The determination of ferric iron in plants by HPLC using the microbial iron chelator desferrioxamine E

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Abstract

Common methods for plant iron determination are based on atomic absorption spectroscopy, radioactive measurements or extraction with subsequent spectrophotometry. However, accuracy is often a problem due to background, contamination and interfering compounds. We here describe a novel method for the easy determination of ferric iron in plants by chelation with a highly effective microbial siderophore and separation by high performance liquid chromatography (HPLC). After addition of colourless desferrioxamine E (DFE) to plant fluids, the soluble iron is trapped as a brown-red ferrioxamine E (FoxE) complex which is subsequently separated by HPLC on a reversed phase column. The formed FoxE complex can be identified due to its ligand-to-metal charge transfer band at 435 nm. Alternatively, elution of both, DFE and FoxE can be followed as separate peaks at 220 nm wavelength with characteristic retention times. The extraordinarily high stability constant of DFE with ferric iron of $K = 10^{32}$ enables extraction of iron from a variety of ferrous and ferric iron compounds and allows quantitation after separation by HPLC without interference by coloured by-products. Thus, iron bound to protein, amino acids, citrate and other organic acid ligands and even insoluble ferric hydroxides and phosphates can be solubilized in the presence desferrioxamine E. The "Ferrioxamine E method" can be applied to all kinds of plant fluids (apoplasmic, xylem, phloem, intracellular) either at physiological pH or even at acid pH values. The FoxE complex is stable down to pH 1 allowing protein removal by perchloric acid treatment and HPLC separation in the presence of trifluoroacetic acid containing eluents.

Abbreviations: DFB – desferrioxamine B; DFE – desferrioxamine E; FoxB – ferrioxamine B; FoxE – ferrioxamine E; HPLC – high performance liquid chromatography

Introduction

Early efforts to relate lime-induced chlorosis leaf symptoms to the amount of leaf iron yielded variable results. Several investigators failed to find a consistent correlation between chlorophyll and iron content in leaves and evidence that chlorotic leaves often contained as much or even more iron than green leaves has been reported (Abadia 1992). A distinction between active and inactive leaf iron has been suggested, a phenomenon known as the 'chlorosis paradox' (Morales *et al.* 1998, Römheld 2000, Kosegarten *et al.* 2001). Methods to determine metabolically active

iron by atomic absorption spectroscopy using fresh, frozen or oven-dried leaf tissue have been used with varying success (Abadía *et al.* 1984; Pierson & Clark 1984). Although differences in apoplasmic and xylem fluid Fe concentrations according to variable plant Fe status have been observed (López-Millán *et al.* 2000, 2001), there is very little information available concerning the Fe concentration of such fluids to use it as a means of defining the iron status of plants grown under low-iron and high-iron stress. Most Fe(II)-specific chelators such as phenathroline derivatives (BPDS), ferrozine or 2,2′ bipyridyl require prior reduction and thus cannot distinguish between ferric and ferrous iron. Mössbauer spectroscopy is the method of choice when oxidised and reduced iron compounds need to be determined, although its sensitivity is not very high and large amounts of samples are required. Mössbauer spectroscopy, revealed that iron predominantly occurred in the ferric form in duckweed (*Lemna gibba* L.*)*, stock plant (*Matthiola incana* (L.) R.Br.) and soybean (*Glycine max* (L.) Merr.) leaves, while some minor ferrous component was observed in pea (*Pisum sativum* L.) leaves (Goodman & DeKock 1982). Most iron in veins and interveinal areas of wild-type tomato leaves (*Lycopersicon esculentum* Mill.) was present in the ferric form (Yoshimura *et al.* 2000). Spectra taken from rice plants (*Orizya sativa* L.) showed that leaf iron existed as Fe(III) and that the level of Fe(II), was so low that it could not be detected via Mössbauer spectroscopy (Kilcoyne *et al.* 2000).

Iron taken up by root cells is translocated in the xylem to aerial plant parts as a ferric citrate complex (Tiffin 1966). The Fe(III)–citrate complex arrives at the leaf apoplast where it is reduced by a ferric chelate reductase at the cell plasma membrane (Brüggemann *et al.* 1993) and it is consequently transported into the leaf mesophyll via an iron-regulated metal transporter (Eide *et al.* 1996).

