Plant Soil
DOI 10.1007/s11104-011-1118-y

REGULAR ARTICLE

Fertilizer properties of DCHA/Fe^{3+}

Paloma Nadal · Sonia García-Marco · Rosa Escudero · Juan J. Lucena

Received: 11 July 2011 / Accepted: 22 December 2011

Abstract
Aims The suitability of the non-symmetrical chelating agent DCHA (2-{2-[(2-hydroxybenzyl)amino]ethylamino}-2-{2-hydroxyphenyl}acetic) to improve Fe nutrition in plants is investigated in order to confirm the good results deriving from its chemical reactivity in agronomic systems achieved by analytical and modeling studies. Moreover, the factors affecting the efficacy of this new Fe chelate, that it is predicted to combine a good stability in nutrient solution and calcareous soils, are explored.
Methods The role of DCHA/Fe^{3+} as substrate for the Fe chelate reductase (FCR) activity in cucumber (*Cucumis sativus* L. cv. Ashley) plants and its efficacy to provide Fe to chlorotic soybean (*Glycine max* L. cv. Stine 0408) plants in both hydroponic and soil culture were determined.
Results The chelate DCHA/Fe^{3+} presented an intermediate behavior between o,oEDDHA/Fe^{3+} and o,pEDDHA/Fe^{3+} as substrate of the FCR. In the hydroponic experiment, nutritional indexes indicated a faster and higher re-greening of the plants treated with DCHA/Fe^{3+} and o,pEDDHA/Fe^{3+} than with o,oEDDHA/Fe^{3+}. In the soil experiment, plants treated with o,oEDDHA/$^{57}Fe^{3+}$ showed the highest ^{57}Fe concentration in leaves and no differences were observed between o,pEDDHA/$^{57}Fe^{3+}$ and DCHA/$^{57}Fe^{3+}$.
Conclusions The chelate DCHA/Fe^{3+} has adequate fertilizer properties since it is able to correct the Fe chlorosis and to maintain good nutritional status of plants over time both in hydroponic and soil cultures. This is related to its ability to serve as substrate for the FCR and its good stability in solution and in soil conditions observed in this and previous studies.

Keywords Iron chlorosis · Iron chelates · DCHA/Fe^{3+} · Iron chelate reductase · Hydroponic · Soil cultures

Abbreviations

BPDS	Bathophenanthroline disulfonic acid
DAT	Days after first treatment
DCHA	2-{2-[(2-hydroxybenzyl)amino] ethylamino}-2-(2-hydroxyphenyl)acetic acid
DTPA	Diethylenetriaminepentaacetic acid

Responsible Editor: Hans Lambers.

Electronic supplementary material The online version of this article (doi:10.1007/s11104-011-1118-y) contains supplementary material, which is available to authorized users.

P. Nadal · S. García-Marco (✉) · J. J. Lucena
Department of Agricultural Chemistry, Universidad Autónoma de Madrid,
C/Francisco Tomás y Valiente nº 7,
28049 Madrid, Spain
e-mail: sonia.garcia@upm.es

R. Escudero
Department of Organic Chemistry,
Universidad Complutense de Madrid,
28040 Madrid, Spain

Present Address:
S. García-Marco
EUIT Agrícola, Universidad Politécnica de Madrid,
Ciudad Universitaria s/n,
28040 Madrid, Spain

Published online: 04 February 2012

EDTA	Ethylenediaminetetraacetic acid
FAAs	Flame Atomic Absorption Spectroscopy
FCR	Fe chelate reductase
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
ICP-MS	Inductively coupled plasma mass spectroscopy.
o,oEDDHA	Ethylenediamine-N,N'bis(o-hydroxy-phenylacetic) acid
o,pEDDHA	Ethylenediamine-N(o-hydroxyphenylacetic)-N'(p-hydroxyphenylacetic) acid
p,pEDDHA	Ethylenediamine-N(p-hydroxyphenylacetic)-N'(p-hydroxyphenylacetic) acid

Introduction

Iron (Fe) chlorosis is a complex nutritional disorder, manifested as intervenial young leaf yellowing and associated with an insufficient plant Fe uptake, which affects the development and decreases the yield and quality of many sensitive crops (Inskeep and Bloom 1984; Álvarez-Fernández et al. 2006; Rombolá and Tagliavini 2006) growing primarily on calcareous soils (Hansen et al. 2006) and resulting in significant economic looses.

Nowadays, Fe chelates applied to the soil are the most effective agricultural practice to correct this nutritional disorder (Chen and Barak 1982; Sanz et al. 1992; Lucena 2000). Among all soil-applied Fe fertilizers, o,oEDDHA/Fe^{3+} and its analogues are the most efficient (Norvell 1991; Lucena 2006). The industrial synthesis of EDDHA/Fe^{3+} commercial products usually produces a mixture of 3 regioisomeric compounds: o,oEDDHA, o,pEDDHA, and p,pEDDHA (Gómez-Gallego et al. 2002). The relative position of the hydroxy group in the benzene ring of these molecules has been shown to have a crucial importance on the formation of the ferric chelate. The symmetric positional isomer o,oEDDHA, with a coordination number of 6 and with 2 hydroxy groups in the ortho-position of the benzene ring, forms soluble Fe(III) chelates of relatively low reactivity in soils and high stability in either neutral or alkaline solutions. However, in the asymmetric o,pEDDHA isomer, since one of the hydroxy groups is moved to the para-position of the benzene ring, the resultant ferric chelate shows a coordination number of 5, and its complexation affinity to Fe is weaker than that of o,oEDDHA (Yunta et al. 2003a).

