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The use of microbial siderophores for foliar iron application studies

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Abstract

Experiments were conducted to assess the distribution of foliar applied Fe-containing compounds using microbial siderophores. Fe was measured in leaf fluid obtained by centrifugation according to a determination method based on Fe chelation by desferrioxamine E and HPLC separation on a reversed phase column. To avoid sample Fe contamination, treatments were only applied to a part of the leaf following a systematic and reproducible procedure and iron concentration was exclusively determined in fluid obtained from non-treated leaf surfaces. The increase in leaf fluid Fe concentration associated with the distribution of leaf applied Fe-siderophores, Fe–EDTA and $FeSO_4 \times 7H_2O$ was evaluated using *Vicia faba* L., *Nicotiana tabacum* L. and *Citrus madurensis* Lour. plants. The method proved useful to investigate the process of leaf Fe penetration and its distribution within the plant. Evidence of the penetration and distribution of leaf applied Fe-rhizoferrin, Fe-coprogen hydrolysis products and Fe-dimerum acid is presented in this study.

Abbreviations: DFE – desferrioxamine E; FW – fresh weight; FoxE – ferrioxamine E; HPLC – high performance liquid chromatography.

Introduction

Iron (Fe) deficiency chlorosis is a common physiological disorder affecting plants grown on high pH, calcareous soils worldwide. Although Fe is very abundant on earth, its bioavailability is often restricted by the very low solubility of Fe(III)-oxides under aerobic conditions (Schmidt, 2003). Preserving crop quality and yields under soil conditions inducing Fe deficiency requires the use of correcting methods, among which is foliar fertilisation. Treatment with foliar Fe sprays involves a reduction in production costs and a lower environmental impact. However, variable effects after foliar Fe treatment to chlorotic plants have been reported due to the many factors involved in the process of leaf penetration, plant translocation and cell Fe uptake (Fernández, 2004). Given the limited knowledge concerning the process of leaf Fe penetration, this strategy alone cannot currently be considered a reliable alternative for full correction of plant Fe deficiency (Álvarez-Fernández et al., 2004).

All primary aerial plant parts are covered by a cuticle, which is the principal barrier for the exchange of water and ions between plant and environment (Schönherr and Schreiber, 2004). Leaf surfaces have been found to have a great micro-structural diversity (Barthlott and Neinhuis, 1997). The occurrence of water films on leaf surfaces as a consequence of atmospheric

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deposition has been shown (Burkhardt and Eiden, 1994). The role of stomata regarding leaf penetration of foliar applied solutions is also not fully understood (Fernández et al., 2003), but may represent a major pathway for the uptake of foliar sprays (Eichert et al., 2002).

Many factors related to Fe uptake, transport and its distribution within the plant remain obscure but there is evidence that Fe deficiency may impair such mechanisms (Larbi et al., 2001). The role of the leaf apoplast with regard to Fe delivery to the cell is not clear. The existence of a leaf cell plasma membrane-bound reductase similar to the one in the root system was shown (Brüggemann et al., 1993) and some of its characteristics have been described (Larbi et al., 2001). Fe deficient leaves were found to have increased apoplastic pH values as compared with Fe sufficient leaves (Kosegarten et al., 2001; López-Millán et al., 2001). Such increase in apoplastic pH has been suggested to depress the activity of the Fe^{III} leaf reductase, thereby hindering symplastic Fe uptake (Kosegarten et al., 1999, 2001). However, the significance of apoplastic pH changes and the factors inducing such physiological response remain unclear and are subject of much debate (Kosegarten et al., 2001; Nikolic and Römheld, 2003).

Under Fe starvation conditions microorganisms and graminaceous species produce and excrete low molecular weight, high-affinity Fe(III) chelators known as siderophores and phytosiderophores, respectively (Fett et al., 1998). Siderophores are produced for the solubilisation, transport and storage of Fe in microorganisms in the presence of other metal ions (Winkelmann and Drechsel, 1997). The outstanding properties of many microbial siderophores render them suitable for therapeutical and analytical purposes. Some investigations evaluated the potential of siderophores as Fe carriers for plants supplied via the root system (Bar-Ness et al., 1991; Crowley et al., 1992). In nutrient culture, Fe-rhizoferrin was found to be an efficient Fe source for tomato, barley and corn plants (Shenker et al., 1992; Yehuda et al., 1996). More recently, Hördt et al. (2000) obtained evidence of plant Fe utilisation after root treatment with ferric mono- and di-hydroxamate siderophores.

