



Organic acids and Fe deficiency: a review

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Abstract

Organic acid concentrations often increase with iron deficiency in different plant parts such as roots, leaves and stem exudates. The review summarises data available on the changes in the concentrations of organic anions in plants with iron deficiency and the effects of these changes in plant metabolism. The paper reviews data available in the literature on the changes in xylem and apoplasmic fluid composition with iron deficiency, both in plants grown in controlled conditions and in the field, and discusses the possible ways of iron complexation and transport in these compartments. The characteristics of the iron reduction and uptake by the iron-deficient leaf mesophyll cells are also discussed, with especial emphasis in the possible roles of organic acids in these processes. Both the possible causes and functions of the organic acid concentration increases in iron-deficient plants are reviewed.

Abbreviations: CA – carbonic anhydrase; FC-R – ferric chelate reductase; G3PDH – glyceraldehyde 3-phosphate dehydrogenase; ICDH – isocitrate dehydrogenase; PEP – phosphoenol pyruvate; PEPC – phosphoenol pyruvate carboxylase; PFK – phosphofructokinase; PK – pyruvate kinase

Introduction

Iron deficiency (Fe chlorosis) occurs in many plant species grown in calcareous soils. This review summarises data published on the association of chlorosis with organic acids. We review data on the changes in organic acid concentrations caused by Fe deficiency in different plant parts, such as leaves, roots, xylem sap and leaf apoplasmic fluid. We summarise data on Fe speciation in different plant compartments and mechanisms of Fe uptake for leaf cells. The possible causes and functions of the organic acid increase that occurs with chlorosis are also discussed. For additional information, readers may also consult classic reviews on Fe chlorosis, such as those from Bindra (1980), Landsberg (1984), Brown and Jolley (1986), Chaney and Bell (1987), Welkie and Miller (1993) and Schmidt (1999).

Iron deficiency has been generally shown to cause increases in the organic acid concentrations in roots, stem exudates and leaves of different plant species.

Increases in organic acid concentrations in roots of Fe-deficient plants are fairly ubiquitous, and occur both in Strategy I and Strategy II plant species. In leaves, however, organic acid concentration increases do not always occur and are sometimes restricted to citrate.

Changes in the concentrations of organic acids in leaves induced by iron deficiency

Iron chlorosis was first reported to cause increased leaf organic acid concentrations. Schander (1939, 1943a,b) was probably the first author to report on such increases, followed by Iljin (1943, 1944). McGeorge (1949) found increases in citric acid in Fe-deficient leaves of different plant species. Iljin (1951) reported increases in organic acids such as citric and malic in 'expressed leaf sap' of moderately chlorotic leaves of several plant species with respect to the controls. In severely chlorotic leaves, citric acid in leaf sap increased with respect to the green controls, whereas malic acid decreased. These early data are difficult to

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interpret quantitatively because analytical techniques used then were not very specific. Also, the basis used to express the data by Iljin (1951), mg of organic acids per g dry weight of expressed sap, makes difficult to convert those data into currently used concentration units.

De Kock and Morrison (1958) found that Fe deficiency in mustard decreased total leaf citrate and malate concentrations, resulting in increases in the citrate:malate ratios. Increases in leaf citrate concentrations were later found in Fe-deficient soybean (Su and Miller, 1960), mustard (Palmer et al., 1963), *Macadamia* (Gilfillan and Jones, 1968) and apple leaves (Sun et al., 1987). Leaf malate increased in *Macadamia* (Gilfillan and Jones, 1968) and apple leaves (Sun et al., 1987) and was decreased in soybean (Su and Miller, 1960) and mustard (Palmer et al., 1963).

Organic acids in Fe-deficient sugar beet leaves increased from the control values of 16–37 nmol m⁻² (López-Millán et al., 2001b). Moderately chlorotic leaves (200 μmol Chl m⁻²) had, relative to controls, 12- and 7-fold increases in citrate and malate concentrations, respectively. Organic acids in pear leaves increased from 20 to 40 nmol m⁻² with Fe deficiency (López-Millán et al., 2001c). Moderately chlorotic leaves had, relative to controls, 15- and 1.7-fold increases in citrate and ascorbate concentrations, respectively, and 1.3-fold decreases in malate concentrations.

Changes in the concentrations of organic acids in roots induced by iron deficiency

Bedri et al. (1960) were the first, to our knowledge, to show that organic acid concentrations increase in the roots of several plant species with Fe deficiency. The same authors also reported that a large part of the ¹⁴C fixed by roots was in the organic acid fraction, mainly in the form of malate. Scheffer et al. (1965) found increased root organic acid concentrations in Fe-deficient corn. Brown (1966) confirmed that the concentration of citrate in root sap increased with Fe deficiency in soybean. Citrate and to a lesser extent malate increased with Fe deficiency in sunflower roots (Venkat Raju et al., 1972).

