Metabolization of iron by plant cells using O-Trensox, a high-affinity abiotic iron-chelating agent

Catherine CARIS, Paul BARET, Claude BÉGUIN, Guy SERRATRICE, Jean-Louis PIERRE and Jean-Pierre LAULHÈRE*

Laboratoire d'Etudes Dynamiques et Structurales de la Sélectivité, Université J. Fourier, URA CNRS 0332, BP 53-38041 Grenoble, Cedex 9, France

A synthetic siderophore, O-Trensox (L), has been designed and synthesized to improve iron nutrition of plants. The affinity for iron of this ligand [$p\text{Fe(III)} = 29.5$ and $p\text{Fe(II)} = 17.9$] is very high compared with EDTA. In spite of its high and specific affinity for iron, O-Trensox was found to be able to prevent, and to reverse, iron chlorosis in several plant species grown in axenic conditions. It also allows the iron nutrition and growth of *Acer pseudoplatanus* L. cell suspensions. The rate of iron metabolization was monitored by ^{59}Fe radioiron. Ferritins, the iron storage proteins, are shown to be the first iron-labelled proteins during iron metabolization and to be able to further dispatch the metal. Using Fe(III)-Trensox, the rate of iron incorporation into ferritin was found to be higher than when using Fe-EDTA, but

slower than with Fe-citrate, the natural iron carrier in xylem. During a plant cell culture, the extracellular concentrations of iron complex and free ligand were measured; changes in their relative amounts showed that the iron complex is dissociated extracellularly and that only iron is internalized. This suggests a high affinity for iron of a putative carrier on the plasmalemma. In contrast with Fe-citrate and Fe-EDTA complexes, Fe(III)-Trensox is not photoreducible. Its ability to induce radical damage as a Fenton reagent was tested using supercoiled DNA as target molecule. Unlike Fe-citrate and Fe-EDTA, Fe(II)-Trensox and Fe(III)-Trensox were proven to be harmless even during ascorbate-driven reduction, while Fe-EDTA and Fe-citrate generate heavy damage to DNA.

INTRODUCTION

Siderophores are iron-chelating agents which are excreted by plants or micro-organisms to render iron soluble in the environment and favour the uptake of this metal. Their ability to fulfil this function is primarily related to their powerful and selective affinity for the metal: species able to synthesize ligands of high affinity are potential winners in the nutritional competition for iron.

From an ecological point of view, nutritional competition for iron in soils controls the settling of species and crops. The niche of a species may be limited by the stability constants of the siderophore it can use [1]. Similarly, from a pathological point of view, bacterial siderophores often allow the success of infections by inducing an iron deficiency in the host plant [2].

Some plants (grasses) are able to synthesize their own nutritional siderophores (phytosiderophores) whose affinities for iron are weaker [3] than those of bacterial siderophores [4,5]. Plant roots may also use bacterial siderophores shared with the rhizospheric microflora as nutritional agents, as far as they are able to internalize iron from those tightly bound complexes. They generally need the cooperation of the microflora to take up iron from microbial siderophores [6] but, conversely, microbes can divert iron from plant siderophores [7–9]. The result of competition for natural siderophores in soils is complex [10–12] and not documented enough to explain their consequences on the iron nutritional input of crops. Micro-organisms 'can be an unpredictable confounding factor' in the study of iron uptake from siderophores [13].

A few abiotic siderophores are used in agriculture for prevention or treatment of chlorosis and also in synthetic media for plant cells and tissue cultures.

The most common synthetic iron-complexing agents belong to

the EDTA family. In plant nutrition, this family is not completely satisfying, since the products are photosensitive and, as Fenton reagents [14], induce deleterious cellular peroxidation. Moreover, they ineffectively chelate iron which precipitates with phosphates [15]. Generally, such abiotic siderophores are not internalized by plants. The release of iron from abiotic iron chelates follows the extracellular reduction [16,17]. They should be designed to allow the dissociation of the complex to the benefit of the target crop. Consequently, the affinity for Fe(II) of the nutritional siderophore may be as critical as that for Fe(III).

We have designed and synthesized [18] an abiotic siderophore, named herein Trensox {Tris-*N*-(2-aminoethyl)-[8-hydroxyquinoline-5-sulphonato-7-carboxamido]amine} (Figure 1). It is a tripodal ligand based on 8-hydroxyquinoline subunits. We first

Figure 1 Chemical structure of O-Trensox (named Trensox in the text)

Abbreviations used: FZ, Ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulphonic acid]; O-Trensox, Tris-*N*-(2-aminoethyl)-[8-hydroxyquinoline-5-sulphonato-7-carboxamido]amine; NHE, normal hydrogen electrode.

