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REGULAR ARTICLE

Effectiveness of N,N′-Bis(2-hydroxy-5-methylbenzyl) ethylenediamine-N,N′-diacetic acid (HJB) to supply iron to dicot plants

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Abstract Iron chlorosis is commonly corrected by the application of EDDHA chelates, whose industrial synthesis produces o,oEDDHA together with a mixture of regioisomers and other unknown by-products. HJB, an o,oEDDHA analogous, is a new chelating agent with a purer synthesis pathway than EDDHA. The HJB/Fe^{3+} stability constant is intermediate between the racemic and meso o,oEDDHA/Fe^{3+} stereoisomers. This work studied the efficacy of HJB as a Fe source in plant nutrition. No significant differences between o,oEDDHA/Fe^{3+}, HJB/Fe^{3+} and HBED/Fe^{3+} were observed when they are used as substrates of the iron-chelate reductase of mild chlorotic cucumber plants. Chelates prepared with the stable isotope ^{57}Fe were used in both soil and hydroponic experiments. In the hydroponic experiment, nutrient solutions with low doses of chelates were renewed weekly. Soybean plants treated with o,oEDDHA/$^{57}Fe^{3+}$ recorded the highest results in biomass, SPAD index and Fe nutrition. In the soil experiment, chelates were added once at a rate of 2.5 mg Fe per kg of a calcareous soil. Soybean plants treated with HJB/$^{57}Fe^{3+}$ recorded a higher biomass and SPAD index in young leaves than the plants treated with o,oEDDHA/$^{57}Fe^{3+}$; however, ^{57}Fe and total Fe concentrations in leaves were lower. The results of both pot experiments are associated with a faster ability by o,oEDDHA to provide Fe to the plants and with a more continuous supply of Fe from HJB/Fe^{3+}. HJB/$^{57}Fe^{3+}$ effectively alleviated the Fe-deficiency chlorosis of soybean with a longer lasting effect than o,oEDDHA/$^{57}Fe^{3+}$.

Keywords Iron chelates · Fertilisers · o,o-EDDHA · HJB · HBED · Cucumber · Soybean · Calcareous soil · Chlorosis.

Abbreviations

BPDS	Bathophenanthroline disulfonic acid or 4,7 diphenyl- 1,10 phenanthroline disulfonic acid
FC-R	Iron chelate reductase
HBED	N,N′-Bis(2-hydroxybenzyl)ethylenediamine-N,N′-diacetic acid
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HJB	N,N′-Bis(2-hydroxy-5-methylbenzyl) ethylenediamine-N,N′-diacetic acid
MES	2-Morpholinoethanesulfonic acid.

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o,o-EDDHA	Ethylenediamine-N,N′bis(o-hydroxyphenylacetic) acid
o,pEDDHA	Ethylenediamine- N(o-hydroxyphenylacetic) - N′(p-hydroxyphenylacetic) acid
o,pEDDHA	Ethylenediamine- N(p-hydroxyphenylacetic) - N′(p-hydroxyphenylacetic) acid
EDDHMA	Ethylenediamine-N,N′bis(o-hydroxymethylphenylacetic) acid
DTPA	Diethylenetriaminepentaacetic acid
EDTA	Ethylenediaminetetraacetic acid
ICP-MS	Inductively coupled plasma mass spectroscopy

Introduction

Iron chlorosis is a widespread agricultural problem, especially in crops grown in calcareous soils, where calcium carbonate buffers soil solution pH in the range of 7.5 – 8.5 (Lindsay and Schwab 1982) and high bicarbonate concentration is present (Lucena 2000). In soil, the solubility of Fe is controlled by Fe oxides (Lindsay 1991) and the most soluble Fe oxide limits total soluble inorganic Fe concentration to around 10^{-10} M in calcareous soils, much lower than that required (10^{-8} M) for optimal plant growth (Römheld and Marschner 1986).

Fe-efficient dicots and non-grass monocots employ the Strategy I response to mobilize iron from the soil. This response includes an increase in the reduction of Fe(III)-chelates to Fe(II) at the root surface, and transport of Fe(II) across the root epidermal cell membrane. The genes that encode the iron-regulated ferric chelate reductase (*FRO2*) and the ferrous iron transporter (*IRT1*) (Connolly et al. 2003) have been identified in Arabidopsis. The isolation of these genes will have implications for the generation of crops with an improved Fe acquisition mechanism. Until this goal is achieved, the most efficient practice to overcome iron deficiency in plants is the application of synthetic iron chelates (Chen and Barak 1982). Among all soil-applied iron fertilizers, synthetic Fe(III)-chelates, mainly Fe(III)-chelates of polyamine — carboxylic acids with phenolic groups, such as ethylenediamine di(*o*-hydroxyphenylacetic) acid (EDDHA), are the most effective and commonly used. EDDHA is industrially prepared by a Mannich-like reaction between phenol, ethylenediamine and glyoxylic acid (Petree et al. 1978). This pathway is known to produce around 4–6% Fe chelated with *o*,*o*-EDDHA as the main component, together with a mixture of positional isomers that have been identified as *o*,*p*-EDDHA and *p*,*p*-EDDHA (Cremonini et al. 2001; Gómez - Gallego et al. 2002) accompanied by other unknown by-products that are also able to complex Fe^{3+} (Hernández-Apaolaza et al. 2006). The o, oEDDHA positional isomer is found to form the most stable complexes with iron (Hernández-Apaolaza et al. 1997; Yunta et al. 2003a) and presents two regioisomers (meso and racemic) with different agronomic efficacy (Cerdán et al. 2006); o,pEDDHA has now been included as an authorized chelating agent in the European Regulation on fertilizers (EC Regulation No. 2003/2003) due to its good agronomical behaviour (García-Marco et al. 2006); p, pEDDHA does not form the Fe^{3+} complex because the two p-hydroxyphenyl groups are sterically impeded from binding Fe^{3+} (Yunta et al. 2003a), and the other by-products have a limited value as iron fertilizers (Hernández-Apaolaza et al. 2006).