The aim of this study was introduce a new method for assessing the penetration and translocation of leaf applied Fe-siderophores, Fe–EDTA and $FeSO_4 \times 7H_2O$ after Fe determination by the FoxE Method (Fernández and Winkelmann, 2005). Aware of the excellent transport properties of Fe-dimerum acid, its ability to penetrate and to be distributed within the trifoliate bean leaf was evaluated under light and dark conditions.

Materials and methods

Plant culture

Experiments were developed with 1 to 2 months-old *Vicia faba* var. 'Marona' seedlings, 4 to 7 months-old *Nicotiana tabacum* var. 'Virginia' seedlings and 3 years-old *Citrus madurensis* cuttings grown in sand culture. Plants were grown at 25 °C under incandescent light, 16 h-light/8 h dark photoperiod and 60–80% relative humidity. Plants were daily watered with full-strength Arnon and Hoagland (1952)'s solution without Fe (pH 8 reached by addition of 10 mEq/L $NaHCO_3$). The composition of the nutrient solution was: 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 $MgSO_4 \times 7H_2O$, 2.86 mg/L H_3BO_3, 1.81 mg/L $MnCl_2 \times 4H_2O$, 0.08 mg/L $CuSO_4 \times 5H_2O$, 0.22 mg/L $ZnSO_4 \times 7H_2O$ and 0.09 mg/L H_2MoO_4 ($MoO_3 + H_2O$).

For the development of foliar application experiments, plants were transferred from the greenhouse to an air-conditioned laboratory, at 20–22 °C and 40–50% relative humidity.

Fe solutions for foliar treatment

Several Fe-containing compounds at different concentrations were applied to plant leaves (Table 1). Purified Fe-rhizoferrin, Fe-dimerum acid, DFE and solutions containing hydroxamate mixtures (based on Fe-dimerum acid and a lower Fe-fusarinin concentration) i.e. coprogen hydrolysis products (Hördt et al., 2000) were obtained from EMC micro-collections GmbH, Tübingen, Germany (Biophore Research Products, www.siderophores.info).

All foliar treatment solutions contained 1 g/L of the non-phytotoxic, C8/18-alkyl polyglucoside surfactactant Glucopon CSUP (Cognis) (Schönherr, 2001) and were applied at pH 5.

Table 1. Fe-containing compounds and concentrations applied to plant leaves

Fe-containing compound	Concentration range used (m*M*)	Stability constant (in aqueous solution at 25 °C, I = 0.1 *M*)
Fe(III)–citrate (1:20)	1–15	10^{12a}
Fe(III)–EDTA	5	$10^{25.7a}$
$FeSO_4 \times 7H_2O$	5	–
Fe-rhizoferrin	1	$10^{25.3b}$
Fe-dimerum acid	1–10	$10^{21.5b}$
Fe-coprogen hydrolysate	5	$10^{21.5b}$

[a]According to Furia (1972).
[b]According to Albrecht-Gary and Crumbliss (1998).

Leaf treatment and sample collection

Fe-containing solutions were applied to the distal part of the leaf/central bean leaflet including the tip as described by Fernández et al. (2004). The treated area corresponded to approximately 25% of the entire leaf/leaflet surface. Fe-containing compounds were applied via limiting the treated area with a 3.5×3.5 cm^2 transparent plastic foil stuck to the leaf tip with Tesa paper film. The piece of plastic covering the upper leaf surface was sealed with silicone (Bayer). 50 μL of the treatment solution were injected into the space between the plastic layer and the lower leaf side with a pipette.

By the end of the experimental period, leaves were detached and all treated leaf surfaces were discarded. In all instances, surface Fe contamination of the non-treated part of the receptor leaf was prevented by excising the treated area plus a margin of 0.5–1 cm.

In the case of *V. faba* plants (having trifoliate leaves), the tip of the central leaflet (receptor leaflet) was treated. Fluid from the non-treated area of the detached, receptor leaflet and from the remaining leaflets of the trifoliate leaf (neighbouring leaflets) was obtained and measured separately. A part of a *N. tabacum* or a *C. madurensis* leaf was treated and fluid was obtained from the non-treated part of detached, treated leaves. Fe translocation in tobacco was evaluated by obtaining fluid from the whole aerial plant parts excluding the treated leaf.

