Effects of Iron Deficiency on the Composition of the Leaf Apoplastic Fluid and Xylem Sap in Sugar Beet. Implications for Iron and Carbon Transport[1]

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The effects of iron deficiency on the composition of the xylem sap and leaf apoplastic fluid have been characterized in sugar beet (*Beta vulgaris* Monohil hybrid). pH was estimated from direct measurements in apoplastic fluid and xylem sap obtained by centrifugation and by fluorescence of leaves incubated with 5-carboxyfluorescein and fluorescein isothiocyanate-dextran. Iron deficiency caused a slight decrease in the pH of the leaf apoplast (from 6.3 down to 5.9) and xylem sap (from 6.0 down to 5.7) of sugar beet. Major organic acids found in leaf apoplastic fluid and xylem sap were malate and citrate. Total organic acid concentration in control plants was 4.3 mM in apoplastic fluid and 9.4 mM in xylem sap and increased to 12.2 and 50.4 mM, respectively, in iron-deficient plants. Inorganic cation and anion concentrations also changed with iron deficiency both in apoplastic fluid and xylem sap. Iron decreased with iron deficiency from 5.5 to 2.5 μM in apoplastic fluid and xylem sap. Major predicted iron species in both compartments were $[FeCitOH]^{-1}$ in the controls and $[FeCit_2]^{-3}$ in the iron-deficient plants. Data suggest the existence of an influx of organic acids from the roots to the leaves via xylem, probably associated to an anaplerotic carbon dioxide fixation by roots.

When grown under limited iron supply, many plant species develop iron-acquisition mechanisms that are not expressed or under-expressed when iron supply is sufficient. The most widespread iron-acquisition mechanism in plants, Strategy I, has been found in dicotyledonous and non-graminaceous monocotyledonous species (Marschner et al., 1986; Römheld and Marschner, 1986; Bienfait, 1988; Brown and Jolley, 1988). This Strategy involves morphological changes, such as increased formation of lateral roots, root hairs, and transfer cells, all of them increasing root surface for iron uptake (Kramer et al., 1980; Landsberg, 1982; Schmidt, 1999). Strategy I also includes physiological changes, such as the development of an increased proton excretion, which decreases rizosphere pH (Brown, 1978), a release of reducing and/or chelating substances such as phenolics and flavins (Welkie and Miller, 1960; Susín et al., 1994), and a two-step mechanism for iron uptake, in which Fe(III) is first reduced by a plasma membrane-bound ferric chelate reductase (FC-R) enzyme (Moog and Brüggemann, 1994; Susín et al., 1996; Robinson et al., 1999) and then absorbed as Fe(II) (Chaney et al., 1972; Eide et al., 1996; Fox and Guerinot, 1998). Once iron enters the root cell it must be transported to the leaves. Iron is thought to be transported in the xylem as Fe(III), probably complexed by citrate (Tiffin, 1966; Brown and Chaney, 1971; White et al., 1981a; Cataldo et al., 1988). The mechanism of iron uptake by leaf cells has been much less studied than the corresponding processes in the roots.

The apoplastic compartment occupies 5% or less of the plant tissue volume of aerial organs (Steudle et al., 1980; Parkhurst, 1982) and root cortexes (Vakhmistrov, 1967). Solute concentrations in the apoplast of aerial organs are determined by the balance of import via xylem, absorption by cells, and export by phloem. Due to the small apoplastic volume, relatively small changes in these fluxes could result in large changes in the apoplastic composition. The apoplast contains enzymes (Li et al., 1989; Pinedo et al., 1993), high concentrations of metabolites such as ascorbic acid (Polle et al., 1990; Luwe et al., 1993), and sugars (Tetlow and Farrar, 1993), plays important roles in the transport and storage of mineral nutrients (Starrach and Mayer, 1989; Wolf et al., 1990; Zhang et al., 1991), and is involved in signal transmission (Hartung et al., 1992). Studies have been made on the composition of the apoplast under different conditions (Clarkson, 1984; Blatt, 1985; Bowling, 1987; Grignon and Sentenac, 1991; Speer and Kaiser, 1991; Tetlow and Farrar, 1993; Canny, 1995). Primary reactions that lead to symptoms of nutrient deficiency or

[1] This work was supported by the Comisión Interministerial de Ciencia y Tecnología (grant no. AGR97–1177 to A.A.), the Dirección General de Investigación Científica y Técnica (grant no. PB97–1176 to J.A.), and the Commission of European Communities (grant nos. AIR3–CT94–1973 and PL971176 to J.A.). A.F.L.-M. and F.M. were supported by a pre-doctoral fellowship and a scientist research contract from the Spanish Ministry of Science and Education, respectively.

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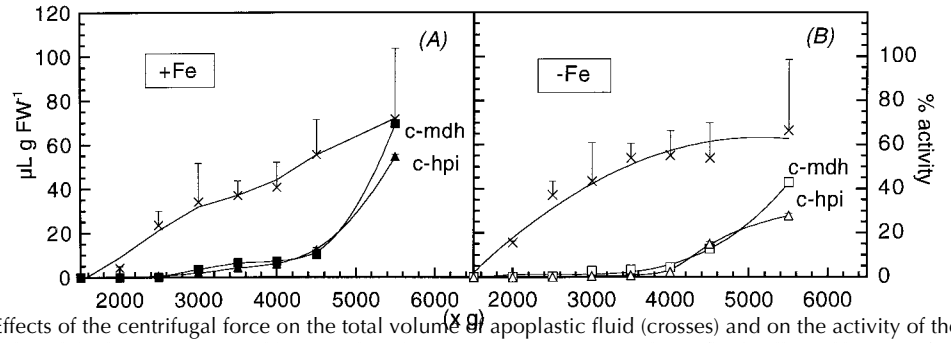

Figure 1. Effects of the centrifugal force on the total volume of apoplastic fluid (crosses) and on the activity of the cytosolic enzymes malate dehydrogenase (■) and hexose phosphate isomerase (▲) in apoplastic fluid collected by centrifugation from iron-sufficient (A) and iron-deficient (B) sugar beet leaves. Data are means ± SE of 10 replications.

toxicity take place in the apoplast (Mengel and Geurtzen, 1988; Speer and Kaiser, 1991).

