Photoprotective Mechanisms of ‘Concord’ Grape Leaves in Relation to Iron Supply

Brandon R. Smith and Lailiang Cheng¹

Department of Horticulture, Cornell University, Ithaca, NY 14853

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ABSTRACT. The objective of this study was to quantify how photoprotective mechanisms in the leaves of ‘Concord’ grapevines (*Vitis labruscana* Bailey) respond to a range of iron (Fe) supply. Own-rooted, 1-year-old container-grown vines were fertigated twice weekly for 11 weeks with a complete nutrient solution containing 1, 10, 20, 50, or 100 μM Fe from ferric ethylenediamine di (*o*-hydroxyphenylacetic) acid (Fe-EDDHA). Leaf total Fe content did not increase in response to Fe supply; however, “active” Fe (extracted with 2,2′-dipyridyl) and chlorophyll (Chl) increased on a leaf area basis as applied Fe increased. At the lowest active Fe level, leaf absorbance and the efficiency of excitation transfer (F_v/F_m) was lower, and nonphotochemical quenching (NPQ) was significantly greater. Photosystem II (PSII) quantum efficiency decreased curvilinearly, and the proportion of PSII reaction centers in the open state (qP) decreased linearly as active Fe content decreased. On a Chl basis, the xanthophyll cycle pool size [violaxanthin (V) + antheraxanthin (A) + zeaxanthin (Z)], lutein, and β -carotene increased curvilinearly as active Fe decreased, and neoxanthin (Neo) increased at the lowest Fe level. On a leaf area basis, as active Fe decreased, V+A+Z and β -carotene decreased curvilinearly, and lutein and Neo decreased linearly. At noon, conversion of V to A and Z increased as active Fe decreased. On a Chl basis, activities of antioxidant enzymes superoxide dismutase (SOD), monodehydroascorbate reductase (MDAR), and dehydroascorbate reductase (DHAR) increased curvilinearly, and glutathione reductase (GR) activity increased linearly as active Fe levels declined. Ascorbate peroxidase (APX) and catalase (CAT), on a Chl basis, were relatively constant. On a leaf area basis, a decrease in active Fe increased SOD and MDAR activity, whereas APX, CAT, DHAR and GR activity decreased. Antioxidant metabolites ascorbate (AsA), dehydroascorbate (DAsA), reduced glutathione (GSH) and oxidized glutathione (GSSG) also increased in response to Fe limitation when expressed on a Chl basis, whereas on a leaf area basis AsA and DAsA decreased and GSH increased curvilinearly. The GSH:GSSG ratio increased as active Fe declined, whereas the AsA:DAsA ratio did not change. In conclusion, both photoprotective mechanisms, xanthophyll cycle-dependent thermal dissipation and the ascorbate-glutathione antioxidant system, are enhanced in response to Fe deficiency to cope with excess absorbed light. In a low soil pH tolerant species such as *V. labruscana*, the foliar antioxidant system was upregulated in response to excess absorbed light from Fe deficiency-induced chlorosis, and there was no evidence of an increase in oxidative stress from high rates of applied Fe-EDDHA.

Iron deficiency in crops is a global problem that affects plant productivity and human nutrition. Many horticultural and agronomic crops, including grapes (*Vitis* L.), are frequently plagued by Fe-deficiency (Chaney, 1988). Fe-deficiency induced chlorosis impacts grape production throughout Europe and the northwest United States (Tagliavini and Rombola, 2001; Davenport et al., 2003). Tolerance to Fe-deficiency varies by scion and rootstock genotype, and *Vitis vinifera* L. and *V. berlandieri* Planch. are generally less susceptible to Fe-deficiency than *V. labruscana* and *V. riparia* Michx. (Bavaresco et al., 2003; Tagliavini and Rombola, 2001; Winkler et al., 1974).

Fe uptake in dicotyledons and nongraminaceous monocots, so-called Strategy I plants, is typically accomplished by reducing Fe(III)-chelates with a root plasma membrane (PM) bound ferric chelate reductase (FCR), and transporting the liberated Fe²⁺ ion across the PM by Fe²⁺ transporters (Chaney et al., 1972; Robinson et al., 1999; Vert et al., 2002). Under severe Fe limitation, Fe-efficient plants can increase Fe solubility and uptake by increasing: 1) root H⁺ efflux by activation of H⁺-ATPase, 2) root FCR activity, 3) Fe²⁺ transporter activity, and 4) exudation of

Fe(III)-chelating organic acids (Bienfait, 1987; Kochian, 1991). Fe-inefficient species generally lack, or have decreased levels of these Fe uptake mechanisms, and frequently suffer from Fe-chlorosis (Brown and Jones, 1976).