It has been demonstrated that o,pEDDHA/Fe^{3+} is faster at re-greening Fe chlorotic plants than o,oEDDHA/Fe^{3+} in hydroponics (García-Marco et al. 2006; Rojas et al. 2008). However, its large reactivity with soil materials limits its persistence to a few days after application (Schenkeveld et al. 2007). Therefore, a combination of the long-lasting effect of the o,oEDDHA/Fe^{3+} and the fast action of o,pEDDHA/Fe^{3+} would be a good mixture in order to solve Fe chlorosis and to maintain a good Fe nutrition in plants. According to Mckenzie et al. (2005), products containing both o,oEDDHA/Fe^{3+} and o,pEDDHA/Fe^{3+} isomers (when the ratio o,p to o,o isomer is higher than 0.8:1) present some advantages: the short-term supply of Fe is improved, while the long-term supply is maintained, as for known products comprising only the o,oEDDHA/Fe^{3+} isomer.

Since the properties of the commercial products based on polyaminecarboxilic acids currently used are not entirely satisfactory, a new chelating agent, DCHA (2-{2-[(2-hydroxy benzyl)amino] ethylamino}-2-{2-hydroxyphenyl}acetic) (Fig. 1), has been synthesized by Prof. Sierra's Research Group (Sierra et al. 2008) to provide an alternative product for the treatment of Fe chlorosis. This chelating agent is a non-symmetrical ethylene diamino hidroxyphenyl

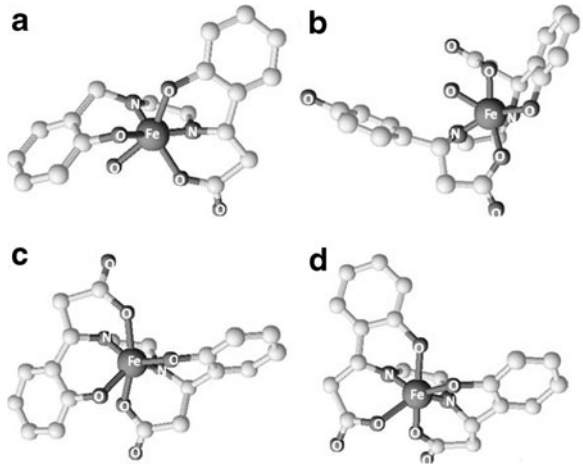

Fig 1 Spatial structures of **a** DCHA/Fe^{3+}, **b** o,pEDDHA/Fe^{3+}, **c** rac-o,oEDDHA/Fe^{3+}, **d** meso-o,oEDDHA/Fe^{3+}

acetic acid, that maintains both hydroxyphenyl groups but lack one of the 2 carboxylic groups present in the o,oEDDHA molecule.

Chelating agent characterization, including purity, protonation, and Ca^{2+} (DCHA/Ca^{2+} K≈10^{5}), Mg^{2+} (DCHA/Mg^{2+} K≈10^{5}), Fe^{3+}, and Cu^{2+} (DCHA/Cu^{2+} K≈10^{22}) stability constants, together with its ability to maintain Fe in solution in different agronomic conditions, were determined by López-Rayo et al. (2010). The results indicated that DCHA/Fe^{3+} (K≈10^{28}; López-Rayo et al. 2010) has an intermediate stability between o,oEDDHA/Fe^{3+} (K≈10^{35}; Yunta et al. 2003b) and o,pEDDHA/Fe^{3+} (K≈10^{29}; Yunta et al. 2003a) chelates, based on the percentage of Fe that remains chelated in nutrient solution and in soil conditions with limited and unlimited Cu calculated by modeling. Since Cu^{2+} shows a higher affinity with these chelates than Ca^{2+} and Mg^{2+}, the Fe^{3+} chelated displacement by the presence of high available Cu^{2+} concentrations in soil is usually studied (Yunta et al. 2003a; López-Rayo et al. 2010).

The aim of this work is to confirm agronomically the good results deriving from analytical and modeling data and to learn the factors affecting the efficacy of this new Fe chelate that is predicted to combine a good stability in nutrient solution and calcareous soils (due to the presence of 2 phenolate groups in its structure) and a fast action to relieve Fe chlorosis (due to its open nature similar to o,pEDDHA/Fe^{3+}). The effectiveness of DCHA was tested through 3 crop trials where the role of DCHA/Fe^{3+} as substrate for the FCR in Fe-stressed cucumber plants and its efficacy to provide Fe to soybean plants in hydroponic and soil cultures were studied.

Materials and methods

Iron chelates

The chelating agents used in this work were: o,oEDDHA 92.9% (Promochem), o,pEDDHA 59.6% (Syngenta Crop Protection), o,oEDDHA 86.9%, and DCHA 73.4% synthesized in the facilities of the Bio-Organometallic Chemistry Research Group (Universidad Complutense of Madrid, Spain) led by Prof. Sierra. The titrimetric purity of each product was determined as described in Yunta et al. (2003b). The results were expressed with respect to the acidic forms. Also, pure Na_2EDTA 99% (86% as free acid) (Titriplex III, Merck) was used in some experiments.

For the Fe chelate solutions preparation, ligands were dissolved in sufficient NaOH (1:3 molar ratio). Then, an amount of either $Fe(NO_3)_3{\cdot}9H_2O$ (Merck) or a $^{57}Fe^{3+}$ solution, prepared by dissolving ^{57}Fe (95.3%, Isoflex) in HNO_3 (Suprapur, Merck) calculated to be 5% in excess of the molar amount of ligand, was slowly added. During the chelation, pH was maintained between 6.0 and 8.0 and was finally adjusted to 7.0. Solutions were left to stand overnight to allow Fe excess precipitation as (hydr)oxides. Final solutions were filtered through a 0.45-μm cellulose membrane (Millipore) and made up to volume to obtain the desired concentration with type I water (electrical conductivity max: 0.056 μS cm^{-1} at 25°C; electrical resistivity min: 18.0 MΩ cm at 25°C; total organic C max: 100 μg L^{-1}; Na max: 1 μg L^{-1}; Cl^{-}max: 1 μg L^{-1}; total Si max: 3 μg L^{-1}). Light exposure was avoided during both the chelate solutions preparation and storage in order to avoid chelate photodecomposition (Hill-Cottingham 1955; Gómez-Gallego et al. 2005).