Iron trafficking in the apoplast is mandatory for iron uptake processes by root cells (Longnecker and Welch, 1990; Zhang et al., 1991). However, little is known so far about the changes induced in the leaf apoplast by iron deficiency. Once in the leaf apoplast, Fe(III) has been shown to be reduced by a mesophyll plasma membrane-bound FC-R similar to that present in roots (Brüggemann et al., 1993; de la Guardia and Alcántara, 1996; Nikolic and Römheld, 1999; González-Vallejo et al., 2000). It has been suggested that iron reduction and transport across the plasma membrane of mesophyll cells is a crucial step that could be impaired by iron deficiency through an increase of apoplastic pH (Mengel, 1995; Kosegarten et al., 1999). It has recently been shown that mesophyll protoplasts have lower FC-R activity on a protoplast surface basis when iron-deficient (González-Vallejo et al., 2000).

The aim of this work was to investigate the effects of iron deficiency on the composition of the apoplast and xylem sap of the model plant sugar beet (*Beta vulgaris* Monohil hybrid) to understand the role of these compartments in the transport and acquisition of iron by leaf cells.

RESULTS

Apoplastic Fluid Isolation

The volume of apoplastic fluid obtained by centrifugation increased gradually when the centrifugal forces increased, in both iron-deficient and control, iron-sufficient sugar beet leaves (Fig. 1). The activities of cytosolic marker enzymes in apoplastic fluid were low at centrifugal forces lower than 4,000*g* and increased markedly thereafter. When using seven successive steps of centrifugation, the activities of malate dehydrogenase (c-mdh) were less than 7% and 4% of the total leaf homogenate activities at 4,000*g* in control and iron-deficient sugar beet leaves, respectively (Fig. 1). At higher centrifugation forces the activities of c-mdh reached values of 70% and 43% of the total leaf homogenate activities, indicating loosening or rupture of cell membranes. Similar results were obtained for cytosolic hexose phosphate isomerase (c-hpi) activity at centrifugal forces of 4,000*g* or lower, with activities equivalent to less than 7% and 10% of the total leaf homogenate activities in control and iron-deficient sugar beet, respectively. When using only two centrifugation steps at 2,500 and 4,000*g* the c-mdh and c-hpi activities in apoplastic fluid were 2% to 3% of those found in total leaf

Table I. *Activities of the cytosolic marker enzymes c-mdh and c-hpi in apoplastic fluid and xylem sap (in nmol mL^{-1} s^{-1}) and whole extracts of leaves and petioles (in µmol g^{-1} FW s^{-1}) of iron-sufficient (300 µmol Chl m^{-2}) and iron-deficient (50 µmol Chl m^{-2}) sugar beet*

Values in brackets represent the percentages with respect to the maximum activities in total homogenates. Data are the mean ± SE of five replications.

Material	Iron-Sufficient		Iron-Deficient	
	c-mdh	c-hpi	c-mdh	c-hpi
Leaf homogenate	0.472 ± 0.200	84 ± 10	0.626 ± 0.321	57 ± 3
Apoplastic fluid	0.013 ± 0.004 (3%)	2.5 ± 1.8 (3%)	0.014 ± 0.010 (2%)	1.7 ± 0.5 (3%)
Petiole homogenate	0.071 ± 0.009	10.8 ± 5.7	0.174 ± 0.030	24.6 ± 9.0
Xylem sap	<0.001 (0%)	0.07 ± 0.01 (0.6%)	0.001 ± 0.001 (0.6%)	0.24 ± 0.01 (1%)

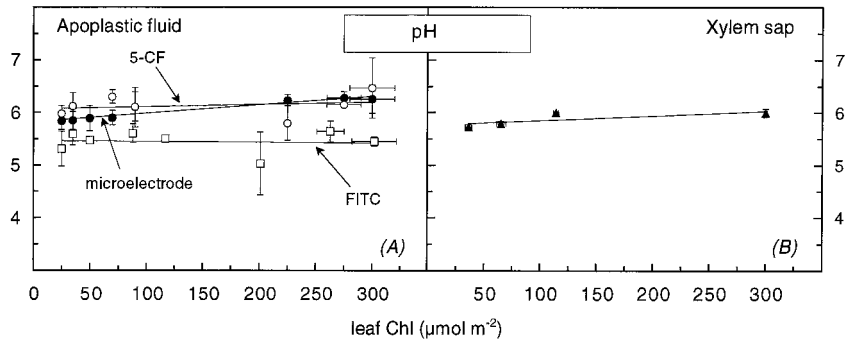

Figure 2. Effects of iron deficiency on the xylem sap and apoplastic pH in sugar beet leaves. Measurements were made with a microelectrode in apoplastic fluid (● in A) and xylem sap (▲ in B) obtained by centrifugation and in vivo by fluorescence with the dyes 5-CF (○ in A) and FITC-dextran (□ in A). Data are means ± SE of three replications.

homogenates (Table I). In routine experiments apoplastic fluid was collected at 4,000*g*, after carrying out a preliminary centrifugation of the leaves at 2,500*g* to discard fluid containing the xylem sap of the main vein. In the case of xylem sap obtained from the centrifugation of petioles the activities of the cytosolic marker enzymes were always 1% or less of those found in total petiole homogenates (Table I). Contamination by cytosolic enzymes was always assessed in each sampling.

Apoplastic Fluid and Xylem Sap pH

The apoplastic pH of sugar beet leaves was measured using two different methods, pH determination in the apoplastic fluid obtained by centrifugation and in vivo estimation by means of fluorescent dyes (Hoffman et al., 1992; Fig. 2). When determined with a microelectrode the pH of the apoplastic fluid was slightly decreased by iron deficiency from approximately 6.3 in control leaves to 5.9 in markedly iron-deficient leaves (Fig. 2A). The pH of sugar beet xylem sap obtained by centrifugation decreased with iron deficiency from 6.0 to 5.7 (Fig. 2B).

In vivo pH measurements were carried out with the fluorescent dyes 5-carboxyfluorescein (5-CF) and fluorescein isothiocyanate (FITC)-dextran (Fig. 2A). The pH values estimated in vivo using 5-CF were very similar to those found by direct pH measurement in the apoplastic fluid, with values of approximately 6.5 in control leaves and 6.0 in markedly iron-deficient leaves. With FITC-dextran, pH was in the range of 5.4 to 5.6 in all leaves. The different pH values obtained using 5-CF and FITC-dextran are possibly related to their different size and permeability through biological membranes, since the larger size of FITC-dextran may difficult its access to the whole of the apoplastic space.