The chelating agent EDDHMA (ethylenediamine di N-N′(2- hydroxy-methylphenylacetic) acid) is an EDDHA analogous (Fig. 1) with methyl groups bound to the phenolates, which increase the stability of the chelate but have a similar agronomic behaviour (Álvarez-Fernández et al. 2005). HBED (N,N′-Bis(2-hydroxybenzyl)ethylenediamine-N,N′-diacetic acid) has a similar structure to o,oEDDHA (Fig. 1) and forms a very stable Fe chelate (Chaney 1988). HBED has traditionally been used as an oral drug to remove excessive Fe from humans (Brittenham 1992; Bergeron et al. 2002), but HBED/Fe^{3+} has never been used in agriculture due its high stability constant. HJB (N,N′-Bis(2-hydroxy-5-methylbenzyl)ethylenediamine-N,N'-diacetic acid) (Fig. 1) is similar to HBED but with methyl groups bound to the phenolates as in EDDHMA. Its synthesis may produce purer commercial products (approximately 9% chelated Fe) than the EDDHA pathway synthesis (less than 6%). Our research group (López-Rayo et al. 2009) has characterized and studied the stability of HJB and HBED in comparison with o, oEDDHA. The purity of the chelating agents, their protonation constants, Ca(II), Mg(II), Fe(III) and Cu(II) stability constants and their ability to maintain Fe in

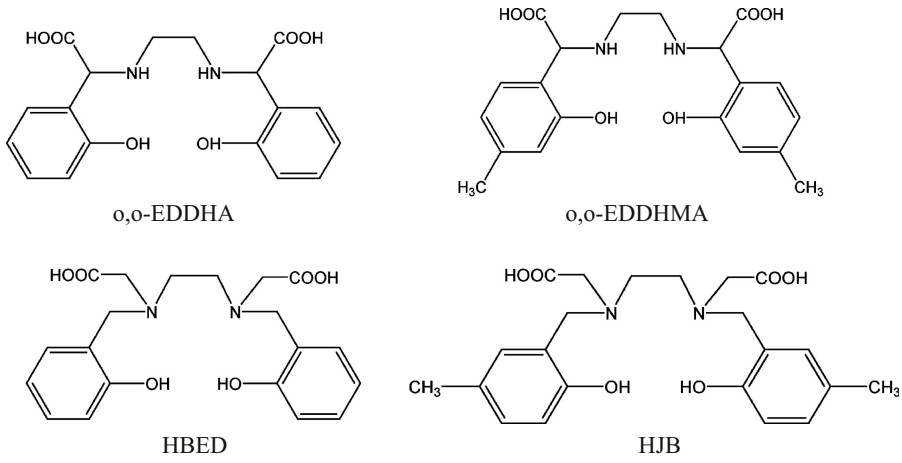

Fig. 1 Structures of the chelating agents described in the text

solution under different conditions have been determined. The results obtained indicated that HJB (log K=34.45) and HBED (log K=39.01, Ma et al. 1994) had similar and higher stability with Fe, respectively, than o,o-EDDHA (log K=35.09, Yunta et al. 2003a).

The objective of this work is to evaluate the efficiency of HJB/Fe^{3+}, a new and very stable chelate, to supply iron to plants. In this paper we report the role of HJB/Fe^{3+} as substrate of the FC-R in cucumber plants (a Fe-efficient plant) and its efficacy to provide Fe to soybean (a Fe-inefficient plant) in hydroponic and calcareous soil cultures.

For years, the absorption and translocation of Fe in plants has been studied using different approaches. Many authors suggest the use of radioactive isotopes, such as ^{59}Fe (Cesco et al. 2002). However the use of this isotope requires specific laboratory facilities and trained staff, and does not allow long-term trials because isotope activity drops over time. Mössbauer spectroscopy using ^{57}Fe has also been used in the study of the Fe chemistry in plants (Kilcoyne et al. 2000); however, the concentration of Fe in the plant tissues is normally too low to be detected by this technique.

Rodríguez-Castrillón et al. (2008) have developed a new method for studying the ^{57}Fe in plant tissues by ICP-MS using isotope pattern deconvolution. The main advantage of this technique lies in its high precision and low detection thresholds. Most of the elements and isotopes in the periodic table can be analyzed with this technique. This is the reason ICP-MS has been used in this work to study the micronutrient absorption by plants treated with Fe chelates enriched with ^{57}Fe.

Materials and methods

HJB/Fe^{3+} as substrate for FC-R in Fe-stressed cucumber plants

The chelating agents used in this experiment were: o, oEDDHA (Promochem), HJB provided by PPC ADOB and HBED (Strem Chemicals). The titrimetric purity of each product was determined (Yunta et al. 2003a) and the results obtained, expressed as acid content, were 92.94±0.42%, 82.01±0.37% and 89.72±0.43%, respectively.

For the preparation of the iron chelate solutions, ligands were dissolved in sufficient NaOH (1:3 molar ratio) and an amount of $FeCl_3 \cdot 6H_2O$ (Merck), calculated to be 5% in excess of the molar amount of ligand, was then slowly added. During the chelation process pH was maintained between 6.0 and 8.0 and adjusted to 7.0 at the end. Solutions were left to stand overnight to allow excess Fe to precipitate as oxides. Final solutions were filtered through 0.45 μm Millipore membrane and made up to volume with water.

Light exposure was avoided during the preparation and storage of the chelate solutions because of the potential photodecomposition of chelates (Hill-Cottingham 1955, Gómez-Gallego et al. 2005).

Cucumber plants (Strategy I) were used as they are efficient and induce FC-R when iron is limited. Cucumber seeds (*Cucumis sativus* L. cv. Ashley) were germinated in standard seed germination papers moistened with a macronutrient solution in diffuse light in a growth chamber for 7 days. Uniform seedlings were selected and the stems of two

individual plants were wrapped together with polyurethane foam and placed in a 12 L polypropylene bucket (12 pairs of plants per bucket). The buckets contained a continuously aerated EDTA buffered nutrient solution (Degryse et al. 2006) with the following composition: macronutrients (mM) 1.0 Ca $(NO_3)_2$, 0.9 KNO_3, 0.3 $MgSO_4$, and 0.1 KH_2PO_4; cationic micronutrients (μM) 5.0 EDTA/Fe^{3+}, 2.5 $MnSO_4$, 1.0 $CuSO_4$, 10 $ZnSO_4$, 1.0 $CoSO_4$, 1.0 $NiCl_2$, and 115.5 $EDTANa_2$; anionic micronutrients (μM) 35 NaCl, 10 H_3BO_3, and 0.05 Na_2MoO_4 and 0.1 mM HEPES. The pH was adjusted to 7.5 with KOH 1 M.