* To whom correspondence should be addressed.

present its physicochemical properties (full details will be published elsewhere), then we characterize its bioavailability and biostability (antioxidant properties). We compare its properties, in iron nutrition and metabolization into ferritins, with those of citrate, a natural iron transporter in xylem [19], and with those of EDTA. We also show that ferritins are the major proteins in which iron is first incorporated, and from which it may be subsequently dispatched.

MATERIAL AND METHODS

Physicochemical measurements

Fluorescence studies

These were performed with a Perkin-Elmer Luminescence LS 50 on 50 μM Trensox; the pH was adjusted to pH 7.5 with a Tris-maleate/NaOH buffer.

Electrochemical experiments

Electrochemical experiments were carried out using a PAR model 273A potentiostat. All experiments were run under an argon atmosphere at room temperature in an aqueous medium, using 1 M NaClO_4 as the supporting electrolyte. The pH was adjusted with diluted aqueous solutions of NaOH or perchloric acid. A standard three-electrode electrochemical cell was used. Potentials are referred to an Ag/AgCl, KCl-saturated reference electrode, the working electrode was a platinum disc electrode (diam. 5 mm) polished before each record with diamond paste (2 μm). The electrochemical method used is cyclic voltamperometry.

Stability constants measurements

Ligand deprotonation constants and ferric complex stability constants were determined by UV-visible spectrometry [20]. Spectrophotometric titrations gave the $\text{p}K_a$ of the ligand. The UV-visible spectra of a solution containing equal amounts of ligand and Fe(III) (about 2.5×10^{-4} M) were monitored as a function of the pH (adjusted with HClO_4 or NaOH) in the range 1–11. For these measurements the ionic strength was maintained at 0.1 M with NaClO_4 . The spectrophotometric data were treated by the program LETAGROP-SPEFO [21], which uses a non-linear, least-squares method to compute the complexation constants.

Photoreduction of Fe(III) complexes

Solutions of ferric complexes of citrate, EDTA (sodium salt) and Trensox were prepared with a molar ligand (L):iron ratio of 2:1 and an iron concentration of 0.1 mM. The pH was adjusted to 4.8 or 6 with acetate buffer. Ferrozine (FZ) was added in large excess (9 mM) in order to trap the Fe(II) during the photoreduction (1200 lux, daylight). This method allows the quantification of Fe(II) by spectrophotometry ($\epsilon_{\text{Fe(II)}-\text{FZ}}$ 28000 $\text{M}^{-1} \cdot \text{cm}^{-1}$ at $\lambda = 562$ nm). With Fe(III)-Trensox, iron reduction was also tested by changes in the absorption spectrum (595 nm).

Axenic plant cultures for *Arabidopsis thaliana*, *Zea mays* and *Nicotiana tabacum*

Seeds were made axenic by immersion in a solution containing 5% calcium hypochloride and Triton X-100 (10 μl for 100 ml) and were then washed extensively with sterile distilled water. The seeds were dried in a sterile hood. Germinations and growth took place in sterile glass vials containing 40 ml of Murashige and

Skoog (MS) [22] medium previously prepared solidified with purified agar at 23 °C, under continuous light.

Hydroponic axenic cultures of peas

The germination of axenic pea seeds (*Pisum sativum* cv Serpette Guilloteau) was performed in sterile conditions on MS solid medium. Seedlings were transferred into a nutrient solution in a sterile hood. Hypocotyls were tightly wrapped in sterile hydrophobic cotton wool. A stream of sterile filtered air was bubbled into the nutrient solution, a constant overpressure of sterile air was maintained around the nutrient medium and hypocotyls to prevent the contamination of the nutrient solution. Microporous tape closed any air leaks. Three days after the transfer, desferrioxamine B was added (concentration 100 μM) in order to accelerate the appearance of chlorosis. Growth was then allowed for 10 days before observing chlorotic symptoms. After the introduction of Fe-Trensox (100 μM ; molar ratio of ligand:Fe of 2:1) in fresh medium, the recovery was observed after 3 days.

Plant cell cultures

Acer pseudoplatanus L. cells were grown in suspension in liquid medium defined by Lamport and modified by Lescure [23]. Iron is provided by 10 μM Fe-EDTA complex (molar ratio of ligand:Fe of 1:1). Material and culture medium were sterilized by autoclaving at 121 °C for 15 min or by filtration (0.22 μm -pore-size). Cell suspensions (100 ml) are cultivated in 300 ml Erlenmeyer flasks and are corked with cellulose stoppers and aluminium foil. Cells are maintained in suspension at 90 rev./min and 25 °C.