The increases in organic acid concentrations in roots with Fe deficiency occur in both Strategy I and Strategy II species (Landsberg, 1981). In Strategy II species (e.g. barley, oat and millet) these increases were 3.9- to 8.8-fold for citrate and 1.5- to 2.4-fold

for malate. In Strategy I, species (e.g. pea, sugar beet and bean) organic acid concentration increases were 3.8- to 11.9-fold for citrate and 1.9- to 2.2-fold for malate (Landsberg, 1981). In bean roots, the concentrations of citrate and malate increased 6- and 4-fold, respectively, with Fe deficiency, whereas the concentrations of these acids in the leaves of the same plants and in the phloem sap were not changed by Fe deficiency (de Vos et al., 1986). Sun et al. (1987) found 3.5- and 2-fold increases in citrate and malate in Fe-deficient apple roots. Rabotti et al. (1995) reported that Fe-deficient cucumber had 4.8- and 2.7-fold increases in the root concentrations of citrate and malate, respectively.

The increases in organic acid concentrations in Fe-deficient roots are not restricted to citrate and malate. Alhendawi et al. (1997) found that increases in bicarbonate in the nutrient solution led to chlorosis and to increases of citrate, malate, aconitate and succinate in the roots of barley, sorghum and maize. In sugar beet roots the concentrations of several organic acids increase with Fe deficiency (López-Millán et al., 2000a), with citrate and malate increasing 26- and 16-fold, respectively.

Relationships between the increases in organic acids in roots and other responses to Fe deficiency

In several species, the concentrations of organic acids in roots were higher in Fe-efficient genotypes than in Fe-inefficient ones (Brown and Ambler, 1970; Brown et al., 1971; Clark et al., 1973; Fournier et al., 1992). Links between organic acid increases and other root responses to Fe deficiency, such as enhanced activity of ATPase and ferric chelate reductase (FC-R) have been proposed. Generally, under Fe deficiency all three responses occur in Strategy I species. Accumulation of organic acids, however, does not always develop in parallel with the increases in FC-R and ATPase activities (Bienfait et al., 1989; Fournier et al., 1992; Landsberg, 1981; Schmidt, 1999; van Egmond and Aktas, 1977).

The accumulation of organic acids in roots does not seem to be spatially localised in a strict manner as is the case for FC-R activity. In a recent study, different root parts with or without increased Fe-reducing activities from Fe-deficient and Fe-sufficient control sugar beet plants have been examined (López-Millán et al., 2000a). The distal root parts of the Fe-deficient plants, 0–5 mm from the root apex, can reduce Fe(III)-

chelates, have enhanced proton extrusion rates and contain concentrations of flavin sulfates near $700 \mu\text{M}$, three characteristics that are absent in the 5–10 mm root sections of Fe-deficient plants and the whole root of Fe-sufficient plants. In the Fe-deficient plants, large pools of carboxylic acids were found in root sections with and without increased FC-R activities (López-Millán et al., 2000a). Possibly, the mobility of root-tip synthesised organic acids towards the xylem could explain this.

Changes induced by iron deficiency on the concentrations of organic acids in the xylem

Several papers published in the 1960s by Brown and co-workers reported organic acid concentration increases in xylem exudates of plants with Fe deficiency. Iron was first suggested to be transported bound with malate in the xylem of soybean (Lingle et al., 1963; Tiffin and Brown, 1961). Later, citrate was found to increase markedly in stem exudates of Fe-deficient plants. For instance, Fe deficiency increased approximately 3-fold the concentrations of citrate in stem exudate of the Fe-efficient Hawkeye soybean (Brown, 1966; Brown and Tiffin, 1965).

Different papers from the same laboratory supported that Fe and citrate were associated in some way. Brown and Tiffin (1965) found that the concentration of citrate in stem exudate of soybean increased considerably upon Fe resupply. Working with sunflower, Tiffin (1966a) found citrate concentrations in xylem exudate of 30 and $890 \mu\text{M}$ after Fe resupply to Fe-sufficient and Fe-deficient sunflower plants, respectively. After Fe resupply, citrate:Fe ratios were 2–3 in Fe-deficient plants and 15 in the Fe-sufficient controls. Since in these early papers stem exudates were collected for 10–20 h, citrate concentrations were probably underestimated (because ion concentrations in stem exudates decrease markedly with collection time; see for instance White et al., 1981a). Malate and citrate were shown to co-migrate with Fe during paper electrophoresis of sunflower stem exudate (Tiffin, 1966a). Citrate concentrations in stem exudate of Fe-deficient plants resupplied with Fe were 200–400, 400 and $120 \mu\text{M}$ in sunflower, soybean and tomato, respectively (Tiffin, 1966b). Citrate is usually present in large molar excess of Fe in xylem exudates, with the possible exception of Fe-deficient plants resupplied with Fe (Tiffin 1966a,b). Tiffin (1970) confirmed that citrate and Fe migrate at similar positions in pa-

per electrophoresis of soybean xylem exudates. Brown and Chaney (1971), Brown et al. (1971), Tiffin (1971) and Clark et al. (1973) further confirmed the Fe: citrate association in stem exudates from soybean, tomato and maize.

Cataldo et al. (1988) indicated that the distribution of Fe-organic acid complexes in soybean xylem exudate may vary with plant age. Bialczyk and Lechowski (1992) found increased citrate and malate concentrations in xylem sap of tomato grown in the presence of bicarbonate in nutrient solutions. Pich et al. (1994) found large increases in citrate in stem exudates of the chloronerva tomato mutant. Alhendawi et al. (1997), however, concluded that increases in bicarbonate in the nutrient solution did not lead to increases in translocation of organic acids to the shoots of sorghum and maize in spite of the increases in organic acid biosynthesis in the roots. Rombolà (1998) found increases in the concentrations of several organic acids in Fe-deficient kiwifruit.