Plants were grown for 14 days in this nutrient solution in a Dycometal-type CCK growth chamber provided with fluorescent and sodium vapour lamps with a 16 h, 30°C and 50% humidity day and 8 h, 25°C and 70% humidity night regime. Water was added every 2 days, and the nutrient solution was renewed every 7 days.

The amount of iron added (5 μM) was found to be the most adequate to produce green cucumber plants, but with high FC-R activity (stressed plants) in an assay with similar experimental conditions (Lucena and Chaney 2006).

The measurement of FC-R activity was made as in Lucena and Chaney (2006) at pH 6 (FC-R activity is greater at pH 6 than at a higher pH (Susín et al. 1996)). Na_2BPDS (300 μM) was used as the Fe^{2+} trapping and colorimetric reagent. The experiment began 2 h into the daylight period. At time zero, 5 mL of the corresponding treatment solution was added so that the final concentration was 100 μM. Aliquots of 3 ml were withdrawn at 0, 10, 20, 60 and 120 min. Seven replicates were prepared for each treatment. Two replicate blanks per chelate, consisting of solutions without plants, were included in order to correct reduction rates for slow photoreduction.

The $Fe(II)(BPDS)_3$ was calculated as in Lucena and Chaney (2006) after the determination of the absorbances at 535 nm (maximum of the Fe(II) $(BPDS)_3$) and at 480 nm (near the maximum absorbance of o,oEDDHA/Fe^{3+}, HJB/Fe^{3+} and HBED/Fe^{3+}) to consider the contribution of the treatments applied to total absorbance.

The fresh weight of the roots was measured at the end of the experiment. The slope of the plots of Fe^{2+} ($\mu mol \cdot g^{-1}$ fresh root) plotted against time (h) was used as the Fe^{2+} reduction rate for each pair of plants. Data were expressed as the mean reduction rate and the standard error, corresponding to seven plant replications for each treatment.

Efficacy of HJB/$^{57}Fe^{3+}$ to provide Fe to soybean plants in hydroponic culture

Soybean plants (*Glycine max* L. cv Stine 0480) were used in this experiment as they are considered susceptible to chlorosis, being taken as a model for crops normally treated with chelates. The seeds were washed with water for 30 min and then placed in trays between cellulose paper sheets soaked with type I water. The trays were kept in darkness in a growth chamber, at 30 °C and 60% of humidity for 2 days . Afterwards, the seedlings were placed in 10 L containers filled with a 1/5 strength EDTA buffered nutrient solution (the same composition as in the FC-R experiment) for 5 days. On the 6 day, in order to induce iron chlorosis, the seedlings were transferred to 2 L buckets containing an aerated full-strength EDTA buffered nutrient solution (Degryse et al. 2006), but without iron chelate, and 0.4 g of solid $CaCO_3$. Plants were grown under these conditions until severe symptoms of iron deficiency were observed (5 days), whereupon several treatments were applied.

Three treatments (o,oEDDHA/$^{57}Fe^{3+}$, HJB/$^{57}Fe^{3+}$ and HBED/$^{57}Fe^{3+}$) at two different doses (5 μM and 10 μM) and a Control —Fe (no iron added) were assayed. Doses are lower than those required by the plants, but are adequate for finding differences between chelates of high stability. For the preparation of the iron chelate solutions, ligands were dissolved as in the previous experiment, but in this case the Fe was added as ^{57}Fe (provided by Isoflex) (95.38%) dissolved in HNO_3 Suprapur (Merck).

Three replicate pots per treatment were prepared. Plants were kept for 21 days under these conditions. The nutrient solution was renewed weekly. The chlorophyll index (SPAD 502, Minolta) was measured in all the leaves every 2 days. Samples were taken after 7 days (Sampling 1, two pairs of plants) and after 21 days (Sampling 2, one pair of plants) as of the first treatment application, washed following the procedure described by Álvarez-Fernández et al. (2001), and the fresh and dry weights of leaves, stems and roots were determined separately. Micronutrient concentrations were then determined in the plant

organs after dry mineralization by atomic absorption spectrophotometry (Perkin-Elmet Analyst 800) and ^{57}Fe in leaves and roots was analysed by ICP-MS.

Efficacy of HJB/$^{57}Fe^{3+}$ to provide Fe to soybean plants in soil culture

Isotopic exchange between chelates and soil

Prior to the biological experiment, an interaction experiment with ^{57}Fe chelates was performed to test whether isotopic exchange occurs between native Fe (mainly ^{56}Fe) from the soil and the ^{57}Fe from the chelates. The aim of this test was to evaluate the suitability of using enriched ^{57}Fe chelates in the soil-plant experiment. A calcareous soil from Picassent (Valencia, Spain) was used as incubation substrate and later in the pot experiment. Soil characteristics are presented in Table 1. In brief, it is a calcareous soil that has a sandy loam texture, low organic matter content and normal Cu availability. o,oEDDHA/$^{57}Fe^{3+}$ and HJB/$^{57}Fe^{3+}$ were used in this experiment.

Table 1 Chemical and physical characteristics of the soil used in the interaction and biological experiments

Properties	Soil
Sand (g ·kg^{-1})	435
Silt (g ·kg^{-1})	80
Clay (g ·kg^{-1})	485
Texture	Sandy clay
pH (H_2O)	7.70
pH (KCl)	7.10
E.C extract 1:5 (dS·m^{-1})	0.270
M.O. Oxidized (g ·kg^{-1})	9.2
N Kjeldahl (g ·kg^{-1})	0.3
C/N	30.7
$CaCO_3$ Total (g ·kg^{-1})	380
Active lime (g ·kg^{-1})	89
Macronutrients (NH_4Ac, $cmol_c \cdot kg^1$)	
Ca	5.13
Mg	2.92
K	0.51
Na	0.14
Micronutrients (Lindsay DTPA extract, mg·kg^1)	
Fe	2.34
Mn	1.87
Cu	0.73
Zn	2.48

For the experiment, two grams of soil were weighed in 40 mL polyethylene flasks. Five mL of each chelate solution containing 4×10^{-4} M of chelating agent and 5 mL type I water were added. Chelate blanks (without soil) and soil blanks (without chelate but with 10 mL water) were also prepared. All the samples were shaken at 56 cycles min^{-1} at 25 °C in the dark, for 1 h, 3 h, 7 h, 1 day, 3 days, 7 days and 30 days. After the incubation time, the solutions were filtered through 0.45-µm Millipore membranes. Samples for each time and product tested were prepared in duplicate. Total Fe concentration in the filtrate was determined by AAS and ^{57}Fe by ICP-MS after their acidification with HNO_3 Suprapur (Merck).

