

CO₂ Assimilation, Photosynthetic Enzymes, and Carbohydrates of ‘Concord’ Grape Leaves in Response to Iron Supply

Li-Song Chen¹, Brandon R. Smith, and Lailiang Cheng²

Department of Horticulture, Cornell University, Ithaca, NY 14853

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ABSTRACT. Own-rooted 1-year-old ‘Concord’ grapevines (*Vitis labruscana* Bailey) were fertigated twice weekly for 11 weeks with 1, 10, 20, 50, or 100 μM iron (Fe) from ferric ethylenediamine di (*o*-hydroxyphenylacetic) acid (Fe-EDDHA) in a complete nutrient solution. As Fe supply increased, leaf total Fe content did not show a significant change, whereas active Fe (extracted by 2,2'-dipyridyl) content increased curvilinearly. Chlorophyll (Chl) content increased as Fe supply increased, with a greater response at the lower Fe rates. Chl a : b ratio remained relatively constant over the range of Fe supply, except for a slight increase at the lowest Fe treatment. Both CO₂ assimilation and stomatal conductance increased curvilinearly with increasing leaf active Fe, whereas intercellular CO₂ concentrations decreased linearly. Activities of key enzymes in the Calvin cycle, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), NADP-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoribulokinase (PRK), stromal fructose-1,6-bisphosphatase (FBPase), and a key enzyme in sucrose synthesis, cytosolic FBPase, all increased linearly with increasing leaf active Fe. No significant difference was found in the activities of ADP-glucose pyrophosphorylase (AGPase) and sucrose phosphate synthase (SPS) of leaves between the lowest and the highest Fe treatments, whereas slightly lower activities of AGPase and SPS were observed in the other three Fe treatments. Content of 3-phosphoglycerate (PGA) increased curvilinearly with increasing leaf active Fe, whereas glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), and the ratio of G6P:F6P remained unchanged over the range of Fe supply. Concentrations of glucose, fructose, sucrose, starch, and total nonstructural carbohydrates (TNC) at both dusk and predawn increased with increasing leaf active Fe. Concentrations of starch and TNC at any given leaf active Fe content were higher at dusk than at predawn, but both glucose and fructose showed the opposite trend. No difference in sucrose concentration was found at dusk or predawn. The export of carbon from starch breakdown during the night, calculated as the difference between dusk and predawn measurements, increased as leaf active Fe content increased. The ratio of starch to sucrose at both dusk and predawn also increased with increasing leaf active Fe. In conclusion, Fe limitation reduces the activities of Rubisco and other photosynthetic enzymes, and hence CO₂ assimilation capacity. Fe-deficient grapevines have lower concentrations of nonstructural carbohydrates in source leaves and, therefore, are source limited.

Iron (Fe) deficiency-induced leaf chlorosis frequently occurs in grapevines (*Vitis* spp.) grown on calcareous soils (Mengel et al., 1984), which significantly decreases grapevine growth (Bavaresco et al., 1992). Fe in these soils, although abundant, is often precipitated as insoluble Fe(III) oxides and hydroxides and therefore is less available for the roots (Lindsay and Schwab, 1982). Fe uptake in dicotyledons is accomplished via reduction of Fe(III)-chelates by a root plasma membrane bound ferric chelate reductase (FCR), before Fe²⁺ is taken up by Fe²⁺ transporters (Chaney et al., 1972; Robinson et al., 1999; Vert et al., 2002). Once it enters the root cells, Fe is transported through the xylem primarily as Fe(III)-citrate, and upon reaching the leaf apoplast, FCR activity is required again for uptake into mesophyll cells (Bruggemann et al., 1993; Larbi et al., 2001). Plants grown on calcareous soils have a higher pH in the leaf apoplast (Lopez-Millan et al., 2001; Mengel et al., 1994), which decreases the reduction of Fe(III)-citrate to Fe²⁺ by FCR (Gonzalez-Vallejo et al., 2000), leading to a greater immobilization of Fe in leaves.

Although considerable efforts have been made to understand the mechanisms of Fe uptake by roots and genotypic differences in susceptibility to chlorosis (Bavaresco et al., 1993; Brancadoro et al., 1995; Dell'Orto et al., 2000), and to test agronomic and chemical means to prevent and cure Fe chlorosis (Tagliavini and Rombolà, 2001), very little work has been done to study how Fe deficiency affects leaf carbon assimilation and to understand the underlying mechanisms in grapevines, especially *Vitis labruscana*. Compared with *Vitis vinifera* L., *V. labruscana* requires a lower optimum pH (Bates et al., 2002) and is more susceptible to high pH-induced Fe deficiency.