The ratios of organic anions:Fe in xylem sap are usually increased by Fe deficiency. In Fe-sufficient soybean and tomato concentrations of citrate and malate in stem exudate were approximately 0.3–1.7 and 0.6–0.9 mM, whereas Fe concentrations were 6–7 μM (White et al., 1981a). Therefore, citrate:Fe ratios in Fe-sufficient soybean and tomato were approximately 300 and 45, respectively. Iron deficiency increased citrate concentrations in xylem exudate of faba bean 26-fold (Nikolic and Römheld, 1999). This led to increases in the citrate:Fe ratios from approximately 8 to 400–600 with Fe deficiency. Iron deficiency in sugar beet was associated with increases in total xylem sap organic acid concentration from 9 to 50 mM, with major organic acids being malate and citrate (López-Millán et al., 2000b). Iron concentrations in xylem sap decreased with chlorosis from 5.5 to 2.0 μM . Therefore, in sugar beet the citrate:Fe ratios increased from approximately 35 to 2000, under Fe deficiency conditions.

Iron deficiency also caused other changes in the ionic composition of the xylem sap. Iron deficiency resulted in a slight decrease in xylem sap pH of tomato and sugar beet from 6.8–7.0 to 6.3–6.5 and from 6.0 to 5.7, respectively (Bialczyk and Lechowski, 1992; López-Millán et al., 2000b), whereas in faba bean xylem sap pH did not change (Nikolic and Römheld, 1999). In white lupine, 3 mM bicarbonate in the nutrient solution slightly increased the pH of the xylem sap from 5.7 to 6.1, although the increase was not statistically significant (Pissaloux et al., 1995).

In sugar beet, Fe deficiency led to slight increases in xylem sap total inorganic cations (Ca, K and Mg) from 116 to 124 mM, whereas total inorganic anions (Cl^- , NO_3^- , SO_4^{2-} and PO_4^{3-}) decreased from 78 to 38 mM (López-Millán et al., 2000b).

Changes induced by iron deficiency on the concentrations of organic acids and other characteristics of the leaf apoplasmic fluid

Three recent papers have investigated the changes in leaf apoplasm composition in faba bean (Nikolic and Römheld, 1999), sugar beet grown in hydroponics (López-Millán et al., 2000b) and in pear trees grown in the field (López-Millán et al., 2001c). Iron deficiency increased citrate concentrations in apoplasmic fluid of faba bean 3–5-fold (Nikolic and Römheld, 1999). In sugar beet total apoplasmic fluid, organic acid concentration increased from 4 to 12 mM, with major organic acids being malate and citrate (López-Millán et al., 2000b). Citrate concentrations in sugar beet were similar in the apoplasmic fluid and in the xylem sap, whereas malate concentrations were lower in apoplasmic fluid than in xylem sap. Iron concentrations in sugar beet apoplasmic fluid decreased with Fe deficiency from 5.5 to 2.5 μM . The major organic acids found in leaf apoplasmic fluid in field-grown pear were malate, citrate and ascorbate, and the total concentration of organic acids increased with Fe deficiency from 2.9 to 5.5 mM (López-Millán et al., 2001c). Iron concentration decreased from 4 to 1.6 μM in pear apoplasmic fluid with Fe deficiency.

As a consequence of these changes the citrate:Fe ratios in leaf apoplasmic fluid increase markedly with Fe deficiency. Citrate:Fe ratios increase with Fe deficiency from 100 to 800–1400 in faba bean (Nikolic and Römheld, 1999), from 120 to 1750 in sugar beet (López-Millán et al., 2000b) and from 150 to 900 in pear (López-Millán et al., 2001c).

The pH of the leaf apoplasm has been hypothesised to increase with Fe deficiency (Mengel, 1994). The pH of faba bean apoplasmic fluid did not change with Fe deficiency (Nikolic and Römheld, 1999). Iron deficiency caused a slight decrease in the pH of the sugar beet leaf apoplasm, from 6.3–6.5 to 5.9–6.0 as estimated from direct pH measurements in apoplasmic fluid obtained by centrifugation and by fluorescence of leaves incubated with 5-carboxyfluorescein and fluorescein isothiocyanate-dextran (López-Millán et al., 2000b). In pear leaves, however, Fe deficiency caused

an increase in the apoplasmic pH from 5.5–5.9 to 6.5–6.6 (López-Millán et al., 2001c). In the apoplasm of detached, young, Fe-deficient sunflower leaves localised increases in pH to values higher than 6.3 were found by fluorescence microscopy (Kosegarten et al., 1999).

Iron deficiency was associated with other changes in the composition of the leaf apoplasmic fluid of sugar beet (López-Millán et al., 2000b) and pear (López-Millán et al., 2001c). In sugar beet, total apoplasmic inorganic cation concentrations increased under Fe deficiency from 32 to 67 mM, whereas total apoplasmic inorganic anion concentrations increased under Fe deficiency from 30 to 53 mM. In pear the total apoplasmic inorganic cation concentration increased from 15 to 20 mM with Fe deficiency, whereas the total concentration of inorganic anions did not change.