Biological experiment

For the biological experiment, soybean seeds were germinated as in the previous hydroponic experiment. The seedlings were then transplanted to 1 L pots filled with 1 kg soil:sand (2:1 v:v) mixture (see soil and sand characteristics in Tables 1 and 2) (1 kg mixture contains approximately 0.70 kg soil). The pots were placed in the growth chamber with the same daily growth cycle as in the previous experiments.

The pots were initially irrigated up to 80% saturation and then daily with the amount of solution necessary, determined by weight loss, to return to the 80% water saturation capacity. Irrigation involved a macronutrient nutrient solution (double concentrate) similar to that used before but without micronutrients and with 0.1 g L^{-1} of lime and 0.1 g L^{-1} of sodium bicarbonate. Pots were placed on plates to control leaching.

The experiment consisted of two treatments (o,oEDDHA/$^{57}Fe^{3+}$ and HJB/$^{57}Fe^{3+}$) and one control without iron. Five replicate pots per treatment were used. Treatments were applied 7 days after transplanting when the plants showed slight chlorosis symptoms. A dark plastic film covered the soils to avoid photodegradation of the iron chelates (Hill-Cottingham 1955; Gómez-Gallego et al. 2005) and to avoid algae development.

The concentration of Fe in the treatments was 2.5 mg of ^{57}Fe per kg of soil (1.75 mg/pot). For the addition of the chelate solutions, 50 mg L^{-1} of Fe in the form of o,o-EDDHA/$^{57}Fe^{3+}$ or HJB/$^{57}Fe^{3+}$ were

Table 2 Chemical and physical characteristics of the sand used in the biological experiment

Properties	Sand
$CaCO_3$ (g ·kg^{-1})	975
SiO_2 (g ·kg^{-1})	11.0
Al_2O_3 (g ·kg^{-1})	1.8
$MgCO_3$(g ·kg^{-1})	11.5
Fe_2O_3(g ·kg^{-1})	0.55
Density (g/mL)	2.60
Lime (CaO) (g ·kg^{-1})	548.2

prepared, and then 35 mL of these solutions and the water necessary to achieve 80% saturation were added in the centre of the pots.

The SPAD Index was determined, after the treatment application, every 2 days throughout the experiment. Three sampling times were: 2 days, 1 week and 3 weeks after the treatment application. At each sampling time, one plant shoot was taken. Leaves and stems were separated and washed, first with Tween 80 in 0.1 M HCl for 30 seconds (Álvarez-Fernández et al. 2001), and then with abundant distilled water, and subsequently weighed and dried. Total Fe and ^{57}Fe were determined in leaves after dry digestion procedure by AA and ICP-MS, respectively.

After the plant experiment had been completed, the soluble and available ^{57}Fe remaining in the soils were determined. The complete pot contents (including roots) were submerged in 1 L distilled water and shaken until complete disaggregation of the substrate. Roots were taken apart to be analyzed. Forty mL of the substrate-water mix was centrifuged and the supernatant filtrated. HNO_3 Suprapur (Merck) was added to the supernatant to obtain a concentration of 10 g L^{-1}. Total Fe and ^{57}Fe were determined in these extracts. The remaining solid in the centrifuge tube was extracted for 20 min with 25 mL of Soltanpour and Schwab (1977) extractant (DTPA+ammonium bicarbonate) and then filtered. The extraction was made in triplicate and the extracts joined in a single extract and volume amounted to 100 mL. HNO_3 was added to eliminate excess bicarbonate and allow an acid media for the analytical determinations of ^{57}Fe by ICP-MS and total Fe by AA. Roots were washed with Tween 80 at 0.1% in HCl 0.1 M solution (Álvarez-Fernández et al. 2001), weighed, dried and oven mineralized to determine total Fe and ^{57}Fe.

Statistical analysis

Results were statistically treated using the Statistical Package Social Science PC 14.0. Statistical comparison of means have been carried out to reveal the differences between chelates using Duncan's Multiple Range Test (α=0.05).

Results

HJB as substrate for FC-R in Fe-stressed cucumber plants

Table 3 presents the Fe(III) reduction rate (µmol Fe (II) · g^{-1} root fresh weight · hour^{-1}) for the different iron chelates used as substrates. There are no significant differences between o,oEDDHA/Fe^{3+}, HJB/Fe^{3+} and HBED/Fe^{3+} when they are used as substrates of the FC-R (Duncan Test, α=0.05). The similar behaviour found for o,oEDDHA/Fe^{3+}, HJB/Fe^{3+} and HBED/Fe^{3+} is well correlated with the similar stability of the chelates, in good agreement with the findings published by Lucena and Chaney (2006). This could be related to the similar structure of the three molecules (six bonds with the same type of donor groups between the chelating agent and the Fe^{3+}) that implies a comparable behaviour when they are used as substrates of the FC-R.

Efficacy of HJB/$^{57}Fe^{3+}$ to provide Fe to soybean plants in hydroponic culture

In this experiment, Fe deficient soybean plants were used and three different Fe chelated treatments with two different doses were applied. Changes in the SPAD index (ΔSPAD) between the readings at each

Table 3 Rate of Fe^{3+} reduction (µmol of Fe^{2+} h^{-1}g^{-1}) in root dry weight (DW) and fresh weight (FW) basis from iron chelates used for cucumber plants. ns: No significant differences between treatments according to the Duncan test (α=0.05)

Treatments	µmol of Fe^{2+} h^{-1}g^{-1}	
	DW	FW
o,oEDDHA/Fe^{3+}	10.0 ns	0.27 ns
HJB/Fe^{3+}	7.3	0.23
HBED/Fe^{3+}	13.3	0.32

time and the reading at the beginning of the treatments (t=0) gives a quantitative measurement of the plants' recovery from chlorosis and the relative effectiveness of the Fe fertilization treatment (Pestana et al. 2003). During the experiment, SPAD readings were taken for all leaf stages, although only values measured for the second leaf stage (the youngest fully open leaf at the start of the treatments, t = 0) have been presented in Fig. 2 since they were the most representative of the whole plant. Figs. S1 and S2 in the supporting information present the visual aspect of the plants, providing the same information as the SPAD index.