Methods to correct iron chlorosis have received incresing attention during recent years. Investigations concerning Fe-chelate application to chlorotic plants have been started 50 years ago (Heck & Bailey 1950). Soil application of synthetic Fe–EDTA or Fe–EDDHA is a common agricultural practice which may not be economical on large scale corrections of plant iron deficiency. Another key factor which should be considered when synthetic metal chelators are used in agriculture, is a potential array of environmental problems related to persistence and waste and ground water metal enrichment. In this sense, Hördt *et al.* (2000) showed that plants were able to utilise Fe supplied as mono- and dihydroxamate siderophores which do not pose a threat to the environment.

Application of iron sprays to chlorotic plants is another practice when soils inducing plant Fe deficiencies are present. However, many factors related to leaf penetration of the sprayed Fe-containing solution, Fe translocation and the ultimate cell Fe uptake are still unsolved. Evaluation of foliar applied Fe-containing compounds is generally accompanied by measuring leaf chlorophyll increase over time (Fernández *et al.* 2003). The FoxE method has already been successfully applied to iron-application studies with plants and to evaluate penetration of different foliar Fe sprays (Fernández *et al.* 2005). According to these findings, the Fox E method is especially suitable to measure the iron content of apoplastic fluids from leaves at different time intervals during treatment with different foliar iron sprays. For the purpose of solubilizing and extracting Fe from plant fluids, the microbial iron chelator, desferrioxamine E, was selected being both acid resistant and possessing an extraordinary high formation constant ($K \sim 10^{32}$). Ferrioxamines are a large hydroxamate siderophore family produced by *Streptomyces* and several entobacterial genera. These compounds are linear or cyclic trihydroxamates with repeating units of 1-amino-5-hydroxylaminoalkane (pentane or butane) and succinic or acetic acid (Winkelmann & Drechsel 1997). However, while previous methods used the linear desferrioxamine B (DFB), the present Fe determination procedure employs the cyclic desferrioxamine E (DFE) which has a 100-fold higher formation constant with ferric iron and better chemical stability (Boukhalfa & Crumbliss 2002). Thus, the use of DFE as a means to determine the amount of soluble iron in biological fluids by HPLC has the advantage of rapid conversion of all kinds of ionic and chelated iron into the ferric form of FoxE and the subsequent quantitation and separation from other chromophoric compounds present in biological fluids.

Materials and methods

Plant culture

Experiments were carried out with 1–2-months-old *Vicia faba* var. 'Marona seedlings' (15–20 seeds per pot), 4–7-months-old *Nicotiana tabacum* var. ''Virginia'' seedlings and 3-years-old *Citrus madurensis* cuttings (Fe-deficient plants) grown in sand culture (neutral quartz–sand). Seeds were germinated in a mist chamber. Experimental plants were grown at 25 °C under incandescent light, 16-h-light/8-h dark photoperiod and 60–80% relative humidity. Iron concentrations in the tissue fluid of pea (*P. sativum*) and tomato (*L. esculentum*) plants grown from seed in sand were also determined. Plants were watered daily with full-strength Arnon and Hoagland's solution with (pH 5.5) and without iron (pH 8 reached by addition of 10 mEq/l $NaHCO_3$) via the root. The composition of Arnon and Hoagland (1952)'s nutrient solution is 1.02 g/l KNO_3, 0.492 g/l $Ca(NO_3)_2$, 0.3 g/l $NH_4H_2PO_4$, 0.49 g/l $MgSO_4$ $7H_2O$, 2.86 mg/l H_3BO_3, 1.81 mg/l $MnCl_2$ $4H_2O$, 0.08 mg/l $CuSO_4$ $5H_2O$, 0.22 mg/l $ZnSO_4$ $7H_2O$, 0.09 mg/l H_2Mo O_4 (MoO_3 + H_2O) and 10.8 μM $FeSO_4$ $7H_2O$ when iron was added. Tobacco plants (3-months old) developed chlorosis 3 weeks after the onset of the pH treatment. Chlorotic leaves appeared in bean plants approximately 5 weeks after first watering with a pH 8 solution.