In the xylem, Fe is transported primarily as Fe(III)-citrate, and FCR activity has been shown to be a requirement for uptake into mesophyll cells (Brüggemann et al., 1993; Tiffin, 1970). A high leaf apoplast pH is associated with Fe-deficiency in the leaves of field-grown pear trees (*Pyrus communis* L.) and has been found to decrease FCR activity (Gonzalez-Vallejo et al., 2000; López-Millán et al., 2001). In field-grown plants suffering from Fe-deficiency, there is often a poor correlation between total Fe content and chlorosis, termed the “chlorosis paradox,” and chlorotic leaves often contain higher total Fe than chlorophyll-sufficient leaves. Extraction of so-called “active” Fe from leaves with Fe(II)-chelators or dilute acids is often a better measure of Fe status than total Fe (Abadía et al., 1984; Chen et al., 2004).

In the leaves, Fe can play a pivotal role in photosynthesis and oxidative stress due to the requirement of Fe for chlorophyll synthesis, photosynthetic electron transport, and enzyme activation via the ferredoxin-thioredoxin system (Miller et al., 1995; Terry, 1983). Fe limitation affects the utilization of light energy due to a decrease in the number of photosynthetic units, and a reduction in light absorption (Morales et al., 2000; Spiller and Terry, 1980). Under conditions of high photon flux density (PFD), the decline in photosynthetic capacity accompanying Fe-deficient leaves can lead to an excess of absorbed light (Abadía et al.,

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¹Corresponding author: Fax: +1-607-255-4355; email: LC89@cornell.edu

1999). Excess absorbed PFD can then result in the production of potentially harmful reduced reactive oxygen species (ROS). High concentrations of free or loosely-bound Fe can also contribute to the production of ROS, particularly through the Fenton reaction (Halliwell and Gutteridge, 1984). In the presence of free Fe^{2+} , the dangerous hydroxyl radical ($\cdot\text{OH}$) can be produced from H_2O_2 , and the resulting oxidized Fe^{3+} can be reduced by superoxide, promoting continuation of the reaction (Briat, 2002).

To deal with the damaging effects of excess absorbed light and the increased production of ROS, plants have developed photoprotective mechanisms (Niyogi, 2000). The two major photoprotective mechanisms are 1) singlet oxygen ($^1\text{O}_2$) quenching and thermal dissipation via the xanthophyll cycle, and 2) scavenging of ROS through the ascorbate-glutathione cycle. In the xanthophyll cycle, high light and a low lumen pH activates violaxanthin deepoxidase, which converts violaxanthin to the intermediate antheraxanthin, then to the epoxide-free zeaxanthin, both of which are able to dissipate excess excitation energy as heat (Demmig-Adams and Adams, 1996; Li et al., 2004).

During conditions that promote direct electron transfer to O_2 , the ascorbate-glutathione cycle becomes an important ROS scavenging system (Asada, 1999; Noctor and Foyer, 1998). In this process, the univalent reduction of O_2 results in the formation of superoxide (O_2^-), and SOD dismutates O_2^- to O_2 and H_2O_2 (Noctor and Foyer, 1998). APX then converts H_2O_2 to H_2O using AsA (Nakano and Asada, 1981), and the resulting MDA radical is reduced back to AsA in the thylakoids by ferredoxin (Fd), or in the stroma by MDAH using reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) (Hossain et al., 1984; Miyake and Asada, 1994). MDA can also spontaneously disproportionate to DHA, which is reduced to AsA by DHAR using GSH (Noctor and Foyer, 1998). The resulting oxidized GSSG is then reduced by GR using NAD(P)H. In addition to scavenging ROS from the direct photoreduction of O_2 , the ascorbate-glutathione cycle has also been shown to be upregulated under conditions of Fe-mediated oxidative stress (Kampfenkel et al., 1995).

Our objective was to quantify how photoprotective mechanisms in the leaves of 'Concord' grapevines respond to a range of Fe supply. This was accomplished by measuring xanthophyll carotenoids, chlorophyll fluorescence quenching, and enzymes and metabolites in the ascorbate-glutathione cycle.

Materials and Methods

PLANT CULTURE AND FE TREATMENTS. A detailed experimental design was described in Chen et al. (2004). Briefly, own-rooted 1-year-old 'Concord' grapevines pruned to one shoot were grown outdoors in 19.8-L plastic containers, using a commercially blended medium (MetroMix 560 with Coir; Scotts Co., Marysville, Ohio). There were five replicate plants per Fe treatment in a completely randomized design. Eleven weeks after transplanting, recent fully expanded leaves were chosen for analysis.