Negative values of ΔSPAD imply a lack of recovery from chlorosis, while positive values mean that the treatment is effective. With the low dose, plants treated with o,oEDDHA/$^{57}Fe^{3+}$ showed the highest increase in SPAD during the experiment that corresponds to higher plant biomass. The plants treated with HJB/$^{57}Fe^{3+}$ had a lower ΔSPAD than the plants receiving the other treatments, but significantly different to the control plants. HBED/$^{57}Fe^{3+}$ had an intermediate behaviour between o,oEDDHA/$^{57}Fe^{3+}$ and HJB/$^{57}Fe^{3+}$. With the high dose, still lower than normally used in a hydroponic culture, the tendency was similar. The plants treated with o,oEDDHA/$^{57}Fe^{3+}$ showed the greatest recovery from chlorosis, followed by HBED/$^{57}Fe^{3+}$ and HJB/$^{57}Fe^{3+}$.

Two-way ANOVA analysis considering doses and Fe source as factors revealed that there was no

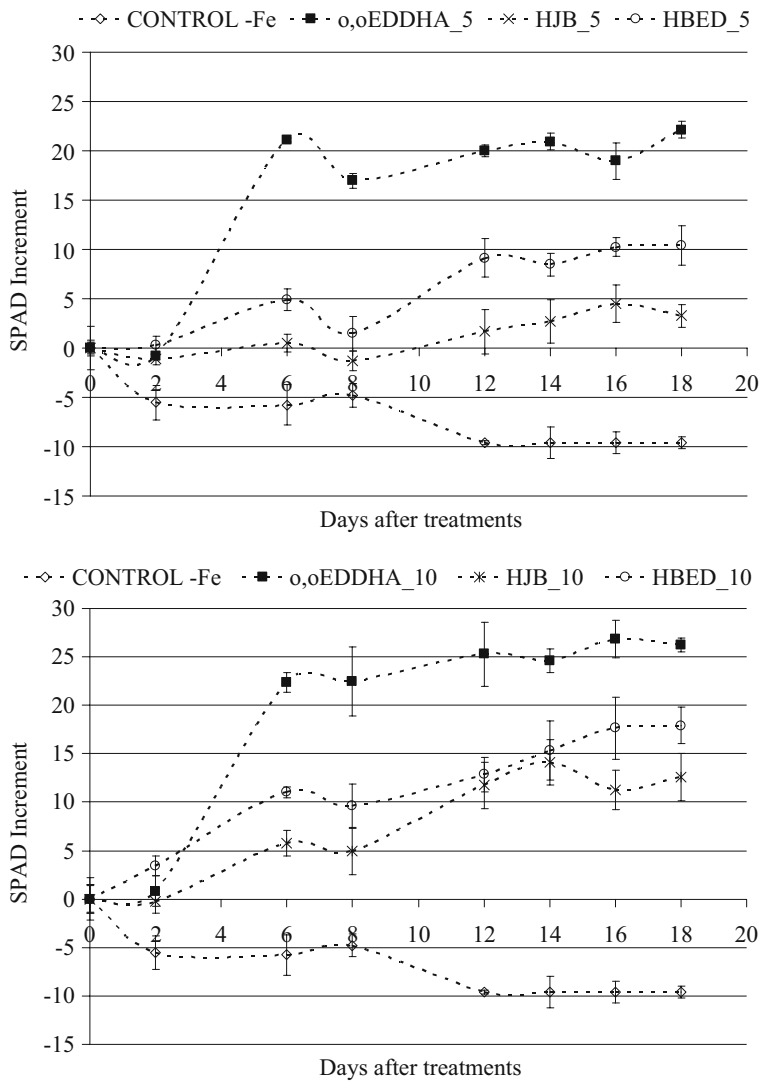

Fig. 2 Variation of the SPAD Index (ΔSPAD) for the 2nd leaf stage (youngest fully open leaf) with time in the hydroponic experiment

interaction between factors when plant dry weight and Fe and ^{57}Fe concentrations were considered, so the average values for these parameters are presented in Table 4. The plants treated with chelates recorded a higher biomass at the end of the experiment than the untreated plants. The total biomass of the treated plants follows the order: o,oEDDHA/$^{57}Fe^{3+}$ > HBED/$^{57}Fe^{3+}$ >HJB/$^{57}Fe^{3+}$. In addition, greater doses of treatment provide a higher plant biomass.

Iron nutrition status was also assessed by leaf and root micronutrient concentration (Total Fe and ^{57}Fe in leaves and roots are presented in Table 4). In this hydroponic experiment, iron nutrition has been evaluated considering the Fe uptake from the chelates prepared with the isotope ^{57}Fe, so the iron uptake could be differentiated from the chelate and from the pre-treatment nutrient solution or from the seed.

When comparing ^{57}Fe content for the treatments in the second sampling time, significant differences have been found between treatments and the control -Fe.

At the end of the experiment, the plants treated with o,oEDDHA/$^{57}Fe^{3+}$ presented higher levels of total Fe and ^{57}Fe in leaves than HJB/$^{57}Fe^{3+}$ and HBED/$^{57}Fe^{3+}$. The plants treated with the high dose (10 μM) recorded the highest level of Fe and ^{57}Fe in leaves and roots.

Since the 1950's, soil application of Fe chelates is known to negatively affect Mn uptake in a large number of plant species including soybean (Ghasemi Fasaei et al. 2003) due to better Fe nutrition. The Fe/Mn ratio in leaves is considered to be a better iron nutritional index than total Fe for several crops (Pestana et al. 2003). Since Mn chelation is negligible (speciation of our NS with Vminteq reveals that less than 0.6% of the Mn in the NS can be chelated, even when the Fe concentration is reduced to half the initial by plant uptake), an increase of the Fe/Mn ratio implies a recovery from iron chlorosis. Interaction between Fe source and doses is observed when the two-way ANOVA analysis was performed for the Fe/Mn ratio in leaves, so a one-way analysis was made revealing that 10 μM o,oEDDHA/$^{57}Fe^{3+}$ presented the highest value (1.22). The 5 μM o,oEDDHA/$^{57}Fe^{3+}$ (0.85) and 10 μM HBED/$^{57}Fe^{3+}$ (0.74) provide intermediate Fe/Mn ratios. The rest of the treatments presented lower values and no differences could be observed between the two doses of HJB/$^{57}Fe^{3+}$ studied.