Organic Anion Composition

Organic anions were quantified by HPLC. Peaks corresponding to oxalate, cis-aconitate, citrate, 2-oxoglutarate, malate, succinate, and fumarate were identified in apoplastic fluid and xylem sap (Fig. 3). Succinate co-eluted with another unidentified compound with absorption maxima at 261 and 205 nm.

Apoplastic fluid from control and iron-deficient sugar beet contained concentrations of citrate, malate, and succinate in the millimolar range, and of cis-aconitate, 2-oxoglutarate, and fumarate in the micromolar range (Fig. 4). Iron deficiency caused a general increase in organic anion concentrations in the apoplastic fluid, reaching a maximum in chlorotic leaves with approximately 35 μmol chlorophyll (Chl) m^{-2}. Maximum increases in the concentration of the three major anions in apoplastic fluid were 6-fold for citrate (from 0.7 to 4.4 mM), 3-fold for malate (from 0.7 to 2.2 mM), and 1.8-fold for succinate (from 1.4 to 2.6 mM; Fig. 4A). For the minor organic anions the maximum increases in concentration were 4-fold for cis-aconitate (from 72 to 300 μM), 4.6-fold for 2-oxoglutarate (from 32 to 145 μM), and 11-fold for fumarate (from 0.8 to 9 μM; Fig. 4B).

The major organic anions in xylem sap were also citrate, malate, and succinate (Fig. 5). The concentrations of citrate, malate, and succinate in xylem sap increased with iron deficiency 24-fold (from 0.2 to 4.7 mM), 14-fold (from 2.1 to 30.2 mM), and 2-fold (from 3.5 to 7.0 mM), respectively (Fig. 5A), when compared with the controls. The highest organic anion concentrations were found in leaves with approximately 35 μmol Chl m^{-2}. Cis-aconitate increased with iron de-

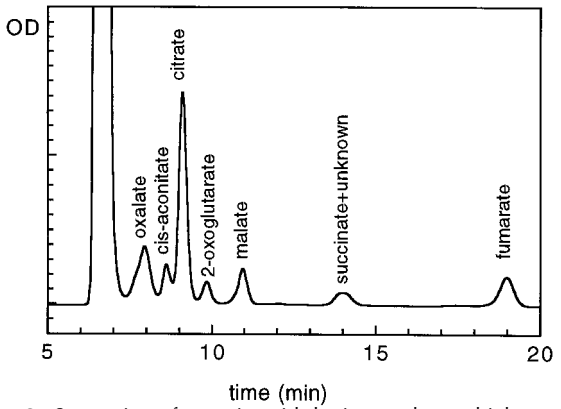

Figure 3. Separation of organic acids by ion-exchange high pressure liquid chromatography. Organic acids were detected at 210 nm.

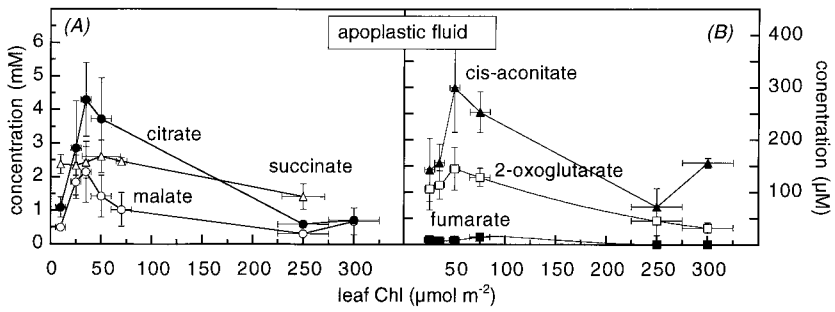

Figure 4. Effects of iron deficiency on the organic anion concentrations in apoplastic fluid of sugar beet leaves. A, Major organic anions (in millimolars): ●, citrate; ○, malate; and △, succinate. B, Minor organic anions (in micromolars): □, 2-oxoglutarate; ▲, cis-aconitate; and ■, fumarate. Data are means ± SE of 10 replications.

ficiency 47-fold (from 4 to 190 μM), 2-oxoglutarate 14-fold (from 43 to 630 μM), and fumarate 23-fold (from 27 to 615 μM; Fig. 5B).

Amino Acid Composition

Amino acid concentrations in apoplastic fluid were always in the micromolar range. Iron deficiency caused increases in the apoplastic concentration of total amino acids of approximately 40%. Major amino acids in apoplastic fluid were Asp, Ser, Glu, Gln, Pro, Ala, Val, and γ-amino-n-butyric acid (Table II). The concentration of Asp in apoplastic fluid decreased with iron deficiency, whereas those of Gln, Ala, and Ser did not change significantly and those of Glu, Val, Pro, and γ-amino-n-butyric acid increased. Among the minor amino acids in apoplastic fluid, the concentration of Lys and Asn decreased with iron deficiency, whereas those of Thr, Gly, and Ile did not change significantly and those of Leu and His increased (Table II).

Sugars

Iron deficiency caused changes in the sugar concentrations of the apoplastic fluid (Fig. 6A). Moderate iron deficiency caused a 4.6-fold decrease in Suc concentration (from 3.7 to 0.8 mM), although severely deficient leaves had Suc concentrations similar to the controls (2.6 mM). Glc concentrations decreased with iron deficiency (from 16.4 to 9.0 mM), whereas Fru concentrations increased 2-fold with iron deficiency (from 4.5 to 9.1 mM).

The sugar concentrations were higher in xylem sap than in apoplastic fluid. In xylem sap moderate iron deficiency caused a 2-fold increase in Fru and a marked decrease in Glc (Fig. 6B). In severely deficient leaves, however, the concentrations of Fru and Glc were, respectively, 60% lower and 2-fold higher of those found in the control leaves. The concentration of Suc did not show major changes with iron deficiency (Fig. 6B).