The photosynthetic apparatus of plants is one of the most Fe-enriched cellular systems (Behrenfeld et al., 1996; Varotto et al., 2002), with chloroplasts alone containing about 80% of the leaf iron (Terry and Abadía, 1986; Terry and Low, 1982). Fe is involved in Chl synthesis, photosynthetic electron transport, and regulation of Calvin cycle enzymes (Briat et al., 1995; Buchanan, 1991; Terry, 1980; Varotto et al., 2002). Studies with herbaceous plants have shown that Fe deficiency causes a marked decrease in photosynthetic capacity (Abadía et al., 1999; Morales et al., 1998; Srivastava and Luthra, 1993; Terry, 1980; Terry and Abadía, 1986). This is accompanied by a decrease in the number of granal and stromal lamellae per chloroplast (Spiller and Terry, 1980) and all membrane components, including electron carriers of the photosynthetic transport chain (Spiller and Terry, 1980;

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¹Current address: Department of Horticulture, Fujian Agriculture and Forestry University, Fuzhou 350002, P.R. China.

²To whom reprint requests should be addressed: fax: +1-607-255-0599; e-mail: LC89@Cornell.edu

Terry, 1980) and light-harvesting pigments (Abadía et al., 1999; Gogorcena et al., 2001; Morales et al., 1994). Fe deficiency also decreases the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC4.1.1.39) (Taylor and Terry, 1986; Winder and Nishio, 1995). However, it is controversial whether Rubisco limits photosynthesis in Fe-deficient leaves, because Rubisco activity does not decrease to a similar extent as CO₂ assimilation (Arulanantham et al., 1990; Tayler and Terry, 1984; 1986; Terry, 1980; Winder and Nishio, 1995). Reports on the effect of Fe deficiency on the activity of other photosynthetic enzymes are not consistent. Arulanantham et al. (1990) found that Fe deficiency decreased the total extractable activities of PRK (EC2.7.1.19), GAPDH (EC1.2.1.12), and FBPase (EC3.1.3.11), whereas Taylor et al. (1982) observed an increase in the activities of the same enzymes in sugar beet (*Beta vulgaris* L.). In addition, there has not been a detailed examination of how Fe deficiency affects leaf carbohydrate metabolism (Aralanantham et al., 1990; Srivastava and Luthra, 1993).

The objective of this work was to determine how CO₂ assimilation, key enzymes involved in the Calvin cycle and in starch and sucrose synthesis, and levels of nonstructural carbohydrates in grapes leaves respond to a wide range of Fe supply.

Materials and Methods

PLANT CULTURE AND FE TREATMENTS. Own-rooted 1-year-old 'Concord' grapevines were pruned to two nodes and transplanted into 19.8-L plastic containers, using a commercially blended medium (MetroMix 560 with coir; Scotts Co., Marysville, Ohio) consisting of composted pine bark, coconut coir pith, sphagnum peat moss, processed bark ash, and perlite. Vines were grown outdoors at Cornell Orchards in Ithaca, N.Y. (42°26'N, 76°29'W; elevation 500 m). At budbreak, extra shoots were removed and only one shoot was allowed to grow on each plant.

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-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 blended based on a modified Johnson's nutrient solution (Johnson et al., 1957), and were applied at a concentration of 10 μM Mn (MnSO₄·H₂O), 2 μM Zn (ZnSO₄·7 H₂O), 0.50 μM Cu (CuSO₄·5 H₂O), 25 μM B (H₃BO₃), and 0.50 μM Mo (NaMoO₄).

There were five replicates per Fe treatment with two plants each in a completely randomized design. The mean pH of the medium was 6.6 \pm 0.1, measured weekly using the saturated paste method (Warncke, 1986). Eleven weeks after transplanting, recent fully expanded leaves were chosen for analysis.

GAS EXCHANGE MEASUREMENTS. Measurements were made with a CIRAS-1 portable photosynthesis system (PP Systems, Herts, U.K.) at ambient CO₂ (360 $\mu\text{mol}\cdot\text{mol}^{-1}$) at noon under a photon flux density (PFD) of 1750 \pm 50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, leaf temperature of 26.3 \pm 0.1 °C, and ambient water vapor pressure of 1.88 \pm 0.04 kPa.

ASSAY OF ENZYMES IN THE CALVIN CYCLE AND CARBOHYDRATE METABOLISM. Leaf discs (1 cm² in size) were taken from the same leaves used for gas exchange measurements at noon under full sun (PFD of 1750 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). They were frozen in liquid N₂, then stored at -80 °C until assay.

Rubisco, GAPDH, PRK, FBPase, and SPS (EC2.4.1.14) were extracted according to Chen and Cheng (2003a). Two frozen leaf discs were ground with a precooled mortar and pestle in 1.5 mL extraction buffer containing 50 mM Hepes-KOH (pH 7.5), 10 mM MgCl₂, 2 mM ethylenediaminetetraacetic acid (EDTA), 10 mM dithiothreitol (DDT), 1% (v/v) Triton X-100, 5% (w/v) insoluble polyvinylpyrrolidone (PVPP), 1% (w/v) bovine serum albumin (BSA), 10% (v/v) glycerol and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The extract was centrifuged at 13,000 g_n for 5 min in an Eppendorf microcentrifuge, and the supernatant was used immediately for enzyme activity assay.