Glycine max cell cultures (cultivar Mandarin, line Sbe4) were grown at 30 °C in the dark in B5 medium with 5 μM 2,4-dichlorophenoxyacetic acid, 0.2% casein hydrolysate and 100 μM Fe-EDTA (sodium salt) as described by Leguay and Jouanneau [24]. To obtain cells grown with limiting, normal and superabundant nutritional iron (see Figure 6), cells were grown for 7 days in media containing respectively 5, 50 or 500 μM iron supplied as Fe-EDTA. Then the cells were grown for 3 days in Fe-free medium and, when in exponential growth phase, were supplied with 5, 50, or 500 μM radiolabelled iron as Fe-citrate. After 1, 3, 6, 12 or 24 h, apoplastic iron was eliminated by four successive washing and draining steps, on nylon grids by centrifugation at 70 g for 15 s. The iron content and the specific radioactivity of iron in cells was measured; ^{59}Fe labelling of the cells was quantified by grinding the cells mechanically and by scintillation counting. Native protein extraction, gradient gel electrophoresis on 4–20% PAGE, autoradiography, quantification of labelled proteins in autoradiograms were done as described in [2]. Densitometric scanning of autoradiograms was done using a CCD camera system ('the imager' Apligene) and quantified using the N.I.H. image 1.44 b11 computer program. Plant ferritins used as standards for electrophoretic mobility were purified as described in [25]. They were characterized by their subunit composition, iron content, absorbance spectrum (as in [26]), and their *in vitro* labelling with ^{59}Fe was performed as previously described [27].

^{59}Fe incorporation (see Table 1) was performed in 12-well plates with lids (Corning Glass Works, New York), in axenic conditions, under usual culture conditions: 30 °C, 100 rev./min rotating agitation, in the dark, using 3 ml cell suspensions containing 300 mg of fresh cells. Results correspond to the mean of three repeats.

Figure 2 Absorption spectra of (a) Fe(III)-Trensox and Fe(II)-Trensox in aqueous medium at pH 7.5 and (b) mixtures of Fe(III) and Trensox in increasing molar ratios from 0.1 to 1, in increments of 0.1

(a) Peaks are at 446 and 605 nm for the ferric complex; shoulder at 460 and peaks at 440 and 595 nm for the ferrous form. (b) Arrows indicate increasing increments.

Trensox and Fe-Trensox concentrations in cell culture medium

Five cultures of *Acer pseudoplatanus* L. were grown in classical medium containing 10 μ M Fe-Trensox under iron-buffered conditions (molar ratio Trensox:Fe of 2:1). Each day, an aliquot (5 ml) of the decanted culture medium was taken under sterile conditions and immediately frozen. In used supernatants, Trensox could not be detected by its absorbance at its isosbestic points (323 and 395 nm, Figure 2b) because of the interference of absorbing material released by plant cells. Fe-Trensox concentration was estimated at 561 nm [maximum absorbance wavelength of Fe(III)-Trensox in the culture medium]. In order to estimate the free ligand concentration in the nutrient medium, the excess of ligand was saturated with Fe(III); to avoid iron hydroxide precipitation, the pH of the medium was first lowered to 3 with 1% HCl. The saturation was reached by addition of an iron solution [$\text{Fe}_2(\text{SO}_4)_3$ in 0.1% HCl], then the pH was raised to 6.5 with Tris-maleate buffer. After agitation, the absorbance was read at 561 nm as for Fe(III)-Trensox.

Radical damage of DNA

The activity of Fenton-type reactions was measured on supercoiled circular DNA as the target molecule for hydroxyl radicals [28]. Radical damage to DNA induces changes in its electrophoretic mobility: the first nick in one strand of supercoiled

DNA relaxes the molecule and slows down its electrophoretic migration on agarose gels. The following nicks will not modify the migration pattern until two neighbouring nicks on opposite strands linearize the closed circular molecule. Further pairs of neighbouring nicks will generate linear fragments. The peroxidation of DNA by Fenton reactions was estimated by the percentage of supercoiled, relaxed, linear, or fragmented DNA after the reaction.

Fe(III) solutions were prepared with $\text{Fe}_2(\text{SO}_4)_3$. Fe(II) solutions were prepared by dissolution of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in N_2 -saturated water. The Fenton reaction was tested in a 35 μ l assay containing 60 ng of Bluescript plasmid DNA (2.8 kb), 1 mM H_2O_2 , 10 mM Tris-maleate buffer (pH 6.5), 100 μ M Fe(II) or Fe(III) and 200 μ M ligand (citrate, EDTA, or Trensox). When used, ascorbate was present at 400 μ M. After 60 min, the reaction was stopped by addition of 5 μ l of an electrophoresis buffer [0.05% FZ, 2 M urea, 50% (v/v) glycerol, 0.01% Bromophenol Blue]. After electrophoresis on 1% agarose gels in TBE buffer, fluorometry of DNA-ethidium bromide was recorded on an even UV transilluminator and using a CCD camera system ('the imager' Apligene). Each conformational band of DNA was quantified using the N.I.H. image 1.44 b11 computer program.