DCHA/Fe^{3+} as substrate for FCR activity in Fe-stressed cucumber plants

Cucumber seeds (*Cucumis sativus* L. cv. Ashley) were germinated on standard seed germination papers moistened with macronutrient solution [1.0 mM Ca$(NO_3)_2$, 0.9 mM KNO_3, 0.3 mM $MgSO_4$, 0.1 mM KH_2PO_4] in diffuse light in a growth chamber for 7 days with a 16 h/30°C and 50% humidity day and 8 h/25°C and 70% humidity night regime. Uniform seedlings were selected and bunches of 2 individual plants were wrapped together with polyurethane foam and placed in a 12-L polypropylene bucket (12 pairs of plants per bucket) containing 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 $MgSO_4$, 0.1 KH_2PO_4; EDTA buffered cationic micronutrients (μM): 5.0 EDTA/Fe^{3+}, 2.5 $MnSO_4$, 1.0 $CuSO_4$, 1.0 $ZnSO_4$, 1.0 $CoSO_4$, 1.0 $NiCl_2$, 115.5 Na_2EDTA; anionic micronutrients (μM): 35 NaCl, 10 H_3BO_3, 0.05 Na_2MoO_4; 0.1 mM HEPES and 2.4 g $CaCO_3$ for pH buffering at 7.5 to simulate calcareous soil conditions.

Plants were grown for 14 days in the above-described nutrient solution in a Dycometal type CCK growth

chamber provided with fluorescent and sodium vapor lamps with the same conditions describe above. Deionized water was added every 2 days and the nutrient solution was renewed on a weekly basis. The amount of Fe added (5 μM) was found by Lucena and Chaney (2006) to be the most adequate to produce green cucumber plants but with a high FCR activity (stressed plants) in an experiment with similar experimental conditions.

The FCR activity measurement was made in accordance with Lucena and Chaney (2006) at pH 6.0 (FCR activity dramatically decreases at pH >6.5; Susín et al. 1996). In brief, the assay solution contained Na_2BPDS (300 μM) and macronutrient solution. The experiment was initiated within the following 2 h after the daylight period. After transplanting a bunch of 2 plants into 200 mL assay solution, 5 mL of the corresponding treatment solution (o,oEDDHA/Fe^{3+}, o,pEDDHA/Fe^{3+} or DCHA/Fe^{3+}) was added (time 0) so that the final concentration was 100 μM Fe. Aliquots of 3 mL were sampled at 0, 10, 20, and 60 min, respectively. Six replicates for each treatment were arranged. In addition, 2 replicate blanks per chelate, consisting of solutions without plants, were included in order to correct reduction rates for slow photoreduction.

The $(BPDS)_3/Fe^{2+}$ concentration was calculated as in Lucena and Chaney (2006) by absorbances determination at 535 nm (maximum absorbance of the $(BPDS)_3/Fe^{2+}$) and at 480 nm (near the maximum absorbance of o,oEDDHA/Fe^{3+}, o,pEDDHA/Fe^{3+}, and DCHA/Fe^{3+}) to consider the contribution of the applied treatments on the total absorbance. The concentration of each chelate was calculated by solving the two-equation system (e.g., for o,oEDDHA/Fe^{3+}):

$$A_{535} = a_{FeBPDS535} \times [Fe(BPDS)_3] + a_{o,oEDDHA/Fe535} \times [o,oEDDHA/Fe]$$

$$A_{480} = a_{FeBPDS480} \times [Fe(BPDS)3] + a_{o,oEDDHA/Fe480} \times [o,oEDDHA/Fe]$$

where A_{535} and A_{480} are the absorbencies measured for each sample at 535 and 480 nm, respectively; $a_{FeBPDS535}$, $a_{FeBPDS480}$, $a_{o,oEDDHA/Fe535}$, and $a_{o,oEDDHA/Fe480}$ are the molar absorption coefficients in the experimental conditions and $[Fe(BPDS)_3]$ and (o,oEDDHA/Fe). [o,oEDDHA/Fe]= concentration of this Fe chelate.

The fresh weight of the roots was determined at the end of the experiment. The slope of the plots of produced Fe(II) (μmol g^{-1} fresh root) against time (h) was used as the Fe(III) reduction rate for each pair of plants. Data were expressed as the mean reduction rates, including the standard error corresponding to 6 plant replicates per treatment.

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

Soybean plants (Glycine max L. cv. Stine 0408) were used in this experiment, since they are susceptible to Fe chlorosis and are considered as model plants for Fe chelate treatment. Seeds were placed in trays between cellulose paper moistened with deionized water and germinated in a growth chamber in darkness during 2 days at 30°C and 60% moisture.

Afterward, the seedlings were placed in 10-L containers (27 seedlings per container) filled with a 1/5 strength EDTA buffered nutrient solution, with the same composition as in the above-described FCR experiment, for 6 days. On the 7th day, to induce Fe chlorosis, seedlings were transferred to 12-L polypropylene buckets containing an aerated full-strength EDTA buffered nutrient solution but without any Fe source, and 1 g of solid $CaCO_3$ was added to simulate calcareous soil conditions (7.5–8.0 pH). Plants were grown under these conditions for 7 days until visual symptoms of Fe deficiency were observed. Plants were placed in 2-L pots: 3 pairs of plants per pot and 2 Fe doses (5 and 10 μM) as o,oEDDHA/Fe^{3+}, o,pEDDHA/Fe^{3+}, EDTA/Fe^{3+}, and DCHA/Fe^{3+} were compared. Pots were covered with black plastic to prevent fungal proliferation. Besides the treatments applied, the nutrient solution used contained macronutrients and anionic micronutrients as for the FCR experiment and cationic micronutrients added at (μM) 1.0 $MnSO_4$, 0.5 $CuSO_4$, 0.5 $ZnSO_4$, 0.1 $NiCl_2$, and 0.1 $CoSO_4$. Volume was made up to 2 L with deionized water every 2 days, and the complete nutrient solution renewed weekly. Treatments, Fe chelates at 2 Fe doses and a Control (−Fe) without any Fe source, were replicated 4 times in a completely randomized design. The growth chamber conditions were the same as those used in the FCR experiment.