Total Rubisco activity was measured after incubating the leaf extract in the assay solution without ribulose-1,5-bisphosphate (RuBP) for 15 min at room temperature (Cheng and Fuchigami, 2000).

GAPDH and PRK activities were determined according to Leegood (1990).

Stromal FBPase was assayed in a mixture (1 mL) containing 50 mM Tris-HCl (pH 8.2), 10 mM MgCl₂, 1 mM EDTA, 0.1 mM fructose 1,6-bisphosphate (FBP), 0.5 mM NADP, four units of phosphoglucose isomerase (PGI, EC5.3.1.9) and two units of glucose-6-phosphate dehydrogenase (G6PDH, EC1.1.1.49). The reaction was initiated by adding the enzyme extract (Holaday et al., 1992; Leegood, 1990). Cytosolic FBPase was assayed according to Holaday et al. (1992) with some modifications. The enzyme activity was measured in 1 mL reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 2 mM MgCl₂, 0.1 mM FBP, 0.5 mM NADP, four units of PGI, and two units of G6PDH. The reaction was initiated by adding the enzyme extract.

SPS was assayed according to Grof et al. (1998). Sixty microliters of leaf extract was incubated for 15 min at 30 °C with 100 mM Hepes-KOH (pH 7.5), 100 mM KCl, 6 mM EDTA, 30 mM uridine 5'-diphosphoglucose (UDPG), 10 mM fructose-6-phosphate (F6P), 40 mM glucose-6-phosphate (G6P) in a total volume of 100 μL . At the end of the 15-min incubation period, the reaction was stopped by adding 100 μL ice-cold 1.2 N HClO₄ and held on ice for another 15 min. The reaction mixture was neutralized by adding 60 μL of 2 M KHCO₃, held on ice for 15 min, then centrifuged at 13,000 g_n for 1 min. An aliquot (130 μL) of the supernatant was assayed for uridine 5'-diphosphate (UDP) by coupling to oxidation of NADH with lactate dehydrogenase (LDH, EC1.1.1.27) and pyruvate kinase (EC2.7.1.40). The reaction mixture (1 mL) contained 50 mM Hepes-NaOH (pH 7.0), 5 mM MgCl₂, 0.3 mM NADH, 0.8 mM phosphoenolpyruvate (PEP), 14 units LDH, and four units pyruvate kinase. The reaction was started by adding pyruvate kinase (Stitt et al., 1988). Controls without F6P and G6P were carried through for all the samples.

AGPase (EC2.7.7.27) was extracted and assayed as described in Chen and Cheng (2003a). The only modification was that reduced glutathione (GSH) was not included in the extraction buffer.

EXTRACTION AND ASSAY OF G6P, F6P, AND PGA. Forty centimeter-squared leaf tissues (\approx 1 g) were taken at noon under full sun (PFD of 1750 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), frozen in liquid N₂, and stored at -80 °C until assay. Metabolites were extracted and measured according to Chen et al. (2002) with some modifications (Chen and Cheng, 2003a).

ANALYSIS OF NONSTRUCTURAL CARBOHYDRATES. Three leaf discs (total of 3 cm²) were taken at dusk and at predawn from the same leaf, frozen in liquid N₂ and stored at -80 °C until assay. Sucrose, glucose, fructose, and starch were extracted and assayed according to Chen and Cheng (2003a).

IRON AND CHL ANALYSIS. A large portion of the total Fe pool can be immobilized in the leaf, and extracting “physiologically active” or loosely bound Fe with weak acids or Fe(II) chelators often provides a more accurate diagnosis of leaf Fe status (Abadía et al., 1984; Gezgin and Er, 2001; Katyál and Sharma, 1980). In this study, active Fe was extracted and assayed according to Abadía et al. (1984) with some modifications. Three 1-cm² discs were punched from each leaf, frozen in liquid N₂, and stored at -80 °C until analysis. Each disc was cut into ≈5-mm² pieces using stainless steel scissors. Leaf tissue was 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. Active Fe values were calculated from a standard curve using Fe atomic absorption standard solution. For total Fe analysis, leaves were washed with 0.1% Triton X-100 and dried at 70 °C for 5 d. Dried samples were ground through a 0.5-mm screen in a Cyclotec sample mill, dry-ashed (Greweling, 1976), and measured for total Fe using ICP emission spectrometry. Leaf Chl was extracted and measured according to Arnon (1949).

Results

LEAF TOTAL FE, ACTIVE FE, AND CHL.

Leaf total Fe content did not show a significant change, whereas active Fe content increased curvilinearly as Fe supply increased (Fig. 1 A and B). Leaf Chl content increased as Fe supply increased, with a greater response at the lower Fe rates (Fig. 1C). Leaf chlorosis was most obvious in plants supplied with 1 μM Fe, whereas slight chlorosis developed in the 10 μM Fe treatment. The ratio of Chl a:b remained relatively constant as Fe supply decreased, except for a slight increase at the 1 μM Fe treatment (Fig. 1D).