Inorganic Ion Composition

The concentrations of inorganic cations (calcium, potassium, and magnesium) in apoplastic fluid of sugar beet leaves increased with iron deficiency with a similar trend to that found for the organic anions. The maximum potassium, calcium, and magnesium concentrations were found in apoplastic fluid of iron-deficient leaves with approximately 50 μmol Chl m^{-2} (Fig. 7A). Potassium, calcium, and magnesium in apoplastic fluid were 30, 0.5, and 2 mM in control plants and reached maximal concentrations of 50, 10, and 7 mM in iron-deficient plants. Therefore, the largest increase in apoplastic fluid concentrations with iron deficiency was 20-fold for calcium, followed by 3.5-fold for magnesium, and 1.7-fold for potassium.

Nitrate, Cl^-, and SO_4^{2-} concentrations in the apoplastic fluid increased with iron deficiency (Fig. 7B). Increases were maximal in iron-deficient leaves with approximately 100 μmol Chl m^{-2}. The increases were 1.6-fold for Cl^- (from 9 to 14.4 mM), 1.5-fold for NO_3^- (from 15 to 23 mM), and 11.4-fold for SO_4^{2-} (from 1.4 to 16 mM). Phosphate decreased 2.2-fold with iron deficiency, from 4.5 to 2.0 mM.

The concentrations of magnesium and potassium were higher in xylem sap than in the apoplastic fluid, whereas calcium had the opposite behavior (Fig. 8A). Xylem sap concentrations of calcium and magnesium

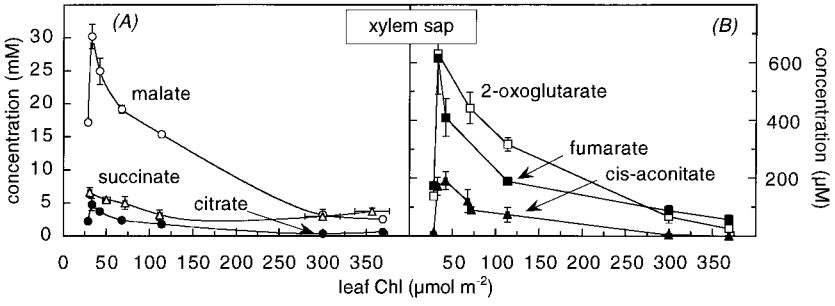

Figure 5. Effects of iron deficiency on the organic anion concentrations in xylem sap from sugar beet leaves. A, Major organic anions (in millimolars): ●, citrate; ○, malate; and △, succinate. B, Minor organic anions (in micromolars): □, 2-oxoglutarate; ▲, cis-aconitate; and ■, fumarate. Data are means ± SE of 10 replications.

Table II. *Concentrations of amino acids (in micromolar) in sugar beet apoplastic fluid from iron-deficient (−iron, 50 μmol Chl m^{-2}) and iron-sufficient (+iron, 300 μmol Chl m^{-2}) leaves*

Data are means ± SE of five replications.

Amino Acid	Iron-Sufficient	Iron-Deficient	
	(+Iron)	(−Iron)	−Iron/+Iron
Asp	262 ± 20	177 ± 28	0.7
Ser	121 ± 42	182 ± 56	1.5
Glu	320 ± 3	538 ± 83	1.7
Gln	450 ± 180	396 ± 90	0.9
Pro	<1	77 ± 43	–
Ala	303 ± 130	243 ± 52	0.8
Val	60 ± 55	510 ± 50	8.5
GABA	56 ± 11	166 ± 12	3.0
Thr	44 ± 15	39 ± 8	0.9
Asn	59 ± 10	33 ± 3	0.5
Gly	56 ± 8	48 ± 12	0.8
Ile	16 ± 16	9 ± 4	0.6
Leu	<1	14 ± 7	–
Lys	31 ± 0.5	12 ± 0.1	0.4
His	<1	4 ± 2	–
Total amino acids	1,778	2,448	1.4

increased with iron deficiency, whereas those of potassium were quite constant (80–130 mM). The increases were 2.4-fold for magnesium (from 4.9 to 11.9 mM) and calcium (from 0.8 to 1.9 mM).

Nitrate, Cl^-, and HPO_4^{2-} concentrations in the xylem sap decreased with iron deficiency (Fig. 8B). The decreases were 2.6-fold for NO_3^- (from 49 to 19 mM), 3-fold for Cl^- (from 24 to 8.1 mM), and 1.8-fold for HPO_4^{2-} (from 2.3 to 1.3 mM). Sulfate increased 5-fold with iron deficiency (from 1.7 to 8.4 mM).

When expressed in meg L^{-1}, total cation/anion concentrations in apoplastic fluid were 33/34 in iron-sufficient plants and 67/74 in iron-deficient plants. Total cation/anion concentrations (in meg L^{-1}) in xylem sap were 120/80 in iron-sufficient plants and 120/115 in iron-deficient plants.

Iron Concentrations

The concentration of iron was in the micromolar range in leaf apoplastic fluid and xylem sap of sugar beet (Fig. 9). Apoplastic iron was approximately 6 μM in leaves with 370 and 170 μmol Chl m^{-2}, then decreased to 2.5 μM in leaves with 70 to 100 μmol Chl m^{-2}, and increased again in extremely chlorotic leaves (30 μmol Chl m^{-2}) to values similar to those found in control leaves. Iron concentrations in xylem sap decreased linearly with iron deficiency from 5.6 μM in iron-sufficient leaves to approximately 1.9 μM in extremely deficient sugar beet plants.

Chemical Speciation

Iron was predicted by chemical speciation to be complexed mainly by citrate in both apoplastic fluid and xylem sap. In iron-sufficient and deficient leaf apoplastic fluid iron was distributed evenly between $[FeCitOH]^{-1}$ and $[FeCit_2]^{-3}$ (Table III). Iron deficiency caused changes in the predicted distribution of total iron between both complexes. With iron deficiency the amount of iron predicted to exist as $[FeCitOH]^{-1}$ decreased from 50% to 24%, whereas the $[FeCit_2]^{-3}$ form would increase from 46% to 76%. The predicted amount of iron-malate complexes in the apoplastic fluid was always very low, with the most abundant species ($[FeMal]^{+1}$) being in the 10^{-13} M range (not shown).