Influence of organic acids on long distance iron transport in plants

The possibility that organic acids may maintain Fe in soluble forms within the plant was postulated many years ago (Rogers and Shive, 1932). This hypothesis was proposed because Fe(III) can form stable, water-soluble complexes with organic acids. In the first chemical speciation papers published most of the Fe (>96%) was predicted to be bound to citrate (Mullins et al., 1986; White et al., 1981a,b). Although only the existence of uncharged citrate-Fe complexes was considered in the calculations, it was recognised that the existence of negatively-charged complexes was most likely (Clark et al., 1986; Mullins et al., 1986; White et al., 1981a,b), as indicated by electrophoresis techniques (Tiffin, 1967). Negatively charged Fe-containing complexes are essential for efficient Fe movement through the xylem. Recently, the major Fe species predicted to exist by chemical speciation in xylem sap and apoplasmic fluid were FeCitOH^- and FeCit_2^{-3} in control and Fe-deficient sugar beet, respectively (López-Millán et al., 2000b). In leaves of field-grown pear trees, however, the major Fe species predicted for the apoplasmic fluid was FeCitOH^- both in Fe-sufficient and Fe-deficient leaves (López-Millán et al., 2001c).

Some negative effects of organic acids in Fe transport have been discussed in the literature. For instance, the possibility that, at high pH, ferric citrate may form large, non-diffusible polymers has been considered frequently in reviews (Bienfait and Scheffers, 1992;

Moog and Brüggemann, 1995; Schmidt, 1999), based on the experiments of Spiro et al. (1967a). These experiments, however, were made with equimolar concentrations of citrate and Fe and at pH values of 8–9, a situation unlikely to occur in most plant compartments. In fact, when similar experiments were made with a citrate:Fe ratio of approximately 20:1 excess citrate competed effectively with the formation of the citrate-Fe polymers, therefore inhibiting completely polymerisation even at very high pH values (Spiro et al., 1967b).

Bienfait and Scheffers (1992) postulated that in plants grown in calcareous soils (that have a high citrate:Fe ratio) citrate photodestruction would lead to formation of ferrous Fe. This was derived from experiments carried out *in vitro* with solutions containing 10 μM Fe and 1 mM citrate. In these cases, addition of 10 μM phosphate led to a decrease in Fe(II). From the data available on apoplasmic fluid composition, however, citrate photodestruction in leaves does not appear to be of physiological significance. For instance, in sugar beet the concentrations of citrate in the xylem and leaf apoplasmic fluid are similar, approximately 500 μM and 5 mM in Fe-sufficient and Fe-deficient plants, respectively (López-Millán et al., 2000b). Also, in leaf apoplasmic fluid of pear trees, grown in the field, citrate concentrations are quite high, approximately 700 μM and 1.5 mM in Fe-sufficient and Fe-deficient leaves, respectively (López-Millán et al., 2001c). In faba bean, apoplasmic fluid citrate concentrations of 3.4 and 18 mM have been reported in Fe-sufficient and Fe-deficient leaves, respectively (Nikolic and Römheld, 1999).

Iron acquisition by leaf mesophyll cells: leaf ferric chelate reductase

Brüggemann et al. (1993) first found that Fe(III) reduction is a prerequisite for Fe uptake by leaf disks, since Fe(II) chelators inhibit Fe uptake. This has been recently confirmed with sunflower leaf disks by Nikolic and Römheld (1999). This Fe reduction process is light-dependent and seems capable of using as substrates artificial Fe chelates such as Fe(III)-EDTA and natural chelates such as Fe(III)-citrate and Fe(III)-malate.

Up to now three different systems have been used to study Fe acquisition by mesophyll tissue: leaf disks, plasma membrane preparations and protoplasts. A light-dependent FC-R activity was found in excised

leaf pieces or disks of *Vigna unguiculata* (Brüggemann et al., 1993), sunflower (de la Guardia and Alcántara, 1996), *Valerianella locusta* and *Prunus persica* (Grünewald, 1996) and *Beta vulgaris* (Grünewald, 1996; Larbi et al., 2001). These studies include data obtained with Fe(III)-citrate (Brüggemann et al., 1993; Grünewald, 1996; Larbi et al., 2001) and Fe(III)-malate (Larbi et al., 2001). Activities obtained with leaf disks or pieces, however, may include other reducing activities not related with the plasma membrane (PM) of mesophyll cells. These include those related to leakage of reducing compounds, such as organic anions, at the leaf wound (Larbi, 1999; Larbi et al., 2001). For instance, organic anions are capable of inducing the photochemical reduction of Fe(III) (Abadía et al., 1984). Also, organelles exposed to the media at the wound edge may have their own FC-R activity. For instance, it has been reported that chloroplasts reduce Fe from Fe(III) chelates (Bughio et al., 1997a,b). Most studies with leaf pieces (Brüggemann et al., 1993; de la Guardia and Alcántara, 1996; Nikolic and Römheld, 1999) have not distinguished between PM-associated FC-R and other activities, and only one study has attempted to discriminate the different sources for Fe reduction (Larbi, 1999; Larbi et al., 2001).