Leaf Chl content was linearly correlated with leaf active Fe content (Fig. 2).

LEAF GAS EXCHANGE. As leaf active Fe increased, both CO₂ assimilation (Fig. 3A) and stomatal conductance (Fig. 3B) increased curvilinearly, whereas the calculated intercellular CO₂ concentration decreased (Fig. 3C).

ACTIVITIES OF KEY ENZYMES IN THE CALVIN CYCLE AND STARCH AND SUCROSE SYNTHESIS. Activities of key enzymes in the Calvin cycle, Rubisco, GAPDH, PRK, and stromal FBPase and a key enzyme in sucrose synthesis, cytosolic FBPase, all increased linearly with increasing leaf active Fe (Fig. 4A–E). Compared with the highest active Fe leaves, decreases in enzyme activity and CO₂ assimilation in the lowest active Fe leaves were in the order: stromal FBPase > Rubisco > CO₂ assimilation, cytosolic FBPase > GAPDH > PRK (Fig. 3A and Fig. 4A–E). No significant difference in the activities of AGPase and SPS was found between the lowest and the highest Fe treatments, whereas slightly lower activities of AGPase and SPS were observed in

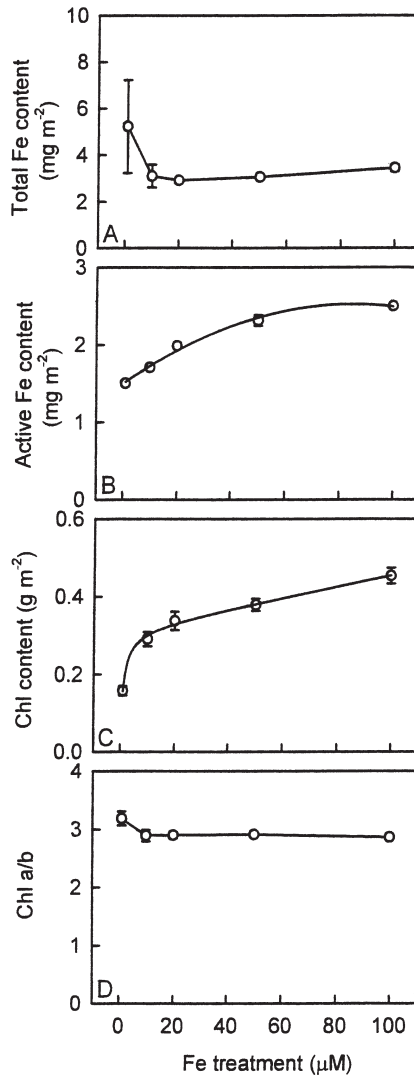


Fig. 1. Effects of Fe supply on total Fe (A), active Fe (B), chlorophyll (Chl, C), and the ratio of Chl a:b (D) in ‘Concord’ grape leaves. Each point is mean ± standard error (n = 5). In (A): Total Fe data were analyzed by analysis of variance with respect to Fe supply, $P > 0.05$. Regression equations: (B) $y = 1.4950 + 0.0243x - 0.0001x^2$ ($r^2 = 0.991$, $P < 0.01$); (C) $y = 0.3143x/(1.0217 + x) + 0.0014x$ ($r^2 = 0.996$, $P < 0.01$).

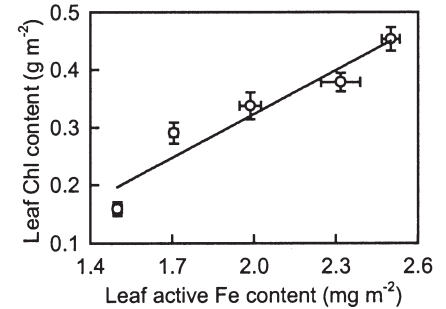


Fig. 2. Leaf Chl content in relation to active Fe content. Regression equation: $y = -0.185 + 0.254x$ ($r^2 = 0.915$, $P < 0.05$). Each point is mean ± standard error for the leaf active Fe content (horizontal, n = 5) and the dependent variable (vertical, n = 5).