Each plant was supplied twice weekly with 1 L of a complete nutrient solution including micronutrients, and 1, 10, 20, 50, or 100 μM Fe from Fe(III)-EDDHA. Macronutrients were from a blended water-soluble fertilizer (17N-2.2P-14.1K-3Ca-1Mg; GreenCare Fertilizers, Chicago) and were applied at a concentration of 16 mM N, 0.9 mM P, 4.5 mM K, 0.9 mM Ca, and 0.5 mM Mg. Micronutrients were applied at a concentration of 10 μM Mn ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$), 2 μM Zn ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), 0.50 μM Cu ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), 25 μM B (H_3BO_3), and 0.50 μM Mo (NaMoO_4). The mean pH of the medium was 6.6 ± 0.1 .

LEAF ABSORPTANCE AND CHLOROPHYLL FLUORESCENCE. Leaf reflectance and transmittance was measured with a LI-1800 spectroradiometer, using an 1800-12S integrating sphere attachment (LI-COR, Lincoln, Neb.) as described in Cheng et al. (2000). Leaf absorbance was calculated as 1-reflectance-transmittance.

Chlorophyll fluorescence was measured with a pulse-modulated fluorometer (FMS2; Hansatech Instruments, Norfolk, U.K.) under natural conditions, either at a midday PFD of $1805 \pm 20 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, or at predawn. The fibre optic of the FMS2 was positioned using the PFD/temperature leaf clip at a 60° angle from the upper surface of the leaf, and the distance between the fiber optic and the leaf surface was kept constant for both the predawn and the midday measurements. Dark-adapted leaves were measured at predawn for minimum fluorescence (F_o) and maximum fluorescence (F_m). During midday measurements, stable steady-state fluorescence (F') readings were obtained before further measurements were taken. Maximum fluorescence (F_m') under natural light exposure was obtained by imposing a 1 s saturating flash of $\approx 6000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PFD at the leaf surface to reduce all PSII centers. Minimal fluorescence (F_o') was calculated according to Oxborough and Baker (1997) as: $F_o' = F_o / ((F_v/F_m) + (F_o/F_m'))$. The maximum PSII efficiency of dark-adapted leaves was calculated as $F_v/F_m = (F_m - F_o)/F_m$ (van Kooten and Snel, 1990). Thermal energy dissipation was estimated from nonphotochemical quenching (NPQ) as $F_m/F_m' - 1$ (Bilger and Bjorkman, 1990). Efficiency of excitation transfer, F_v'/F_m' , was calculated as $(F_m' - F_o')/F_m'$, the photochemical quenching coefficient (qP , related to the proportion of PSII centers in "open" state) was estimated as $(F_m' - F')/(F_m' - F_o')$ (Baker et al., 2001), and the PSII quantum efficiency (similar notation, ΦPSII) was calculated as $(F_m' - F')/F_m'$ (Genty et al., 1989).

ANALYSIS OF LEAF PIGMENTS. Immediately before midday Chl fluorescence measurements, disks (1 cm^2 in size) were punched from the leaves and frozen in liquid N_2 . Frozen leaf disks were stored at -80°C until analysis of leaf pigments. V, A, Z, Neo, lutein, and β -carotene were extracted from one leaf disk (1 cm^2) and analyzed by high-performance liquid chromatography (HPLC) following the same procedure described by Cheng (2003). Leaf Chl was extracted and measured according to Arnon (1949).

EXTRACTION AND ASSAY OF ANTIOXIDANT ENZYMES AND METABOLITES. Antioxidant enzymes, and AsA and DAsA, were measured as described by Chen and Cheng (2003). GSH and GSSG were determined according to Griffith (1980), with some modifications. Two leaf disks (total of 2 cm^2) were ground in 1 mL ice-cold 5% (w/v) trichloroacetic acid (TCA) and 5% (w/v) insoluble polyvinylpyrrolidone. Extract was centrifuged at 13,000 g_n for 10 min at 2°C , and the supernatant was centrifuged again under the same conditions. The resulting supernatant was used for analysis. Total GSH was determined at 412 nm in a 1-mL reaction mixture containing 150 mM sodium phosphate buffer (pH 6.9), 6.3 mM EDTA, 1.2 mM 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB), 0.2 mM NADPH, 0.5 units GR (Sigma-Aldrich, St. Louis), and 20 μL plant extract. The reaction was initiated with the addition of GR. To determine GSSG, 20 μL of plant extract was incubated in 20 μL 2-vinylpyridine for 1 h to completely derivatize all GSH. GSSG was then assayed as above. Both GSH and GSSG were measured against an internal standard of 10 μM GSSG, and final concentration was determined from a standard curve of GSSG.