The same Fe-containing solution was applied to seven leaves per treatment. Consequently, seven detached, non-treated leaf parts were centrifuged together providing a common fluid. Similarly, seven neighbouring leaflets (bean) or aerial parts (tobacco) were collected per treatment. Measurements were repeated twice.

Light treatments were applied in a chamber with Osram Lumilux Plus Eco, fluorescent tubes (approximately 1 m away from plants) at 25 °C and 30–50% RH. The effect of supplying treatments under complete darkness was assessed by keeping plants in a dark room, for the time-span of the trial. Plants were conditioned to light or dark conditions 2–3 h before treatment. Plants were well irrigated prior to foliar treatment and throughout the whole experimental period.

Sample preparation

Leaf fluid was obtained by leaf centrifugation at $2500 \times g$ and $4000 \times g$ as described by López-Millán et al. (2001), with some modifications (Fernández et al., 2004). Eppendorf tubes containing apoplastic and xylem fluids were stored at 4 °C or −70 °C for a later use. Fluid from *C. madurensis* leaves was obtained after centrifugation at $8000 \times g$ for 15 min.

Fe determination by the Ferrioxamine E (FoxE) method

Fe present in plant fluids was measured after addition of DFE and HPLC separation as described by Fernández and Winkelmann (2005). Calibration curves were made by FoxE spectrophotometic reading (Ultrospec III, UV/VIS Spectrophotometer, Pharmacia) at 220 and 430 nm ($\lambda_{max} = 430$ nm, molar extinction coefficient (ε) = 2750 M^{-1} cm^{-1}). Fe-saturated, FoxE solutions were measured on a Shimadzu HPLC System (Shimadzu, Duisburg, Germany) at 220 and 435 nm detector wavelengths ($R^2_{220\ nm} = 0.9985$ and $R^2_{430\ nm} = 0.9974$). Samples were separated on a reversed-phase Nucleosil 100 C18 column (20×250 mm, 7 μm, Grom Herrenberg, Germany) with a gradient of acetonitrile/water (6–40%) containing 0.1% trifluoroacetic acid (TFA) over 20 and 35 min (flow rate 1 mL/min). Prior to measurement, plant fluids plus DFE were incubated at 65 °C for 30 min. The Fe concentration of plant fluid was always determined at 435 nm detector wavelength.

Contact to any Fe source was avoided and the HPLC system was thoroughly cleaned prior to sample analysis.

Calculation of Fe increment after foliar treatment

An estimate of the Fe increment in leaf fluid associated with foliar treatments was obtained as described below. Calculations aimed at correcting differences related to the leaf area treated or to the leaf weight providing the fluid.

Correlations leaf area-leaf fresh weight (FW) and fresh weight-volume of fluid obtained by centrifugation were obtained for data gathered from tobacco, citrus and bean leaves.

The Fe concentration in leaf fluid ($[Fe]_{leaf\ fluid}$) prior to and after foliar Fe application was calculated as follows (equation (1)):

$$[Fe]_{leaf\ fluid}(\text{mg Fe} \times (100\,\text{g FW})^{-1} = \frac{55.85(\text{g mol}^{-1}) \times M_{fluid}(\text{m}M) \times V_{fluid}(\text{L}) \times 100}{FW_{fluid}(\text{g})} \quad (1)$$

where $M_{fluid} = [Fe]$ determined in leaf fluid (mM); V_{fluid} = volume of fluid obtained by leaf centrifugation (L); FW_{fluid} = leaf fresh weight providing the fluid (g).

Similarly, the amount of Fe covering the treated leaf surface after treatment ($Fe_{supplied}$) corresponded to (equation (2)):

$$[Fe]_{supplied}(\text{mg Fe} \times (100\,\text{g FW})^{-1} = \frac{55.85(\text{g mol}^{-1}) \times M_{solution}(\text{m}M) \times V_{supplied}(\text{L}) \times 100}{FW_{treated}(\text{g})} \quad (2)$$

where $M_{solution} = [Fe]$ of the applied solution (mM); $V_{supplied}$ = volume of treatment solution applied per leaf surface (L); $FW_{treated}$ = leaf fresh weight corresponding to the treated surface (g).