A partial characterisation of the FC-R activities has been carried out with leaf PM materials in the presence of the detergent Triton X-100, which opens PM vesicles and is supposed to induce maximal FC-R activities. This has been done with PM from control and Fe-deficient leaves of *Vigna unguiculata* (Brüggemann et al., 1993) and *Beta vulgaris* (González-Vallejo et al., 1998, 1999) and also with PM-enriched microsomes of Fe-sufficient kiwifruit (Rombolà et al., 2000a). Fe(III)-chelates used include Fe(III)-citrate (Brüggemann et al., 1993; González-Vallejo et al., 1998, 1999; Rombolà et al., 2000a) and Fe(III)-malate (González-Vallejo et al., 1998, 1999; Rombolà et al., 2000a). The total FC-R activity of Triton X-100-treated PM, however, has been recently suggested to include a significant contribution of cytoplasmic side *cis* electron transport activities (i.e. all electron transport components situated at the cytoplasmic side of the membrane), in addition to the physiologically relevant *trans*-membrane activity (Schmidt and Bartels, 1998). Studies on FC-R activities of PM published so far have not discriminated between *cis* and *trans* activities.

It is now clear that FC-R activities of leaf mesophyll cells, conversely to what occurs in roots, are not enhanced by Fe deficiency. This has been conclusively

shown with leaf disks (Brüggemann et al., 1993; de la Guardia and Alcántara, 1996; Larbi, 1999; Larbi et al., 2001; Nikolic and Römheld, 1999; Rombolà et al., 2000a), protoplasts (González-Vallejo et al., 2000) and isolated plasma membranes (Brüggemann et al., 1993; González-Vallejo et al., 1999).

The FC-R activity of mesophyll protoplasts isolated from Fe-sufficient and Fe-deficient sugar beet leaves has been characterised recently (González-Vallejo et al., 2000). Measurements were made in an ionic environment similar to that of the leaf apoplasm. The FC-R activity of Fe-sufficient and Fe-deficient protoplasts was dependent on light, and Fe deficiency markedly decreased the FC-R activity per protoplast surface unit. The optimal pH for the activity of the FC-R in mesophyll protoplasts was in the range 5.5–6.0, typical of the apoplasmic space. Beyond pH 6.0 the activity of the FC-R in mesophyll protoplasts decreased markedly in both Fe-sufficient and Fe-deficient protoplasts.

Modulation of the leaf ferric chelate reductase activity by pH and other factors

The first factor proposed to modulate leaf FC-R and Fe acquisition was apoplasmic pH (Mengel, 1994). The rationale for this was that an increase of the leaf apoplasmic pH in Fe-deficient plants would produce a marked decrease in mesophyll cell FC-R activity. New data have been obtained recently, both on the pH dependence of the leaf FC-R and on the real pH values of the leaf apoplasm. Data published so far on the pH dependence of the FC-R enzyme reflect the different pH dependence of the different materials used to study FC-R enzyme activities (isolated plasma membranes in González-Vallejo et al., 1999, isolated protoplasts in González-Vallejo et al., 2000 and leaf disks in Larbi, 1999 and Larbi et al., 2001). Since the use of leaf disks and PM preparations involve methodological difficulties that make interpretation of data complex, perhaps the safest approach is to rely on data obtained with protoplasts that show a FC-R pH optimum of approximately 5.5–6.0 (González-Vallejo et al., 2000). The optimal pH values for Fe(III) reduction with leaf disks, 6.0–6.7 in sugar beet (Larbi, 1999; Larbi et al., 2001) and 6.0 in faba bean (Nikolic and Römheld, 1999), could be associated with causes of reduction other than the FC-R (Larbi, 1999; Larbi et al., 2001). Also, the high optimal pH range (6.8–7.0) for the activity of the FC-R in leaf PM preparations

(Brüggemann et al., 1993; González-Vallejo et al., 1999) could be due to the contribution of cytoplasmic side FC-R activities.

The pH of the bulk apoplasmic fluid (obtained by centrifugation) does not seem to have dramatic changes with Fe deficiency, although it may increase to values close to 6.5 in some plant species such as pear and tomato. Apoplasmic fluid pH did not change in faba bean with respect to the control (Nikolic and Römheld, 1999), whereas it decreased by 0.4 units in sugar beet (López-Millán et al., 2000b) and increased by approximately 0.7–1 unit in pear (López-Millán et al., 2001c) and tomato leaves (López-Millán et al., 2001d). These differences may be related to growth conditions, including factors such as light intensity and availability of other nutrients, or be species-dependent. One possible cause for this could be an enhancement of the leaf PM ATPase under Fe deficiency conditions, similar to that occurring in roots, which could be more marked in sugar beet than in other species (López-Millán et al., 2000b). Therefore, very different experimental approaches suggest that the changes observed in apoplasmic pH with Fe deficiency – from 5.5 to 6.5 – may decrease FC-R activities by approximately 50% (González-Vallejo et al., 2000; Kosegarten et al., 1999, 2000).