The major predicted complexes of iron in xylem sap were $[FeCitOH]^{-1}$ and $[FeCit_2]^{-3}$ in iron-sufficient and deficient samples (Table III). Iron predicted to be present as $[FeCitOH]^{-1}$ decreased from 66.2% to 4.2% with iron deficiency, whereas the $[FeCit_2]^{-3}$ species increased from 25.9% to 95.8%. The predicted amount of iron-malate complexes in xylem sap was very low, with the most abundant species ($[Fe_2Mal_3(OH)_2]^{-2}$) being in the 10^{-10} M range (not shown).

DISCUSSION

We have characterized the effects of iron deficiency on the chemical composition of two plant sites crucial for long-distance iron transport, the xylem sap, and the leaf apoplastic fluid in the model plant sugar beet. The major change caused by iron deficiency at both sites is an increase in the concentrations of organic anions, especially malate and citrate. This increase was accompanied by other changes in inorganic anion concentrations and was balanced by an increase in cations, especially potassium. The changes found are consistent with previously reported effects of iron de-

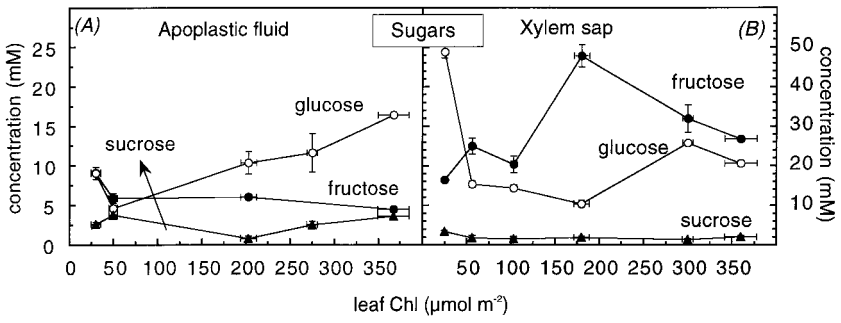

Figure 6. Effects of iron deficiency on the concentration of sugars in leaf apoplastic fluid (A) and xylem sap (B) of sugar beet plants. Glc (○), Fru (●), and Suc (▲) were in millimolars. Data are means ± SE of five replications.

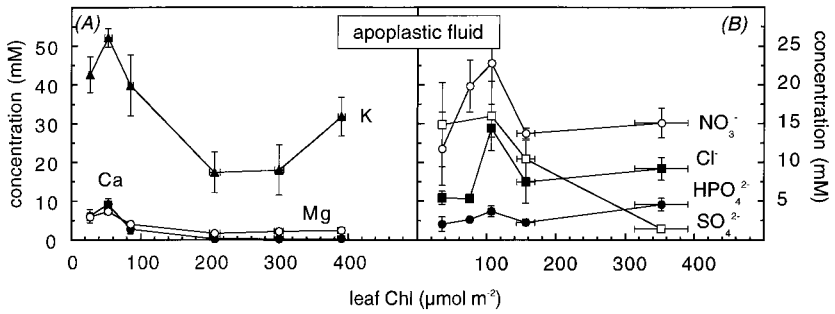

Figure 7. Effects of iron deficiency on the concentration of cations and anions in apoplastic fluid from sugar beet leaves. A, Potassium (▲), calcium (●), and magnesium (○). B, Chloride (■), phosphate (●), nitrate (○), and sulfate (□). All data are in millimolars. Data are means ± SE of 10 replications.

ficiency on bulk concentrations of organic acids and cations in plant shoots (for review, see Welkie and Miller, 1993; Alhendawi et al., 1997). Sugar and amino acid concentrations are also affected by iron deficiency.

Iron-citrate complexes were the major predicted iron chemical species in xylem sap and apoplastic fluid of sugar beet. This agrees with previous data obtained from tomato and soybean stem exudates (White et al., 1981a, 1981b) and supports that citrate plays a major role in long distance iron transport, as proposed by Tiffin (1966). Our data, however, indicate that iron deficiency causes significant changes in the chemical speciation of iron-citrate in sugar beet. Under iron sufficiency conditions the major predicted iron species was $[FeCitOH]^{-1}$ in xylem sap (66% of total iron) and in apoplastic fluid (50% of total iron). Conversely, under iron deficiency the major species was $[FeCit_2]^{-3}$ in xylem sap (96% of total iron) and apoplastic fluid (76% of total iron). From our data it seems that the citrate:iron molar ratio could be the major factor controlling iron speciation. Other factors that change significantly with iron deficiency, such as malate concentrations, pH, and the concentrations of other cations and anions have only minor effects on iron speciation. The formation of citrate-iron polymers (Spiro, 1967a) is frequently assumed to occur in plant shoots (Bienfait and Scheffers, 1992; Moog and Brüggemann, 1994; Schmidt, 1999), although no experimental evidence is available in support of this theory. The formation of citrate-iron polymers in the xylem and leaf apoplast of sugar beet is unlikely because of the high citrate:iron ratios found in these compartments. Excess citrate competes effectively with the formation of the citrate-iron polymers, therefore inhibiting polymerization (Spiro et al., 1967b).

The concentration of iron in apoplastic fluid was approximately 5.6 to 5.9 μM in severely deficient (30 μmol Chl m^{-2}) and control sugar beet leaves, whereas in leaves with 100 μmol Chl m^{-2} it was approximately 2.2 μM. The relatively high iron concentrations in the apoplast of severely iron-deficient leaves suggest that iron deficiency is associated to a progressive impairment of the iron acquisition mechanisms in mesophyll cells. This agrees with the low FC-R activity of iron-deficient sugar beet protoplasts reported recently (González-Vallejo et al., 2000).

The large citrate:iron molar ratios found in the leaf apoplastic fluid of iron-deficient plants may significantly impair iron uptake by mesophyll cells. The citrate:iron molar ratios increased with iron deficiency from 120 to 1,750 in apoplastic fluid and from 35 to 2,000 in xylem sap of sugar beet. The activity of the FC-R leaf PM enzyme has been recently shown to decrease markedly when the citrate:iron ratio increases, activities decreasing 5-fold when the citrate:iron molar ratio increased from 100 to 500 (González-Vallejo et al., 1999). It should be also mentioned that increases in the malate:iron molar ratio above 10 do not affect the activity of the leaf PM FC-R (González-Vallejo et al., 1999). The marked decrease in FC-R activities at high citrate:iron ratios could be related to the fact that the major chemical species under these conditions is the strongly charged $[FeCit_2]^{-3}$ species, which may experience a strong electrostatic repulsion with the negatively charged PM.