RESULTS

Physicochemical characterization of Trensox

Spectrophotometric properties

The UV-visible absorption spectra of Fe(III)-Trensox and Fe(II)-Trensox in distilled water at pH 7.5 (Tris-maleate buffer 50 μ M) are shown in Figure 2(a). Trensox exhibits a maximum absorbance at 343 nm (ϵ 11 500 $\text{M}^{-1} \cdot \text{cm}^{-1}$) (Figure 2b). The green ferric complex of Trensox has two absorption bands at 440 nm (ϵ 5200 $\text{M}^{-1} \cdot \text{cm}^{-1}$) and 595 nm (ϵ 5200 $\text{M}^{-1} \cdot \text{cm}^{-1}$). The ferrous complex presents one absorption band at 576 nm with ϵ 1700 $\text{M}^{-1} \cdot \text{cm}^{-1}$ (Figure 2a). Two isosbestic points at 323 nm and 395 nm appear during the Fe(III) saturation of Trensox, which can be used for the spectrophotometric determination of Trensox independently of its iron load (Figure 2b).

Fluorescence

The chelating agent Trensox alone is fluorescent in aqueous solution around pH 7, whereas the Fe(III) complex of Trensox does not present any fluorescence. The fluorescence spectrum (Figure 3) presents an excitation maximum at 343 nm and a maximum emission at 473 nm.

Redox potential of Fe(III)-Trensox/Fe(II)-Trensox

In aqueous solution, Fe(III)-Trensox/Fe(II)-Trensox exhibits a stable redox potential of +0.087 V versus the normal hydrogen electrode (NHE) between pH 4.2 and pH 10.5. The system is rapid, quasi-reversible and monoelectronic (results not shown).

Stability constants of the Fe(III) and Fe(II) complexes of Trensox

The spectrophotometric behaviour of the ferric complex was investigated as a function of pH in the range 1–11. This allowed us to determine the stepwise deprotonation equilibria in these complexes upon increasing pH. The refinement of the data with the LETAGROP program has provided the more probable equilibria in solution upon increasing pH leading to the value of 30.9 for $\log \beta_{110}$ (β_{110} is related to the equilibrium $\text{mM} + \text{IL} + \text{hH} \rightleftharpoons \text{M}_m\text{L}_l\text{H}_h$). The same value of $\log \beta_{110}$ was also determined from the spectrophotometric competition with EDTA.

Figure 3 Excitation and re-emission spectra of Trensox in aqueous solution at pH 7.5

Taking into account the redox potentials for the Fe(III)–Trensox/Fe(II)–Trensox ($E_c = +0.087$ V versus NHE) and Fe(III)/Fe(II) ($E_r = +0.77$ V versus NHE), the $\beta_{110[\text{Fe(II)-L}]}$ can be calculated at 298 K from $E_b = E_c + 0.06 \log(\beta_{110[\text{Fe(III)-L}]} / \beta_{110[\text{Fe(II)-L}]})$ yielding $\log \beta_{110[\text{Fe(II)-L}]} = 19.3$.

Since the β_{110} values do not reflect the competition of H^+ for the ligand, the pFe values are usually used in the comparison of the biological efficiency of ligands. They are defined in conventional conditions [29] ($\text{pFe} = -\log[\text{Fe}]$ at pH 7.4; [ligand] = 10^{-5} M; $[\text{Fe}] = 10^{-6}$ M.). These values for Trensox are: $\text{pFe(III)} = 29.5$ and $\text{pFe(II)} = 17.9$.

Cupric ions compete for the ligand Trensox with ferric ions (partial exchange) as shown spectrophotometrically. It must be emphasized that the same is true for mugineic acid, a major phytosiderophore [3]. On the other hand, Zn^{2+} , Mg^{2+} , Ca^{2+} , Ni^{2+} and Al^{3+} do not displace Fe^{3+} from ferric Trensox.

Photoreduction of Fe(III) complexes

The photoreductibility of Fe(III) complexes was tested in the presence of FZ to allow Fe(II) quantification and to prevent its further oxidation. Under visible light, only Fe(III)–citrate developed a fast photoreduction. The differential photosensitivity of Fe(III)–citrate, Fe(III)–EDTA and Fe(III)–Trensox was shown in UV light at 366 nm. Figure 4 shows the kinetics of photoreduction of the ferrisiderophores: Fe(III)–Trensox does not generate Fe(II) when exposed to UV light. This was also controlled in the absence of FZ by absorption spectra of Fe–Trensox after irradiation.