For comparison, the Fe concentration present in leaf fluid and covering the treated leaf surface were expressed in μg Fe per 100 cm^2. The Fe increment in leaf fluid in relation to the Fe-solution covering the leaf surface was calculated according to equation (3):

$$\text{Fe increase in leaf fluid}(\%) = \frac{([Fe]_{fluid} - [Fe]_{non\text{-}treated})}{[Fe]_{supplied}} \times 100 \quad (3)$$

where $[Fe]_{fluid}$ = Fe (μg/100 cm^2) in fluid obtained from the non-treated area of detached, receptor leaves/leaflets or from neighbouring leaves/leaflets; $[Fe]_{non\text{-}treated}$ = Fe (μg/100 cm^2) in fluid obtained from non-treated leaves; $[Fe]_{supplied}$ = Fe (μg/100 cm^2) covering the treated surface.

Experiments were designed as a completely randomised block. Results were statistically analysed using analysis of variance (ANOVA).

Results

Fe increment after foliar treatment

Results obtained after bean, tobacco and *C. madurensis* leaf treatment with several Fe sources in the light are summarised in Table 2. An estimate of the increase in Fe concentration associated with foliar Fe application as compared with non-treated leaves was calculated following equations (1)–(3). Highest Fe increments in fluid obtained from the non-treated part of detached, receptor leaves were related to 1 mM Fe-rhizoferrin and 5 mM $FeSO_4 \times 7H_2O$ application to bean. Highest Fe distribution rates to neighbouring leaves were observed after 1 mM Fe-rhizoferrin and 15 mM Fe(III)–citrate application to bean and tobacco, respectively. However, excluding the mentioned cases, the rate of fluid Fe increment after foliar Fe application varied between 0.2 and 1.4% for the non-treated area of detached, receptor leaves and from 0.005 to 0.4% for detached, neighbouring leaflets as compared with non-treated leaves.

Light-dark effect

Aware of the good transport properties of Fe-dimerum acid, the effect of applying this Fe-siderophore to bean leaves under light and dark conditions was investigated (Figure 1). A major effect of applying Fe-dimerum acid in the light was observed in terms of increased fluid Fe concentration of the non-treated part of detached, receptor leaflets and to a lower extent of neighbouring leaflets. Within 8 h after treatment, Fe distribution from the area of application to the non-treated part of dark-leaflets treated also

Table 2. Fluid Fe increment associated with foliar application of Fe-containing solutions within 1 day after treatment. Data are means ± SE ($n = 2$)

Plant species	Fe-treatment	[Fe] fluid non-treated part of receptor leaf (μg/100 g FW)	[Fe] fluid neighbouring leaves (μg/100 g FW)	Fe increase in fluid from non-treated part receptor leaf (%)	Fe increase in fluid from neighbouring leaves (%)
Bean	Not-treated	5.1 ± 0.3	5.1 ± 2.3		
Bean	1 m*M* Fe–rhizoferrin	126.9 ± 5.7	65.4 ± 3.1	5.7 ± 0.26	3.22 ± 0.16
Bean	5 m*M* $FeSO_4 \times 7H_2O$	798.6 ± 41.5	5.6 ± 0.3	5.08 ± 0.25	0.005
Bean	5 m*M* Fe–EDTA	42.1 ± 2	6.6 ± 0.4	0.7 ± 0.04	0.01
Bean	5 m*M* Fe–coprogen hudrolysate	90.5 ± 5.2	15.9 ± 0.8	1.23 ± 0.06	0.16 ± 0.01
Bean	1.5 m*M* Fe–dimerum acid	30.9 ± 1.4	30.5 ± 1.6	0.37 ± 0.02	0.36 ± 0.02
Bean	10 m*M* Fe(III)–citrate	70.7 ± 4.3	5.7 ± 0.3	0.9 ± 0.05	0.008
Bean	10 m*M* Fe–dimerum acid	20.0 ± 0.76	16.6 ± 0.9	0.01	0.2 ± 0.01
Citrus	Not-treated	14.86 ± 0.77	–	–	–
Citrus	10 m*M* Fe(III)–citrate	43.1 ± 2.1	–	0.6 ± 0.04	–
Tobacco	Not-treated	1.22 ± 0.1	1.63 ± 0.1	–	–
Tobacco	15 m*M* Fe(III)–citrate	13.81 ± 0.7	3.3 ± 0.2	3.4 ± 0.17	2.4 ± 0.12