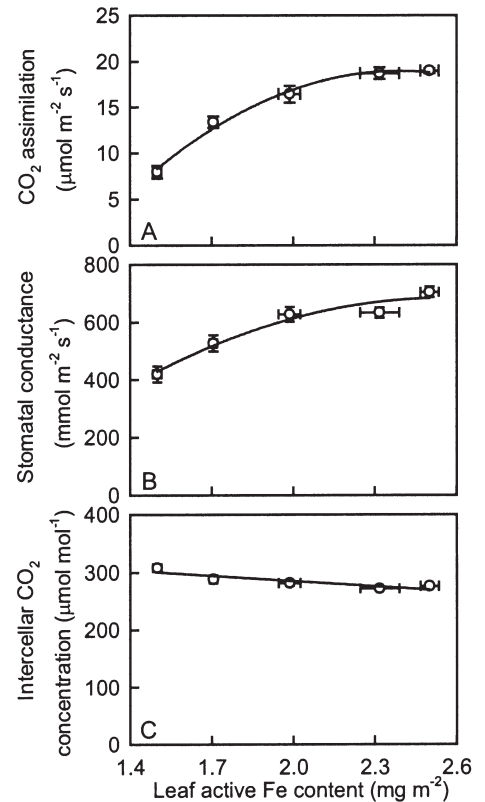


Fig. 3. CO₂ assimilation (A), stomatal conductance (B), and intercellular CO₂ concentration (C) in relation to active Fe content in ‘Concord’ grape leaves. Each point is mean ± standard error for the leaf active Fe (horizontal, n = 5) and the dependent variable (vertical, n = 5). Regression equations: (A) $y = -57.8 + 64.2x - 13.4x^2$ ($r^2 = 0.991$, $P < 0.01$); (B) $y = -819.7 + 1177.5x - 230.6x^2$ ($r^2 = 0.953$, $P < 0.05$); (C) $y = 346.0 - 30.4x$ ($r^2 = 0.806$, $P < 0.05$).

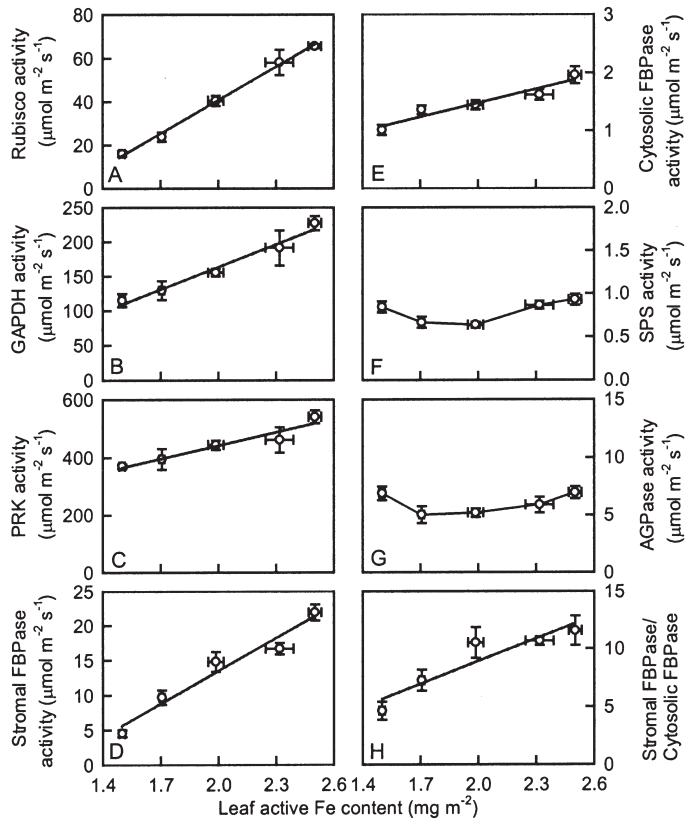


Fig. 4. Activities of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, **A**), NADP-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, **B**), phosphoribulokinase (PRK, **C**), stromal fructose-1,6-bisphosphatase (FBPase, **D**), cytosolic FBPase (**E**), ADP-glucose pyrophosphorylase (AGPase, **F**), and sucrose phosphate synthase (SPS, **G**), and ratio of stromal FBPase activity: cytosolic activity (**H**) in relation to active Fe content in 'Concord' grape leaves. Each point is mean \pm standard error for the leaf active Fe (horizontal, $n = 5$) and the dependent variable (vertical, $n = 5$). Regression equations: (**A**) $y = -62.15 + 51.39x$ ($r^2 = 0.996$, $P < 0.01$); (**B**) $y = -55.46 + 109.31x$ ($r^2 = 0.975$, $P < 0.01$); (**C**) $y = 134.67 + 153.32x$ ($r^2 = 0.921$, $P < 0.01$); (**D**) $y = -17.96 + 15.72x$ ($r^2 = 0.956$, $P < 0.01$); (**E**) $y = -0.14 + 0.80x$ ($r^2 = 0.922$, $P < 0.01$); (**H**) $y = -4.11 + 6.51x$ ($r^2 = 0.868$, $P < 0.05$).

the middle three Fe treatments (Fig. 4 F and G).

As the stromal FBPase activity decreased to a larger extent than the cytosolic FBPase in response to Fe limitation, the ratio of stromal FBPase activity: cytosolic FBPase activity decreased as leaf active Fe decreased (Fig. 4H).