IRON ANALYSIS. Active Fe was assayed according to Abadía et al. (1984) as described by Chen et al. (2004). Three 1- cm^2 disks were shaken for 24 h in 1.2 mL of 80 mM 2,2'-dipyridyl-HCl (pH 3.0) in 10% methanol. Extract was passed through a 0.45- μm

syringe filter and 1 mL of solution was assayed at 522 nm. Total Fe was measured from dried leaves using an inductively coupled plasma emission spectrometer (model 975, Plasma Atomcomp; Thermo Jarrell Ash Corp., Franklin, Mass.).

Results

FOLIAR FE AND FRESH MASS. An increase in Fe application rate did not increase leaf total Fe content (Fig. 1A). Active Fe extracted from the fresh leaves, however, rose curvilinearly as nutrient solution Fe levels increased (Fig. 1B). The leaf specific fresh mass increased curvilinearly as active Fe content increased, but lacked a relationship with total Fe content (data not shown).

TOTAL CHLOROPHYLL AND LEAF ABSORPTANCE. Total chlorophyll was closely related to leaf active Fe content (Fig. 2A), yet showed no relationship to total Fe content (data not shown). Leaf absorbance declined curvilinearly as active Fe decreased (Fig. 2B), with only small differences at the higher active Fe levels. Leaf absorbance decreased the most from the second to the lowest active Fe level, from 90.7% to 83.3%.

XANTHOPHYLL CYCLE, LUTEIN, β -CAROTENE, AND NEOXANTHIN. On a leaf area basis, the xanthophyll pool size [V+A+Z] and β -carotene decreased curvilinearly, and lutein and Neo decreased linearly as active Fe decreased (Fig. 3A–D). When expressed on a leaf Chl basis, V+A+Z and lutein increased curvilinearly and β -carotene increased linearly as active Fe levels declined (Fig. 3E–G). Neoxanthin was higher only at the lowest Fe level (Fig. 3H) when expressed on a leaf Chl basis.

At noon, the amount of A+Z on a Chl basis increased as active Fe content decreased (Fig. 4A). The percentage of the xanthophyll pool present as A+Z decreased linearly from 97% to 88% as active Fe content decreased from 1.43 to 2.54 mg·m⁻² (Fig. 4B). At predawn, the majority of the xanthophyll cycle pool was present

Fig. 1. Effect of iron (Fe) supply on total Fe (A) and active Fe (B) content of grape leaves. Each point is mean $\pm$ SE (n = 5). Analysis of variance for (A) $P > 0.05$, and regression equation for (B) $y = 1.4685 + 0.0237x - 0.0001x^2$ ($r^2 = 0.987$, $P < 0.05$).

Fig. 2. Total chlorophyll (Chl) content (A) and leaf absorbance (B) of grape leaves in relation to active Fe content. Each point is mean $\pm$ standard error (n = 5). Regression equations for (A) $y = -768.7495 + 925.0537x - 153.9393x^2$ ($r^2 = 0.999$, $P < 0.01$); and (B) $y = 38.6244 + 45.4246x - 9.8228x^2$ ($r^2 = 0.975$, $P < 0.05$).

as V, and the percentage of A+Z in the xanthophyll pool ranged from 25% at the lowest active Fe level to 19% at the highest active Fe level (data not shown).

CHLOROPHYLL FLUORESCENCE VARIABLES. At the lowest active Fe level, NPQ was greater, and F_v'/F_m' was lower (Fig. 5 A and B). As active Fe decreased, qP decreased linearly and the PSII quantum efficiency increased curvilinearly (Fig. 5 C and D). Maximum PSII efficiency decreased only slightly at the lowest active Fe level (Fig. 5E).

RELATIONSHIP BETWEEN XANTHOPHYLL CYCLE AND CHL FLUORESCENCE VARIABLES. At midday, NPQ did not increase as leaf A+Z content on a Chl basis increased, except at the A+Z content of 361 mmol·mol⁻¹ Chl, there was a significant increase in NPQ (Fig. 6A). A similar trend was found when NPQ was plotted against midday A+Z/V+A+Z, where NPQ was greater only at the highest percentage of A+Z/V+A+Z (data not shown). F_v'/F_m' decreased curvilinearly, and qP and PSII quantum efficiency decreased linearly as leaf A+Z content on a Chl basis increased (Fig. 6B–D).

ANTIOXIDANT ENZYMES AND METABOLITES. On a leaf area basis, SOD and MDAR activity increased, whereas the activity of APX, CAT, DHAR and GR decreased as leaf active Fe decreased (Fig. 7A–F). On a Chl basis, SOD, MDAR, and DHAR activity increased curvilinearly, and GR activity increased linearly as active Fe levels declined (Fig. 7 G, J, K, and L). APX and CAT activity, when calculated on a Chl basis, were relatively constant (Fig. 7H–I).

