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Prevention of stress in iron metabolism of plants

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INTRODUCTION

Iron metabolism in plants is characterized by a dual requirement: (i) to have iron available in quantities sufficient for growth and for activities of essential processes, and (ii) to keep its concentrations low enough to prevent iron toxicity. This review is concerned with the ways in which plants may fulfil both conditions.

Iron is extensively used as an electron carrier, as in cytochromes, ferredoxins, reductases and oxidases, but also in enzymes that do not catalyse a net electron transfer, such as aconitase. Synthesis or activation of these enzymes requires (as far as is known) the ferrous (Nakazawa *et al.* 1969; Bentle *et al.* 1976; Jones 1983; Kennedy *et al.* 1983) or ferric ion, under reducing conditions (Pagani *et al.* 1984).

UPTAKE OF IRON BY PLANTS

Higher plants have two known ways ('Strategies', Römheld & Marschner 1986a, Römheld 1987a, b) of mobilizing and taking up iron from the soil (Fig. 1). Dicots and non-grass monocots mobilize iron by acidification of the rhizosphere, and the dissolved ferric iron and its chelates can be reduced by a plasma membrane-bound enzyme system (Chaney *et al.* 1972). The resultant ferrous ion is easily taken up. Plants that grow in

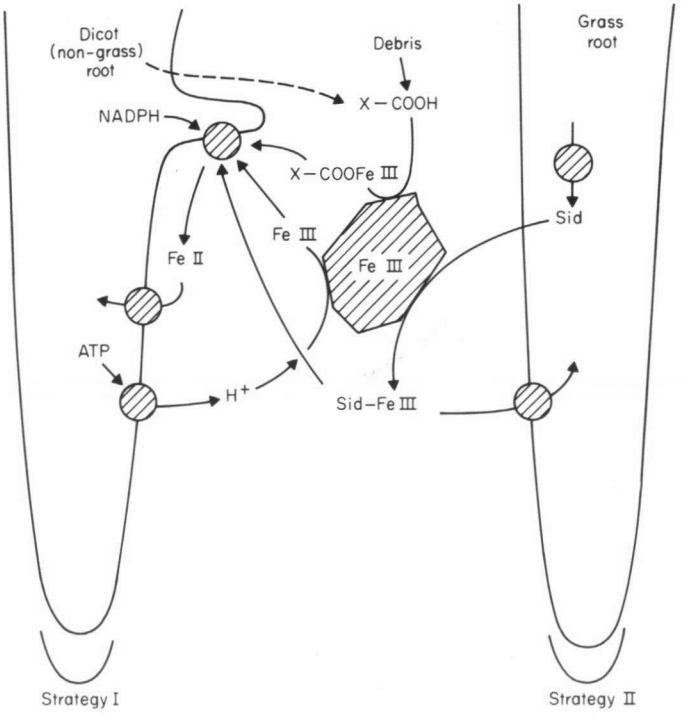

Fig. 1. Two mechanisms ('Strategies') for iron uptake. Strategy I can use the ferric-siderophore complex from Strategy II (Römheld & Marschner 1986a, b).

nutrient solution with a ferric chelate turn chlorotic when a ferrous chelator is added to the solution. During iron deficiency in the plant, proton excretion, ferric reduction and ferrous uptake capacity are all activated or induced. Moreover, the roots form extra root hairs and laterals. This complex of reactions has been called Strategy I for iron efficiency.

Strategy II, used by grasses, has been discovered recently (Takagi *et al.* 1984). Roots of grasses excrete iron-binding compounds ('phytosiderophores') which, after mobilizing ferric ions from the soil, can be taken up as such, presumably by a carrier system in the root cells' plasma membranes. Both phytosiderophore excretion and the carrier system are activated or induced by iron deficiency.

Metabolic events in roots of iron-deficient plants

During iron deficiency, both grasses and the other higher plants accumulate organic acids in their tissues, mainly malate and citrate (Iljin 1950). It is not known how the deficiency causes the accumulation. It may be instructive to have a look at microbiology, as many micro-organisms excrete citric acid upon iron deficiency. The most intensively studied organism in this respect is *Aspergillus niger*, which produces most of the world's industrially made citric acid, generally assumed to be in response to iron or other metal deficiency (Lockwood 1975). In this mould, manganese deficiency causes a deregulation of protein metabolism, which leads to intracellular accumulation of NH_4^+ (Ma *et al.* 1985). This, in turn, makes a key enzyme in glycolysis (phosphofructokinase I) insensitive to feedback inhibition by citrate (Habison *et al.* 1979). The result is uncontrolled glycolysis and accumulation of phosphenolpyruvate (PEP) and pyruvate, which are carboxylated to

oxaloacetate. The final product is citric acid; the capacity of the mitochondria to metabolize citrate being insufficient (Kubicek & Röhr 1978; cf. Ackrell *et al.* 1984). In the case of plant tissues no such effects have been reported.

In grasses, acid accumulation in roots results in a shift in the uptake pattern of anions and cations (van Egmond & Aktas 1977). Grasses tend, in general, to excrete OH^- (or HCO_3^-) when growing on nitrate as a nitrogen source, and this tendency is diminished upon iron deficiency. The net excretion of protons by an iron-deficient grass growing on nitrate has not been shown, except in a special case where nitrate reductase activity in the roots had been lowered by preculture on NH_4^+ (Landsberg 1979).

In dicots, which have a more acid uptake pattern (van Egmond & Aktas 1977), acid accumulation can be accompanied by a net proton excretion. During proton excretion the production of organic acids, and CO_2 fixation, is increased (Landsberg 1986).

Strategy I. Rhizosphere acidification, ferric reduction, ferrous uptake

Proton excretion in parallel with acid production is performed by transfer cells that are formed in the rhizodermal layers as a response to iron deficiency (Kramer *et al.* 1980). The labyrinth-like wall, lined with the proton-excreting plasma membrane, is oriented to the outside of the root. In the plasma membrane an ATPase pumps out the protons (Römheld *et al.* 1984). Due to the large surface of the plasma membrane in the transfer cells and the large numbers of ATP-supplying mitochondria, the plasma membrane ATPase can drive an extremely fast proton excretion on the basis of root fresh or dry weight (Römheld *et al.* 1984). The acids formed during a wave of proton excretion are partly stored in the roots themselves and partly exported to the shoot via the xylem (de Vos *et al.* 1986; Landsberg 1986; cf. Tiffin 1966).

The cells that excrete the protons are also the site of ferric reduction (Landsberg 1986). Ferric ions, dissolved by the low local pH, diffuse to the root surface or are taken there by the transpiration stream, which is strongest during the period of maximal proton excretion (Sijmons & Bienfait 1986). The plasma membrane contains a reduction system which can reduce ferric ions and its chelates. Its activity is strongly increased upon iron deficiency (Turbo reductase, Bienfait 1985; Cakmak *et al.* 1987). The reduction system has a low specificity and attacks many ferric chelates (Bienfait *et al.* 1983; Chaney 1989), with the exception of ferrioxamine (Bienfait *et al.* 1983; Römheld & Marschner 1983b). In its strong complex with desferrioxamine, ferric probably has too low an affinity for electrons (Nomoto *et al.* 1987). The redox potential of the Turbo electron donation site depends on the potential of the redox agent that keeps the plasma membrane system reduced. According to Sijmons *et al.* (1984) this redox agent is NADPH because the level of NADPH dropped within 2 min after addition of a reducible iron salt. Recently, however, the Beltsville group (Luster *et al.* 1988) reported that iron deficiency increases the NADH-oxidizing capacity of tomato roots. It is possible that a large cytosolic pool of NADPH gives its electrons to a smaller (undetectable) pool of cytosolic NAD, which then reacts with the ferric reductase in the plasma membrane.