CONTENTS OF G6P, F6P, AND PGA. Leaf G6P and F6P contents (Fig. 5 A and B) and the ratio of G6P:F6P (Fig. 5C) remained unchanged over the range of leaf active Fe content examined. Leaf PGA content increased curvilinearly with increasing leaf active Fe content (Fig. 5D).

NONSTRUCTURAL CARBOHYDRATES AT DUSK AND PREDAWN. Contents of glucose, fructose, sucrose, starch, and total nonstructural carbohydrate (TNC) at both dusk and predawn increased curvilinearly with increasing leaf active Fe (Fig. 6A–E). Starch content and TNC at any given leaf active Fe level were higher at dusk than at predawn (Fig. 6D and E), whereas both glucose and fructose showed the opposite trend (Fig. 6A and B). No difference in sucrose content was found between dusk and predawn (Fig. 6C). The ratio of starch:sucrose at both dusk and predawn increased as leaf active Fe content increased with the ratio being higher at dusk than at predawn (Fig. 6F). A similar trend was found in the ratio of starch:soluble sugars (glucose + fructose + sucrose) (Fig. 6G).

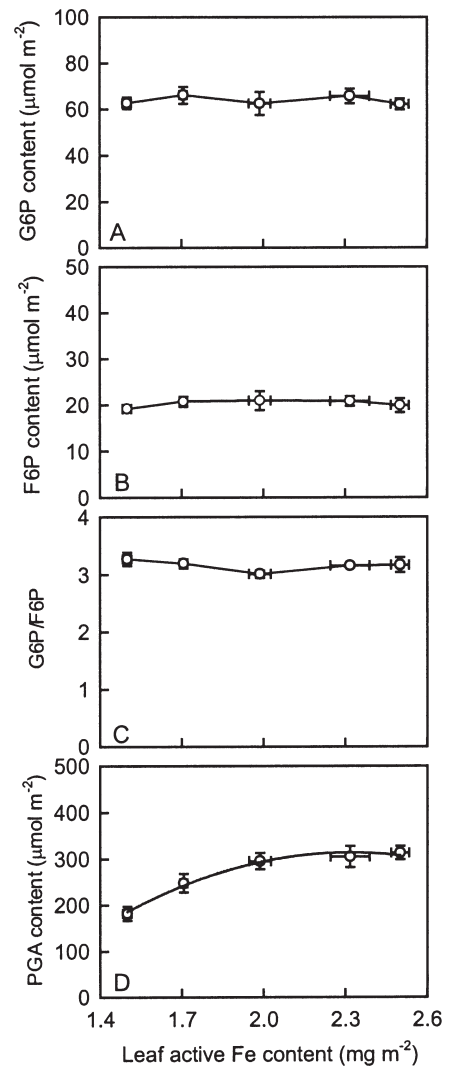


Fig. 5. Glucose-6-phosphate (G6P) content (**A**), fructose-6-phosphate (F6P) content (**B**), G6P:F6P ratio (**C**), and 3-phosphoglycerate (PGA) content (**D**) in relation to active Fe content in 'Concord' grape leaves. Each point is mean \pm standard error for the leaf active Fe (horizontal, $n = 5$) and the dependent variable (vertical, $n = 5$). Regression equations: (**D**) $y = -725.95 + 898.55x - 194.12x^2$ ($r^2 = 0.986$, $P < 0.05$).

Carbon export from starch degradation during the night, calculated as the difference between dusk and predawn values, increased as leaf active Fe content increased (Fig. 6H).

Discussion

Our finding that chlorosis developed in response to Fe limitation, yet without a corresponding significant change in the total Fe content of 'Concord' grape leaves (Fig. 1A), is similar to that observed in leaves of *Vitis vinifera* (Römhald, 2000) and *Pyrus pashia* 'Buch-Ham' (An and Fan, 2003). The exact cause of this "chlorosis paradox" still remains unclear, but it may be related to inhibition of leaf growth (Häussling et al., 1985; Römhald, 2000) and inactivation of Fe by high leaf apoplast pH (Mengel, 1994; Mengel and Geurtzen, 1986). In our study, the average area of the leaves used for gas exchange measurements in the 1- μM Fe treatment was about 35% smaller than the ones in the highest Fe