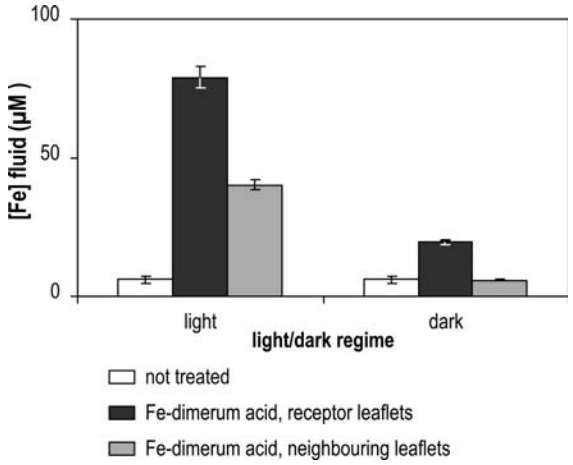

Figure 1. Fluid Fe concentration of non-treated and 5 m*M* Fe-dimerum acid treated bean leaves, 8 h after solution application. Treatments were supplied under light and dark conditions. Results correspond to fluid obtained from the detached, non-treated area of receptor leaflets and from neighbouring leaflets. Data represent means ± SE of four independent trials.

took place, but at a lower rate as compared with light-treated leaves. In the dark, no significant Fe translocation to neighbouring leaflets was observed, while a significant fluid Fe increase was measured in light-treated, neighbouring leaflets. Subsequently, Fe transport from the treated leaflet to neighbouring leaflets appeared to be strongly inhibited by darkness in contrast to plants treated in the light. Dark conditions inhibited more strongly Fe translocation to neighbouring leaflets than leaf Fe penetration and its distribution within the treated leaflet.

Discussion

A new procedure to gain evidence of the penetration of Fe-containing compounds through plant leaves was introduced due to inconsistencies related to Fe measurement of Fe-treated leaves (Fernández, 2004). The method was directed towards avoiding the risk of Fe contamination due to Fe sticking to the treated leaf surface, however obtaining an estimate of the penetration and distribution of leaf applied Fe-containing solutions. Fe increments associated with foliar treatment were measured in fluid obtained from detached, non-treated plant parts sometime after solution application. Only about ¼ of the whole lower leaf area was treated, the area of application being discarded by the end of trials. After excising the treated area leaving a security margin, Fe distribution from the site of treatment was assessed in the remaining approximately 50% of the total leaf surface. Fluid Fe concentration was accurately measured after addition of desferrioxamine E and HPLC separation on a reversed phase column (Fernández and Winkelmann, 2005). Apoplastic and xylem fluids (López-Millán et al., 2001) were

obtained from bean and tobacco plants, while *C. madurensis* leaves were centrifuged at $8000 \times g$ to yield some fluid.

The chance for foliar penetration was maximised via treating the lower leaf side and adding 1 g/L Glucopon 215 SCUP to improve leaf wetting and lowering surface tension below 30 mN/m (Schönherr, 2001). Solutions were applied at pH 5 to avoid altering the exchange properties of the leaf surface (Fernández et al., 2004). Most foliar treatments induced a 0.2–1.2% Fe increase in fluid obtained from the non-treated part of receptor leaves. Higher fluid Fe concentrations (5%) were recorded after $FeSO_4 \times 7H_2O$ and Fe-rhizoferrin application. This may be due to the lower molecular weight of both substances in contrast to foliar penetration of larger Fe-complex molecules, as noted by Schönherr (2002).

The ability of Fe to be distributed from the treated leaf was evaluated. Fe translocation was found to be chiefly associated with Fe-siderophore application as shown after Fe-rhizoferrin, Fe-dimerum acid or Fe-coprogen hydrolysate treatment. A slight Fe increase in fluid obtained from neighbouring leaflets related to Fe–EDTA application was recorded (0.01%). However, Fe supplied as $FeSO_4 \times 7H_2O$ had a reduced capacity to be distributed from the treated leaflet. Results are in agreement with the findings of Basiouny and Biggs (1971) suggesting that Fe–chelates move into and through the plant system better than inorganic Fe-containing compounds. More recently, Hüve et al. (2003) investigated Fe transport in *Vicia faba* after application of radiolabelled 0.1 m*M* Fe-EDTA to the injured midrib of the upper surface of a leaflet. Two hours after treatment ^{59}Fe was detected in the non-treated second leaflet of the bifoliate leaf.