Iron nutrition and metabolism of ^{59}Fe

Growth and iron nutrition of plants

Since recent information underlines the unpredictable cooperation of micro-organisms in the uptake of iron by plants [13], we have studied iron nutrition in axenic conditions, i.e. using axenic seedlings in sterile nutrient medium. *Arabidopsis thaliana*, maize or tobacco seedlings, when grown in iron-free solidified medium, develop a strong chlorosis. *Arabidopsis thaliana* and tobacco seedlings developed actively when supplied with Fe–EDTA and Fe–Trensox; young *Arabidopsis thaliana* seedlings

Figure 4 Photoreduction of Fe(III) complexes of Trensox, citrate and EDTA in UV light (366 nm)

The reduction is measured by the absorbance of Fe(II)–FZ at 562 nm. Key to symbols: ○, citrate (pH 6); ●, citrate (pH 4.8); □, EDTA (pH 6); ■, EDTA (pH 4.8); △, Trensox (pH 6); ▲, Trensox (pH 4.8).

provided with Fe–Trensox displayed a faster growth and greening in the first 2 weeks as compared with controls fed with Fe–EDTA. Chlorotic *Arabidopsis thaliana* or pea seedlings recovered after 3 days and 8 days respectively, when the nutrient solution was supplemented with Fe–Trensox (molar ratio Trensox:Fe of 2:1). In all species, regreening began with the youngest leaves and spread down to older ones.

In *Zea mays*, on the contrary, Fe–Trensox induced an iron chlorosis more rapidly than observed in the iron-free medium control.

Growth and iron nutrition of cell suspensions

The growth rate of cell suspensions of *Acer pseudoplatanus* was compared in the absence of iron or when provided using Fe–EDTA and Fe–Trensox (Figure 5). The growth rate of the cells supplied with Trensox is slightly higher than when supplied with EDTA.

Suspensions of *Acer pseudoplatanus* cells were supplied with ^{59}Fe to allow the measurement of iron uptake for 17 h. After the labelling, cells were washed four times to release the apoplastic iron. The results are shown in Table 1. When supplied with Trensox, the activity of iron uptake was intermediate between that of citrate and EDTA. In the presence of FZ, the iron nutrition was only reduced and not suppressed.

Kinetics of labelling of iron proteins

To identify iron proteins which reflect an actual metabolism of newly incorporated ^{59}Fe , a preliminary experiment was done using Fe(III)–citrate, on cells previously fed at three nutritional levels of iron. The results are shown in Figure 6. The bulk of the iron uptake occurred in less than 6 h. ^{59}Fe bound to the native proteic fraction was analysed using a 5–20% gradient gel of polyacrylamide and autoradiography. Ferritin was identified on the autoradiogram by a co-migrating sample of purified pea

Table 1 Intracellular incorporation of ^{59}Fe into cell suspensions of *Acer pseudoplatanus* and into ferritins, using EDTA, Trensox and citrate as iron ligands, in the presence or in the absence of FZ, for 17 h

Chelating agent Presence of FZ...	Incorporation of ^{59}Fe into cells ($\mu\text{M/g}$)		Incorporation of ^{59}Fe into ferritins (relative absorbance units)	
	—	+	—	+
EDTA	2.65 ± 0.18	1.50 ± 0.07	88	71
Trensox	7.51 ± 0.48	3.53 ± 0.10	175	126
Citrate	18.70 ± 1.70	9.78 ± 0.82	308	252

Figure 5 Kinetics of growth of *Acer pseudoplatanus* L. cell suspensions (mg of drained cells per ml of medium)

Mean values from five independent repeats are shown. ●, No iron; ■, Fe(III)–EDTA; ▲, Fe(III)–Trensox.

ferritins which had been radiolabelled *in vitro* (Figure 6, lane 14). The only heavily labelled protein in all chronological and physiological conditions was ferritin. In normal or plethoric iron nutritional conditions (Figure 6: lanes 6 to 13), the label remained in ferritin for 24 h. In prestarved cells (lanes 1 to 5), ferritin labelling reached a maximum after 3 h, but after 6 h, the cellular specific radioactivity of iron being stable, the labelling of ferritin decreased to the benefit of the other iron proteins, and represented a decreasing part of the labelling of total iron proteins (82 %, 50 %, 19 % at 1, 3 and 24 h respectively). This shows that in prestarved cells, ferritin functions as an intermediate in the dispatching of iron, while it only accumulates iron in normal or abundant iron nutritional conditions. Ferritin appears to be the first and the main protein involved in the early steps of iron metabolism.

In order to compare the metabolism of iron from different chelates, cell suspensions were fed with Fe–citrate, Fe–EDTA or Fe–Trensox for 17 h, then washed and analysed as above. The only ^{59}Fe -labelled protein was ferritin, as expected, when cells have not been prestarved for iron. Table 1 shows the labelling of ferritin iron. When iron is carried by Fe–Trensox, the radio-labelled iron in ferritins was found to be intermediate between the labelling of ferritin when iron was carried by Fe–EDTA or Fe–citrate. The nutritional ligand does not seem to influence the further metabolism of the internalized iron.