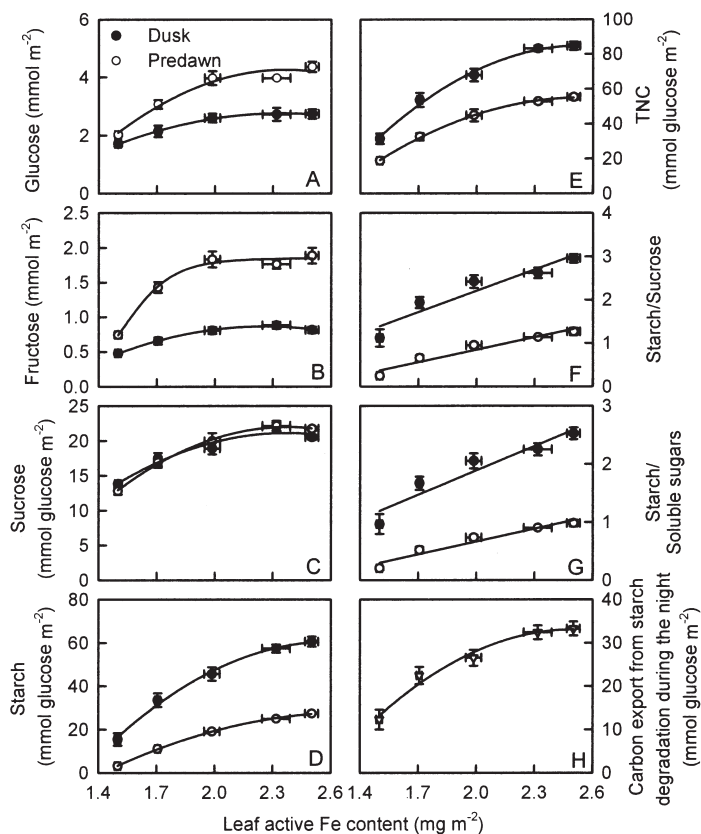


Fig. 6. Glucose (A), fructose (B), sucrose (C), starch (D), total nonstructural carbohydrate (TNC, E) contents, the ratios of starch : sucrose (F) and starch : soluble sugars (glucose + fructose + sucrose) (G) at dusk and at predawn, and carbon export from starch degradation during the night (H) in relation to active Fe content in 'Concord' grape leaves. Each point is mean \pm standard error for the leaf active Fe (horizontal, $n = 5$) and the dependent variable (vertical, $n = 5$). Regression equations for glucose at dusk: $y = -5.43 + 6.99x - 1.49x^2$ ($r^2 = 0.997$, $P < 0.01$), and at predawn: $y = -11.97 + 13.73x - 2.90x^2$ ($r^2 = 0.964$, $P < 0.05$); for fructose at dusk: $y = -2.62 + 3.09x - 0.68x^2$ ($r^2 = 0.995$, $P < 0.01$), and at predawn: $y = 1.85/[1 + (x/1.55)^{-13.09}]$ ($r^2 = 0.988$, $P < 0.05$); for sucrose at dusk: $y = -32.69 + 45.43x - 9.59x^2$ ($r^2 = 0.952$, $P < 0.05$), and at predawn: $y = -45.04 + 56.46x - 11.89x^2$ ($r^2 = 0.997$, $P < 0.01$); for starch at dusk: $y = -185.43 + 189.02x - 36.30x^2$ ($r^2 = 0.995$, $P < 0.01$), and at predawn: $y = -94.21 + 89.66x - 16.45x^2$ ($r^2 = 0.999$, $P < 0.01$); for TNC at dusk: $y = -226.17 + 244.52x - 48.07x^2$ ($r^2 = 0.994$, $P < 0.01$), and at predawn: $y = -159.58 + 169.03x - 33.29x^2$ ($r^2 = 0.999$, $P < 0.01$); for starch : sucrose at dusk: $y = -1.08 + 1.64x$ ($r^2 = 0.908$, $P < 0.05$), and at predawn: $y = -1.07 + 0.96x$ ($r^2 = 0.941$, $P < 0.01$); for starch : soluble sugars at dusk: $y = -0.90 + 1.39x$ ($r^2 = 0.911$, $P < 0.05$), and at predawn: $y = -0.81 + 0.74x$ ($r^2 = 0.943$, $P < 0.01$); for carbon export: $y = -91.23 + 99.36x - 19.86x^2$ ($r^2 = 0.979$, $P < 0.05$).

treatment (data not shown). The concentrating effect caused by reduction in leaf area may help to explain why total Fe levels did not decrease, but it does not explain why active Fe was much lower under Fe limitation.

Considering that EDDHA can be directly taken up by beans (*Phaseolus vulgaris* L.) and other plants (Jeffreys et al., 1961; Wallace and Wallace, 1983), we suggest that the difference in total and active Fe observed in our study was the result of inactivation of Fe by a high apoplast pH, which was overcome with a potential rise of Fe(III)-EDDHA in the plant. Under low Fe(III)-EDDHA supply, the roots most likely mine for Fe from the growing medium using adaptive Strategy I mechanisms that increase Fe uptake (Römheld, 1987). This Fe is then chelated as Fe(III)-citrate and delivered to the mesophyll cells, where it is likely immobilized by high apoplast pH (Mengel, 1994), due to the low stability of Fe(III)-citrate at high pH (Norvell, 1991). When

the supply of Fe(III)-EDDHA is adequate, more Fe might be translocated as the pH stable Fe(III)-EDDHA complex and thus not immobilized by high apoplast pH. The mechanism by which EDDHA enters the root cells is not clear, but Fe(III)-EDDHA either delivers Fe to FCR in a more efficient manner than Fe(III)-citrate, or the entire Fe(III)-EDDHA complex is available for utilization in the mesophyll cells. Our finding that CO_2 assimilation was closely correlated with active Fe confirms that active Fe, rather than total Fe, provides a more accurate diagnosis of Fe status in grape leaves (Nikolic and Kastori, 2000).