Sample preparation

Leaf fluid was obtained from leaves by centrifugation as described by Dannel *et al.* (1995) and modified by López-Millán *et al.* (2001). Young fully expanded leaves were excised at the base of the rachis with a razor blade. The terminal leaflet (having a longer petiole) was also excised and leaflets were fixed on a 0.18×0.01 m strip of Parafilm M (American National Can, Chicago, USA), with the cut rachis remaining out of the plastic foil. Thereafter, the foil bearing the leaflets was rolled and tightly secured with 2 or 3, 0.03×0.01 m Parafilm strips. The side showing the rachis ends of such roll was put into a 1.5 ml Eppendorf tube, the cap of which had been cut. The roll tightly fitted into the Eppendorf tube so that the cut rachis ends hang out of the plastic foil to allow fluid dropping into the tube. The leaf roll partially inserted into the Eppendorf tube was then placed into a 50 ml plastic test tube, which was also put into a 250 ml centrifugation bottle without cap. Leaf rolls arranged in such a manner were centrifuged at low speed ($2500 \times g$) for 15 min at 4 °C (Sorvall RC-2 B Superspeed centrifuge, Kendro Laboratory Products). The Eppendorf tubes contained leaf fluid, which has been associated with xylem sap (López-Millán *et al.* 2001). A further centrifugation step at $4500 \times g$ for 15 min at 4 °C yielded the apoplasmic fluid (López-Millán *et al.* 2001). Eppendorf tubes containing the resulting fluids were closed and stored at 4 °C or −70 °C for further HPLC analysis.

Iron determination by the ferrioxamine E method

Iron from plant fluids, was analyzed by HPLC after addition of desferrioxamine E (DFE) obtained from EMC micro-collections GmbH, Tübingen, Germany (Biophore Research Products, www.siderophores.com) Calibration curves were made spectrophotometrically (Ultrospec III, UV/VIS Spectrophotometer, Pharmacia) using the extinction coefficient $\varepsilon = 2750$ M^{-1} cm^{-1} at 435 nm and subsequent analysis by HPLC (Shimadzu HPLC System, Duisburg, Germany). Stock solutions of DFE were made by dissolving 10 μmol/ml in a water–methanol mixture (1:1), warming up the suspension until the solution is clear and adjusting the concentration in a photometer using the molar extinction coefficient. Samples were separated on a C18-reversed-phase (ReproSil-Pur 120, ODS-3, 5 μm, 4×250 mm, Dr Maisch, Ammerbuch, Germany) using a gradient of acetonitrile/water (6–40%) containing 0.1% trifluoroacetic acid (TFA) over 20 or 35 min and a flow rate 1 ml/min (Konetschny-Rapp *et al.* 1988). While at 435 nm detector wavelength the increase of the ferric form (FoxE) can be measured, at a detector wavelength of 220 nm both, the increase of the ferric and the decrease of the desferri- form (DFE) can be followed, allowing calculation of the amount of iron chelated Stock solutions of DFE (0.3, 0.5 and 0.7 mM) were prepared in water–methanol. Leaf fluid (10–60 μl) were mixed with 10 μl of a DFE stock solution and diluted as required. Excess DFE addition to samples did neither alter nor improve HPLC iron measurement. Leaf fluid samples containing DFE were incubated for 30 min at 60 °C and thereafter

centrifuged for 15 min at 14,000 rpm. prior to HPLC separation. Contact to any iron source was avoided at all stages and the HPLC system was thoroughly cleaned prior to sample FoxE determination.

Chlorophyll measurement

The chlorophyll status of leaves was measured as follows: Fresh tissue samples were frozen at $-70\ °C$ and thereafter ground on a porcelain mortar. Chlorophylls were extracted in 80% acetone and the concentration of chlorophyll *a* and *b* was calculated after spectrophotometric reading (664 nm for chlorophyll *a* and 647 nm for chlorophyll *b*) (Ultrospec III, UV/VIS Spectrophotometer, Pharmacia) (MacKinney 1941). Fresh weight of leaves providing the fluid was always determined and the volume of fluid extracted after leaf centrifugation was also recorded for quantitative calculation. Total iron determination from chlorophyll extracts of bean leaves (10 × 1 cm ∅ leaf discs weighing 0.1 g) was performed after evaporation to dryness. The remaining residue was dissolved in 150 μl 10% $HClO_4$, 100–200 μl H_2O_2 (to have a transparent solution) and distilled water to reach a final volume of 1500 μl. The evaporation glass containing this solution was closed with a cap prior to incubation at 65 °C for 1–2 h. The spectrum of fresh and dried leaves between 360 and 750 nm was obtained (SP8-300 UV/VIS Spectrophotometer, Pye Unicam) from 0.5 g fresh-green and dried-chlorotic *C. madurensis* tissue, after extraction in 20 ml water or 20 ml 1 N HCl and shaking for 3 h.