Data obtained with sugar beet protoplasts suggest that an intrinsic decrease in the FC-R activity per protoplast surface area in Fe-deficient leaves could be determinant in causing major decreases in FC-R activity (González-Vallejo et al., 2000). Furthermore, this effect was larger at pH 6.0–6.5 (more than 80% decrease relative to the controls) than at pH 5.5 (70% decrease). The origin of this decrease in intrinsic FC-R activity is still unknown, although it could be related to the availability of reducing equivalents in the Fe-deficient protoplasts. This effect would occur in addition to the 50% FC-R decrease due to the increase in apoplasmic pH, resulting in a total Fe deficiency-associated decrease of more than 90% compared to the controls.

Possibly, a third factor decreasing leaf FC-R activities to an even larger extent is a large citrate:Fe ratio. The citrate:Fe molar ratios (1400–1750) found in the leaf apoplasmic fluid of Fe-deficient plants (López-Millán et al., 2000b, 2001c; Nikolic and Römheld, 1999, 2000) may impair significantly Fe uptake by mesophyll cells. The activity of the FC-R leaf PM enzyme has been recently shown to decrease markedly when the citrate:Fe ratio increases, with activities decreasing 5-fold when the citrate:Fe molar ratio in-

creased from 100 to 500 (González-Vallejo et al., 1999). This effect does not occur with malate, since malate:Fe molar ratios above 10 did not affect the activity of the leaf PM FC-R (González-Vallejo et al., 1999). Increases in the citrate:Fe ratio from 1.1 to 1000 produced approximately 80% decreases in Fe uptake by sunflower disks (Nikolic and Römheld, 1999; however, these experiments were carried out in darkness, whereas leaf PM FC-R is known to be largely light-dependent; see González-Vallejo et al., 2000). In sugar beet, the marked decrease in FC-R activities at high citrate:Fe 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. The inhibition of the reduction of Fe(III)-citrate at high citrate:Fe ratios could be also ascribed to a fast re-oxidation of the Fe(II) formed by excess citrate (Bienfait and Scheffers, 1992).

Causes of the organic acid increase in plants

The first explanation for the organic acid increase under Fe deficiency was a depression of aconitase activity (Bacon et al., 1959; De Kock, 1981). Aconitase activity, however, does not decrease consistently with Fe deficiency in roots (de Vos et al., 1986; López-Millán et al., 2000a; Pich and Scholz, 1993), whereas in leaves it may either decrease (de Vos et al., 1986) or increase (López-Millán et al., 2001b,c) when compared to the controls. In lemon juice sac cells, Fe deficiency causes a decrease only in one, possibly cytosolic, aconitase isoform (Sadka et al., 2000).

Landsberg (1981, 1986) reported that since in Fe-deficient roots organic acid increases coincided with an enhanced proton extrusion, cytoplasm alkalinisation associated with proton efflux could be responsible for the activation of PEPC in the cellular pH-stat mechanism (Davies, 1973). A second hypothesis (de Vos et al., 1986) is that Fe deficiency may cause an alteration in the glycolytic pathway, as reported in fungi (Habison et al., 1979). Under Fe deficiency, PFK would lose its regulation by citrate and PK would be inhibited by citrate. This would cause an accumulation of PEP, resulting in organic acid increases via PEPC. In this case cytoplasm acidification produced by the increases in organic acids would drive H^+ extrusion. In any case, an increased PEPC activity leading to organic acid accumulation would maintain the ionic

balance of the root cell cytoplasm (pH-stat theory; Davies, 1973).

The increase in organic acids resulting from Fe deficiency was proposed to be related to increases in root C fixation as early as in 1959 by researchers in Wallace's laboratory (Rhoads et al., 1959). Bedri et al. (1960) and Rhoads and Wallace (1960) reported enhanced assimilation of bicarbonate by roots of Fe-deficient plants. In the latter paper authors found that enhanced assimilation of bicarbonate occurred not only in the presence of Ca carbonate, but also when Fe shortage occurred in the absence of carbonate. Several reports have confirmed that Fe-deficient roots show an increased CO_2 fixation (Bienfait, 1988, 1989; Bienfait et al., 1989; Landsberg, 1986; Miller et al., 1990; Rabotti et al., 1995). Miller et al. (1990) found that C was incorporated in Fe-deficient roots into organic acids, carbohydrates and amino acids, with citrate being the organic acid with the highest labeling.

The increase in CO_2 fixation with Fe deficiency in plant roots is likely related to the increases in PEPC activity. This increase in PEPC activity was first described in root extracts of Fe-deficient plants by Huffaker et al. (1959). Recent papers have reported PEPC activity increases in root extracts with Fe deficiency of 1.85-, 2.3-, 6- and 7- to 14-fold in pepper (Landsberg, 1986), kiwifruit (Rombolà, 1998), cucumber (Rabotti et al., 1995; de Nisi and Zocchi, 2000) and sugar beet (López-Millán et al., 2000a; Andaluz et al., 2001), respectively. Notably, the increase in PEPC with Fe deficiency may be different for different genotypes within the same species (Rombolà et al., 2000b). The activity of other enzyme that could modulate C fixation in roots, CA, does not change with Fe deficiency (López-Millán et al., 2000a). A possible Fe-dependent inhibitory step for PEPC, which may be decreased under Fe stress, was postulated by Welkie and Miller (1993). Root PEPC activities decrease by 50% after 24 h of Fe resupply in sugar beet (López-Millán et al., 2001a).