The E of the $NADPH/NADP^+$ pool is probably poised at around $-0{\cdot}37$ V (Sijmons *et al.* 1984), and this sets a lower limit to the E at the electron donation site of the Turbo system, on the other side of the plasma membrane. It is therefore to be expected that compounds with an E_0, between pH 3 and 6, below $-0{\cdot}40$ V will not be readily reduced. Thus, ferric rhodotorulate (E_0' $-0{\cdot}36$ V, Nomoto *et al.* 1987) is reduced (Miller *et al.* 1985) but ferrioxamine is not (E_0' $-0{\cdot}47$ V). See Bienfait (1988a) for a discussion on this subject.

Castignetti & Smarrelli (1986; see also Smarrelli & Castignetti 1988) reported that NADH can reduce several ferric siderophores via nitrate reductase, amongst those ferrioxamine. Theoretically, such a system might attain high reduction rates if part of the electrons went to a high-potential acceptor, in an obligatory coupled mechanism, thus different from that described by Cakmak *et al.* (1987). A model for a plasma membrane-bound nitrate reductase was proposed in which electrons could be given to extracellular acceptors (Jones & Morel 1988). A Jones/Morel nitrate reductase should then reduce ferrioxamine outside the cell, which is in contrast with the findings of Römheld & Marschner (1983b) and Bienfait *et al.* (1983). However, if the low-potential electrons are available inside the cell, the system might function as a 'Standard' reductase (see later, and Bienfait & Lüttge 1988) and at best be able to reduce ferrioxamine during or after passage through the membrane.

Strong ferric chelators are mostly weak ferrous chelators, so that both the reduction of a ferric chelate and of a free ferric ion result in a free ferrous ion. This is easily taken up by the root (Kliman 1937; Chaney *et al.* 1972). Moreover, the divalent metal uptake capacity is increased upon iron deficiency (Römheld *et al.* 1982; Young & Terry 1983).

During rhizosphere acidification, roots may also release organic compounds, probably by leakage of the root cells (Brown & Ambler 1973; Marschner *et al.* 1974; Olsen & Brown 1980). These compounds may stimulate iron uptake by solubilizing soil iron (Julian *et al.* 1983; Hider 1986; Lehmann *et al.* 1987; Erich *et al.* 1987), or by serving as substrates for microbial growth, which lowers the local O_2 level and thus increases the lifetime of ferrous iron.

Stimulation of proton excretion and ferric reduction go together (Landsberg 1986). Lubberding *et al.* (1988) proposed the following explanation: during proton excretion, citrate accumulates in the transfer cells and in the vacuoles of the neighbouring cells. Citrate, via aconitase, can be isomerized to isocitrate and this drives the NADP couple to a strongly reduced state via cytosolic isocitrate dehydrogenase (see Fig. 2); ferric reduction can now proceed at a high rate. Aconitase is an Fe–S enzyme, but its activity does not diminish at the stage of iron deficiency where iron efficiency reactions are developed (de Vos *et al.* 1986).

After uptake, ferrous ions are transported to the protoxylem where they are soon oxidized on their way to the shoot (Ambler *et al.* 1971). Citrate functions as the ferric chelator in the xylem (Tiffin 1972; White *et al.* 1981); it is already present as an earlier by-product of proton excretion which made the iron ions available for uptake (cf. Tiffin 1986).

We do not know how mesophyll cells take up iron, but they probably use the same system as the roots. *Lemna* cells reduce Fe-EDTA and this activity is increased upon iron deficiency (Lass *et al.* 1986). Suspension cells derived from soybean cotyledons reduce iron in ferric-EDTA and other complexes (Cornett & Johnson 1988), and the ferrous chelator bathophenanthroline disulphonate inhibits uptake (Sain & Johnson 1986). Some plant species are chlorotic when grown under low-pressure sodium light, which contains little low-wavelength light (Brown *et al.* 1979). The leaves contain normal amounts of iron (Brown *et al.* 1979; Jolley *et al.* 1987) but the chloroplasts show typical symptoms of iron chlorosis (Pushnik *et al.* 1987). Ferric chelates of the carboxylate type are generally yellow and thus absorb blue light; this may lead to electron transfer from the carboxylate group to ferric, so that ferrous, CO_2 and an organic radical result. The results of Brown *et al.* (1979) suggest, therefore, that mesophyll cells take up the ferrous form only, and that species that turn chlorotic under low-pressure sodium light are not reducing ferric citrate at the leaf cell surface themselves, but depend on photoreduction instead.

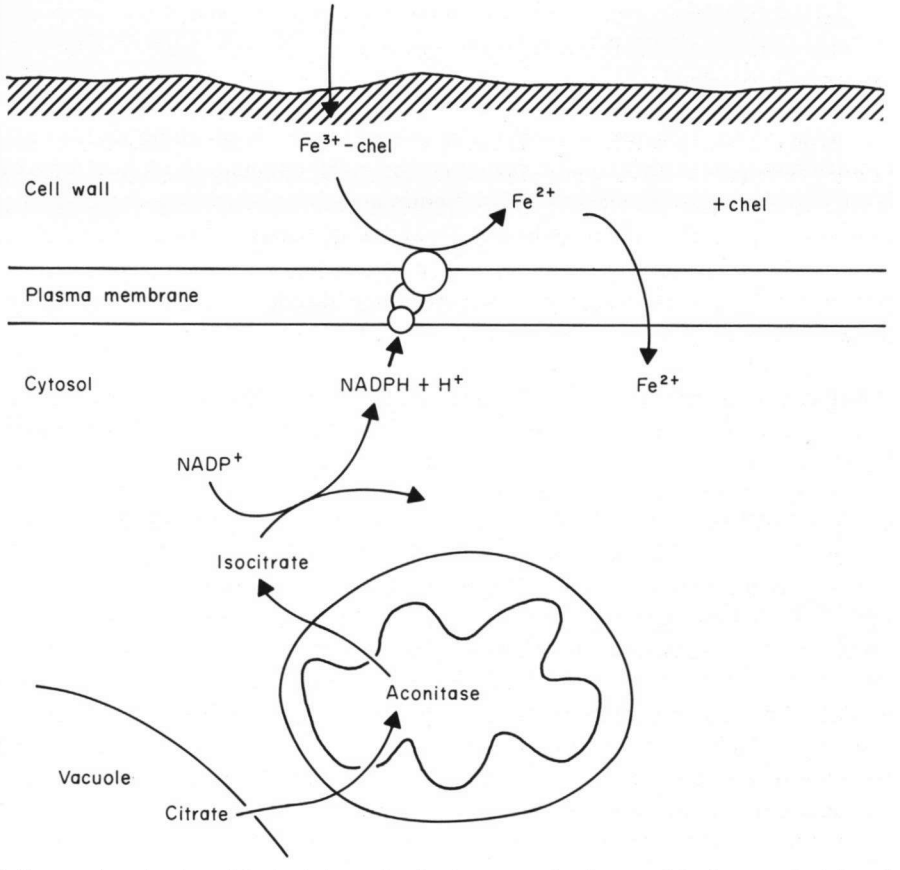

Fig. 2. Proposed mechanism of ferric chelate reduction by roots using Strategy I for iron uptake. The primary electron donor, citrate, accumulates in the cell concomitant with proton excretion.