Results

As shown in Figures 1 and 2 the HPLC-chromatograms of the ferric complex, FoxE, and the iron-free chelator, DFE, revealed well separated peaks using a gradient of 6–40% acetonitrile ($+0.1\%$ TFA). While at 435 nm (Figure 1) only the iron complexes can be detected, using a wavelength at 220 nm (Figure 2) allows measurement of both the ferric and the iron-free chelator. Thus, Fe binding by DFE yields the FoxE method suitable for Fe determination, since distinct and well-separated peaks at defined retention times are obtained. The absolute amount of iron injected onto the column
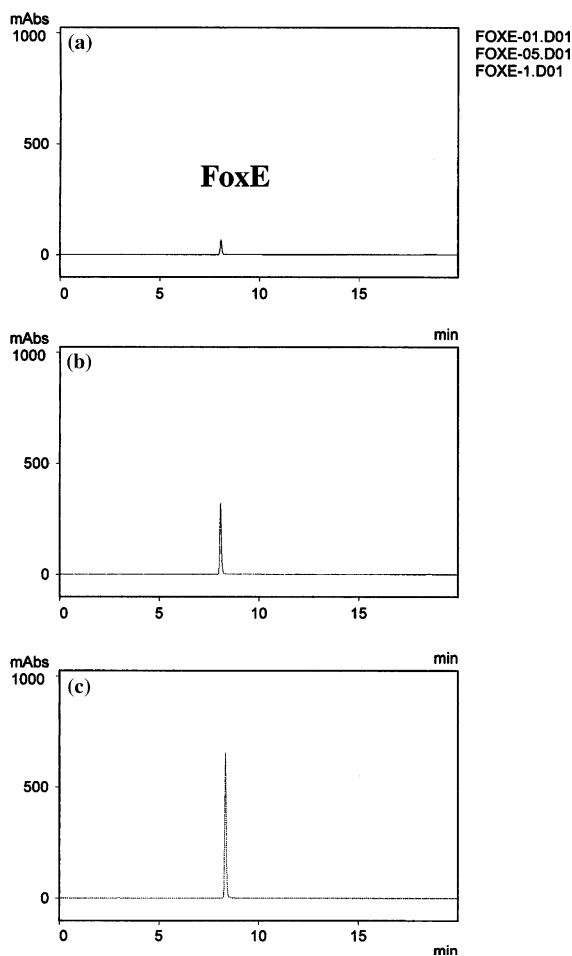

Figure 1. HPLC chromatogram of ferrioxamine E (ferric complex) recorded at a detector wavelength of 435 nm. (a) 0.1 mM, (b) 0.5 mM, (c) 1 mM corresponding to (a) 2 nmol, (b) 10 nmol, (c) 20 nmol, respectively per peak (attenuation 12).