Figure 6 Incorporation of ^{59}Fe into ferritins

Kinetics of incorporation of ^{59}Fe from citrate into iron proteins of soya cells. Cells were cultivated for 7 days at three levels of iron nutrition: 5, 50 and 500 μM Fe–EDTA, and transferred to an iron-free medium for 3 days. Then they received 5, 50, or 500 μM iron as ^{59}Fe (III)–citrate (L:Fe = 2:1). Autoradiograms of ^{59}Fe proteins: lanes 1 to 5, preculture using 5 μM EDTA (1, 3, 6, 12 and 24 h labelling respectively); lanes 6 to 9, preculture using 50 μM EDTA (3, 6, 12 and 24 h labelling); lanes 10 to 13, preculture using 500 μM EDTA (3, 6, 12 and 24 h labelling); lane 14, pure pea seed ferritin labelled with ^{59}Fe .

Kinetics of uptake of Trensox and Fe–Trensox by plant cell cultures

In an initial approach to the mechanism of iron uptake by plant cells, daily measurements of Trensox and Fe–Trensox concentrations were made on the nutrient solution after separation from the cells (Figure 7). Fe(III)–Trensox disappeared quickly. In order to saturate and quantify the free ligand, Fe(III) was added to the samples of nutrient solution. The amounts of the complex and ligand were found to be stable, showing that Trensox was not internalized by the plant cells. Attempts to detect the fluorescence of Trensox within the cytoplasm of the cells using a confocal microscope were unsuccessful. Some fluorescence was found in the apoplast (cell walls) of cells grown with Fe–Trensox (50 μM Fe, molar ratio Trensox:Fe of 2:1).

Chemical and photochemical Fenton-type radical damage

In order to verify whether the photoreduction was followed by a subsequent peroxidation due to the Fenton reaction, the iron

Figure 7 Concentration of Trensox (○) and Fe-Trensox (●) in culture cell medium

Figure 8 Evaluation of ferrisiderophores as Fenton reagents

The oxidation of Fe(II) complexes generates hydroxyl radicals, introducing nicks that relax then degrade supercoiled DNA.

complexes of citrate, EDTA and Trensox were checked as Fenton reagents during their exposure to visible light.

The oxidative damage was studied using DNA as the target molecule: the addition of ferrous iron to DNA resulted in a peroxidation. The most sensitive modification of the electrophoretic mobility is obtained in the presence of H_2O_2 for the supercoiled-relaxed transition.

The reactions were made simultaneously and comparisons were considered within the same series. Results are shown in Figure 8. The radical damage produced by ferric complexes in the dark (Figure 8a) and in the light (Figure 8b) for the same amount of iron, confirm the phototoxicity of Fe(III)-citrate and of Fe(III)-EDTA, and the innocuity of Fe(III)-Trensox, which is not photoreduced. To evaluate the possible behaviour of Fe(II)-Trensox as a Fenton reagent, we have prepared ferrous complexes with the three ligands and compared the damage done to the DNA by each of them. The results are shown in Figure 8(c). They show that the radical damage caused by the oxidation of Fe(II) complexes in iron-buffered conditions decreases from Fe(II)-citrate to Fe(II)-Trensox in the order citrate $\gg$ EDTA $>$

Trensox, damage with Trensox being close to the 'iron-free' control.

In separate experiments, when the production of ferrous complexes was achieved by the reduction of ferric complexes by ascorbate, citrate and EDTA produced active damage to DNA (Figure 8d). Using Trensox, a very slow radical damage was observed, slightly lower than with the 'iron-free' control experiment. The absence of damage was not due to the absence of reduction: ascorbate is able to reduce Fe(III)-Trensox quantitatively in less than 1 min (results not shown).

It is therefore clear that Fe(II)-Trensox does not induce radical damage with H_2O_2 , neither when introduced into the reaction nor when produced *in situ* by reduction of Fe(III)-Trensox by ascorbate.

DISCUSSION

Bioavailability and toxicity

The bioavailability of a siderophore for plant cells can be characterized by the rate of its iron intracellular absorption. Ferritins are shown to be an early and important step of iron metabolism. Using Trensox, an efficient iron nutrition was obtained in axenic conditions with *Arabidopsis thaliana*, *Pisum sativum*, *Nicotiana tabacum* and *Acer pseudoplatanus* cell suspensions, but not with *Zea mays*. This is an encouraging observation for an eventual use of Fe-Trensox in non-axenic conditions, i.e. in agriculture, for a selective support to iron-deficient dicotyledonous crops.

The lipophilicity of chelates due to 8-hydroxyquinoline ligands has been shown to allow an uncontrolled transmembrane traffic of iron, and consequently to induce cellular damage [30,31]. Sulphonation of the molecule favours its water solubility: the water/chloroform partition ratio for the sulphonated complex (Fe-Trensox) is 1:6. Water solubility allows availability to the plant and prevents transmembrane diffusion. Preliminary studies on Trensox as a clinical iron-detoxifying agent have shown its efficiency and lack of toxicity [32].