Plants were monitored for 21 days. Soil plant analysis development (SPAD) readings with a chlorophyll

meter (Minolta SPAD-502) were taken for all the leaf levels (average of 3 readings per leaf) every 2 days.

Sampling times were arranged to be performed at 7, 14, and 21 days after treatment (DAT), respectively. One pair of plants for every pot was taken in each sampling time. Roots, stems and leaves were separated and thoroughly washed following the procedure described by Álvarez-Fernández et al. (2001). Fresh and dry weights and micronutrient content were determined in each organ of the plants. Samples were dried and ground and, after dry digestion in a muffle furnace (480°C), the ashes were then dissolved in 6 M HCl (Benton 2001). Micronutrients (Fe, Mn, Cu, and Zn) were analyzed by FAAs (Perkin-Elmer Analyst 800).

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

Chelate stability in soil

The aim of this study was to determine the existence of an isotopic exchange between native Fe (mainly ^{56}Fe) from soil and the labeled ^{57}Fe from the chelates and evaluate the suitability of using labeled ^{57}Fe chelates in the next soil–plant experiment. A calcareous soil from Picassent (Valencia, Spain) was used as incubation substrate and later in the pot experiment. In brief, the soil is a highly calcareous soil (pH in H_2O 7.70, total $CaCO_3$ 380 g kg^{-1} and active lime 89 g kg^{-1}) that has a sandy loam texture, low organic matter content (O.M. oxidized, 9.2 g kg^{-1}), and normal Cu availability (Lindsay DTPA extract, 0.73 mg kg^{-1}). Three ^{57}Fe chelates were used in this experiment: o,oEDDHA/$^{57}Fe^{3+}$, o,pEDDHA/$^{57}Fe^{3+}$, and DCHA/$^{57}Fe^{3+}$ to study their behavior.

The methodology employed was as described by Nadal et al. (2009). For the experiment, 2 g 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. After the incubation time (1, 3, and 7 h, 1 , 3, 7, and 30 days) the supernatant was filtered and the pH was determined with an ion-meter (Orion Research). Two replicates per selected chelate at each sampling were arranged. Total Fe concentration in the filtrate was determined by FAAs (Perkin-Elmer Analyst 800) and ^{57}Fe by ICP-MS (Varian 820) using ^{57}Fe standards and correcting Ca and Ar interferences by means of a collision cell quadrupole ICP-MS instrument, after acidification of the samples with HNO_3 (Suprapur; Merck).

Soil culture

Soybean seeds were germinated as in the previous hydroponic trial. Afterwards, seedlings were transplanted into 1-L pots (3 plants per pot) filled with 1 kg soil:sand mixture containing approximately 0.70 kg of the same soil employed in the previous assay and 0.30 kg of sand (total $CaCO_3$ 975 g kg^{-1}, 1–3 mm size). Both soil and sand were the same as previously used by Nadal et al. (2009) and their main characteristics are described in that work. The experiment was carried out in the same growth chamber and under similar conditions to the ones previously described. Pots were irrigated until 80% of their saturated conditions (250 mL) 2 days before transplanting and then daily with the amount of solution necessary, determined by weight loss, to achieve again the 80% of the water holding capacity of the soil. Plants were irrigated with an aerated macronutrient solution (2 times concentrate) similar to that used in the above-described hydroponic experiment with 0.1 g L^{-1} of lime and 0.1 g L^{-1} of sodium bicarbonate (pH 8–8.5). Trays were placed under the pots for lixiviate recovering.

The experiment was designed to test 3 iron chelates (o,oEDDHA/$^{57}Fe^{3+}$, o,pEDDHA/$^{57}Fe^{3+}$, and DCHA/$^{57}Fe^{3+}$) and one Control (−Fe) without any exogenous Fe source. Five replicate pots per treatment were prepared. Treatments were applied 7 days after transplanting when visual chlorosis symptoms were observed. A dark plastic film was used for soils covering to avoid photodegradation of the Fe chelate and to avoid algae proliferation.

The Fe concentration in the treatments was calculated to be 2.5 mg of ^{57}Fe per kg of soil (43.9 μmol kg^{-1}). For the addition of the chelate solutions, 50 mg L^{-1} of Fe in the form of o,oEDDHA/$^{57}Fe^{3+}$, o,pEDDHA/$^{57}Fe^{3+}$, or DCHA/$^{57}Fe^{3+}$ were prepared and 35 mL of these solution were added in the center of the pot.

The SPAD Index was determined, after treatment application, every 2 days during the experiment. Plant material was sampled 3 times at 2, 7 and 21 DAT, respectively. One plant shoot was sampled at each sampling time. Leaves and stems were separated and washed as described in Álvarez-Fernández et al. (2001), weighed and dried. Total Fe and labeled ^{57}Fe

were determined in leaves after dry digestion procedure by FAAs (Perkin-Elmer Analyst 800) and ICP-MS (Varian 820), respectively, as indicated above in the isotopic exchange experiment.

At the end of the plant experiment, both water soluble and DTPA extractable (Soltanpour and Schwab 1977) Fe fractions in soil in each pot were obtained and labeled ^{57}Fe, and total Fe in soil and roots determined as in Nadal et al. (2009).

Statistical analysis

Differences among treatments were determined using a one-way analysis of variance (ANOVA) with Fe treatment as factor. For leaf mineral status in the hydroponic experiment, a two-way analysis was chosen with Fe treatment and Fe doses as factors. Significant differences were established at $p<0.05$ using the Duncan test. Statistical analyses were performed with SPSS statistical software (v.19.0; SPSS, Chicago, IL, USA).