Efficacy of HJB/$^{57}Fe^{3+}$ to provide Fe to soybean plants in soil culture

Isotopic exchange

Table 5 shows the concentration (mg/L) of ^{57}Fe that remained in solution over time with the two chelates

Table 4 Effect of the different Fe chelate treatments and doses on dry weight of plants, total Fe and ^{57}Fe concentration in soybean plants in the hydroponic experiment at the end of the experiment. Data for Fe sources are the average of the different doses and data for doses are the average of the different treatments. Different letters in the same column and factor indicate significant differences between chelate or dose according to the Duncan test (α=0.05)

Factors	Dry weight (g pair of plants^{-1})	Concentration in leaves (μmol g^{-1} DW)		Concentration in roots (μmol g^{-1} DW)	
		Fe	^{57}Fe	Fe	^{57}Fe
Fe sources					
Control -Fe	1.44 d	0.34 d	0.01 d	0.86 b	0.02 c
o,oEDDHA	10.05 a	1.07 a	0.76 a	1.07 ab	0.52 b
HJB	3.43 c	0.50 c	0.28 c	0.99 ab	0.57 b
HBED	5.41 b	0.64 b	0.42 b	1.18 a	0.69 a
Doses					
0	1.44 c	0.34 c	0.01 c	0.86 b	0.02 c
5 μM	5.36 b	0.64 b	0.39 b	1.01 ab	0.51 b
10 μM	7.23 a	0.84 a	0.59 a	1.15 a	0.68 a

Table 5 Concentration of ^{57}Fe in solution along time in the soil-chelate interaction experiment

		Time						
		1 h	3 h	7 h	1 d	3 d	7 d	30 d
[^{57}Fe] (mg/L)	o,oEDDHA	23.9	23.7	23.3	23.7	23.3	23.5	23.1
	HJB	22.0	22.1	21.7	22.0	22.3	22.0	21.8

assayed. The percentage of ^{57}Fe over the total Fe from o,oEDDHA/$^{57}Fe^{3+}$ and HJB/$^{57}Fe^{3+}$ that remained in solution after 30 days was very high. The constancy of the results is indicative of very little or no exchange between the added ^{57}Fe and the native ^{56}Fe, with no substitution of the chelated ^{57}Fe by any other element. In addition, the retention of the chelate is negligible. These results indicate that it is possible to discriminate the source of Fe (soil or chelate) that soybean plants take up in the soil-plant experiment if chelated ^{57}Fe is used.

Biological experiment

SPAD readings were taken for all leaf stages, although only values measured for the 3rd leaf stage (the youngest fully developed leaf at the start of the treatment period, t=0) and 5th level (youngest leaf level at the end of the experiment) have been presented in Fig. 3. In the 3rd level, differences between treatments and control -Fe could be observed. However, in the 5th level, the plants treated with HJB/$^{57}Fe^{3+}$ recorded the highest SPAD Index at

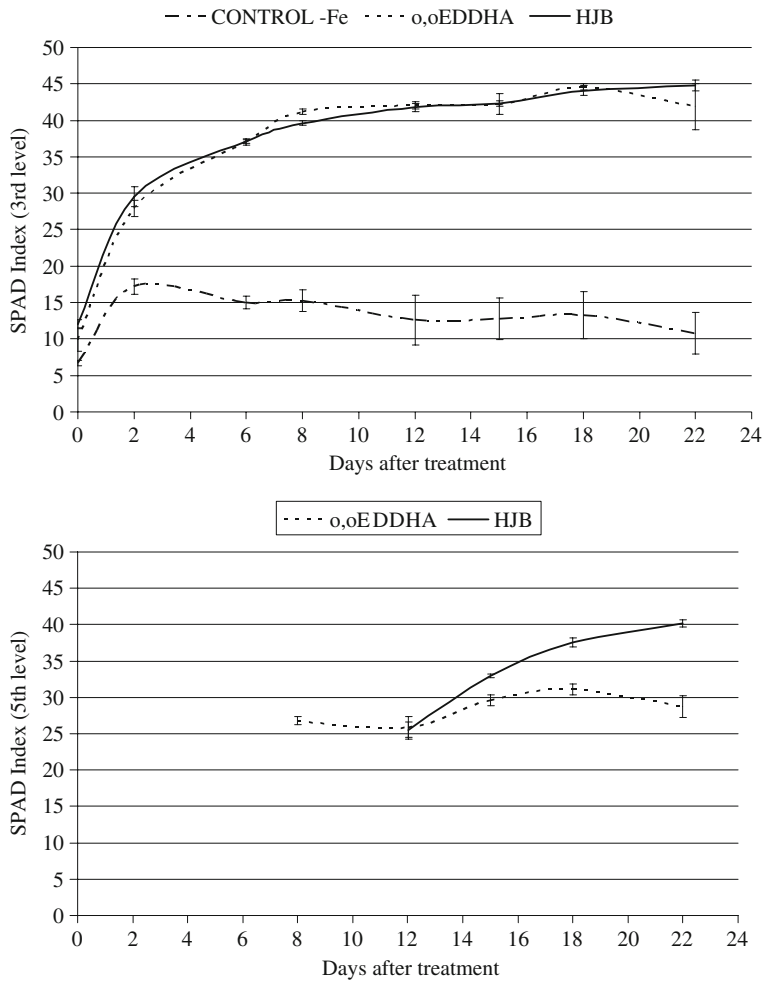

Fig. 3 Evolution of SPAD index for the 3rd and 5th leaf stages along time in the soil experiment

the end of the experiment (Fig. 3) that corresponds to the higher biomass of the plants treated with this product. Control -Fe plants did not reach this leaf level. It is clear from the visual aspect (Fig. S3 in the supporting information) that o,oEDDHA/$^{57}Fe^{3+}$ presents some chlorosis symptoms at the end of the experiment, while HJB/$^{57}Fe^{3+}$ treated plants look healthy.

Table 6 presents plant dry weight (stems and leaves) for the third sampling time for each treatment. The first sampling time (data not shown) was done only 2 days after treatment addition, so no differences were expected in such a short time. In the second sampling time (1 week after treatment application), differences between treated and control —Fe plants were observed (data not shown). In the third sampling time, the plants treated with HJB/$^{57}Fe^{3+}$ had a higher biomass than plants treated with o,oEDDHA/$^{57}Fe^{3+}$. At the end of the experiment, the roots (of the three plants) were collected and weighed. No differences between treatments were observed, and both of them had a higher root weight than the control -Fe plants.