In this context, it should be mentioned that there is a growing literature on the capacity of cells from different tissues, including leaf cells (Dhamawardhane *et al.* 1989), to reduce extracellular ferricyanide. Ferricyanide reduction is also shown by tissues, such as grass roots, that do not reduce ferric chelates like Fe-EDTA (Federico & Giartosio 1983; Qiu *et al.* 1985). This activity is not influenced by the iron status of the plant and is not known to play a role in iron uptake by any kind of plant. The possible function of an apparently basic capacity of cells to donate electrons to ferricyanide ('Standard reductase', Bienfait 1985) is unknown and subject to speculation (Bienfait & Lüttge 1988).

H. Marschner remarked (personal communication) that the need for iron by the leaf cells must, for a large part, be fulfilled during growth, i.e. before significant leaf extension has taken place. This means that iron which arrives via the xylem with the transpiration stream, may be too late to avert a degree of chlorosis that is irreversible. Phloem iron may therefore play an important role in the determination of the leaf cell's iron status (cf. Branton & Jacobson 1962). Iron in the phloem of *Ricinus communis* is continuously cycled through the ferric and ferrous form (Maas *et al.* 1988) so that the unloading cell does not need a Turbo reductase for iron uptake; but such an activity may be required at the place of entry of iron into the phloem.

A reaction to iron deficiency, for which no function has yet been found, is the release of flavins by the roots of some species (sunflower, tabacco) (Welkie & Miller 1960; Nagaraja & Ulrich 1966). Recently, Welkie & Miller (1988) found, in grafting experiments with tomato and tobacco, that in those combinations in which the roots produced flavins upon iron deficiency, leaf flavin and chlorophyll levels where highest. Unfortunately, no data on leaf iron content were given. Some algae synthesize a flavoprotein to replace ferredoxin upon iron deficiency (Zumft & Spiller 1971; Sandmann & Malkin 1983), and a search for flavodoxins in the leaves of low-iron tobacco might, therefore, be worthwhile.

Strategy I: Regulation

Iron deficiency is easily recognized as leaf chlorosis, and, in plants grown on water culture, chlorosis and iron efficiency reactions develop more or less synchronously. This synchronism suggests that the leaves send a signal to the roots which induces them to make extra laterals, root hairs, organic acids, etc. (Landsberg 1986). However, roots grown from normal potato tubers, and roots attached to small stem fragments grown on culture solution were both able to develop rhizodermal transfer cells and ferric reduction capacity upon iron deficiency (Bienfait *et al.* 1987). Thus, leaves are needed for the development of iron-efficiency reactions. In the iron-inefficient genotypes that were tested, e.g. by grafting experiments, the deficiency was located in the rootstock (Brown *et al.* 1958; Brown *et al.* 1971; Bell *et al.* 1962).

For net proton excretion, an unimpaired phloem connection between roots and leaves (Landsberg 1986) or tuber (Bienfait *et al.* 1987) was necessary; the sugar supply via the nutrient solution cannot replace the phloem connection (Landsberg 1986; cf. Bloom & Caldwell 1988 and Bowling *et al.* 1978). The collection of phloem sap from iron-deficient bean shoots yielded more sugar in a 2-h period than from control plants (Maas *et al.* 1988). This observation suggests a stimulation of the sugar stream to the roots during iron deficiency.

Phloem also transports iron; the shoot can therefore influence the iron status of the roots, and consequently, its development of iron efficiency reactions.

The present data indicate that the root's iron status, determines how far it develops the apparatus for iron efficiency reactions, and that this may be influenced by the shoot via the phloem iron concentration; the degree of expression of these reactions is influenced by the phloem sugar content in the shoot.

An interesting mutant of tomato plants may be used to gain more insight into the regulation of iron efficiency reactions. This mutant is unable to develop any of the known biochemical or morphological (Römheld & Marschner 1983a) iron efficiency reactions, i.e. the formation of extra root hairs, development of rhizodermal transfer cells (Landsberg 1981), proton excretion, ferric reduction (Brown *et al.* 1971; Brown & Ambler 1974), and is heavily chlorotic when grown on normal soils, if it grows at all (Wann & Hills 1973). Only when supplied with large amounts of ferric chelate does it turn green, probably by passive uptake via small leaks in the endodermal layer (initiation points of laterals), and then it is indistinguishable from the wild type. The recessive mutation is in a single nuclear gene called *FER*. The mutant does not develop transfer cells in the roots upon iron deficiency, but makes them elsewhere at sites of heavy sugar transport (D. Kramer, personal communication). Furthermore, roots of the iron-deficient mutant do not make root hairs when submerged, in contrast to the iron-deficient wild-type; but they make normal root hairs, regardless of their iron status, when they are not in the water. Thus, the mutation leaves the ability of the plant to make the necessary structures intact,

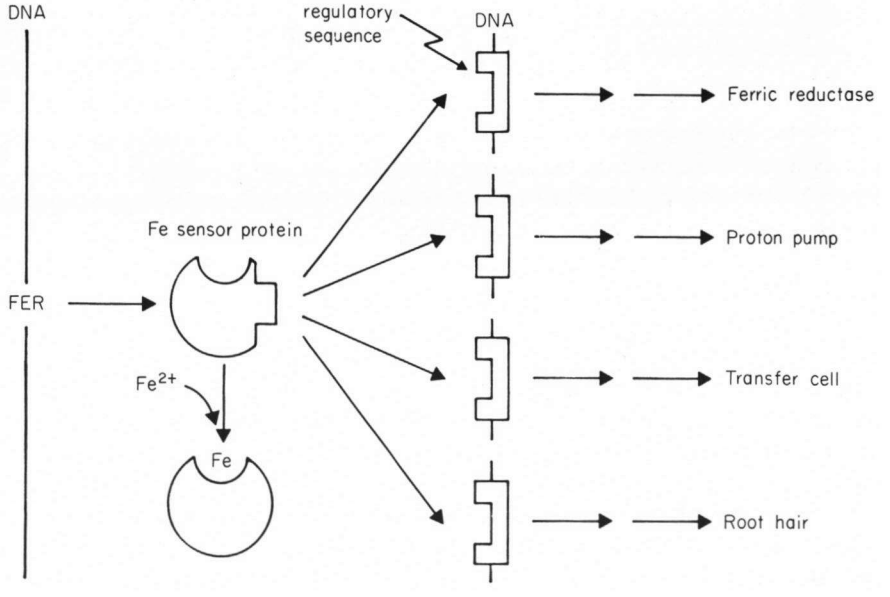

Fig. 3. Hypothesis for the regulation of iron-efficiency reactions in tomato plants. The *FER* gene encodes a regulatory protein that can bind to common sequence elements which activate genes involved in iron-efficiency reactions, inducing transcription. The regulatory protein can bind ferrous ions and, in doing so, changes its conformation so that it can no longer bind to the genes' regulatory sequences. From Bienfait (1988b).

but iron deficiency does not turn on the relevant synthesis processes. The variety of developmental and metabolic reactions that are affected by the *FER* gene indicates that *FER* codes for a factor regulating the expression of several other genes.