was 2×10^{-9} mol of FoxE (Figure 1a). By changing the attenuation of the detector, the sensitivity can be further increased giving peak signals even at 1×10^{-11} mol. A major concern with the FoxE method was the question whether or not compounds present in plant fluids would interfere with the HPLC separation of FoxE. Therefore, plant fluids were mixed with DFE and run together in the HPLC (Figure 3). Chromatograms corresponding to Fe-sufficient bean fluid (2500 × *g*) were recorded as follows: Figure 3a) leaf fluid with no DFE addition. A peak appearing at 9.7 min related to endogenous leaf compounds is observable, Figure 3b) leaf fluid plus 115 μM DFE. Peaks corresponding to FoxE and DFE can be seen after 9.1 and 10.6 min, respectively, and Figure 3c) leaf fluid
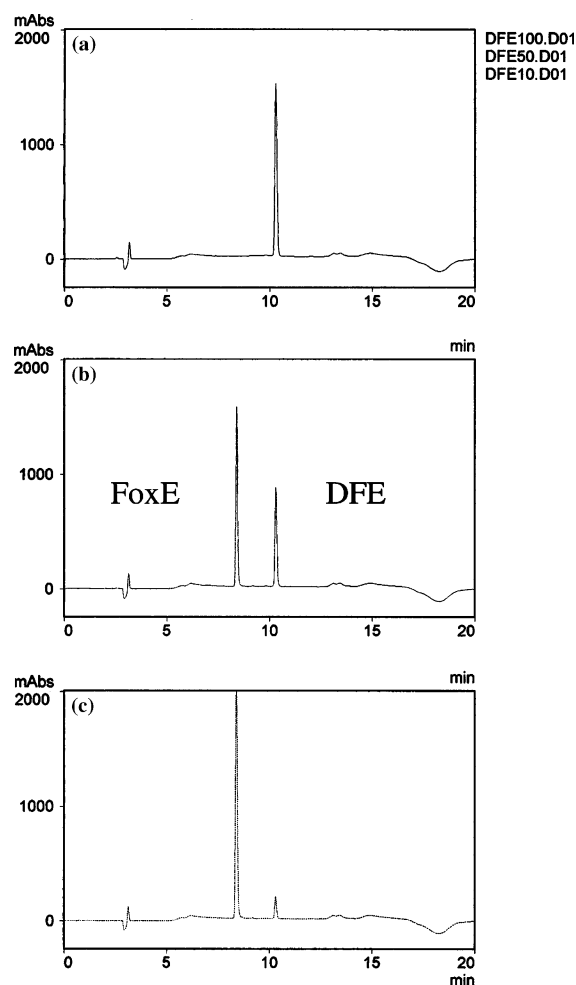
Figure 2. HPLC chromatogram of ferrioxamine E (FoxE) with the addition of desferrioxamine E (DFE) recorded at a detector wavelength of 220 nm (attenuation 12). (a) 1 mM DFE, (b) 0.5 mM DFE + 0.5 mM FoxE and (c) 1 mM Fox-E + 0.1 mM DFE.

plus 50 μM Fe and 165 μM DFE. In Figure 3, a clear increase in FoxE and a decrease in DFE associated with higher sample Fe concentrations can be observed, suggesting that no interference with chromophores occurs. While HPLC detection of FoxE at 435 nm in plant fluids yielded clean base lines, runs at 220 nm wavelength showed many absorbing compounds. Thus, the concentration of ferri- and desferrioxamine E present in samples can be individually calculated from calibration curves of the area size of the DFE and FoxE peaks as shown in Figure 4. In general, the detection at 220 nm is approximately 6 times more sensitive than that at 435 nm, but has the disadvantage of

having more interfering compounds. Overlapping peaks originating from the plant fluids can be identified when the ratio between added DFE and formed FoxE is calculated. Thereby, an increase in the FoxE peak in the chromatogram is always accompanied by a corresponding decrease of the DFE peak. As seen in the chromatogram of Figure 3b and c, addition of a surplus of iron to plant fluids gave a clear increase of the FoxE signal. Incubation of leaf fluid plus DFE probes at 65 °C provided higher iron concentrations as referred to samples left standing at room temperature. Since no significant iron increment was observed between 30 and 60 min, incubation at 65 °C, for 30 min became a standard practice.

Although detection at 220 nm proved useful to estimate optimal DFE concentrations for iron determination in fluids of different origin, the selected wavelength for routine measuring of leaf sample iron was 435 nm. This choice was based on minimizing interference in the HPLC due to the absorbance of leaf extracts in the UV. Although most of the obtained leaf fluids were almost transparent (chiefly fluid obtained from tobacco, pea, tomato and citrus leaves), browning of bean fluid was eventually observed.