Total AsA (AsA+DAsA), AsA, and DAsA decreased on a leaf area basis as active Fe declined, whereas GSH increased curvilinearly and GSSG remained relatively constant (Fig. 8A–E). On a Chl basis, total AsA, AsA, DAsA, GSH, and GSSG all increased curvilinearly as active Fe levels declined (Fig. 8G–K). The ratio of AsA/DAsA remained constant across active Fe content, however the GSH/GSSG ratio increased as active Fe decreased (Fig. 8 F and L).

Fig. 3. Xanthophyll cycle pool size (A, E), β -carotene (B, F), lutein (C, G), and neoxanthin (D, H) expressed on a leaf area or Chl basis in relation to active Fe in grape leaves collected during midday. Each point is mean $\pm$ SE ($n = 5$). Regression equations for (A) $y = -92.4368 + 180.6737x - 38.6204x^2$ ($r^2 = 0.988$, $P < 0.05$); (B) $y = -101.0800 + 126.6183x - 25.2793x^2$ ($r^2 = 0.997$, $P < 0.01$); (C) $y = -2.5030 + 37.7560x$ ($r^2 = 0.990$, $P < 0.001$); (D) $y = -6.0850 + 14.3382x$ ($r^2 = 0.998$, $P < 0.0001$); (E) $y = 994.0988 - 598.2532x + 112.8140x^2$ ($r^2 = 0.990$, $P < 0.01$); (F) $y = 150.697 - 20.3736x$ ($r^2 = 0.958$, $P < 0.05$); and (G) $y = 540.1507 - 332.5544x + 71.6746x^2$ ($r^2 = 0.915$, $P < 0.05$). Analysis of variance and Tukey's mean comparison test results for (H) $P < 0.05$, with significantly higher Neo at the lowest active Fe level.

Discussion

'Concord' grape leaves developed chlorosis under Fe-limitation, however leaf total Fe did not decrease. This observation is in line with the "chlorosis paradox" phenomenon, where chlorotic leaves can often contain higher total Fe than green leaves (Römhild, 2000). Extraction of active Fe, or the loosely bound fraction of Fe, from the leaves with the Fe(II)-chelate 2,2'-dipyridyl was a better indicator of Fe nutritional status (Fig. 1B and 2A).

The possible physiological mechanism that led to chlorosis paradox observed in this experiment was discussed in detail by Chen et al (2004). Briefly, under adequate Fe-EDDHA supply conditions, most of the Fe taken up by the roots might be translocated as the pH-stable Fe-EDDHA and thus not immobilized by high apoplast pH. At lower Fe-EDDHA application rates, however, absorption of the Fe-EDDHA complex may not have

been enough to satisfy the demand for Fe, and the roots most likely have employed Fe-efficiency mechanisms to increase Fe uptake from the growing medium. As most Fe is transported to the mesophyll cells as Fe(III)-citrate under low Fe-EDDHA supply, a high leaf apoplast pH may have inhibited mesophyll FCR activity required for the reductive assimilation of Fe from Fe(III)-citrate complexes, resulting in Fe deficiency in leaves.

Fe-deficiency did reduce leaf absorbance, but the changes were not proportional to the decrease in active Fe (Fig. 2B). Considering that there was a greater decrease in CO_2 assimilation rate relative to the decrease in leaf absorbance under Fe-deficiency (Chen et al., 2004), changes in leaf absorbance alone were not sufficient for photoprotection during high $PPFD$, and excess absorbed light resulted. In grape leaves with nitrogen deficiency induced chlorosis, similar small decreases in leaf absorbance were found (Chen and Cheng, 2003).

Fig. 4. Midday antheraxanthin and zeaxanthin (A+Z) on a Chl basis (A) and as a percentage of the xanthophyll cycle pool (B) of grape leaves in relation to active Fe. Each point is mean $\pm$ SE ($n = 5$). Regression equations for (A) $y = 1015.0860 - 626.4721x + 117.0711x^2$ ($r^2 = 0.990$, $P < 0.01$); and (B) $y = 108.1826 - 7.7971x$ ($r^2 = 0.996$, $P < 0.001$).