Photoactivity is also an important biochemical parameter in plant physiology. The photoreduction of ferric citrate [33] and other iron complexes, such as ferritin [34], may participate in the filling of ferrous pools and favour the biochemical mobility of iron [35]. Nevertheless, when applied extracellularly, the photoreduction of synthetic siderophores is an undesired property since light-induced Fenton reactions cause the self-destruction of the complex and may result in foliar burns and necrosis. The ferric complex of Trensox is not photoreducible. Moreover we have shown that ferrous Trensox is not a Fenton reagent, therefore almost no radical damage on DNA is observed in the light (Figure 8). The resistance to light of Fe(III)-Trensox should make possible surface treatments to plants and soils and its use in foliar sprays for the correction of chlorosis.

Chelate dissociation, affinities for ferrous and ferric ligands and redox potentials

The total concentration [Trensox + Fe-Trensox] maintained in the nutrient solutions (Figure 7) implies that the dissociation of the complex occurs extracellularly. The decrease in absorbance of Fe(III)-Trensox observed in the nutrient solution of cell cultures on the first days may be due either to the direct uptake of Fe(III), or to extracellular reduction of Fe(III)-Trensox, or both. In this alternative, the relative importance of reduction and Fe(III) uptake could not be determined colorimetrically because of the lack of an Fe(II) ligand able to quantitatively displace Fe(II) from Fe(II)-Trensox.

The general mechanism which has been described as 'strategy 1' for the uptake of iron from abiotic siderophores [17] involves a reduction step. The high value of the redox potential (+0.087 V versus NHE) of Fe-Trensox, as compared with those observed for mugineic acid (-0.102 V versus NHE) and bacterial siderophores [4] implies that Fe(III)-Trensox is much more easily reducible. Since evidence for a transmembrane reduction system in this cell line has been obtained [36], the reduction of the ferric form should not be a limit to iron uptake from Fe-Trensox. Nevertheless, the affinity of Trensox for Fe(II) ($\log\beta_{110[\text{Fe(II)}-L]} = 19.3$; $\text{pFe(II)} = 17.9$) is much higher than that of EDTA and citrate ($\log\beta_{110[\text{Fe(II)}-L]} = 14.3$ and 4.2 respectively); it is also higher than that of the known iron carriers found in plants such as mugineic acid ($\log\beta_{110[\text{Fe(II)}-L]} = 10.1$ [3]). Despite its high affinity for Fe(II), Fe-Trensox is an efficient iron donor.

So Trensox, which is a strong chelating agent both for Fe(III) ($\text{pFe} = 29.5$; $\log\beta_{110[\text{Fe(III)}-L]} = 30.9$) and for Fe(II), was found to be more efficient in iron nutrition and metabolization than EDTA ($\log\beta_{110[\text{Fe(III)}-L]} = 25$). The inability of Trensox to provide iron to *Zea mays* may seem unexpected since the affinity constant of its natural siderophore, mugineic acid ($\log\beta_{110[\text{Fe(III)}-L]} = 32.5-33.3$) [37], is higher than that of Trensox. Thus, the efficiency of Fe-Trensox in iron nutrition is not simply correlated to its stability constants. A more confident prediction of expected efficiencies of phytosiderophores should be facilitated if data on pFe(III) and pFe(II) at soil pH values were available in addition to the stability constants [28a].

An important objection in the single use of stability constants, as an image of the bioavailability of iron, is that they are related to equilibrium states. If iron nutrition were thermodynamically controlled, our results would mean that the affinity ($\log\beta_{110}$) of the membrane iron transporter is higher than 30.9 for Fe(III) and/or 19.3 for Fe(II). Since our results do not fit the predictions of thermodynamic data, we underline the necessary consideration for kinetic aspects involved in nutrition: the dynamic flow of biochemical iron exchange may not allow the achievement of thermodynamic equilibria.

This work is supported by the Centre National de la Recherche Scientifique (URA 332 and GDR 1154) and the Conseil Régional de la Région Rhône Alpes. We are grateful to Dr. P. Michon and Dr. E. Saint Aman for their help on fluorescence and electrochemical measurements. We thank Professor A. L. Crumbliss for very helpful discussions and comments.