FoxE determination of samples containing different Hoagland's macro- (N, P, K, Ca, Mg) and micro- (Cu, Mn, Bo, Mo, Zn) nutrient stock concentrations plus DFE were carried out (data not shown) and no difficulties with the FoxE method in the presence of such cations were observed. However, DFE is known to bind Al^{3+} (known as "aluminoxamine") as reported with desferrioxamine B (Kraemer & Breithaupt 1998). HPLC separation at 220 nm may lead to an aluminoxamine E peak in addition to the FoxE, peak, since both compounds have the same retention time. Subsequently, misleading results can be obtained via HPLC detection of FoxE in the presence of Al at 220 nm. Since aluminoxamine E is transparent *versus* the orange-brown FoxE complex, detection at 435 nm provides only values corresponding to iron containing FoxE. Despite the fact that no significant Al concentrations were found in the plants we used, FoxE concentration of probes was always determined at 435 nm. Thus peaks appearing in chromatograms from samples containing plant fluids plus DFE, can be reliably interpreted as FoxE. We therefore suggest that iron determination by HPLC using the Fox E
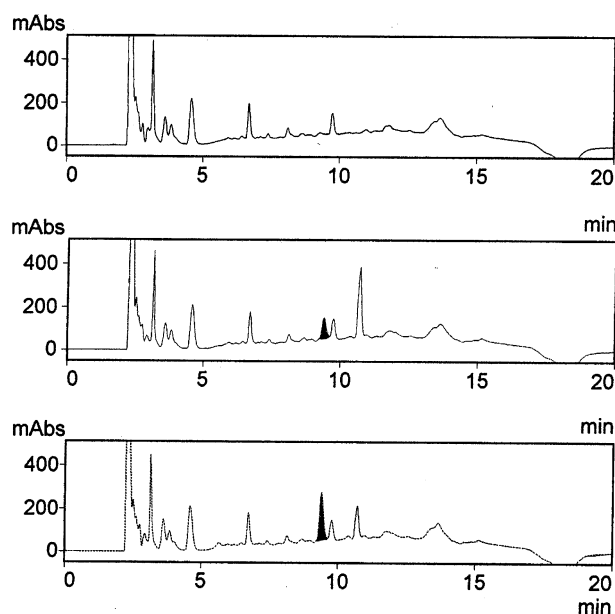
Figure 3. HPLC chromatograms (detection at 220 nm wavelength) of bean leaf fluid in the absence (upper) and in the presence of 0.1 mM DFE (middle). As indicated in black iron is bound by DFE and ferrioxamine E is formed which is enhanced after adding an additional amount of iron (lower). Conditions are as described in Materials and methods.
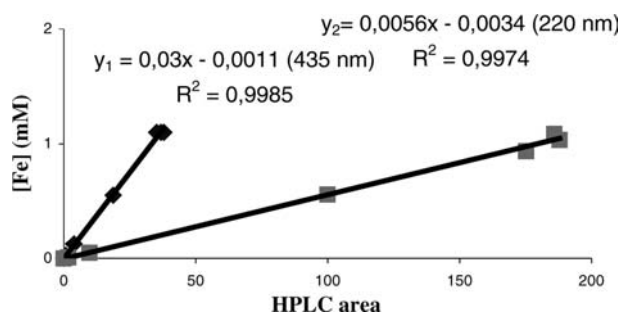

Figure 4. Correlation of iron concentration *versus* peak area as calculated from the ferrioxamine E area after HPLC separation and detection at 220 and 435 nm wavelength.

method should be performed at 435 nm wavelength avoiding interference with absorbing compounds in the plant fluids.

Total iron determination from chlorophyll samples was also attempted by the FoxE method. According to this procedure, bean leaves were found to have an average of 2.1 mg Fe/100 g FW.

Iron concentration of leaf fluid from green and chlorotic broad bean, tobacco and calamondine was determined. Iron in fluid obtained from the aerial part of 1–2-months-old tomato and pea seedlings was also measured. A considerable variation in leaf fluid iron concentration was observed according to factors such as plant and leaf age, frequency of iron root application or leaf location within the plant. Significant differences in iron concentration of green and chlorotic leave fluids were not always observed, chiefly referring to tobacco plants. An average of measured iron concentrations in leaf fluids is presented in Table 1.

Unless exogenous Fe was applied to leaves, the amount of Fe determined in leaf fluid obtained at $2500 \times g$ (initial centrifugation stage ascribed to provide xylem fluid) was normally found to be higher than the Fe concentration in fluid obtained at $4000 \times g$ (associated with apoplasmic fluid). Fe concentration in *C. madurensis* leaf fluid (obtained

Table 1. Iron content in fluids from young, fully expanded leaves, determined after 15 min centrifugation at 2500, 4000 or 8000 $\times g$ (*C. madurensis*) and subsequent HPLC analysis by the FoxE-method.