Thermal dissipation of excess excitation energy, measured as NPQ , was increased only at the lowest active Fe level (Fig. 5A), even though chlorophyll content decreased nearly linearly from $591 \mu\text{mol}\cdot\text{m}^{-2}$ at the highest active Fe level to $238 \mu\text{mol}\cdot\text{m}^{-2}$ at the lowest (Fig. 2A). The efficiency of excitation transfer (F_v'/F_m' , Fig. 5B), however, correspondingly decreased only at the lowest active Fe level, indicating that NPQ is increased to lower the efficiency of excitation transfer to the PSII reaction centers. Midday NPQ in sugar beet (*Beta vulgaris* L.) and pear leaves did not appreciably increase in response to Fe-deficiency as consistently as NPQ from N-deficiency in grapevines and apple (*Malus domestica* Borkh.) (Chen and Cheng, 2003; Cheng, 2003; Morales et al., 1998, 2000). On the other hand, NPQ in maize (*Zea mays* L.) and soybean [*Glycine max* (L.) Merr.] was dramatically increased when plants were grown in a nutrient solution without Fe; however, in this situation the differences in Chl content between Fe-deficient and control plants were either extreme (2.2 vs. $22.4 \text{ g}\cdot\text{m}^{-2}$) or not reported (Jiang et al., 2001, 2003). Our results illustrate that NPQ is increased when Fe-deficiency induced chlorosis decreases to $238 \mu\text{mol}\cdot\text{m}^{-2}$ Chl or $1.4 \text{ mg}\cdot\text{m}^{-2}$ active Fe. Our observation that Fe-deficiency decreases photochemical quenching (qP), and the PSII quantum efficiency of grape leaves at high $PPFD$, yet only marginally effects the maximum PSII efficiency (F_v'/F_m') of dark-adapted leaves (Fig. 5C–E), is in agreement with findings from sugar beet and pear (Morales et al., 1998, 2000). Because PSII quantum efficiency is the product of F_v'/F_m' and qP , the observed curvilinear decrease in the PSII quantum efficiency in response to Fe limitation can ultimately be attributed to the linear decrease in the proportion of open reaction centers (qP), as F_v'/F_m' decreased only at the lowest active Fe level.

An increase in the proportion of closed reaction centers under high light (a decrease in photochemical quenching), without a corresponding decrease in F_v'/F_m' , will result in the transfer of excess excitation energy to O_2 , forming $^1\text{O}_2$. De-epoxidized xanthophylls

Fig. 5. Nonphotochemical quenching (NPQ) (A), efficiency of excitation transfer (F_v'/F_m') (B), photochemical quenching coefficient (qP) (C), PSII quantum efficiency (D), and maximum PSII efficiency (F_v'/F_m') (E) in relation to active Fe in grape leaves. Each point is mean $\pm$ SE ($n = 5$). Regression equations for (C) $y = 0.1165 + 0.1739x$ ($r^2 = 0.942$, $P < 0.01$); and (D) $y = -0.6493 + 0.8003x - 0.1634x^2$ ($r^2 = 0.986$, $P < 0.05$). Analysis of variance and Tukey's mean comparison test results for (A) $P < 0.001$, with greater NPQ at the lowest active Fe level; (B) $P < 0.001$, with lower F_v'/F_m' at the lowest active Fe level; and (E) $P < 0.001$, with lower F_v'/F_m' at the lowest active Fe level.

(A and Z), β -carotene, and lutein can protect PSII from photodamage by quenching $^1\text{O}_2$ and de-exciting ^3Chl (Niyogi, 2000; Niyogi et al., 1997). On a leaf area basis, all measured xanthophyll carotenoids in our leaves decreased in response to Fe-deficiency, with a smaller decrease in the V+A+Z pool compared to β -carotene,

Fig. 6. Nonphotochemical quenching (NPQ) (A), efficiency of excitation transfer (F_v'/F_m') (B), photochemical quenching coefficient (qP) (C), and PSII quantum efficiency (D) in relation to the level of antheraxanthin and zeaxanthin (A+Z) on a Chl basis in grape leaves at midday. Each point is mean $\pm$ SE ($n = 5$). Regression equations for (A) $y = 6.2337 - 0.0394x + 0.0001x^2$ ($r^2 = 0.998$, $P < 0.01$); (B) $y = 0.2229 + 0.0025x - 0.0000x^2$ ($r^2 = 0.979$, $P < 0.05$); (C) $y = 0.7281 - 0.0010x$ ($r^2 = 0.925$, $P < 0.01$); and (D) $y = 0.5056 - 0.0009x$ ($r^2 = 0.951$, $P < 0.01$).

lutein, and Neo (Fig. 3A–D). On a Chl basis, the molar ratio of the V+A+Z pool nearly doubled (197 to 372 mmol·mol⁻¹ Chl), and β -carotene and lutein also increased as active Fe decreased from the highest active Fe level to the lowest (Fig. 3E–H). The A+Z content on a Chl basis, and the percentage of the V+A+Z pool present as A+Z (Fig. 4A–B) increased in response to Fe-deficiency, indicating that xanthophyll conversion increased to dissipate excess absorbed *PFD* in chlorotic leaves.