^{57}Fe, total Fe, Mn, Cu and Zn concentration in leaves (μmol/ g DW) are presented for the different treatments at the third sampling time (Table 6). The plants treated with o,oEDDHA/$^{57}Fe^{3+}$ presented higher levels of ^{57}Fe than HJB/$^{57}Fe^{3+}$. For total Fe, the differences observed were very similar to those observed using the ^{57}Fe data. In all the samplings, iron treatments provided more Fe to plants than control -Fe, and the plants treated with o,oEDDHA/$^{57}Fe^{3+}$ presented a higher level of iron in leaves than plants treated with HJB/$^{57}Fe^{3+}$. Fe nutrition has negatively affected Mn nutritional status. In Fe-chelate treated plants, the Mn concentration found in the third sampling time is close to the lower limit of Mn sufficiency range (>15 ppm). Cu complexation by the chelating agents is a process that could affect Cu uptake. Cu nutrition has been very affected in o,oEDDHA/$^{57}Fe^{3+}$ treated plants, while HJB/$^{57}Fe^{3+}$ ones have been less affected. Zn concentration in plants has been similarly affected.

The results of the solubility and availability of ^{57}Fe in soil at the end of the experiment are presented in Fig. 4. Differences between the treated pots and the control pots are shown. No significant differences between treatments could be observed in the amount of ^{57}Fe available. However, the pots treated with HJB/$^{57}Fe^{3+}$ recorded a higher concentration of soluble ^{57}Fe than the pots treated with o,oEDDHA/$^{57}Fe^{3+}$. The higher uptake of ^{57}Fe in the plants treated with this o,oEDDHA/$^{57}Fe^{3+}$ would explain the decrease in the fraction of soluble ^{57}Fe for this treatment with respect to HJB/$^{57}Fe^{3+}$.

Discussion

The objective of this work is to evaluate the efficiency of a new and very stable chelate, HJB/$^{57}Fe^{3+}$, to supply iron to plants and to relate its efficiency to its chemical characteristics.

For the FC-R experiment, green Fe-stressed cucumber plants were used to simulate a more realistic agronomic condition than using severely chlorotic plants grown without iron. No differences could be observed in the reduction rate from the chelates assayed. Lucena and Chaney (2006) concluded that the reduction rate for the different Fe(III)-chelates depends on chelate stability. The stability of the HJB/Fe^{3+} (log K°=34.45±0.04, (López-Rayo et al., 2009)) is slightly lower than o,oEDDHA/Fe^{3+} (35.09±0.28,

Table 6 Effect of the different Fe chelate treatments on the dry weight of leaves, roots and stems, total Fe, ^{57}Fe, Mn, Cu and Zn concentration and Fe/Mn mass ratio in soybean plants in the soil experiment in the third sampling time. Different letters in the same column indicate significant differences between treatments according to the Duncan test (α=0.05)

	Biomass			Leaves (μmol/g DW)					Leaves
Treatments	Leaf (g)	Stem (g)	Root (g)[a]	Fe	^{57}Fe	Mn	Cu	Zn	Fe/Mn
Control -Fe	0.96 c	0.63 c	0.53 b	0.26 c	0.05 c	1.07 a	1.02 a	1.00 a	0.25 c
o,oEDDHA/$^{57}Fe^{3+}$	1.77 b	1.55 b	0.73 a	1.35 a	1.38 a	0.26 b	0.30 c	0.33 c	5.46 a
HJB/$^{57}Fe^{3+}$	2.30 a	1.89 a	0.87 a	0.71 b	0.64 b	0.31b	0.49 b	0.50 b	2.41 b

[a] at the end of the experiment the roots of the three plants were obtained for the three sampling times

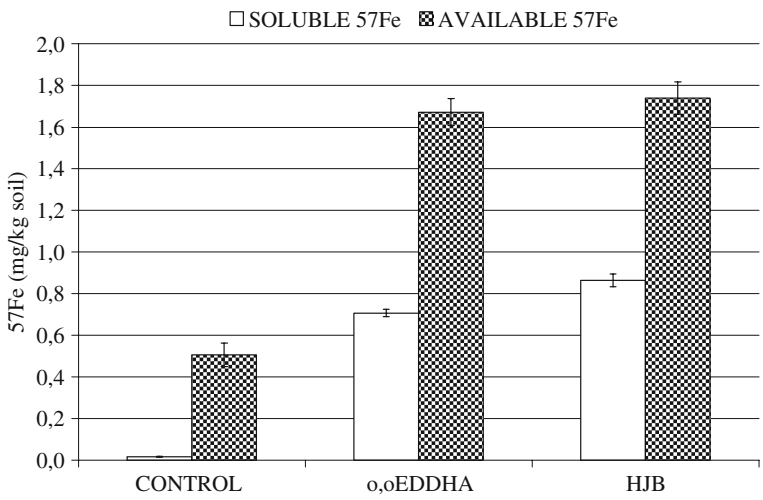

Fig. 4 ^{57}Fe present in the soluble and available fractions of the soils after the plant experiment

Yunta et al. 2003a), and HBED/Fe^{3+} (log K°=39.01, Ma et al. 1994) is appreciably stronger and more selective for Fe^{3+} than EDDHA/Fe^{3+}. However, they all have a similar behaviour as substrates of the iron chelate reductase in mild chlorotic cucumber plants, in agreement with Lucena and Chaney (2006), who observed no significant differences between o,oEDDHA/Fe^{3+} and HBED/Fe^{3+} when they were used as substrates of the FC-R.

The uptake of ^{57}Fe by soybean from o,oEDDHA/$^{57}Fe^{3+}$, HBED/$^{57}Fe^{3+}$ and HJB/$^{57}Fe^{3+}$ was evaluated in hydroponic and soil cultures. In these experiments, iron nutrition was studied considering the Fe uptake from the chelates prepared with the isotope ^{57}Fe to differentiate the iron uptake from the chelate and from other sources.

As expected, the three chelates assayed were able to alleviate the iron chlorosis of the plants in high pH conditions, although significant differences between treatments could be observed.