Results

DCHA as substrate for FCR activity in Fe-stressed cucumber plants

The reduction of Fe(III) to Fe(II) is an essential step for Fe uptake by dicotyledonous plants (Chaney et al. 1972). After 20 min from the beginning of FCR experiment, some solutions produced saturation in the spectrophotometric measurements and for that reason just measurements obtained within the first 20 min were used. The Fe reduction rate (μmol Fe(II) g^{-1} root fresh h^{-1}) using the 3 Fe sources (o,oEDDHA/Fe^{3+}, o,pEDDHA/Fe^{3+}, and DCHA/Fe^{3+}) is shown in Fig. 2. The Fe reduction rate for o,pEDDHA was significantly higher than that of o,oEDDHA/Fe^{3+}. The chelate DCHA/Fe^{3+} showed an intermediate behavior between o,oEDDHA/Fe^{3+} and o,pEDDHA/Fe^{3+} as substrate for the FCR enzyme.

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

In this experiment, Fe-deficient soybean plants were used and 4 different Fe chelate treatments (o,oEDDHA/Fe^{3+}, o,pEDDHA/Fe^{3+}, DCHA/Fe^{3+}, and EDTA/Fe^{3+}) at 2 doses (5 and 10 μM) were applied

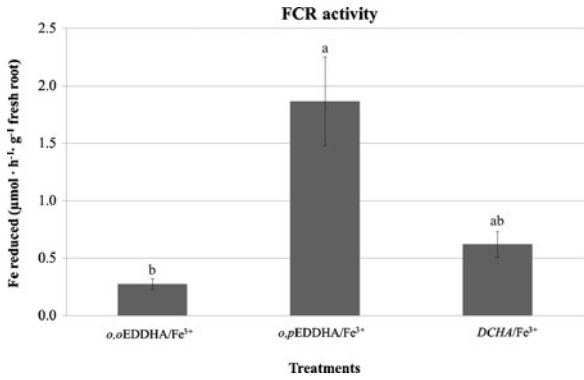

Fig 2 Rate of Fe(III) reduction of Fe(III)-chelates by cucumber plants. *Error bars* represent standard deviations (SD, $n=6$). Different *letters* denote significant differences among treatments ($p<0.05$)

and compared with Control (−Fe) treatment. SPAD readings were taken for all leaf levels during the experiment, but only SPAD values measured for the fully expanded leaf (2nd leaf level on day 4, 3rd leaf level on day 10 and 4th leaf level on day 20) are shown in Table 1.

Plants treated with the 3 phenolic chelates (DCHA/Fe^{3+}, o,oEDDHA/Fe^{3+}, and o,pEDDHA/Fe^{3+}) showed higher SPAD values than those treated with EDTA/Fe^{3+} and Control (−Fe) plants regardless of the dose used and at every determination dates. From the early beginning of the treatments (4 DAT), plants treated with DCHA/Fe^{3+} and o,pEDDHA/Fe^{3+} showed the highest SPAD values. The ANOVA analysis showed that 4 DAT plants treated with DCHA/Fe^{3+} at 5 μM dose presented the highest SPAD values followed by o,pEDDHA/Fe^{3+} and o,oEDDHA/Fe^{3+}. However, when the high dose was used, plants treated with o,pEDDHA/Fe^{3+} showed the highest SPAD values at that time. As the experiment progressed (10 and 20 DAT), plants treated with o,oEDDHA/Fe^{3+} increased their SPAD values without showing significant differences with DCHA/Fe^{3+} and o,pEDDHA/Fe^{3+} at the end of the experiment (Table 1). However, the statistical analysis revealed that the low dose of o,oEDDHA/Fe^{3+} was not able to re-green plants as did DCHA/Fe^{3+} and o,pEDDHA/Fe^{3+} at 10 DAT.

Regarding plant biomass (leaf dry weight; Table 2), no significant differences at any sampling time and any dose could be observed among plants treated with the 3 phenolic chelates (o,oEDDHA/Fe^{3+}, o,pEDDHA/Fe^{3+}, and DCHA/Fe^{3+}). However, significant differences could be observed between them and those plants

Table 1 Time course effect of Fe chelate treatments on SPAD measured on the fully expanded leaf in the hydroponic experiment (the leaf stage is indicated in parentheses)

Treatments	SPAD		
	4 DAT (2nd stage)	10 DAT (3rd stage)	20 DAT (4th stage)
5 μM dose			
Control (-Fe)	3.3±0.4 e	3.4±1.7 c	
DCHA/Fe^{3+}	16.7±0.7 a	32.8±1.1 a	34.8±0.7 a
o,oEDDHA/Fe^{3+}	12.5±0.8 c	26.7±1.1 b	30.2±1.2 a
o,pEDDHA/Fe^{3+}	14.8±0.6 b	30.9±1.3 ab	32.2±0.9 a
EDTA/Fe^{3+}	5.4±0.6 d	6.9±2.2 c	11.4±3.5 b
10 μM dose			
Control (-Fe)	3.3±0.4 d	3.4±1.7 c	
DCHA/Fe^{3+}	19.1±0.6 b	35.7±1.0 a	35.0±1.2 a
o,oEDDHA/Fe^{3+}	18.2±0.6 b	31.6±1.3 a	32.4±2.2 a
o,pEDDHA/Fe^{3+}	21.1±0.7 a	33.1±1.3 a	32.9±1.3 a
EDTA/Fe^{3+}	9.4±1.1 c	16.6±2.3 b	19.9±2.2 b

Data values are mean ± standard error (SE). Different letters in the same column and with the same Fe chelate dose denote significant differences among treatments ($p<0.05$)

SPAD soil plant analysis development, DAT days after treatment

treated with EDTA/Fe^{3+} and Control (−Fe) plants, which showed lower plant biomass.

The leaf mineral status was assessed by the measurement of total Fe, Mn, Cu, and Zn concentrations. Data were analyzed using two-way ANOVA analyses considering dose and Fe source as factors. The average values for these parameters are presented in Tables 3 and 4.