Similar pigment stoichiometries were also observed in moderately Fe-deficient pear leaves, and the ratios of V+A+Z to Chl reached up to 1000 under conditions of severe deficiency (Chl <50 μ mol·m⁻²) (Morales et al., 1994). The ratios of V+A+Z to Chl in response to Fe-deficiency chlorosis are much higher than

values obtained from N-deficient grape leaves, where the V+A+Z content on a Chl basis reached a ratio of only ≈ 210 at the lowest leaf Chl content ($\approx 200 \mu$ mol·m⁻²) (Chen and Cheng, 2003). As the N content of grape leaves decreased, *NPQ* increased curvilinearly to values above 4, and increased linearly in relation to the A+Z content on a Chl basis. In the present study, an increase in the A+Z content on a Chl basis was not associated with a linear increase in *NPQ*, although *NPQ* did rise at the highest A+Z content (Fig. 6A). The reason for the lack of a relationship is unclear, but if Fe availability somehow affects the transthylakoid pH difference or PsbS protein, this may alter the relationship between A+Z and *NPQ*. Nonetheless, more discreet relationships were observed with increases of the A+Z content on a Chl basis and decreases of PSII quantum efficiency and *qP* (Fig. 6C–D).

On a leaf Chl basis, activities of SOD, APX, MDAR, DHAR, and GR increased in low active Fe leaves (Fig. 7G–H, J–L). Similarly, the size of both the ascorbate and glutathione pools on a Chl basis increased as active Fe levels declined (Fig. 8G–K). The increase in these antioxidants on a Chl basis in low active Fe leaves indicates an upregulation of the scavenging of ROS produced from excess absorbed *PFD*. CAT activity on a Chl basis was relatively constant (Fig. 7I), and considering that most of CAT activity is localized in the peroxisomes to remove H₂O₂ from photorespiration (Willekens et al., 1995), it is not surprising that CAT did not change as a photoprotective measure.

The increase in total SOD activity on a leaf area and Chl basis (Fig. 7 A and G) in response Fe-deficiency is consistent with the increased photoreduction of O₂. Cu/Zn-SOD also increased in the upper leaves of Fe-deficient pea (*Pisum sativum* L.), but Iturbe-Ormaetxe et al. (1995) did not find an increase in H₂O₂ concentration. In Fe-deficient sunflower (*Helianthus annuus* L.) leaves, however, Ranieri et al. (1999) measured an increase in both SOD activity and H₂O₂ content in tissue. Although we did not directly measure leaf H₂O₂ concentration in this study, the up-regulation of the ascorbate-glutathione cycle also suggests an increase in H₂O₂ flux in Fe-deficient leaves.

The increase in MDAR activity in response to a decrease in active Fe may be the result of decreased ferredoxin (Fd)-dependent reduction of MDA (Miyake and Asada, 1994) at lower active Fe contents. Fe limitation has been shown to decrease levels of the Fe-S protein Fd in citrus (*Citrus limonum* Risso) (Alcaraz et al., 1985). In situations where Fd content is decreased due to Fe-deficiency, MDAR activity might be up-regulated to maintain the reduction state of the ascorbate pool. In addition, the increase in the size and reduction state of the glutathione pool at lower active Fe (Fig. 8L) is consistent with a decrease in direct photoreduction of MDA by Fd. The elevated ratio of GSH to GSSG at lower active Fe was primarily due to greater GSH content, as GSSG levels remained very low and constant (Fig. 8D–E). Thus if the Fd-dependent photoreduction of MDA decreases as active Fe declines, a larger proportion of the MDA would spontaneously disproportionate to DHA, requiring an increase in the glutathione pool size (higher GSH) to reduce DHA to AsA. In response to Fe limitation, the GSH to GSSG ratio also increased in pea leaves and sugar beet roots on a dry and fresh weight basis (Iturbe-Ormaetxe et al., 1995; Zaharieva and Abadía, 2003). It appears that both reduction of MDA via MDAR and reduction of DHA via DHAR have been up-regulated under Fe deficiency, thereby maintaining a constant reduction state of the ascorbate pool (Fig. 8F).