The increase in PEPC activity coincides spatially in Fe-deficient roots with areas having increased FC-R activity. For instance, the increase in PEPC activity in the swollen root tip area of Fe-deficient pepper plants was 185%, whereas values similar to the controls were obtained in the proximal unswollen root area (Landsberg, 1986). In Fe-deficient sugar beet, the activity of different enzymes involved in organic acid metabolism, including PEPC, were much higher in the root sections with increased FC-R activities than in those

without marked FC-R increases (López-Millán et al., 2000a).

The increase in PEPC activity in roots of Fe-deficient plants may be associated with several factors. First, the absolute amount of the enzyme was increased in root extracts by Fe deficiency (on a protein basis) 35-fold in sugar beet (Andaluz et al., 2001) and 4-fold in cucumber (de Nisi and Zocchi, 2000; Santi et al., 2000). A PEPC polypeptide of 103 kD was particularly enhanced in cucumber roots with Fe deficiency (de Nisi and Zocchi, 2000; Santi et al., 2000). Increases of PEPC activity could be also mediated by post-transcriptional regulation through phosphorylation, as it occurs in the leaves of C4 and CAM species (see Chollet et al., 1996, for a review) and in proteoid roots of P-stressed plants (Gilbert et al., 1998). In sugar beet, the PEPC sensitivity to malate was only slightly lower in extracts of Fe-deficient roots than in those of controls, with 41 and 58% of the initial activity (at pH 7.3) remaining in the presence of 500 μ M malate (Andaluz et al., 2001; López-Millán et al., 2000a). In cucumber, PEPC sensitivity to 100 μ M malate was 50 and 32% in extracts of Fe-deficient and control roots (Santi et al., 2000). The presence of phosphoserine residues could not be detected by immunoblotting techniques in extracts of Fe-deficient sugar beet roots (Andaluz et al., 2001). These data suggest that PEPC may not be regulated at the post-translational level, but further research is needed in this field.

Other factors could further enhance PEPC activity in root cells. For instance, cytoplasmic pH increases could boost PEPC activity, since the optimum pH for the enzyme is quite high. Espen et al. (2000) have recently shown with ^{31}P -NMR techniques, however, that in Fe-deficient cucumber roots cytosolic pH may increase only by approximately 0.02–0.06 units with respect to the controls, whereas the vacuolar pH increased by 0.03–0.20 units. In sugar beet, the increase in PEPC activity does not appear to depend on the level of bicarbonate in the nutrient solution, since root tips of plants grown without Fe in the absence of CaCO_3 also had PEPC activities 40-fold higher (on a fresh weight basis and at pH 8.5) than the controls (López-Millán et al., 2000a).

PEP needed to maintain PEPC activity could possibly come via glycolysis from compounds previously synthesised and/or stored within the plant. Both phloem sap (de Vos et al., 1986; Maas et al., 1988) and root sugar concentrations (Thoiron and Briat, 1999) have been reported to increase with Fe defi-

ciency. In the latter case this may be associated to an enhanced expression in roots of several genes related to carbohydrate biosynthesis (Thoiron and Briat, 1999). Iron deficiency was associated with increases in Glc6P (36–153%) and Fru6P (up to 60%) and with decreases in UDP-Glc (30–70%) (Espen et al., 2000). Accordingly, the activities of enzymes involved in the glycolytic pathway have been shown to increase in Fe-deficient roots. This has been reported for G3PDH (115–120% increases; Espen et al., 2000; Herbig et al., 1996; Rabotti et al., 1995; Sijmons and Bienfait, 1983; Schmidt and Buckhout, 1997), PK and PFK1 (45 and 70% increases, respectively; Espen et al., 2000).

Respiration rate has been shown in some cases to be enhanced by Fe deficiency (Espen et al., 2000; López-Millán et al., 2000a). Transfer cells in roots of Fe-deficient plants are almost devoid of starch and have an increased number of mitochondria (Landsberg, 1994).

The organic acid concentration increases in leaves associated with Fe deficiency are not likely to be mediated via increases in leaf PEPC activity, which is only moderately affected by Fe deficiency (López-Millán et al., 2001b). Therefore, the most likely reason for the increase in leaf organic acid concentrations is the transport, via xylem, of organic acids fixed in the roots of Fe-deficient plants (López-Millán et al., 2000b; Rombolà et al., 2000b).

Possible functions of organic acids

Forty years ago it was generally believed that C fixation by roots and the subsequent organic acid accumulation would be deleterious for plants, and a negative consequence of Fe deficiency “. . . related to the causal mechanism of lime-induced chlorosis” (Rhoads et al., 1959).

The first beneficial effects of the organic acid accumulation described were those related to Fe transport, derived from the association of Fe and citrate in stem exudates, as indicated by the works of Brown and co-workers in the 1960s (Brown and Tiffin, 1965; Tiffin and Brown, 1961). Also, there is a clear body of evidence indicating that excretion of organic acids from plant roots to the rhizosphere is able to improve Fe availability by different mechanisms (Jones, 1998; Ohwaki and Sugahara, 1997; Ström, 1997; Tyler and Ström, 1995).