Plants treated with the 3 phenolic chelates (DCHA/Fe^{3+}, o,oEDDHA/Fe^{3+}, and o,pEDDHA/Fe^{3+}) showed higher Fe concentration in leaf than those treated with EDTA/Fe^{3+} and Control (−Fe) plants at all sampling times except for o,oEDDHA/Fe^{3+} at 7 DAT, which presented similar values to Control (−Fe) and EDTA/Fe^{3+} plants (Table 3). At 7 and 14 DAT, plants treated with DCHA/Fe^{3+} and o,pEDDHA/Fe^{3+} showed higher leaf Fe concentration than plants treated with o,oEDDHA/Fe^{3+}. At the third sampling time (21 DAT), no differences could be observed between o,oEDDHA/Fe^{3+}, o,pEDDHA/Fe^{3+}, and DCHA/Fe^{3+}. At the first and second sampling times, plants treated with the high Fe dose showed the highest Fe concentration in leaf; however, these differences were not significant at 21 DAT.

At the 3 sampling times, plants treated with phenolic chelates showed significantly lower Mn concentrations in leaf than plants treated with EDTA/Fe^{3+} and Control (−Fe) plants (Table 3). At the first sampling time, no

Table 2 Effect of the different Fe chelate treatments on the leaf dry weight (g) at 7, 14, and 21 DAT ± standard error (SE, $n=4$) in the hydroponic experiment

Treatments	Leaf dry weight		
	7 DAT	14 DAT	21 DAT
5 μM dose			
Control (-Fe)	0.45±0.03 b	0.54±0.03 b	0.53±0.07 b
DCHA/Fe^{3+}	0.77±0.10 a	2.14±0.14 a	4.33±0.25 a
o,oEDDHA/Fe^{3+}	0.76±0.05 a	1.86±0.11 a	4.05±0.10 a
o,pEDDHA/Fe^{3+}	0.76±0.08 a	1.83±0.17 a	4.49±0.14 a
EDTA/Fe^{3+}	0.43±0.09 b	0.71±0.21 b	0.94±0.12 b
10 μM dose			
Control (-Fe)	0.45±0.03 b	0.54±0.03 d	0.53±0.07 c
DCHA/Fe^{3+}	0.86±0.12 a	1.92±0.17 ab	4.59±0.28 a
o,oEDDHA/Fe^{3+}	0.88±0.03 a	2.15±0.08 a	4.31±0.12 a
o,pEDDHA/Fe^{3+}	0.98±0.08 a	1.58±0.22 b	4.36±0.31 a
EDTA/Fe^{3+}	0.51±0.14 b	1.02±0.06 c	2.00±0.12 b

Different letters in the same column and with the same Fe chelate dose denote significant differences among treatments ($p<0.05$)

Table 3 Effect of the different Fe chelate treatments on the Fe and Mn concentration (μmol g^{-1} DW) in leaf at 7, 14 and 21 DAT ± standard error (SE, n=4) in the hydroponic experiment

	Fe			Mn		
	7 DAT	14 DAT	21 DAT	7 DAT	14 DAT	21 DAT
Fe treatment						
Control (-Fe)	0.80±0.15 b	0.32±0.01 d	0.36±0.01 b	2.67±0.04 a	2.92±0.06 a	2.68±0.02 a
DCHA/Fe^{3+}	1.36±0.07 a	1.25±0.03 a	1.04±0.07 a	1.61±0.31 b	1.11±0.09 b	0.96±0.09 b
o,oEDDHA/Fe^{3+}	0.94±0.14 b	0.96±0.05 b	1.00±0.06 a	1.92±0.15 ab	1.58±0.07 b	1.23±0.11 b
o,pEDDHA/Fe^{3+}	1.26±0.09 a	1.29±0.06 a	0.95±0.04 a	1.81±0.16 ab	1.19±0.09 b	1.17±0.02 b
EDTA/Fe^{3+}	0.73±0.11 b	0.50±0.04 c	0.42±0.02 b	2.68±0.33 a	3.09±0.03 a	3.10±0.02 a
Fe doses (μM)						
0	0.80±0.15 b	0.32±0.02 c	0.36±0.01 b	2.67±0.04 ns	2.92±0.06 a	2.68±0.08 a
5	0.87±0.09 b	0.96±0.10 b	0.83±0.08 a	2.15±0.26	2.03±0.31 b	1.82±0.33 b
10	1.25±0.08 a	1.07±0.09 a	0.87±0.08 a	1.90±0.13	1.55±0.21 c	1.52±0.19 b

Different letters in the same column denote significant differences between Fe treatments or Fe doses (p<0.05); *ns* not significant

differences could be observed among Fe doses. At the second sampling time, the higher the Fe dose applied, the lower the Mn concentration in leaf was observed. At the end of the experiment (21 DAT), the addition of any Fe dose produced a significant decrease in the Mn concentration in leaf compared with Control (−Fe) plants. Copper and Zn concentrations in leaf (Table 4) were especially affected by the application of phenolic chelates and to a lesser extent by the application of EDTA/Fe^{3+} (Table 4). Regarding Fe doses, Control (−Fe) plants showed the highest Cu and Zn concentrations and, in the case of Cu, the higher the Fe dose, the lower the Cu concentration in leaf was observed.

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

Chelate stability in soil

Figure 3 shows the percentage of soluble ^{57}Fe that remained in soil solution over time from o,oEDDHA/$^{57}Fe^{3+}$, o,pEDDHA/$^{57}Fe^{3+}$, and DCHA/$^{57}Fe^{3+}$.