Although an excess of catalytic Fe in combination with high light would be particularly detrimental to the photosynthetic apparatus, we found no evidence of Fe-toxicity visual symptoms

Fig. 7. Activity of antioxidant enzymes in relation to the active Fe content of grape leaves collected at midday, expressed on a leaf area or leaf Chl basis, respectively: (A, G) Superoxide dismutase (SOD); (B, H) ascorbate peroxidase (APX); (C, I) catalase (CAT); (D, J) monodehydroascorbate reductase (MDAR); (E, K) dehydroascorbate reductase (DHAR); (F, L) glutathione reductase (GR). Each point is mean $\pm$ SE ($n = 5$). Regression equations for (A) $y = 1.7207 - 0.4438x$ ($r^2 = 0.803$, $P < 0.05$); (B) $y = -57.6935 + 85.0020x - 14.3703x^2$ ($r^2 = 0.965$, $P < 0.05$); (C) $y = -93.9577 + 105.3863x - 20.4531x^2$ ($r^2 = 0.952$, $P < 0.05$); (D) $y = 1.3329 + 39.9743x - 12.6999x^2$ ($r^2 = 0.992$, $P < 0.01$); (E) $y = -1.0398 + 2.2608x - 0.4099x^2$ ($r^2 = 0.965$, $P < 0.05$); (G) $y = 20.2608 - 15.5462x + 3.1441x^2$ ($r^2 = 0.976$, $P < 0.05$); (H) $P > 0.05$; (I) $P > 0.05$; (J) $y = 537.1462 - 385.6211x + 74.4148x^2$ ($r^2 = 0.983$, $P < 0.05$); (K) $y = 0.1161 + (8.0371/x)$ ($r^2 = 0.870$, $P < 0.05$); and (L) $y = 52.2771 - 14.7695x$ ($r^2 = 0.856$, $P < 0.05$). Analysis of variance and Tukey's mean comparison test for (F) $P < 0.01$, with significantly decreased GR activity at the lowest two active Fe levels vs. the second highest active Fe level.

Fig. 8. Midday antioxidant metabolite content in relation to the active Fe content of grape leaves expressed on a leaf area or leaf Chl basis, respectively. (A, G) Reduced ascorbate (AsA); (B, H) dehydroascorbate (DAsA); (C, I) total ascorbate (AsA+DAsA); (D, J) reduced glutathione (GSH); (E, K) oxidized glutathione (GSSG); (F) ratio of AsA/DAsA; (L) ratio of GSH/GSSG. Each point is mean $\pm$ SE ($n = 3-5$). Regression equations for (A) $y = 2.0769 + 1.6673x - 0.3176x^2$ ($r^2 = 0.960$, $P < 0.05$); (B) $y = 0.5495 + 0.1277x$ ($r^2 = 0.894$, $P < 0.05$); (C) $y = 3.8464 + 0.5252x$ ($r^2 = 0.946$, $P < 0.01$); (D) $y = 26.7795 + 75.4270x - 25.3027x^2$ ($r^2 = 0.994$, $P < 0.01$); (E) $P > 0.05$; (F) $P > 0.05$; (G) $y = 58.1709 + 42.4207x - 8.8477x^2$ ($r^2 = 0.982$, $P < 0.05$); (H) $y = 12.4818 - 9.5224x + 2.0564x^2$ ($r^2 = 0.962$, $P < 0.05$); (I) $y = 70.6527 - 51.9431x + 10.9041x^2$ ($r^2 = 0.979$, $P < 0.05$); (J) $y = 1230.6186 - 930.6434x + 186.6411x^2$ ($r^2 = 0.981$, $P < 0.05$); (K) $y = 427.7990 - 329.7703x + 69.5584x^2$ ($r^2 = 0.968$, $P < 0.05$); and (L) $y = 5.3095 + 2.4986x - 1.0899x^2$ ($r^2 = 0.999$, $P < 0.001$).

such as chlorotic and necrotic specks on the foliage (Kampfenkel et al., 1995; Smith et al., 2004), and measured no decrease in Chl at the highest Fe levels (Fig. 2A). In fact, the vines grown at the highest Fe supply had the highest CO₂ assimilation capacity (Chen et al., 2004). Fe overload has been associated with a lowered AsA/DAsA ratio (on a FW basis) and a doubling of APX and CAT specific activity in the leaves of *Nicotiana plumbaginifolia* Viv. (Kampfenkel et al., 1995). We found that many of the antioxidant enzymes and metabolites increased on a leaf area basis as active Fe increased, but when expressed on a Chl basis, we cannot conclude that the vines grown at high Fe levels suffered from Fe-catalyzed oxidative stress. NPQ (calculated as qNP) in *N. plumbaginifolia* also increased in response to Fe excess (Kampfenkel et al., 1995), yet we did not find an increase in NPQ at the highest Fe level either.

In conclusion, both photoprotective mechanisms, xanthophyll cycle-dependent thermal dissipation and the antioxidant system, are enhanced in response to iron deficiency to cope with excess absorbed light. In a low soil pH tolerant species such as *V. labruscana*, the foliar antioxidant system was upregulated in response to excess absorbed light from Fe deficiency-induced chlorosis, and there was no evidence of an increase in oxidative stress from high rates of applied Fe-EDDHA.

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