery was $96\% \pm 3.6\%$.

At the end of the season, vines were defoliated and total leaf area per pot was determined. Leaves were dried at 70°C for 7 d and the dry weight recorded. We measured the total cane length for both canes per pot and present the data as average cane length per vine.

Analysis of variance and mean comparison was performed using JMP 5.1 (SAS Institute, Cary, N.C.). Means were compared using LSD at $P = 0.05$. Nonlinear regression was performed using SigmaPlot 9.0 (Systat Software, Point Richmond, Calif.).

Results

Leaf total Fe concentration was similar across most treatments and higher only at the 4.0 mg·L⁻¹ Fe level when compared with the middle three rates (Fig. 1A). Total Fe concentration in the control leaves was not different from the other treatments. Both active Fe concentration and total chlorophyll concentration increased in response to an increase in the rate of applied Fe-EDDHA (Figs. 1B and 2A). Leaf chlorophyll concentration was significantly correlated with active Fe concentration (Fig. 2B) but not with total Fe concentration (data not shown).

CO₂ assimilation was very low without any supplemental Fe and increased rapidly in response to Fe application (Fig. 3A). As applied Fe increased, g_s increased from 109 to 619 mmol·m⁻²·s⁻¹ (Fig. 3B), whereas calculated internal CO₂ concentration decreased from 340 to 246 $\mu\text{mol}\cdot\text{mol}^{-1}$ ($P < 0.001$, data not shown). PSII quantum efficiency was lower in the control plants and increased at rates above 0.5 mg·L⁻¹ Fe (Fig. 3C). Total leaf area, foliar dry weight, and cane length all increased as Fe application increased to 1 mg·L⁻¹ Fe, but above this rate, a further increase in Fe did not significantly increase growth (Fig. 4A–C).

Discussion

This study confirms previous results indicating that foliar total Fe is not a good measure of Fe nutritional status in ‘Concord’ grapevines (Chen et al., 2004). Yet why Fe accumulates in chlorotic leaves and paradoxically fails to alleviate chlorosis is not well understood. Most agree that Fe is transported through the xylem primarily as Fe(III)-citrate, and on reaching the leaf apoplast, a leaf plasma membrane bound ferric chelate reductase is required to cleave Fe from the chelate and reduce it to Fe²⁺ before transport into the mesophyll cytoplasm (Brüggemann et al., 1993). Mengel (1994) suggests that Fe is immobilized in the leaf apoplast when soil HCO₃⁻ or NO₃⁻ raises the pH of the xylem sap and leaf apoplast. There is mixed support for this hypothesis (Nikolic and Römheld, 2002, 2003), and we have found that the pH

Fig. 1. (A) Total Fe concentration ($P \leq 0.05$) and (B) active Fe concentration ($P \leq 0.001$) of ‘Concord’ grape leaves in relation to Fe-EDDHA application rate. Leaf Fe concentration is expressed on a dry weight basis. Each point is the mean of five replicates. Means were separated using Tukey’s HSD test at $P = 0.05$.

of the xylem sap of ‘Concord’ vines does not change when they are grown in substrate pHs ranging from 5.2 to 7.6 (Smith and Cheng, unpublished data). Römheld (2000) suggests that the paradox results from environmental factors that cause an inhibition of leaf expansion. This inhibition of leaf expansion causes a decreased dilution of Fe in the leaf and that total Fe content calculated on a per-leaf basis provides a better indicator of Fe status. This may be true, but the same logic should apply to other nutrients, and it still does not fully explain why chlorosis is not alleviated. In our experiment, low Fe supply decreased the total leaf area per vine (Fig. 4A) as well as midseason individual leaf area (data not shown), indicating that the decrease in leaf expansion is the result of Fe deficiency, not some other environmental factor.

What is clear is that when the paradox exists, extracting active Fe provides a better diagnosis of Fe nutritional status (Figs. 1B and 2B). Active Fe analysis is generally quantified either by acid extraction from dried leaves followed by determination of total Fe in the extract by inductively coupled plasma/atomic absorption spectroscopy (ICP-AA) or by extracting Fe from fresh leaves using Fe(II)-chelates and assaying spectrophotometrically. The ICP-AA method is reliable but can be costly. The traditional Fe(II)-chelate method is less expensive but is not very reliable as a result of a high level of interference, and in many cases, fresh leaves are not readily available. We present a reliable and inexpensive method for quantifying active Fe in dried leaves using a Fe(II)-chelate after acid extraction. By moving the

Fig. 2. (A) Total chlorophyll concentration of 'Concord' grape leaves ($P \leq 0.001$) in relation to Fe-EDDHA application rate and (B) relationship between active Fe concentration and chlorophyll concentration in 'Concord' grape leaves ($Y = -0.865 + 0.588X - 0.012X^2$, $R^2 = 0.91$). Chlorophyll was extracted from fresh leaves but expressed on a dry weight basis. Each point is the mean of five replicates. Means were separated using Tukey's HSD test at $P = 0.05$.

nonpolar Fe(II)-chelate into the upper, immiscible alcohol phase, we can eliminate most interfering compounds by leaving them in the aqueous phase. In addition, we can vary the amount of 1-octanol used so we can concentrate the chromophore and increase resolution and the level of detection.