Schönherr and Huber (1977) showed that above pH 3 plant cuticles are negatively charged. Similarly, cell walls limiting the apoplastic space were found to have charges corresponding to dissociated weak acids (Grignon and Sentenac, 1991). Subsequently, non-charged or electron-charged Fe–chelates can be expected to penetrate the leaf and to be translocated in the apoplast easier than positively charged complexes. Thereby, foliar application of ionic Fe-containing compounds will not only interfere with leaf penetration, but also with Fe movement in the apoplast. This may be one of the reasons for the reduced translocation of $FeSO_4 \times 7H_2O$ from the site of application.

After gaining evidence of the excellent transport properties of Fe-dimerum acid, the effect of light on leaf penetration and Fe translocation within the trifoliate leaf was investigated with *Vicia faba*. Light is a factor known to influence foliar penetration of leaf applied chemicals (Eichert et al., 2002) and leaf mesophyll Fe(III) reduction and uptake (Larbi et al., 2001). No significant Fe translocation from the site of application was recorded when plants were treated and kept under dark conditions, as compared with the same treatments applied in the light. In the dark, Fe translocation to neighbouring leaflets was more restricted than foliar Fe penetration. It was concluded that light is an important factor ruling the translocation of leaf applied Fe in bean and that Fe distribution within the plant may influence the rate of foliar penetration of the applied Fe-containing solution. Leaf applied Fe may initially translocate following the transpiration stream and plant factors such as stomatal aperture, photosynthesis or circadian rhythms may influence leaf penetration and plant translocation. In this regard, Mori (1998) reported that no Fe was translocated to leaves covered with Al foil after ^{59}Fe-3-epihydroxymugineic acid supply to the roots of barley plants. The author suggested that the radial Fe transport in the leaf from the xylem to the apoplast or from the apoplast to the mesophyll cells is light regulated.

The large amounts of Fe recovered in xylem and apoplastic fluid of plants within one day after foliar treatment may indicate that transport is taking place following this path-way as suggested by Seckback (1982). Results represent the total Fe concentration of fluid obtained after leaf centrifugation at 2500 and $4000 \times g$, since no clear trend regarding higher Fe concentrations in apoplastic or xylem fluid was always observed. Hüver et al. (2003) assumed that Fe was transported in the phloem and noted that Fe retranslocation from leaves was not sufficient to meet the demand of the growing tissue probably due to a limitation in the phloem transport system as also suggested by Nikolic et al. (2003). We observed that a restricted proportion of the applied Fe ranging from 0 to 3%, was distributed to non-treated plant parts according to different Fe-carriers.

Results are of importance while trying to evaluate potential Fe-carriers for foliar sprays. Prior to leaf cell uptake and to any physiological response, the Fe-containing foliar solution must penetrate the leaf. Thereby, trials to investigate the rate of penetration of various Fe-containing compounds as a prior step to evaluate the physiological effect of such substances (e.g. via re-greening) will be useful for the selection of suitable Fe-carriers for foliar sprays as shown by Fernández et al. (2004). However, having a high leaf penetration rate is not the sole characteristic to consider since Fe supplied as certain compounds may be readily immobilised in the leaf apoplast due to ionic binging or formation of insoluble Fe compounds.

In conclusion, results suggest that variation in leaf Fe penetration, translocation and cell uptake rates can be expected according to different plant species and the nature of the Fe-containing compound. However, a limited amount of exogenous Fe was found to be distributed from the site of treatment. Fe-siderophore application led to increased Fe distribution rates in contrast to the effect of $FeSO_4 \times 7H_2O$ and Fe–EDTA. Furthermore, Bucheli-Witschel and Egli (2001) reported that the synthetic aminopolycarboxylic acids EDTA, DPTA and HEDTA seem to be either not or only poorly biodegradable, which may represent a major problem for their elimination from waste water and ecosystems. In contrast to some commonly used synthetic chelates, the studied siderophores are not environmental hazardous and may be an alternative means of supplying Fe to plants. However, more research is required to improve the effectiveness of foliar Fe sprays as a strategy to correct plant Fe deficiency.

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