A recent report on root membrane proteins which are controlled by the *FER* gene (Bienfait 1988b). A search for their genes may lead to the identification of regulatory sequences which, depending on the binding of a regulatory protein, control their expression. Figure 3 shows a working hypothesis in which the *FER* gene product is that regulatory protein.

Another interesting mutant is the tomato *Chloronerva*, which cannot make nicotianamine (Fig. 4). It is chlorotic unless it is sprayed with nicotianamine. This compound is thought to be a divalent metal iron carrier in the symplast (Fig. 5) (Scholz *et al.* 1988). The mutant has its iron-efficiency reactions turned on when grown on normal iron levels and containing a high amount of iron. Possibly, the regulatory protein of Fig. 3 cannot be reached by iron without the aid of nicotianamine.

Strategy II. The grasses: phytosiderophore excretion

Grasses with iron deficiency excrete a class of compounds which are shown at the bottom of Fig. 4 (see also Kawai *et al.* 1988a). These 'phytosiderophores' can curl round the ferric ion, like nicotianamine around divalent metal ions (Fig. 5), and in this way protect them against precipitation with OH^-. They are closely related to nicotianamine; the basic difference between nicotianamine and the phytosiderophores is the $-NH_2$ end group which in the siderophores is replaced by $-OH$.

Excretion of the siderophores takes place in the morning (Takagi *et al.* 1984). Thus, in both strategies mobilization of ferric from the soil occurs at the time when transpiration

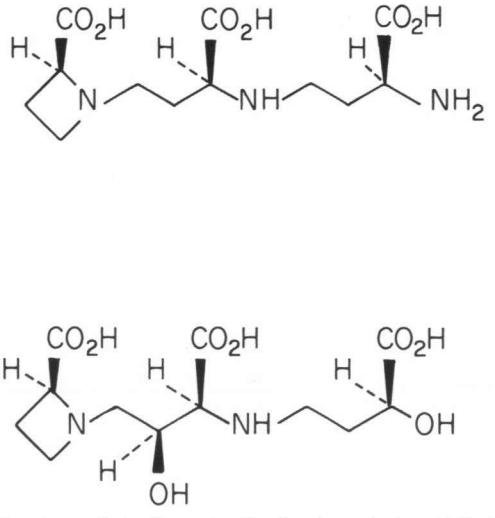

Fig. 4. Structures of nicotianamine (top) and mugineic acid (bottom).

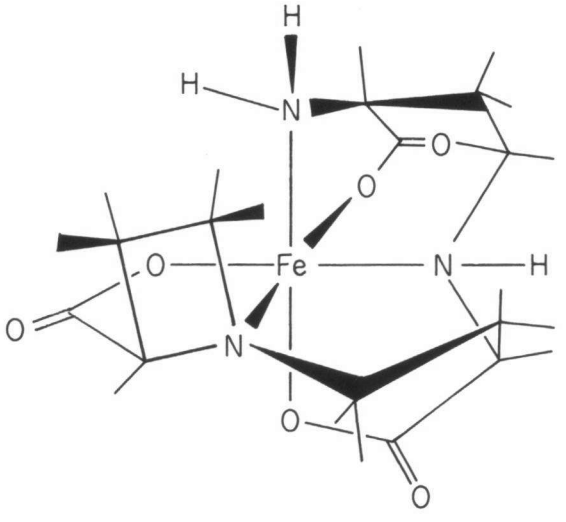

Fig. 5. Nicotianamine curling around a ferrous ion.

increases, i.e. when the chances of returning the siderophore as a ferric complex, or a ferric ion solubilized by acid, are maximal. In the early morning, the roots of iron-deficient oats contain large amounts of vesicles covered with ribosomes; in the course of the day they disappear or shrink away (Nishizawa & Mori 1987). This suggests that protein synthesis is involved in the production of phytosiderophores (Mori *et al.* 1988) that are excreted via exocytosis. The synthesis pathway does not suggest such an involvement, other than for the production of enzymes that makes the siderophores. Possibly the ribosomes synthesize the membrane-bound system that is responsible for the uptake of the ferric-siderophore complex; when this process is ready, the vesicles merge with the plasma membranes.

The affinity of mugineic acid (mugi = wheat, ne = root), the most studied phytosiderophore, for ferric ions is not very high (Nomoto *et al.* 1987) compared with the siderophores of microbial origin, however, phytosiderophores where as efficient as ferrioxamine B at mobilizing iron from a calcareous soil (Awad *et al.* 1988; Takagi *et al.* 1988; Römheld & Marschner 1989; Treeby *et al.* 1989; cf. Cline *et al.* 1983).

Induced synthesis or activation of a ferric phytosiderophore absorption system upon iron deficiency has been shown recently (Marschner *et al.* 1987). The much slower uptake of bacterial ferrated siderophores is also stimulated (Römheld & Marschner 1986b; Crowley *et al.* 1988). Uptake of bacterial siderophore ferric complexes might be a secondary activity of the ferric phytosiderphore carrier.

It is not understood why only grasses have evolved the bacterial-like siderophore excretion and uptake system. It seems easy for the dicots to transfer nicotianamine into dehydromugineic acid, through transamination and reduction, a pathway proposed to be taken by barley (Kawai *et al.* 1988b), but, as it turns out, they do not.

Strategy II: Regulation

Nothing is known about the regulation of phytosiderophore production, excretion and of ferric siderophore absorption. It would be interesting to isolate the *FER* gene from tomato plants and see whether grasses have a comparable gene, or whether they have a regulation system that is completely different from that in tomato plants. In oats, iron efficiency was reported to be mainly due to one gene (McDaniel & Brown 1982).

Strategy III. Uptake of microbial siderophores

Both dicots and grasses are capable of taking up ferric complexes of microbial siderophores. A subject of debate is whether these complexes play a significant role in the iron uptake of plants.

Cline *et al.* (1984) studied the effect of desferrioxamine B (DFOB), a hydroxamate siderophore excreted by the soil mould *Streptomyces pilosus*, on mobilization and uptake of iron from insoluble ferric hydroxide by sunflower plants. DFOB at 5 μM and higher concentrations significantly ameliorated the iron status of the plants. Crowley *et al.* (1988) showed that young roots of oats actively take up iron from ferric DFOB, and that uptake is stimulated upon iron deficiency; 5 μM was sufficient to keep the plants green. In experiments by Becker *et al.* (1985a), 5 μM agrobactin, a catechol siderophore produced by the bacterium *Agrobacterium tumefaciens*, stimulated iron uptake by pea plants which resulted in a significant increase in leaf chlorophyll content. On the other hand, they reported that pseudobactin (not a catechol or hydroxamate), produced by a *Pseudomonas* species, inhibited iron uptake by the same plant (Becker *et al.* 1985b).

Some microbial siderophores can apparently play a significant positive role in iron uptake by plants if present at concentrations of 5 μM or higher.

Levels of extractable siderophore concentrations in soils have been determined; typical values are 10^{-8} to 10^{-7} M (Powell *et al.* 1980; Bossier & Verstraete 1986). When the soil is amended with organic nutrients, which mimic root exudation, these values may rise to about 10^{-5} M (Bossier & Verstraete 1986; Crowley *et al.* 1987). In extracts from rhizosphere soils, levels of hydroxamate siderophores were found to be substantially higher than in the bulk soil (Reid *et al.* 1984); rhizosphere values of 10^{-5} M can be calculated from their data.