Leaves of 'Concord' vines grown at high pH without any supplemental Fe had very low rates of CO₂ assimilation (Fig. 3A) and used only a small proportion of the absorbed light in photosynthesis (Fig. 3C). The decrease in CO₂ assimilation was not the result of stomatal limitation, because Fe-deficient leaves had a higher calculated internal CO₂ concentration compared with the Fe-sufficient leaves. Our previous work showed that the activities of Rubisco and other photosynthetic enzymes were decreased by Fe deficiency (Chen et al., 2004). A small increase in the rate of applied Fe-EDDHA was very beneficial in increasing CO₂ assimilation presumably by overcoming the nonstomatal limitation imposed by Fe deficiency. This conclusion may seem overly simplistic; however, in a companion experiment in which we grew 'Concord' vines from pH 5.2 to 7.6 without any supplemental Fe, leaves had high levels of chlorophyll and corresponding high rates of CO₂ assimilation when the substrate pH was below 6. This indicates that there was sufficient available Fe at low pH from the peat, perlite, and irrigation water. Yet

Fig. 3. (A) CO₂ assimilation ($P \leq 0.001$), (B) g_s ($P \leq 0.001$), and (C) photosystem II quantum efficiency ($P \leq 0.001$) of 'Concord' grape leaves in relation to Fe-EDDHA application rate. Measurements were taken midday at ambient CO₂ (360 μmol·mol⁻¹) under a photon flux density of 1540 ± 68 μmol·m⁻²·s⁻¹ and a leaf temperature of 29.8 ± 0.8 °C. Each point is the mean of five replicates. Means were separated using Tukey's HSD test at $P = 0.05$.

although there may be sufficient levels of total iron in the soil at high pH, 'Concord' grapevines can readily develop Fe deficiency because of low Fe solubility. Thus, the addition of Fe-EDDHA is a highly effective chelate for alleviating Fe chlorosis and increasing CO₂ assimilation at high pH for 'Concord' vines.

Vine growth was very responsive to Fe-EDDHA application. Leaf area and foliar dry weight nearly tripled as Fe-EDDHA increased from 0 to 1.0 mg·L⁻¹ Fe (Figs. 4A and B), and cane length per vine also significantly increased (Fig. 4C). Above 1 mg·L⁻¹ Fe, a further increase in Fe did not significantly increase growth. Our results demonstrate that 1 mg·L⁻¹ Fe from Fe-EDDHA is a critical applied Fe concentration for 'Concord' grapevines grown in containers with a nutrient solution based on an N concentration of 210 mg·L⁻¹. The critical concentration of applied Fe is likely to depend on the nitrogen status and overall growth rate.

There is little published information on the use of Fe-EDDHA to correct Fe deficiency in the vineyard, although it is well known that Fe-EDDHA can effectively cor-

Fig. 4. (A) Total leaf area ($P \leq 0.001$), (B) foliar dry weight ($P \leq 0.001$), and (C) cane length ($P \leq 0.001$) of 'Concord' grapevines in relation to Fe-EDDHA application rate. Data were collected at the end of the growing season. Each point is the mean of five replicates. Means were separated using Tukey's HSD test at $P = 0.05$.

rect chlorosis in other crops. Winkler et al. (1974) applied Fe-EDDHA (1 g·m⁻²) to *Vitis* spp. in the water of a flood irrigation system and were not able to effectively correct chlorosis. They were more successful applying the chelate to dry soil at 15 g per vine over two seasons. Because it is much easier to prevent Fe deficiency rather than correct it, it follows that the best strategy would be to apply Fe-EDDHA before budbreak. McEachern (1982) recommend applying one tablespoon Fe-EDDHA per vine in the spring when chlorosis first appears. However, Tagliavini and Rombolà (2001) found that applying Fe in late summer-early fall was more effective in preventing chlorosis in *Actinidia deliciosa* when compared with applications in the spring, before budbreak. The optimum rate and timing of FeEDDHA application in 'Concord' vineyards to correct Fe deficiency-induced chlorosis remains to be determined.

In conclusion, Fe-EDDHA does effectively alleviate Fe deficiency-induced leaf chlorosis in 'Concord' grapevines grown at high pH. The optimum rate and timing of application under vineyard conditions still need to be worked out. Compared with total

Fe, leaf “active Fe” better indicates the actual Fe status of ‘Concord’ vines.

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