Our sugar beet data do not provide support for the hypothesis (Mengel, 1995; Kosegarten et al., 1999) that apoplast pH changes induced by iron deficiency

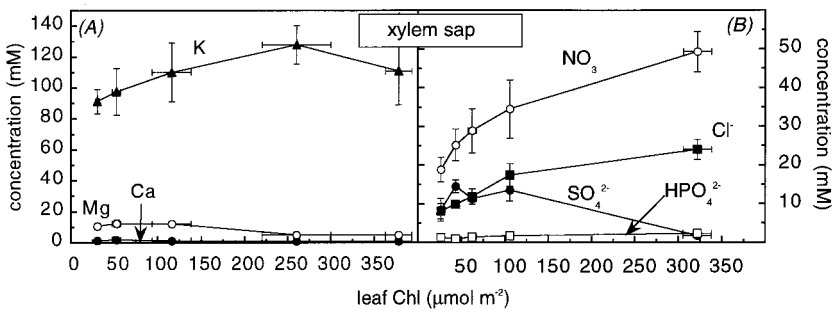

Figure 8. Effects of iron deficiency on the concentration of cations and anions in xylem sap from sugar beet leaves. A, Potassium (▲), calcium (●), and magnesium (○). B, Chloride (■), phosphate (□), nitrate (○), and sulfate (●). All data are in millimolars. Data are means ± SE of 10 replications.

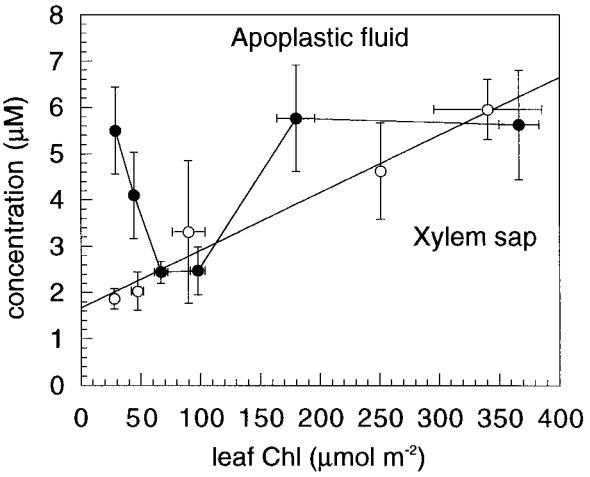

Figure 9. Effects of iron deficiency on the concentration of iron in xylem sap (○) and leaf apoplastic fluid (●) of sugar beet plants. Data are means ± SE of five replications.

could modulate the activity of the FC-R enzyme of the mesophyll cell plasma membrane. Iron deficiency did not increase the bulk apoplastic pH in sugar beet. Conversely, iron deficiency caused small decreases in the pH of the apoplast, as judged from direct pH measurements in apoplastic fluid obtained by centrifugation and in vivo measurements with fluorescent dyes. These pH decreases could possibly originate from an iron deficiency-induced enhancement of the leaf plasma membrane ATPase activity. The apoplastic pH decreases caused by iron deficiency would tend to increase FC-R activities associated to the PM, which were shown to be maximum at a pH of approximately 5.5 in isolated, iron-deficient sugar beet leaf protoplasts (González-Vallejo et al., 2000).

Malate was a major organic anion in iron-sufficient and deficient xylem sap. In the apoplastic fluid, however, malate was present in much lower concentrations than in xylem sap, whereas citrate concentrations were very similar in both compartments. This suggests that the concentration of malate in the apoplast could be depleted by a malate transporter located in the mesophyll cell plasma membrane. Several mechanisms have been reported for malate transport in different cell organelles such as mitochondria and chloroplasts, including the malate-oxalacetate shuttle and various antiport systems with phosphate, tricarboxylates, and 2-oxoglutarate (for review, see Martinoia and Rentsch, 1994). However, there are few references of malate transport mechanisms across the leaf plasma membrane. The mechanisms described so far for the leaf plasma membrane include an anion channel (Martinoia and Rentsch, 1994), which could be part of a plant CO_2 sensor (Hedrich and Marten, 1993). An alternative possibility causing decreases in the malate concentrations in the apoplast could be a high mdh activity associated to the cell plasma membrane, as recently shown in onion roots (Córdoba-Pedregosa et al., 1998).

The high concentrations of organic anions in the xylem sap indicate that anaplerotic, non-autotrophic carbon export from roots could be significant in iron-deficient plants. Concentrations of 30 mM malate, 7 mM succinate, and 5 mM citrate in the xylem sap of iron-deficient plants would be equivalent to approximately 6 μmol carbon m^{-2} s^{-1} from the measured water transpiration rate of 2 mmol m^{-2} s^{-1} in the same leaves. This rate of carbon export could be several fold higher than the rate of photosynthetic CO_2 fixation in the deficient leaves, which could reach values of approximately 3 μmol C m^{-2} s^{-1} at light saturation and less than 1 μmol carbon m^{-2} s^{-1} at the photosynthetic photon flux density occurring in the growth chamber (Terry, 1983). Conversely, in the controls the rates of carbon export from roots would be lower than 1.0 μmol carbon m^{-2} s^{-1}, less than 1% of the maximum leaf photosynthesis in the same leaves, in line with the current view that carbon fixation by roots is negligible under normal conditions (Farmer and Adams, 1991). The occurrence of a significant anaplerotic carbon fixation in the roots of iron-deficient plants could provide an explanation for the relatively small effect of iron deficiency on sugar beet leaf growth under controlled conditions (Terry, 1979), in spite of the markedly reduced photosynthetic rates of the same leaves (Terry, 1980). This non-autotrophic, anaplerotic carbon fixation is associated to an increased phospho*enol*pyruvate carboxylase activity in root tips (Rabotti et al., 1995; López-Millán et al., 2000), which uses bicarbonate, readily available in natural environments leading to iron deficiency such as calcareous soils, as substrate.