It would therefore appear that at the root surface sufficiently high microbial siderophore concentrations may indeed be found to affect the iron status of the plant significantly. However, a serious problem in that siderophores may bind to soil particles which would result in a substantially lower actual free siderophore concentration than the value calculated after large volume or repeated extraction (Powell *et al.* 1980).

Microbial siderophores and Strategies I and II

The availability of microbial ferric siderophores to the Strategy I uptake system depends on the capacity of the root to reduce their ferric complexes (Bienfait 1988a). As mentioned before, ferric-FOB was not reduced by roots in an assay which measured the extracellular production of ferrous ions (Römheld *et al.* 1983b; Bienfait *et al.* 1983). The author has found (unpublished) that in order to produce green plants, ferric FOB must be supplied at 20 μM to French beans, whereas ferric EDTA was sufficient at a concentration of 0·3 μM. Thus, the reductive pathway seems to be the faster uptake system. Microbial siderophores might therefore inhibit iron uptake along the reductive pathway, by competing with the phenolic and organic acid type of compounds that form reducible ferric complexes (Bienfait *et al.* 1983) but that are less efficient ferric binders.

Strategy II depends on ferric solubilization by the phytosiderophores, which, in this respect, are as effective as the microbial siderophores (Awad *et al.* 1988; Takagi *et al.* 1988; Treeby *et al.* 1989). Their binding of ferric, however, is substantially weaker than that of the known microbial siderophores (Crowley *et al.* 1987). Römheld & Marschner (1986b, 1989) have shown that the absorption of ferric FOB by grasses is 100- to 1000-fold slower than that of ferric complexes of the plant's own or related phytosiderophores. Microbial siderophores may therefore, as with Strategy I, inhibit iron uptake by grasses through competition with the phytosiderophores. Such an inhibition probably explains the observations of Cline *et al.* (1984) with sorghum.

In conclusion, microbial siderophores may, in the free form, inhibit iron uptake along the lines of Strategy I and II. In the ferric form they may contribute to iron uptake, provided that the free concentration of their ferric complexes at the root surface, in the steady-state (a resultant of factors such as microbial siderophore excretion, water flow driven by the respiration of the plant, reversible binding to soil particles), is sufficiently high (10^{-6} M or more).

Recent reviews on iron uptake are by Römheld & Marschner (1986a), Römheld (1987a), Chaney (1988) and Bienfait (1988a).

RHIZOBACTERIA AND PLANT DISEASE

A special kind of competition for iron in the rhizosphere is supposed to play a role in growth promotion of crops by Pseudomonads. The Pseudomonads that are effective in this respect excrete siderophores, including pseudobactin, with a very high affinity for ferric ions, and it is thought that they may inhibit the growth of those deleterious micro-organisms that cannot take up iron from the pseudomonad ferric siderophores. Competition is assumed to be for soil iron (Kloepper *et al.* 1980).

It is strange, however, that no cases have ever been reported in which addition of the growth-promoting Pseudomonads to the soil–plant system resulted in chlorosis of the plants. This is particularly remarkable because it was found that pseudobactin inhibits iron uptake by pea and maize plants (Becker *et al.* 1985b). It seems, therefore, that competition for iron takes place remotely from the place where the iron uptake system of

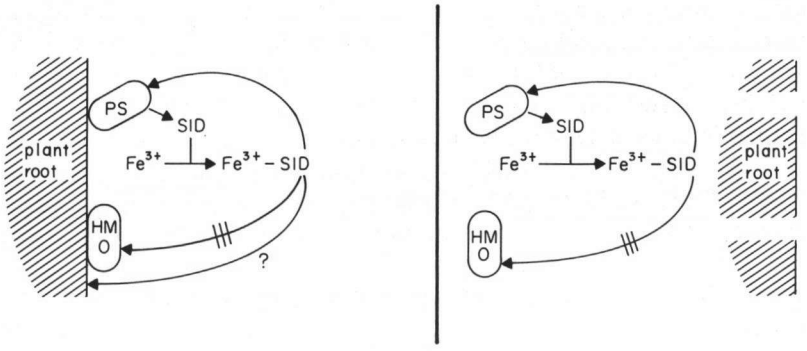

Fig. 6. Competition by harmful micro-organisms (HMO) and Pseudomonads (PS) for iron. Left: in the soil (Kloepper *et al.* 1980; fig. from Schippers *et al.* 1986); the arrow with question mark indicates the uptake of ferric siderophore by the root. Right: in the root tissue (see text); the arrow with question mark has disappeared.

the plant is at work. A plausible site is the region where the ageing root cortex decays, and where pathogenic fungi and bacteria can invade. Here, organic and inorganic nutrients are amply available, including readily mobilized iron in ferredoxin (4 Fe/mole) and phytoferritin (up to 2000 Fe/mole, Laulhere *et al.* 1988). Pseudomonads will, by scavenging this iron with their siderophores, inhibit the growth of the invading pathogens (and flourish themselves). See Fig. 6.

The role of siderophores in plant pathology has recently been treated extensively (Swinburne 1986).

IRON DEFICIENCY

A lack of iron results in diminished synthesis of iron-containing proteins such as Fe–S and haem proteins. This leads to a low capacity of the chloroplasts to reduce NADP and to drive the Calvin cycle for sugar production (Terry 1980). On the other hand, the synthesis of chlorophyll involves at least two iron-requiring steps, one in the synthesis of delta-aminolevulinate (Miller *et al.* 1982), and one in the closing of the cyclopentanone ring in chlorophyll by an iron-requiring oxygenase (Chereskin & Castelfranco 1982). As a consequence, an increase in iron deficiency decreases both the capacity to excite electrons and the capacity to carry them on to $NADP^+$. These parallel responses to iron shortage may well be functional: if the chlorophyll concentration remained high while the capacity of the electron trasport chain went down, a pool of excited electrons, spread over different carriers, would be formed during illumination, which, by reaction with O_2, could give rise to O_2^- radicals. Such a phenomenon can be observed in algae that are illuminated in the absence of CO_2 so that NADPH cannot find sufficient substrate to reduce (Abeliovich *et al.* 1974): superoxide dismutase is not capable of dealing with the avalanche of oxygen radicals, it is broken down itself and the cells die. Diminished synthesis of chlorophyll as a response to iron deficiency is the oldest known indicator of a nutritional disorder (Gris 1844).

Iron-deficient chloroplasts show structural abnormalities such as reduced grana stacking (Stocking 1975; Platt-Aloia *et al.* 1983; see also the review by Terry & Abadia 1986).

Chlorosis by iron deficiency is commonly observed on alkaline soils with a high $CaCO_3$ content, and climatic conditions (cold and wet weather) can play an important role.

Calcifuge species are in general the most sensitive; this sensitivity may be a determining factor for these species not to occur on calcareous soils (for a review see Kinzel 1982). The main inducing factor seems to be HCO_3^- (Boxma 1972; Kolesch *et al.* 1984; Mengel *et al.* 1984), but there may be multiple pathways leading from soil alkalinity to chlorosis. To determine how iron deficiency comes about in a particular case it may be necessary to examine interactions in cation and anion metabolism and the kinetics of growth together.