When plants are grown hydroponically, among chelates of high stability, the less stable Fe(III)-chelates are usually the more effective (Lucena and Chaney 2007) because the effectiveness of Fe(III)-chelates is not affected by the reaction between chelate and soil. However, in this case, o,oEDDHA/$^{57}Fe^{3+}$, with a stability constant intermediately between HJB/$^{57}Fe^{3+}$ and HBED/$^{57}Fe^{3+}$, presents the best results in biomass, SPAD index and Fe nutrition when applied in stressed soybean plants.

The o,oEDDHA/Fe^{3+} contains two diastereoisomers, meso and racemic, which have different characteristics. It was found that the stability constant for EDDHA/Fe^{3+} was 1.71 log units greater for the racemic complex than the meso complex, indicating a 50-fold difference in iron chelating ability (Yunta et al. 2003b). However, HJB/Fe^{3+} presents a stability average between racemic and meso isomers, while HBED/Fe^{3+} presents the highest stability. According to Cerdán at al. (2006) Strategy II plants took up iron from both Fe(o,o-EDDHA) isomers equally; however, Strategy I plants took mainly the iron associated with the meso form (the lowest stability isomer). The same conclusions were reached by Lucena and Chaney (2007) growing cucumber plants in hydroponics using the single isomers, meso or racemic, as iron sources

In our case, it is possible that the presence of the meso isomer in the o,oEDDHA/Fe^{3+} allows a higher Fe uptake from the plants treated with this product in hydroponic cultures and explains the better behaviour of o,oEDDHA/$^{57}Fe^{3+}$ in the conditions assayed.

In agreement with Chaney (1988), HBED/$^{57}Fe^{3+}$ supplied adequate Fe for the growth of Strategy I species at pH 7.5 in hydroponic cultures. However plants treated with HBED/$^{57}Fe^{3+}$ had a lower Fe uptake rate than plants treated with o,oEDDHA/$^{57}Fe^{3+}$, with both behaving similarly when used as substrate of the FC-R. In agreement with Lucena and Chaney (2007), this raises a question about the effect of the chelate, not only on the reduction mechanism, but also on the transporter (IRT1) across the root plasma membrane and through the plant that explains the significant differences between both treatments in the Fe concentration in leaves.

The study of the possible isotopic exchange between chelates and soil revealed the high capacity

of o,oEDDHA/$^{57}Fe^{3+}$ and HJB/$^{57}Fe^{3+}$ to maintain Fe in the soil solution and the low isotopic interchange occurring. So the ^{57}Fe uptake for the plants in the soil experiment, exceeding that of the control, came from the applied chelate. However, the soil analysis at the end of the plant experiment rendered it possible to corroborate the ability of the products tested to maintain the iron available after the 3 weeks of experiment. The different soil:solution ratio in both the isotopic exchange and the pot experiment and the effect of the plant in the latter can explain the differences in the results obtained.

In the soil experiment, plants treated with HJB/$^{57}Fe^{3+}$ recorded the highest SPAD indexes in young leaves (5th level, Fig. 3) and shoot biomass (Table 6) at the end of the experiment. However, plants treated with o,oEDDHA/$^{57}Fe^{3+}$ presented the highest Fe content in leaves, while they showed some chlorosis symptoms and the SPAD Index was lower than for the HJB/$^{57}Fe^{3+}$ in the growing parts of the plant. This can be explained by the different absorption rates that could be determined using the ^{57}Fe data. Plants treated with o,oEDDHA/$^{57}Fe^{3+}$ had a high Fe absorption rate, 0.456 and 0.131 μmol ^{57}Fe plant^{-1} d^{-1}, in the first (1–3 days after the treatment application) and second period (4–8 days) respectively, compared with HJB/$^{57}Fe^{3+}$ (0.123 and 0.034 μmol ^{57}Fe plant^{-1} d^{-1}). On the other hand, in the third period (8 to 21 days) HJB/$^{57}Fe^{3+}$ maintained a higher uptake rate (0.066 μmol ^{57}Fe plant^{-1} d^{-1}) than o,oEDDHA/$^{57}Fe^{3+}$ (0.025 μmol ^{57}Fe plant^{-1} d^{-1}). It can therefore be concluded that HJB/$^{57}Fe^{3+}$ has a longer lasting effect than o,oEDDHA/$^{57}Fe^{3+}$. Due to the low mobility of iron in plants, it is normal for o,oEDDHA/$^{57}Fe^{3+}$ treated plants to show chlorotic symptoms in young leaves and growth reduction at the end of the experiment regarding HJB/$^{57}Fe^{3+}$.

Results in hydroponics are in good agreement with the slower initial effect of the HJB/$^{57}Fe^{3+}$ observed in the soil experiment. Since nutrient solutions are renewed weekly, the possible long-lasting effect could not be assessed in hydroponics.

In these studies, isotope ^{57}Fe is used to confirm the origin of the Fe uptake by the treated plants. The aim is to check iron absorption by the plant from the synthetic chelates in order to determinate its effectiveness in correcting iron chlorosis. The small differences between total Fe and ^{57}Fe concentration in leaves point to the difficulty these chelates have to take iron from the soil, e.g. after having delivered an iron ion to the plant (shuttle effect). The technique used therefore ensures that the Fe quantified in plants came directly from the chelates applied, giving more significant differences than when only total Fe is measured. In fact, the Fe uptake rate determined in the soil experiment and previously discussed can only be related to chelate application when ^{57}Fe is considered. Moreover, the use of this technique allows other analyses to be made, such as the study of Fe mobility in the soil, the chelate capacity to take iron from the soil (shuttle effect), the translocation of Fe in the plant, etc.

According to Lucena (2003), one of the characteristics of an iron chelate that determines its effectiveness in agronomic practice is its ability to maintain Fe in solution. The analysis of the soil at the end of the biological experiment shows a higher increase in the availability of Fe than in the soluble Fe fraction, so it is expected that part of the added Fe with the chelates has been retained in the soil.

Regarding the new product studied, HJB/Fe^{3+}, the results obtained allow verification to be made of its effectiveness to correct iron chlorosis in soybean plants grown in hydroponic cultures and alkaline soils. This product, purer in its composition than the commercial formulations of EDDHA/Fe^{3+}, is an acceptable Fe fertilizer. Field experiments are needed to verify its efficacy. Furthermore, HBED has proven to be a good chelate for plants despite its high stability.

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