In iron-deficient leaves the apoplastic concentrations of cations increased respect to controls, thus tending to balance the organic acid increases. As

Table III. *Predicted distribution of iron among the different iron-chelate species in iron-deficient (−iron) and sufficient (+iron) apoplastic fluid and xylem sap*

Chemical speciation was carried out with the MinteqA2 software. Data are in micromoles. Numbers in brackets are percentages of total iron.

Species	Apoplastic Fluid		Xylem Sap	
	+Iron	−Iron	+Iron	−Iron
$[FeCitOH]^{-1}$	2.79 (49.9%)	0.57 (23.8%)	3.57 (66.2%)	0.10 (4.2%)
$[FeCit_2]^{-3}$	2.59 (46.3%)	1.81 (75.7%)	1.40 (25.9%)	2.30 (95.8%)
$[Fe_2Cit_2(OH)_2]^{-2}$	0.21 (3.7%)	0.01 (0.4%)	0.42 (7.8%)	<0.01

early as 1955 it was reported (Jacobson, 1955) that an increase in cation uptake by iron-deficient roots accounted for the increase in malate concentrations. Concentrations of inorganic cations generally increase in iron-deficient leaves (Nagarajah and Ulrich, 1965; Welkie and Miller, 1993). Also, the increase observed in total amino acid concentration (1.4-fold) in the apoplast of iron-deficient leaves suggests that part of the CO_2 fixed by phospho*enol*pyruvate carboxylase could be incorporated into amino acids. Transamination of oxalacetate may result in increases in Glu (Cramer et al., 1993), such as that observed in the xylem sap of iron-deficient plants. Val, the amino acid having the largest increase with iron deficiency, is synthesized via pyruvate (Goodwin and Mercer, 1983).

In summary, iron deficiency decreases by approximately 0.3 to 0.4 units the pH of the xylem sap and apoplastic fluid of sugar beet leaves. The major increases in organic anion concentrations induced by iron deficiency in apoplastic fluid and xylem sap suggest the existence of an influx of organic anions from the roots to the shoot via xylem, which could be important for the maintenance of basic processes in leaves with low photosynthetic rates. The major predicted iron chemical species in xylem sap and apoplastic fluid of sugar beet plants were iron-citrate complexes, with the citrate:iron ratio being the major factor controlling iron speciation. On the other hand, the large citrate:iron molar ratios found in the leaf apoplastic fluid of iron-deficient plants may impair significantly iron uptake by mesophyll cells. These data indicate the importance of citrate in the long distance iron transport and subsequent uptake by the mesophyll cell.

MATERIALS AND METHODS

Plant Material

Sugar beet (*Beta vulgaris* Monohil hybrid from Hilleshög, Landskröna, Sweden) was grown in a growth chamber with a photosynthetic photon flux density of 350 μmol m^{-2} s^{-1} photosynthetically active radiation at a temperature of 25°C, 80% relative humidity, and a photoperiod of 16 h of light/8 h of darkness. Seeds were germinated and grown in vermiculite for 2 weeks. Seedlings were grown for two more weeks in one-half-strength Hoagland nutrient solution with 45 μM iron and then transplanted to 20-L plastic buckets (four plants per bucket) containing one-half-strength Hoagland nutrient solution (Terry, 1980) with either 0 or 45 μM Fe(III)-EDTA. The pH of the iron-free nutrient solutions was buffered at approximately 7.7 by adding 1 mM NaOH and 1 g L^{-1} of $CaCO_3$. This treatment simulates conditions usually found in the field leading to iron deficiency (Susín et al., 1994). Young, fully expanded leaves from plants grown for 10 d in the presence or absence of iron were used in all experiments.

Chl Determination

Chl concentration was estimated non-destructively with a portable Chl meter (SPAD [portable Chl meter]-502, Minolta, Osaka). For calibration, leaves with different degrees of iron deficiency were first measured with the SPAD and then extracted with 100% (v/v) acetone in the presence of sodium ascorbate and Chl measured spectrophotometrically (Abadía and Abadía, 1993).

Apoplastic Fluid and Xylem Sap Collection

Apoplastic fluid was obtained from whole sugar beet leaves by direct centrifugation as in Dannel et al. (1995) with some modifications. Leaves were excised at the base of the petiole with a razor blade in the growth chamber and transported to the laboratory with the petiole immersed in de-ionized water. Once in the laboratory the petiole was excised under water. Each leaf was then rolled and placed into a plastic syringe barrel with the petiole side at the narrow end of the syringe. Leaf-filled syringes were centrifuged at 4°C and a small volume of apoplastic fluid was obtained from the bottom of the centrifuge tube. Preliminary experiments were carried out to assess contamination by cytoplasmic components by increasing centrifugal force in steps of 500*g* (15 min each) from 1,500*g* to 6,000*g*, the corresponding fluid being collected at each centrifugation step. In the final protocol, a first centrifugation was made at low speed (2,500*g*, 15 min) to remove the xylem sap of the main vein and apoplastic fluid was collected in a second centrifugation step (4,000*g*, 15 min).

For xylem sap isolation sugar beet petioles were excised under water with a razor blade at their base and near the leaf lamina. Three petioles were placed upside down into a plastic syringe barrel and xylem sap was collected by centrifugation for 15 min at 4,000*g* and 4°C.

c-hpi (EC 5.3.1.9) and c-mdh (EC 1.1.1.37) were used as cytosolic contamination markers for apoplastic fluid and xylem sap. The activity of c-hpi was determined using Fru-6-P as substrate, which is converted by c-hpi into Glc-6-P. This is then oxidized by exogenous glucose 6 phosphate dehydrogenase and the simultaneous reduction of $NADP^+$ was measured from the increase in A_{340}. The final reaction mixture (pH 8.0) was 50 mM Tris [tris(hydroxymethyl)-aminomethane], 5 mM $MgCl_2$, 1 mM NaCl, 0.40 mM $NADP^+$, 0.46 U/mL glucose 6 phosphate dehydrogenase, and 1.4 mM Fru-6-P (Bergmeyer et al., 1974). The activity of c-mdh was determined using oxalacetate as substrate and measuring the decrease in A_{340} due to the enzymatic oxidation of NADH. The final reaction mixture (pH 9.5) was 46.5 mM Tris, 0.1 mM NADH, and 0.4 mM oxalacetate (Dannel et al., 1995). The activity of these two markers in leaf apoplastic fluid and xylem sap was checked against the corresponding activities in leaf tissue and petiole total homogenates, respectively. To measure these enzymatic activities one leaf or petiole was homogenized with 2 mL of a buffer (pH 8.0) containing 100 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 30 mM sorbitol, 2 mM dithiothreitol, 1 mM $CaCl_2$, 1% (w/v) bovine serum albumin, and 1% (w/v) polyvinylpyr-

rolidone. The supernatant was collected and analyzed immediately after a 10-min centrifugation at 10,000*g*.