Table 4 Effect of the different Fe chelate treatments on the Cu and Zn concentration (μmol g^{-1} DW) in leaf at 7, 14 and 21 DAT ± standard error (SE, n=4) in the hydroponic experiment

	Cu			Zn		
	7 DAT	14 DAT	21 DAT	7 DAT	14 DAT	21 DAT
Fe treatment						
Control (-Fe)	0.27±0.01 a	0.18±0.01 a	0.31±0.00 a	0.66±0.03 a	0.44±0.07 a	0.73±0.00 a
DCHA/Fe^{3+}	0.07±0.01 c	0.12±0.00 b	0.16±0.01 c	0.42±0.03 c	0.23±0.01 b	0.31±0.05 c
o,oEDDHA/Fe^{3+}	0.04±0.01 c	0.12±0.01 b	0.14±0.02 c	0.42±0.03 c	0.26±0.01 b	0.40±0.03 bc
o,pEDDHA/Fe^{3+}	0.02±0.01 d	0.09±0.01 b	0.07±0.00 d	0.49±0.06 bc	0.24±0.05 b	0.39±0.06 bc
EDTA/Fe^{3+}	0.22±0.03 b	0.12±0.02 b	0.22±0.03 b	0.57±0.06 ab	0.59±0.08 a	0.61±0.10 ab
Fe doses (μM)						
0	0.27±0.01 a	0.18±0.01 a	0.31±0.00 a	0.66±0.03 a	0.44±0.07 a	0.73±0.00 a
5	0.11±0.03 b	0.12±0.01 b	0.16±0.02 b	0.49±0.03 b	0.38±0.07 b	0.42±0.06 b
10	0.07±0.02 c	0.10±0.01 b	0.12±0.01 c	0.45±0.04 b	0.28±0.03 b	0.44±0.05 b

Different letters in the same column denote significant differences between Fe treatments or Fe doses (p<0.05)

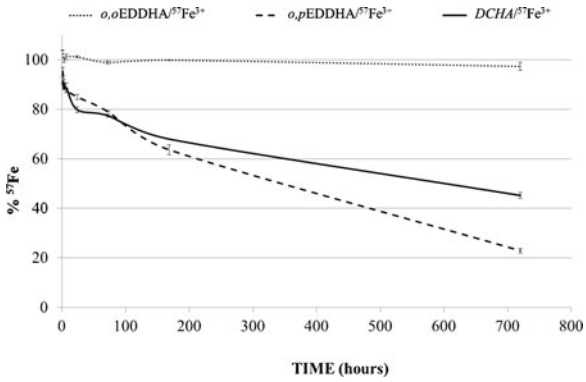

Fig 3 Percentage of ^{57}Fe that remained in solution during time in the soil–chelate interaction experiment

The percentage of ^{57}Fe from o,oEDDHA/$^{57}Fe^{3+}$ that remained in solution after 30 days was very high, indicating that very little or no exchange occured between the added ^{57}Fe and the native Fe (mainly ^{56}Fe) or any other nutrient. However, the percentage of ^{57}Fe with respect to the total Fe from o,pEDDHA/$^{57}Fe^{3+}$ and DCHA/$^{57}Fe^{3+}$ decreased over time, showing isotopic exchange or substitution of the Fe in the chelate by competing cations or chelate reactivity in soil. Comparing total and labeled Fe, an estimation of 30 and 25% of the remaining Fe in solution has been exchanged between the chelate and the soil for DCHA/$^{57}Fe^{3+}$ and o,pEDDHA/$^{57}Fe^{3+}$, respectively, in the 30-day period. Despite the lowest stability of these Fe chelates in soil, it can be considered that most of the Fe in the plant from the fertilizer could be determined by the labeled Fe. Plant accumulation of Fe is the result of all the growth period, and the lowest stability is important only in the last period in which less Fe is taken up by the plants as commented below. Then, chelate stability should not invalidate the results on the soil culture experiment in using ^{57}Fe as a measure of the amount of Fe coming from the fertilizer.

Soil culture

The recovery rate after the treatment applications were estimated by the measure of SPAD for the 3rd leaf level (fully expanded leaf) (Table 5). Control (−Fe) plants showed severe chlorotic symptoms at the end of the experiment (21 DAT), whereas treated plants had regreened and no significant differences among Fe chelate treatments assayed could be observed.

Two-way ANOVA analysis, considering sampling time and Fe source as factors, revealed an interaction between factors when plant dry weight, total Fe and ^{57}Fe concentration in leaves were considered, so the variables were analyzed by separate in each sampling time using a one-way ANOVA analysis.

Leaf dry weight at each sampling time is presented in Table 6. At 2 DAT (first sampling time), no differences among treatments and Control (−Fe) were found. However, at 7 and 21 DAT, treated plants showed higher biomass than Control (−Fe) ones and no statistical differences between the Fe chelates applied were found. Similar results could be observed when root biomass was analyzed at the end of the experiment. These results are in agreement with the trend shown in the previous SPAD results.

The nutritional status was also assessed by measuring leaf micronutrient concentration. Iron nutrition was evaluated considering Fe uptake from ^{57}Fe chelates, so we could differentiate Fe uptake from the chelate and from other sources (e.g., soil, seed, etc.).

Figure 4 revealed that o,oEDDHA/$^{57}Fe^{3+}$ provided the highest levels of ^{57}Fe in leaves in all sampling times; however, this increment did not result in an increase of plant biomass (see Table 6). For the first sampling date (2 DAT), plants treated with DCHA/$^{57}Fe^{3+}$ showed a ^{57}Fe concentration in leaves intermediate between o,oEDDHA/$^{57}Fe^{3+}$ and o,pEDDHA/$^{57}Fe^{3+}$. However, at 7 and 21 DAT, no differences were observed between plants treated with DCHA/$^{57}Fe^{3+}$ and o,pEDDHA/$^{57}Fe^{3+}$. The ^{57}Fe/total Fe ratio absorbed by the plant is a good indicator of the efficiency of chelates to provide Fe to the plant over a long time period. Plants treated with o,oEDDHA/$^{57}Fe^{3+}$ presented a ^{57}Fe/total Fe ratio of approximately 0.94 and 0.78 at 7 and 21 DAT, respectively. However, plants treated with o,pEDDHA/$^{57}Fe^{3+}$ showed a ratio of 0.75 at 7 DAT and 0.59 at the end of the experiment. Plants treated with DCHA/$^{57}Fe^{3+}$ showed an intermediate behavior between o,oEDDHA/$^{57}Fe^{3+}$ and o,pEDDHA/$^{57}Fe^{3+}$ (0.77 and 0.64 at 7 and 21 DAT, respectively).