Alkalinity of calcareous soils inhibits iron uptake, especially in dicots, by buffering against rhizosphere acidification and by diminishing the rate of the ferric reductase with its low pH optimum (Bienfait *et al.* 1983; Römheld & Marschner 1983b). Nevertheless, a chlorosis on alkaline soil does not always implicate a low iron content in the leaf (the disease can be identified when spraying with iron chelates causes regreening). Iron is then apparently inactivated: in the cell, which is not very probable, or in the apoplast. Inactive forms of iron probably occur mainly in the ferric form (Machold *et al.* 1968) and are partly soluble, partly insoluble in 1 N HCl (Oserkowsky 1933; Jacobson 1945). Bicarbonate increases the solubility of phosphates (Greenwald 1945), and iron-chlorosis on calcareous soils is often correlated with high phosphorus levels in the tissues (e.g. Miller *et al.* 1960; Ao *et al.* 1987); efficiency in phosphorus uptake may increase sensitivity to iron chlorosis (Brown & Jones 1975; Elliott & Läuchli 1985). Phosphate may interfere with iron transport (Tiffin 1972), depending on the variety and on bicarbonate levels in the nutrient solution (Coulombe *et al.* 1984). Inactivated iron may therefore partially appear in the form of a ferric phosphate precipitate.

Ion uptake by the mesophyll cells may involve OH^- or HCO_3^- excretion, particularly when nitrogen is present in the form of nitrate, as in the case of root cells. Apoplast solution, which flows from the xylem at an initial pH of 5–6, will then gradually turn more alkaline as it penetrates deeper into the leaf blade (Mengel & Geurtzen 1988). At a certain distance from the veins iron depletion by precipitation may then be so strong that chlorosis appears (De Kock 1955). In the C4 plant sugarcane, iron chlorosis was of more consequence for the mesophyll cells than for the bundle sheath strands (Stocking 1975; Naik *et al.* 1985). If the cells reduce ferric citrate prior to absorption with a system comparable to the Turbo reductase in the roots, high pH in the apoplast diminishes the ferric reduction rate.

Another cause of iron not reaching its proper place in the cells can be that other metal ions e.g. Zn^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}, compete with (probably) ferrous ions for sites on transmembrane carriers or other molecules that function in iron transport such as nicotianamine (metal intoxication). High levels of these metals in soils or nutrient solution cause iron chlorosis (Foy *et al.* 1978).

In nitrogen-fixing legumes, iron deficiency may secondarily cause nitrogen deficiency by inhibiting the development of nodules (O'Hara *et al.* 1988). Nitrogen fixation involves a number of iron-proteins such as leghaemoglobin and nitrogenase; it is the most expensive way, in terms of iron, for the plant to fulfil its need for nitrogen, followed at a distance by nitrate and ammonia (Raven 1988).

IRON TOXICITY

Iron toxicity in general

The ferric ion is practically insoluble at physiological pH values; the solubility product of $Fe(OH)_3$ is 10^{-39} (Biedermann & Schindler 1957). This does not mean that at pH 7 the maximum soluble Fe(III) concentration in water is 10^{-18} M, as more or less hydroxylated

forms of Fe(III) are, at that pH, more soluble than the free ferric ion, e.g. $Fe(OH)_2^+$ (maximally 10^{-10} M) and $Fe(OH)_3$ (maximally $10^{-10.5}$ M) (Lindsay & Schwab 1982). The ferrous ion is more soluble but at a pH above 5 it is easily oxidized by oxygen (Stumm & Lee 1961) to ferric. Chelators can influence the reaction (Theis & Singer 1973), particularly those with different affinities for the ferric and ferrous ion, as they change the E_0' of the ferric/ferrous couple. Thus, 2,2,'-bipyridyl, with a high affinity for ferrous, changes the E_0' to higher values than that of oxygen/water, and the ferrous–bipyridyl complex is stable in aerobic solution. In contrast, citrate, which has a high affinity for ferric, lowers the E_0' and stimulates the oxidation of ferrous ions (Theis & Singer 1973).

Many compounds in the cell have a high affinity for ferric, and cellular iron has therefore a tendency to become oxidized in the presence of oxygen. However, in the cell there are also many compounds with a sufficiently low redox potential, such as ascorbate and reduced glutathione, to be able to reduce even strong ferric complexes. In the aerobic cell iron therefore has a tendency to be oxidized and reduced continuously in a redox mill, thus catalysing a net oxidation of metabolites in quantities largely surpassing that of the iron ions themselves.

The oxidation of ferrous by oxygen gives rise to the formation of the superoxide anion:

$$Fe(II) + O_2 \rightleftharpoons Fe(III) + O_2^{\cdot-}.$$

$O_2^{\cdot-}$ can dismutate to hydrogen peroxide, spontaneously at low pH, and at high pH catalysed by superoxide dismutase (SOD):

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2,$$

and hydrogen peroxide can be broken down by catalase;

$$H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2,$$

or be reduced by ascorbate or glutathione (Foyer & Halliwell 1976; Salin 1987).

In the cell, $O_2^{\cdot-}$ and H_2O_2 are produced continuously (Fridovich 1978) and are therefore always present at significant levels. Ferrous or its chelates readily react with H_2O_2 which results in the production of $OH^{\cdot}$ radicals (Fenton reaction, Walling 1975):

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^{\cdot} + OH^-.$$

The OH radical is extremely reactive. *In vivo* its lifetime is supposed to be very short due to the abundance of potential victims. A well known reaction of $OH^{\cdot}$ is with unsaturated fatty acids (UFA, Kappus 1985):

$$UFA\text{–}H + OH^{\cdot} \rightarrow UFA^{\cdot} + H_2O,$$

$$UFA^{\cdot} + O_2 \rightarrow UFA\text{–}OO^{\cdot},$$

$$UFA\text{–}OO^{\cdot} + \text{second } UFA\text{–}H \rightarrow UFA\text{–}OOH + \text{second } UFA^{\cdot}.$$

In further reactions, which require metal ions (Fe, Cu), UFA–OOH breaks into fragments, and the second $UFA^{\cdot}$ follows the same pathway as the first. In membranes, there is always a next fatty acid. Thus, a single $OH^{\cdot}$ can start a chain reaction of damage in membrane lipids which ultimately leads to leakage or breakdown of the membrane. Moreover, oxygen radicals are often formed by electron carriers in or at membranes (Halliwell 1987), so that the $OH^{\cdot}$ radical has a high probability of meeting a membrane fatty acid as its first potential victim. Perhaps iron can also give rise to lipid peroxidation without an intermediate role for $OH^{\cdot}$ radicals (Minotti & Aust 1987).

Products of iron-induced lipid peroxidation may also have deleterious effects in the cell. One such product, trans-4-hydroxynonenal, attacks sulphydryl compounds such as reduced glutathione and cysteine (Esterbauer 1982). It binds strongly to soluble tubulin and causes enzyme inhibition at micromolar concentrations (Dianzani 1982).

Forms of iron that have been shown to cause radical formation and lipid peroxidation are Fe–ADP (Esterbauer *et al.* 1982; Vianello *et al.* 1987) and Fe-citrate (Baker & Gebicki 1986). Care should be taken in studies with iron-containing incubations (Tadolini 1987a and b; Tadolini & Sechi 1987). Oxygen radical formation is not exclusively caused by free or 'non-physiologically bound' forms of iron, as in Fe–EDTA, Fe–citrate or Fe–ADP. Thus, ferredoxin–Fe(II) is easily oxidized by oxygen (Misra & Fridovich 1971). Iron in ferritin (Biemond *et al.* 1988) and in leghaemoglobin (Puppo & Halliwell 1988) can generate $OH^{\cdot}$ radicals from $O_2^{\cdot-}$.