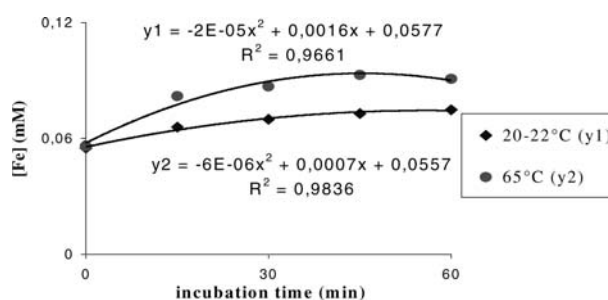| Species	Root treatment	[Fe] (μM) (fluid 2500 $\times g$)	[Fe] (μM) (fluid 4000 $\times g$)	[Fe] (μM) (fluid 8000 $\times g$)
L. esculentum	10 μM Fe	4.19	2.16	–
P. sativum	No Fe, pH 5.5	2.33	1.7	–
N. tabacum	10 μM Fe	4.24	1.52	–
N. tabacum	No Fe, pH 8	3.01	0.99	–
V. faba	10 μM Fe	7.9	3.92	–
V. faba	No Fe, pH 8	3.53	2.03	–
C. madurensis	No Fe, pH 8	–	–	32.59

Figure 5. Effect of incubation temperature and time on iron binding by DFE in leaf fluid samples and subsequent quantitation by HPLC.

at 8000 $\times g$) was higher than Fe values obtained from non-treated leaves centrifuged at a lower speed. Green (supplied with Fe) and chlorotic (pH 8) tobacco leaves used for obtaining fluid had an average of 2.23 and 0.85 mg/100 cm^2 chlorophyll *a*, respectively. Similarly, green bean and chlorotic bean leaves corresponding to the above values had a mean of 1.09 and 2.18 mg/100 cm^2. Plants used for leaf fluid iron determination followed a strict nutrition regime i.e. Hoagland's solution with (pH 5.5) or without Fe (pH 8). For all plants investigated, fluid Fe concentration (adding iron amounts from xylem and apoplasmic fluid) was maintained at relatively low levels which oscillated between 3 and 35 μM. Regardless of the colour state of plant leaves, experiments carried out with tobacco and calamondine revealed that more Fe was found in fluid from young leaves growing on the tip *versus* older leaves located at the bottom of the shoot or the plant. Stopping daily Fe supply via the root system induced a decrease in fluid Fe, however, plants remaining green until the end of the experimental period.

Results concerning Fe determination in different plants and different fluids as shown in Table 1,

revealed that the Fe content of plant fluids was generally in the range of 2–4 μM. Plants treated with 10 μM via the root system showed a xylem (2500 $\times g$) fluid Fe concentration generally twice the amount of Fe recovered in the apoplasmic fluid, indicating that mesophyll cells reduce the concentration of Fe by uptake.

Discussion

A main technical problem for analyzing iron deficiency in plants is the selection of a rapid and reliable method to determine the amount of iron in plant tissues and fluids. Subsequently, a HPLC-based method for the determination of Fe in plants fluids was introduced, owing to the outstanding properties of the siderophore DFE. The actual procedure is similar to the one described by Cramer *et al.* (1984) and Gower *et al.* (1984) with FoxB although a prior extraction of the formed iron complexes is not required in the present method. The affinity of DFE for Fe^{3+} is two orders of magnitude higher than that of DFB, which is $K = 10^{32.5}$ *versus* $K = 10^{30.5}$ for FoxE and FoxB,

respectively (Boukhalfa & Crumbliss 2002). Thus, the method presented in this report has significantly improved Fe determination compared to previous published methods. The higher stability constant of FoxE is attributed to a chelate effect that is due to ligand pre-orientation of Fe^{3+} binding groups, as it occurs with the endocyclic DFE in contrast to the linear acyclic DFB (Albrecht-Gary & Crumbliss 1998; Boukhalfa & Crumbliss 2002). The FoxB molecule has a free amino group which accounts for the basic character of this compound and enables it to form salts with organic and inorganic acids. Therefore, the use of DFE for iron determination in organic fluids proves advantageous as compared with DFB, since no interaction with compounds present in samples can be expected. DFE can extract iron from a variety of soluble iron complexes and the formed FoxE complex is stable down to pH 1 with only slight changes of absorption maximum and extinction coefficient ($\Delta\lambda_{max} = 4, \Delta\varepsilon = -90$) (Konetschny-Rapp et al. 1992).