Organic acids were proposed by Landsberg (1981) to be the source for H^+ released by the roots. Lands-

berg (1986) indicated that one of the possible beneficial effects of the increases in root citrate under Fe deficiency could be to control cytoplasmic pH during enhanced proton extrusion. Organic acid accumulation also occurs in grasses, however, which fail to acidify the rhizosphere under Fe deficiency (Landsberg, 1981). Increases in citrate are usually higher than those of malate. Since the product of the pH-stat mechanism is malate, citrate accumulation could be the primary event induced by Fe deficiency, proton excretion being secondary and occurring or not depending on cation/anion balance (Bienfait, 1996). The increases in citrate were also proposed to replenish the Krebs cycle for increased citrate export from the mitochondria, thus guaranteeing its continuous operation (Landsberg, 1986). Also, Bienfait (1988) indicated that “when the roots accumulate citrate in parallel with a more or less pronounced H^+ extrusion activity, they build up at the same time a capacity to reduce $NADP^+$ ”. Miller et al. (1990) reported increases in CO_2 fixation, organic acid contents and PEPC activity in roots of Fe-deficient plants, and suggested that the increased PEPC activity may feed the TCA cycle via malate. Citrate production in roots of Fe-deficient plants has been proposed to be the source of reducing equivalents through an increase in ICDH (Bienfait, 1988, 1996; Lubberding et al., 1988).

A comprehensive route for C assimilation in roots of Fe-deficient plants has been recently proposed by López-Millán et al. (2000a). A large increase in anaplerotic C fixation in Fe-deficient yellow root tips would be mediated by an increase in root PEPC activity (Andaluz et al., 2001; López-Millán et al., 2000a). Part of this C could be used, through an increase in mitochondrial activity in transfer cells, to increase the capacity to produce reducing power, whereas some could be exported to the shoot via xylem (Bialczyk and Lechowsky, 1992, 1995; Bienfait et al., 1989; López-Millán et al., 2000a; van Beusichem et al., 1988). In sugar beet Fe-deficient roots, flavin sulfates could provide a suitable link between the increased capacity to produce reduced nucleotides and the plasma membrane ferric chelate reductase enzyme(s) (López-Millán et al., 2000a). A significant influx of organic acids from the roots to the leaves could occur via the xylem sap (Bialczyk and Lechowski, 1992, 1995; López-Millán et al., 2000b). A possible function of the export of organic acids to the leaves is the use of these C compounds for basic maintenance processes such as respiration. This would contribute to explaining several characteristics of Fe-deficient plants. For

instance, in sugar beet grown in controlled conditions, Fe deficiency causes a marked reduction in photosynthetic rates (Terry, 1980), but has a relatively small effect on leaf growth (Terry, 1979). Also, Fe-deficient, chlorotic leaves of tree species are able to survive for long periods (months) in field conditions.

Concluding remarks

In summary, it is clear that organic acids accumulate in most Fe-deficient plant parts. The presence of these compounds has an impact on many aspects of the physiology of Fe-deficient plants. Some of them are beneficial, including mechanisms directed to facilitate Fe acquisition by organic acids and proton excretion from roots, and also, to supply the FC-R enzymes with enough reducing power. Also, C export to the leaves via the xylem could facilitate the survival of the shoot parts while roots try to acquire enough Fe from the soil. Presumably this is an emergency mechanism based on the utilization of plant reserves, and could not be maintained for a long time in the absence of external Fe. This mechanism could be very important for plants growing in calcareous soils where an absolute Fe deficiency does not take place and bicarbonate is always present. The presence of a large amount of organic acids in the plant (especially in trees), however, could poison (to a certain extent) other needed physiological processes. For instance, this is the case for Fe acquisition by the leaf mesophyll, that is much affected by high citrate:Fe ratios. Possible research targets include the molecular mechanisms of the activation of the C fixation pathway in roots of Fe-deficient plants through PEPC, as well as studies on the compartmentation of organic acids and other metabolites in roots. The possibility to use PEPC enzymatic activity in screening programs for Fe-efficiency also deserves further investigation.

Many aspects of the responses of plants to iron deficiency with regards to organic acids are still poorly known. These include the mechanism triggering the increased activity of PEPC in Fe-deficient roots, and the relationship between this increase and the regulation of other root responses such as the enhancements of FC-R and ATPase activities. Also, the pathways of organic acids in the Fe-deficient plants, including excretion to the rhizosphere, xylem loading and unloading, and the possible involvement of phloem factors are still largely unknown. The relative importance of the carbon fixation by roots, specially in

severely chlorotic trees, is an important issue. Finally, the role of organic acids in the process of correcting chlorosis has been little studied and is of major importance, because Fe resupply is usually made to plants which are loaded with organic acids.

Experiments to resolve these issues could be assisted by stable isotope techniques, which could aid to trace the pathways and functions of organic acids in plants. This includes total isotope measurements in plant compartments and more detailed analysis of organic acids and related metabolites in those compartments by HPLC–MS techniques.

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