Radical formation by iron is not necessarily a harmful event; in ribonucleotide reductase a free radical, stabilized by iron, is essential for enzyme activity (Harder & Follman 1987); iron specifically protects corn protoplasts from T-toxin of a pathogenic mould (Macrae & Yoder 1987), probably by producing $O_2^{\cdot-}$.

Recent reviews on the role of iron as an inducer of radical formation are by Halliwell & Gutteridge (1988) and Dunford (1987); about the effects of radicals by Halliwell (1987), Leshem (1988) and Thompson *et al.* (1987).

Iron toxicity in plants

Iron toxicity in plants was first mentioned by Ponnamperuma *et al.* (1955). It can be elicited *in vitro* by putting leaves or stems with their cut ends in imitation xylem solutions containing ferrous sulphate (Tanaka *et al.* 1966; Talbot & Etherington 1987). A high iron content of leaves does not automatically mean that they suffer from iron toxicity; high iron contents may very well go together with iron chlorosis (Kinzel 1982). An essential is, probably, whether iron enters the cells. In principle, high amounts of iron in the apoplast can give rise to oxygen radicals, via photoreduction of Fe–citrate or of ferric bound to other carboxylate groups e.g. in the cell wall, followed by oxidation by oxygen. It is questionable, however, whether the lifetime of $O_2^{\cdot-}$ or $OH^{\cdot}$ outside the cell will be sufficient to cause significant damage to the plasma membrane or entry into the cell.

It was recently reported that in homogenates of plants with high iron contents, oxygen radicals were formed at higher rates than in preparations from control plants (Hendry & Brocklebank 1985). However, the extracts were made by homogenizing the iron-containing tissues as such, so that extracellular iron precipitates in and between cell walls could have been partly dissolved by mixing with vacuolar acids. If it is to be shown that in a certain type of tissue iron toxicity works via the production of oxygen radicals in the cell, measures have to be taken to prevent contamination of cellular extracts with extracellular iron during the preparation procedure.

Iron toxicity may occur in plants grown in submerged soils. Oxygen diffuses 10 000 times more slowly in water than in air, and in flooded soils the available oxygen in the water is rapidly used by the respiratory activity of micro-organisms and roots. When oxygen is depleted, micro-organisms start using other compounds as electron acceptors, such as nitrate, sulphate, Fe(III) (Kamura *et al.* 1963; Ottow 1969), and Mn(IV). The reduction products, sulphide, N_2 or ammonia, Mn(II) and Fe(II) accumulate in the soil solution (Ponnamperuma 1984). Depending on the soil and the presence of other potential electron acceptors (nitrate, Munch & Ottow 1977), the period that micro-organisms need to lower the E to levels where Fe(II) is stabilized ($\leqslant +150$ mV) may be a matter of

days; the concentration of soluble Fe(II) can be anything up to a few millimolar (Ponnamperuma 1984).

Uptake of iron by roots in anaerobic zones with high Fe(II) levels escapes control: in dicots, on solubilization and reduction of Fe(III), and in grasses, on solubilization and uptake via a ferric–siderophore carrier.

Aerenchyma and iron plaque

Roots of plants grown in a flooded soil can only do so when the root tips have an adequate supply of oxygen. Several plants can form air channels in their root cortices called aerenchyma (Justin & Armstrong 1987). Ethylene, accumulating in the roots as a consequence of flooding, is considered to be the inducing agent for aerenchyma formation (Drew *et al.* 1979). Oxygen diffuses down a gradient from the above-water tissues (van Raalte 1941; Barber *et al.* 1962). It is not only used by the root tips for growth and for ATP-driven ion uptake, but it also seeps out of the air channels, via the free space, into the rhizosphere. There it may restore more or less aerobic conditions and lead to re-oxidation of reduced compounds, a.o. ferrous and its chelates (Armstrong 1967; Green & Etherington 1977). Thus, in and around roots of submerged plants, a reddish-brown plaque of ferric hydroxide deposits can often be observed as an indication of well-functioning aerenchyma. As a result the ferrous concentration near the roots is lowered. Plaque formation is therefore generally considered to be a defense of the plants against iron toxicity. The capacity to oxidize ferrous ions at the roots can be a determining factor for the distribution of plants over soils with different flooding regimes (Martin 1968; Etherington & Thomas 1986).

The presence of a well-developed aerenchyma may be a prerequisite for plaque formation, it is not a guarantee that the soil solution bathing the cells, where ion uptake takes place, contains a sufficiently low level of ferrous ions (e.g. Chen *et al.* 1980a). This level is the result of a number of variables and processes: the concentration in the soil solution, the form in which it is present (free or chelated) (Theis & Singer 1973; Davison & Seed 1983; Bao & Yu 1987), the local oxygen concentration and the rate of its diffusion into the soil, the transpiration rate of the plant which determines the flux of ferrous ions to the roots (Jones 1971; Laan *et al.* 1989), and the presence of catalytic agents such as micro-organisms (Benckiser *et al.* 1984; Trolldenier 1988), components of the cell wall (Yamada & Ota 1958; Ando *et al.* 1983), and preformed ferric hydroxide giving rise to autocatalytic oxidation kinetics (Tamura *et al.* 1976; Sung & Morgan 1980; see also Macfie & Crowder 1987). The form of iron hydroxide deposited in rice roots was reported to be γ-FeOOH (lepidocrocite) (Bacha & Hossner 1977) and α–Fe–OOH (goethite) (Chen *et al.* 1980b), CO_2 favouring goethite formation (Schwertmann & Fitzpatrick 1977); lepidocrocite stimulates ferrous oxidation (Tamura *et al.* 1976).

A heavy ferric hydroxide plaque might act as a filter for the soil solution before it reaches the root cells (Howeler 1973). Compounds that may be bound are phosphate (Jones 1975; Waldren *et al.* 1987; Willett *et al.* 1988) and heavy metal ions (St.-Cyr & Crowder 1989; Otte *et al.* 1989).

Plaque formation can be studied *in vitro* (Taylor *et al.* 1984). Its iron content is often determined by treating roots with a strong reductant (DCB technique, Taylor & Crowder 1983). The method determines reducible cellular iron as well; with a milder technique extracellular iron can be determined specifically (Bienfait *et al.* 1985; Laan *et al.* 1989).

Plaque formation is not the only factor which determines iron uptake in flooded soils. Other metals such as manganese and zinc influence the uptake and transport of iron to the

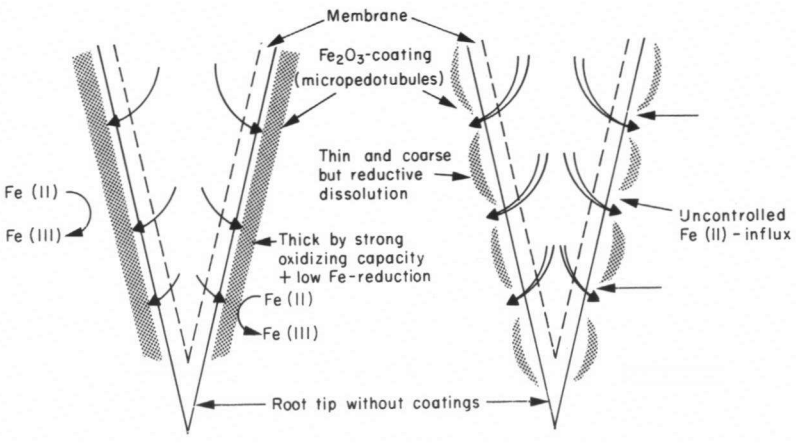

Fig. 7. The sequence of events that may lead to iron toxicity. Left: healthy plants, with a normal supply of potassium, phosphorus or calcium, producing low amounts of exudates, sustain little microbial growth, and have a high oxidation capacity at the root. Right: plants with a deficiency in phosphorus, potassium or calcium. High exudation rates sustain intense microbial growth and respiration so that the oxygen levels are low: insufficient oxidation of ferrous and high iron uptake rates. Modified after Ottow *et al.* (1982).

shoot (Verma & Tripathi 1983; Van der Vorm & Van Diest 1979). Salt stress was reported to decrease the capacity of rice plants to exclude iron at the roots (Tadano 1975). Toxins which accumulate in the anaerobic soil such as H_2S may interfere with root metabolism and aerenchyma development and thereby promote iron toxicity (Tanaka *et al.* 1968).