Iron bound to citrate and other carboxylate ligands and even insoluble ferric phosphates and ferritin can be solubilized and complexed in the presence of DFE. As the equilibrium of the reaction is in favour of the ferric complex, ferrous iron will also be oxidized and complexed to ferrioxamine E under aerobic conditions. Thus the FoxE method measures the total loosely bound iron in plant fluids. Ferrioxamines were reported to have a strong affinity for Al (stability constant of 10^{26}) (Kraemer & Breithaupt, 1998). The overall stability constant of DFB for other divalent ions, such as Cu^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+} and Mg^{2+} was described to be considerably lower, 10^{14}, 10^{10}, 10^{11}, 10^{11}, 10^2 and 10^4, respectively (Keberle 1964) so that any interference with other cations in the plant fluid can be excluded. Similarly no interference with the Fox E method was observed in the presence of plant macronutrients (N, P, K, Ca, Mg). However, DFE proved to have a strong affinity for Al^{3+} yielding an aluminoxamine E complex, which is separated simultaneously with FoxE during HPLC when detected at 220 nm. However, while measuring at 435 nm, only the charge-transfer band of the iron complex of FoxE is determined, the transparent aluminium complex remaining undetectable. According to our experience the analyzed plant fluids did not contain significant amounts of Al. Given the heterogeneity

of compounds and elements present in plant fluids, HPLC detection at 435 nm proved to be a safe way to avoid any metal interference with the Fox E method.

The "FoxE method" proves suitable for measuring plant tissue soluble Fe fractions, since most of the Fe is present as ferric citrate and other ferric carboxylate complexes within the xylem and the apoplast (López-Millán et al. 2000). Leaf fluid was extracted as described by Dannel et al. (1995) and modified by López-Millán et al. (2000). Fluid obtained by leaf centrifugation for 15 min at 2500 and 4000 $\times$ g, has been related to xylem and apoplasmic fluid (López-Millán et al. 2001). The obtained results are within the range described by López-Millán et al. (2000, 2001) for sugar beet and pear leaf. Centrifugation of C. madurensis leaves at 8000 $\times$ g for 15 min was required in order to obtain leaf fluid. Iron concentrations in citrus fluid was much higher than those detected in fluids from other plant species obtained at 2500 and 4000 $\times$ g. Centrifugation at 8000 $\times$ g probably caused cell damage and the consequent release of some symplasmic fluid. However, the method proved useful to assess uptake of iron-containing compounds in citrus leaves. It was observed that iron concentration of fluids from non-treated leaves obtained at 2500 $\times$ g was normally slightly higher than that obtained by centrifugation at 4000 $\times$ g, confirming a decreasing iron gradient from xylem to apoplasmic fluid. Variation of the apoplasmic fluid iron concentration over the growing period was also recorded, chiefly for bean, indicating that apoplasmic iron determination is not always a suitable means of diagnosing plant iron deficiency.

Leaf fluid often contains UV-absorbing endogenous compounds, which make a direct iron determination with ferrous chelators sometimes difficult or are likely to yield misleading results unless suitable blanks are used. Leaf total iron could also be successfully determined by the FoxE method after acetone extraction of chlorophyll. The residual proteins were precipitated with perchloric acid and iron was determined from the supernatant. Thus, the method proved useful to determine all kinds of iron present in neutral and acidic plant fluids. After adding DFE, incubation at 65 °C for 30 min was found to be sufficient in order to complete the iron transfer reaction in solution.

In conclusion, an HPLC-based method for iron determination in plant fluids was established

owing to the outstanding chelating properties of the siderophore DFE. This procedure proved accurate and simple for analysing the iron status of all kinds of plants under normal growth conditions and after treatment with foliar iron sprays.

Acknowledgements

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