pH Measurements

The pH of the apoplastic fluid and xylem sap was measured directly in apoplastic fluid and xylem sap obtained by centrifugation with a microelectrode (Physitemp, Clifton, NJ). Apoplastic pH was also measured in vivo by fluorescence according to Hoffman et al. (1992) with 5-CF and FITC-dextran. The fluorescence emission at 540 nm of these dyes is pH-dependent when excited at 490 nm, but almost pH-independent when excited at 460 nm. Therefore the ratio of fluorescence intensities obtained with excitation at 490 and 460 nm is related to the pH of the compartment where the dye is located. Leaves were excised and the cut end of the petiole was exposed to incubation medium containing 5 μM 5-CF or 500 μM FITC-dextran (4000 D, 0.01 mol FITC per mol Glc; Sigma, St. Louis), 1 mM KCl, 0.1 mM NaCl, and 0.1 mM $CaCl_2$ at pH 5.5. The incubation was carried out for 5 h at 25°C at room ambient light (15–25 μmol photons m^{-2} s^{-1}). The level of autofluorescence was subtracted from total fluorescence. Two leaves per Chl level (each from different plant) were taken and four measurements were carried out in different areas of each leaf.

Organic Anion Analysis

Organic anions were quantified by HPLC with a 300 × 7.8 mm Aminex ion-exchange column (HPX-87H, Bio-Rad, Hercules, CA) in an HPLC Waters system, including a 600E multisolvent delivery system, a 996 photodiode array detector, and Millennium 2010 software. Apoplast and xylem samples were filtered with a 0.45-μm polyvinyl fluoride membrane (LIDA, Kenosha, WI). Samples were injected with a Rheodyne injector (20-μL loop). Mobile phase (8 mM sulfuric acid) was pumped with a 0.6-mL min^{-1} flow rate. Organic anions were detected at 210 nm. Peaks corresponding to cis-aconitate, citrate, 2-oxoglutarate, malate, succinate, and fumarate were identified by comparison of their retention times with those of known standards from Bio-Rad and Sigma. The identity of some peaks was further confirmed by UV-visible and mass spectroscopy. Quantification was made with known amounts of each anion using peak areas.

Amino Acid Analysis

Amino acids were quantified by HPLC (Stein et al., 1957). Chromatography was carried out in an Alpha plus amino acid analyzer (Pharmacia LKB Biotechnology, Uppsala) with a 200- × 4-mm column packed with a cation-exchanger resin (polystyrene divinil-sulfobencene). The mobile phase was citrate buffer with increasing pH. Amino acids were detected at 570 nm after reaction with ninhydrin, and identified by comparison of their retention times with those of standards. Quantification was made from the peak areas.

Sugar Analysis

Sugars (Glc, Fru, and Suc) were analyzed by HPLC with a 300- × 4-mm Spherisorb-NH_2 column (Waters, Milford, MA) and an HPLC Waters system, including a 590 pump, a differential refractometer R401 detector, and Millenium 2010 software. Samples were injected with a Rheodyne injector (20-μL loop). Mobile phase (acetonitrile:water, 860:140) was pumped with a 3.5 mL min^{-1} flow rate. Peaks corresponding to Glc, Fru, and Suc were identified by comparison of their retention times with those of known standards from Sigma. Quantification was made from the peak areas.

Inorganic Ion Analysis

For cation analysis, plant fluids were dried in an oven at 60°C and the residue dissolved in HNO_3 and HCl following the A.O.A.C. procedure (Helrich, 1990). Calcium (after lanthanum addition) and magnesium were determined by atomic absorption spectrophotometry and potassium was determined by emission spectrophotometry. Iron was determined by graphite furnace atomic absorption spectrometry (Varian SpectrAA with Zeeman correction). Each sample was analyzed in triplicate.

Inorganic anions (nitrate, sulfate, chloride, and phosphate) were separated and quantified by HPLC with a 4.6- × 75-mm IC-Pak A HR ion-exchange column (Waters) in an HPLC Waters system, including a 600E pump, a 432 conductivity detector, and Millennium 2010 software. Samples were injected with a Rheodyne injector (50-μL loop). Mobile phase (11 mM borate-gluconate) was pumped with a 1.0-mL min^{-1} flow rate. Quantification was made with known amounts of each anion using peak areas.

Chemical Speciation

Concentrations of the different iron-chelate species were estimated with the software MinteqA2 (U.S. Environmental Protection Agency, Washington, DC) by using the ionic environment of the apoplastic fluid. Chelate formation constants used for citrate and malate were derived from those given by Holden et al. (1991) and Cline et al. (1982), respectively. At an ionic strength of 0 M, the $\log_{10}$ of the chelate formation constants used for the iron-citrate species $[FeCit]^0$, $[FeCitH]^{+1}$, $[FeCitOH]^{-1}$, $[FeCit_2]^{-3}$, and $[Fe_2Cit_2(OH)_2]^{-2}$ were 13.13, 14.43, 10.11, 20.13, and 24.51, respectively. The $\log_{10}$ of the chelate formation constants for the iron-malate species $[FeMal]^{+1}$, $[Fe_2Mal_2(OH)_2]^0$, $[Fe_2Mal_3(OH)_2]^{-2}$, and $[Fe_3Mal_3(OH)_4]^{-1}$ were 8.39, 15.32, 20.33, and 27.75, respectively.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the skillful technical assistance of Aurora Poc in growing the plants and Conchita Fustero and Carmen Lope with the mineral analysis techniques. Thanks are given to Jesús Soriano and Miguel Angel Monesma for their help with the sugar and inorganic

anion analyses and to Drs. Angel Bonilla and Ramón Aragüés for use of HPLC equipment.

Received January 31, 2000; accepted June 10, 2000.

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