REFERENCES

- Crowley, D. E., Wang, Y. C., Reid, C. P. P. and Szaniszlo, P. J. (1991) *Plant Soil* **130**, 179-198
- Neema, C., Laulhère, J. P. and Expert, D. (1993) *Plant Physiol.* **102**, 967-973
- Nomoto, C., Sugiura, Y. and Takagi, S. (1987) in *Iron Transport in Microbes, Plants and Animals* (Winkelmann, G., Van der Helm, D. and Neilands, B., eds.), pp. 401-424. VCH Verlagsgesellschaft, Weinheim, Germany
- Pierre, J. L. and Fontecave, M. (1992) *L'Actualité Chimique*, 297-302
- Crichton, R. R. (1991) in *Inorganic Biochemistry of Iron Metabolism*, E. Horwood Ltd., Chichester, England
- Bar-Ness, E., Hadar, Y., Chen, Y., Shanzer, A. and Libman, J. (1992) *Plant Physiol.* **99**, 1329-1335
- Jurkevitch, E., Hadar, Y., Chen, Y., Chino, M. and Mori, S. (1993) *Bio Metals* **6**, 119-123
- Bar-Ness, E., Hadar, Y., Chen, Y., Römhelt, V. and Marschner, H. (1992) *Plant Physiol.* **100**, 451-456
- Von Wiren, N., Römhelt, V., Morel, J. L., Gucker, A. and Marschner, H. (1993) *Soil Biol. Biochem.* **25**, 371-376
- Baker, R., Elad, Y. and Sneh, B. (1986) in *Iron, Siderophores and Plant Diseases*, Series A Life Sciences (Swinburne, T. R., ed.), vol. 117, pp. 77-84, Plenum Press, New York
- Becker, J. O., Hedges, R. W. and Messens, E. (1986) in *Iron, Siderophores and Plant Diseases*, Series A Life Sciences (Swinburne, T. R., ed.), vol. 117, pp. 61-70, Plenum Press, New York
- Hadar, Y. H., Jurkevitch, E. and Chen, Y. (1986) in *Iron, Siderophores and Plant Diseases*, Series A Life Sciences (Swinburne, T. R., ed.), vol. 117, pp. 43-48, Plenum Press, New York
- Crowley, D. E., Römhelt, V., Marschner, H. and Szaniszlo, P. J. (1992) *Plant Soil* **142**, 1-7
- Cohen, G. (1985) in *CRC Handbook of Methods for Oxygen Radical Research* (Greenwald, R. R., ed.), pp. 55-64, CRC Press Inc., Boca Raton, Florida
- Dalton, C., Iqbal, K. and Turner, D. (1983) *Physiol. Plant* **57**, 472-476
- Chaney, R. L., Brown, J. C. and Tiffin, L. O. (1972) *Plant Physiol.* **50**, 208-213
- Römhelt, V. (1987) in *Iron Transport in Microbes, Plants and Animals* (Winkelmann, G., Van der Helm, D. and Neilands, B., eds.), pp. 353-374, VCH Verlagsgesellschaft, Weinheim, Germany
- Baret, P., Caris, C., Laulhère, J. P. and Pierre, J. L. (1993) *Fr. Patent No.* 93 13310
- Tiffin, L. O. (1966) *Plant Physiol.* **41**, 510-514
- Serratrice, G., Mourral, C., Zeghli, A., Beguin, C., Baret, P. and Pierre, J. L. (1994) *New J. Chem.* **18**, 749-758
- Sillen, L. G. and Warnqvist, B. (1968) *Arkiv Kemi* **31**, 315-339
- Murashige, T. and Skoog, F. (1962) *Plant Physiol.* **15**, 473-497
- Lescure, A. M. (1966) *Physiol. Veg.* **4**, 365-378
- Leguay, J. J. and Jouanneau, J. P. (1987) *Dev. Gene* **8**, 351-364
- Lescure, A. M., Massenet, O. and Briat, J. F. (1990) *Biochem. J.* **272**, 147-150
- Laulhère, J. P., Lescure, A. M. and Briat, J. F. (1988) *J. Biol. Chem.* **263**, 10289-10294
- Laulhère, J. P. and Briat, J. F. (1993) *Biochem. J.* **290**, 693-699
- Toyokuni, S. and Sagripanti, J. L. (1992) *J. Inorg. Biochem.* **47**, 241-248
- Harris, W. R., Raymond, K. N. and Weitz, F. L. (1981) *J. Am. Chem. Soc.* **103**, 2667
- Balla, G., Vercellotti, G. M., Eaton, J. W. and Jacob, H. S. (1990) *J. Lab. Clin. Med.* **116**, 546-554
- Kontoghiorghes, G. J. (1988) *Inorg. Chim. Acta* **151**, 101-106
- Lescoat, G., Chenoufi, N., Zanninelli, G. et al. (1994) *Hepatology* **20**, 182A
- Brown, J. C., Foy, C. D., Bennett, J. H. and Christiansen, M. N. (1979) *Plant Physiol.* **63**, 692-695
- Laulhère, J. P., Labouré, A. M. and Briat, J. F. (1990) *Biochem. J.* **269**, 79-84
- Allnutt, F. C. T. and Bonner, W. D., Jr. (1987) *Plant Physiol.* **85**, 751-756
- Blein, J. P., Canivenc, M. C., De Cherade, X., Bergon, M., Calman, J. P. and Scalla, R. (1986) *Plant Sci.* **4**, 77-85
- Murakami, T., Ise, K., Hayakawa, M., Kamei, S. and Takagi, S. (1989) *Chem. Lett.* 2137-2140