The general nutritional status of a plant may strongly influence its sensitivity to iron toxicity (Howeler 1973). Ottow *et al.* (1982) and Benckiser *et al.* (1984) proposed the following order of events in rice: a bad nutritional status inhibits protein synthesis and shoot growth, causing a stream of unused photosynthate to the root, where the exudation rates are increased. High amounts of exudate stimulate microbial growth with a concomitant heavy demand on oxygen supply, and this finally leads to lower oxygen levels and longer lifetimes of ferrous ions. See Fig. 7.

Phytoferritin: prevention of high cellular iron levels

Plant and animal cells contain a defence system against too high free or loosely bound iron levels. The system involves the inducible synthesis of a hollow protein, called ferritin, that may contain, in its cavity, Fe(III)-oxihydroxide-phosphate to a maximal iron content of 4500 Fe/mol. (mammalian) ferritin (Harrison *et al.* 1987). Animal ferritin consists of 24 subunits of M_r 18 500, plant ferritin (phytoferritin) subunits are 20–50% heavier (van der Mark *et al.* 1983a; Sczekan & Joshi 1987; Laulhere *et al.* 1988). The subunits are arranged in such a way that they surround the cavity but leave open six channels through which iron can enter and leave. Much more is known about animal than about plant ferritin, but as far as comparisons have been made, phytoferritin appeared to behave in essentially the same way, with the exception of its synthesis pathway.

In both plants and animals, ferritin synthesis is induced by increasing cellular iron levels, but the induction mechanism is different. The difference is probably connected to the difference in location of the ferritin in the cell: in animals, ferritin is cytosolic (some ferritin is excreted as a glycosylated form), in plants it is exclusively found in plastids (Seckbach 1982).

The ferritin synthesis pathway, including both synthesis of the protein subunits and their polymerization into the ferritin molecule, can, in animals, take place in one cellular

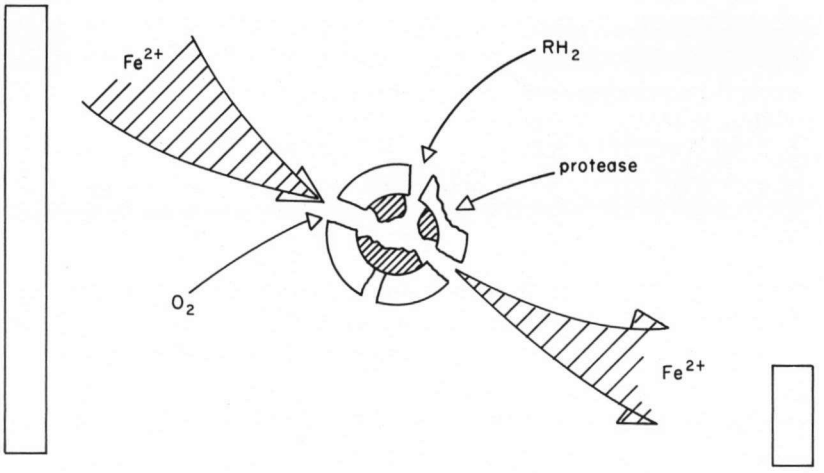

Fig. 8. Ferritin as a buffer for cellular iron. Ferrous ions enter and are oxidized within the protein shell, where they precipitate. Iron can be mobilized by the breakdown of the protein shell or by reduction of the ferric oxihydroxide core. Vertical axis = cellular iron level.

compartment, the cytosol. In animals, control by iron is on the translational level, i.e. in the cytosol. The mRNA is bound and inactivated by protein in the cytosol, until free Fe(II) binds and changes the conformation of the complex in such a way that protein synthesis can proceed (Zähringer *et al.* 1976). In plants, the plastid membranes have to be crossed. This latter process occurs at the level of the subunits, which then combine to the holoprotein after arrival in the plastid (van der Mark *et al.* 1983a). Membrane crossing is incompatible with the animal regulation mechanism. In bean plants, iron controls at the level of mRNA synthesis, i.e. in the nucleus (van der Mark *et al.* 1983b).

Ferritin takes up iron by oxidation of ferrous ions, followed by precipitation of ferric on a ferric–hydroxide–phosphate body in the protein cavity. Two mechanisms have been proposed which probably operate together.

1. Binding of two ferrous ions to the protein on neighbouring sites in one of the channels; oxygen may then attack. The resulting ferric hydroxide precipitates on the core (Crichton & Roman 1978). No oxygen radicals are produced outside the ferritin molecule.

2. Binding of ferrous to the core surface which catalyses its oxidation, followed by precipitation on the spot (Harrison *et al.* 1974).

Mechanism (1) supposedly prevails in ferritin with low iron content; mechanism (2) in ferritin with high iron content (Harrison *et al.* 1987).

For iron release, several mechanisms have been proposed. The best documented case is that of the mould *Phycomyces*, the spores of which contain ferritin. After germination, the protein shell is broken down and iron apparently dissolves. The extent of ferritin breakdown depends on the iron status of the mould, and feedback control was proposed to be realized through iron sensitivity of a specific protease (David 1974). Reduced flavins can release ferritin iron by reduction (Sirivech *et al.* 1974), and this was proposed to occur in mammalian tissues such as liver (Crichton *et al.* 1975). However, reduced flavins are rapidly oxidized by oxygen, and in leaves of plants, where O_2 levels are higher than in liver, such a mechanism is not plausible. Bienfait & van den Briel (1980) proposed reductive release by the monodehydroascorbate radical; superoxide might do the same (Biemond *et al.* 1988). However, there is no evidence yet concerning whether phytoferritin *in vivo* releases its iron through a reductive or a proteolytic mechanism, or both.

Seckbach (1969) tricked *Xanthium* leaves in to making large amounts of ferritin, by first putting them on low iron and then supplying iron so that the plants took it up in large quantities. Until now, nobody has examined the role ferritin might play in resistance against flooding-induced iron toxicity.

Figure 8 shows how ferritin is thought to buffer iron levels in the cell.

CONCLUSION

The interesting aspect of plant iron metabolism is that the handling of iron requires so many and so different activities. Following the course of iron into and through the plant is a journey through the landscape of plant physiology. The most promising area seems to be the regulation of iron efficiency reactions, and the relationship between the Strategies. What caused the grasses and the other higher plants to evolve such different iron uptake systems? Do they have anything in common in the control of their development and activity? Have plants developed anything to profit from the microbial activity at their roots, such as an inducible translocator for a microbial siderophore?

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