The decrease in Chl of 'Concord' leaves in response to Fe limitation (Fig. 1C) will inevitably lead to a decrease in leaf light absorption. However, the decrease in Chl is unlikely to limit light-saturated CO_2 assimilation, as the relationship between leaf Chl and light absorption is curvilinear and a large decrease in Chl only causes a relatively small decrease in light absorption (Chen and Cheng, 2003b). In fact, measurements of Chl fluorescence showed that nonphotochemical quenching of excitation energy was higher in Fe-deficient leaves (Smith and Cheng, unpublished data), indicating that there was a greater excess of absorbed *PFD* in these leaves. Jiang et al. (2001) also found that total Chl did not limit CO_2 assimilation of Fe-deficient maize (*Zea mays* L.) leaves. The slight increase in the Chl a : b ratio in response to Fe limitation (Fig. 1C) could be either due to the preferential photodestruction of Chl b (Terry and Abadía, 1986) or from a reduction of light harvesting antenna complexes relative to reaction center complexes (Ferraro et al., 2003; Nishio et al., 1985).

The decrease in CO_2 assimilation under low Fe supply (Fig. 3A) is caused by nonstomatal limitation, as intercellular CO_2 concentration increased with decreasing active Fe (Fig. 3C). The nonstomatal limitation may come from a decrease of Rubisco activity or insufficient RuBP regeneration. Rubisco activity decreased to a larger extent than CO_2 assimilation in response to Fe limitation (Figs. 3A, 4A). Activities of both GAPDH and PRK were decreased by Fe deficiency, but to a lesser extent than CO_2 assimilation (Figs. 3A, 4B and C). Therefore, PGA reduction and RuBP regeneration may not limit CO_2 assimilation in Fe-deficient grape leaves. Winder and Nishio (1995) suggested that RuBP might actually be in excess in Fe-deficient sugar beet leaves. It is possible that Fe deficiency directly causes the down-regulation of Rubisco expression (Winder and Nishio, 1995) and other Calvin cycle enzymes, which then leads to a decrease in CO_2 assimilation. Alternatively, Fe deficiency may first affect the electron transport chain, especially the photosystem I (PSI) complex (Moseley et al., 2002; Straus, 1994) as PSI is highly enriched in Fe (Terry and Abadía, 1986; Terry and Low, 1982), which then leads to a decrease in electron transport and a corresponding down-regulation of Rubisco and other Calvin cycle enzymes. Further direct evidence is needed to distinguish the two mechanisms.

The finding that glucose, fructose, sucrose, starch, and TNC concentrations of 'Concord' leaves decreased with decreasing Fe supply at both dusk and predawn (Fig. 6A-E) is consistent with the result obtained on sugar beet (Arulanantham et al., 1990). This indicates that Fe limitation decreases the carbon supply of source leaves more than the carbon demand from sink tissues, and that Fe-deficient grapevines are source-limited. Therefore, it can be inferred that the decreased starch and sucrose levels in Fe-deficient leaves result from decreased starch and sucrose synthesis. The preferential decrease in stromal FBPase relative to cytosolic

FBPase (Fig. 4 D, E, and H) in response to Fe limitation is also consistent with the lower starch : sucrose ratio (Fig. 6F) in leaves with lower active Fe. Stromal FBPase plays an important role in starch synthesis, whereas cytosolic FBPase is a key enzyme in sucrose synthesis. Studies with transgenic potato (*Solanum tuberosum* L.) plants that have decreased stromal FBPase (Kofmann et al., 1994) and cytosolic FBPase (Zrenner et al., 1996) clearly showed that decreased stromal FBPase activity led to a decreased ratio of starch to sucrose synthesis, whereas decreased cytosolic FBPase caused an increase in this ratio in the transgenic plants. The decreased starch synthesis in Fe-deficient 'Concord' leaves is also consistent with the lower PGA concentration (Fig. 5D), as AGPase activity is allosterically activated by PGA (Sowokinos, 1981). The fact that no significant difference in the activities of AGPase and SPS was found between the lowest and the highest Fe treatments (Fig. 4 F and G) suggests that AGPase and SPS may be in excess in Fe-deficient leaves. It remains unclear, however, why leaves in the middle Fe treatments had slightly lower activities of AGPase and SPS (Fig. 4 F and G).

In conclusion, Fe deficiency reduces the activities of Rubisco and other photosynthetic enzymes, and hence CO₂ assimilation capacity. Fe-deficient grapevines have lower concentrations of nonstructural carbohydrates in source leaves and, therefore, are source-limited.

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