Total Mn, Cu, and Zn concentration in leaf were also analyzed and the results are shown in Table 7. Concerning Mn status, no differences could be observed between treated and Control (−Fe) plants at 2 DAT, but plants treated with Fe chelates showed significantly lower Mn concentrations in leaf than Control (−Fe) ones at 7 and 21 DAT. Similar results were

Table 5 Time course effect of Fe chelate treatments on SPAD measured on the 3rd soybean leaf level (fully expanded leaf on day 0) in the soil experiment

Treatments	SPAD							
	0 DAT	2 DAT	6 DAT	8 DAT	12 DAT	15 DAT	18 DAT	21 DAT
Control (-Fe)	6.7±0.4	17.2±1.0	15.0±0.8	15.3±1.5	12.6±3.4	12.8±2.8	13.3±3.2	10.8±2.9 b
DCHA/$^{57}Fe^{3+}$	13.8±1.1	30.1±0.7	36.7±0.7	39.4±0.6	41.0±1.3	41.7±1.6	42.3±1.2	43.6±1.4 a
o,oEDDHA/$^{57}Fe^{3+}$	9.9±1.7	27.9±1.0	37.1±0.2	41.1±0.4	42.1±0.5	42.2±1.4	44.6±0.4	41.9±3.1 a
o,pEDDHA/$^{57}Fe^{3+}$	10.0±2.0	30.6±0.5	36.2±0.8	39.8±0.4	41.2±0.6	42.7±0.7	43.9±0.4	45.5±0.7 a

Data values are mean ± standard error (SE). Different letters in the 21 DAT column denote significant differences among treatments ($p<0.05$)

obtained when Zn was analyzed, except for plants treated with o,pEDDHA/Fe^{3+} that showed, at 2 DAT, higher Zn concentrations than Control (−Fe) and DCHA/Fe^{3+}-treated plants. For Cu, no significant differences were found at 2 DAT. However, Cu significantly decreased in plants treated with phenolic Fe chelates with respect to Control (−Fe) plants at 7 DAT. DCHA/Fe^{3+} treated plants presented higher Cu concentration than the other chelates at 21 DAT.

Results of ^{57}Fe solubility and availability in soil at the end of the experiment (21 DAT) are presented in Fig. 5. The amount of ^{57}Fe in the soluble fraction is significantly higher in the pots treated with o,oEDDHA/$^{57}Fe^{3+}$; however, with o,pEDDHA/$^{57}Fe^{3+}$ and DCHA/$^{57}Fe^{3+}$, the Fe available for the plants at the end of the experiment is significantly higher than with the most stable chelate, o,oEDDHA/$^{57}Fe^{3+}$.

Discussion

A previous step in the evaluation of the efficiency of a new chelating agent to provide Fe to plants is to study its behavior when it is used as a substrate of the enzyme FCR. García-Marco et al. (2006) found that structures with only 5 bonds between the Fe(III) and the ligand (open molecules) facilitate the accessibility of Fe for the FCR. Gómez-Gallego et al. (2005) proposed that the reduction of o,oEDDHA/Fe^{3+} by the FCR does not take place on the complex in the octahedral closed form, but on a hexacoordinate open species (formed at the acid pH of the rhizosphere) in which one of the hydroxyl groups in the ortho-position would not be coordinated with the Fe(III), generating a vacant coordination site that facilitates the accessibility of the Fe for the FCR before the reduction step. The chelating agent o,oEDDHA has 6 donor groups able to bind Fe, forming a closed molecule, but o,pEDDHA and DCHA have only 5 donor groups (see Fig. 1), so o,pEDDHA/Fe^{3+} and DCHA/Fe^{3+} are in the required open form (because of their pentacoordinated nature) and could be directly reduced by the enzyme, being better substrates than o,oEDDHA/Fe^{3+}. That was the expected behavior. Nevertheless, in the results obtained in this experiment, o,pEDDHA/Fe^{3+} was the best substrate for FCR, and DCHA/Fe^{3+} showed

Table 6 Effect of the different Fe chelate treatments on dry weight of leaves (g plant^{-1} DW) at 2, 7 and 21 DAT and roots (3 plants, g plant^{-1} DW) ± standard error (SE, $n=5$) in soybean plants in the soil experiment

Treatments	Leaves			Roots
	2 DAT	7 DAT	21 DAT	21 DAT
Control (-Fe)	0.35±0.01 ns	0.58±0.03 b	0.96±0.04 b	0.53±0.02 b
DCHA/$^{57}Fe^{3+}$	0.34±0.05	0.72±0.06 a	1.81±0.10 a	0.82±0.04 a
o,oEDDHA/$^{57}Fe^{3+}$	0.30±0.01	0.76±0.04 a	1.77±0.12 a	0.73±0.03 a
o,pEDDHA/$^{57}Fe^{3+}$	0.37±0.02	0.78±0.04 a	1.98±0.16 a	0.87±0.03 a

Different letters in the same column indicate significant differences among treatments ($p<0.05$); *ns* not significant

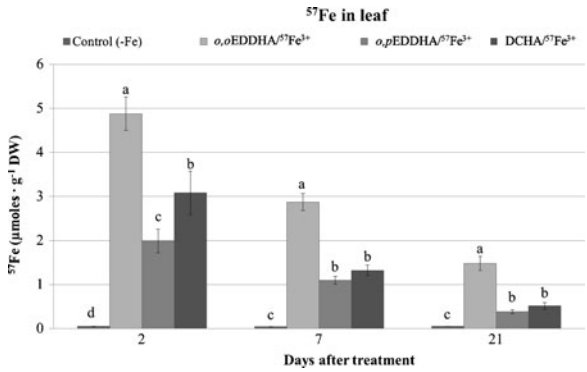

Fig 4 Concentration of ^{57}Fe (μmoles g^{-1} DW) in leaves in the soil culture experiment. Different *letters* in the same sampling time indicate differences between treatments ($p<0.05$)

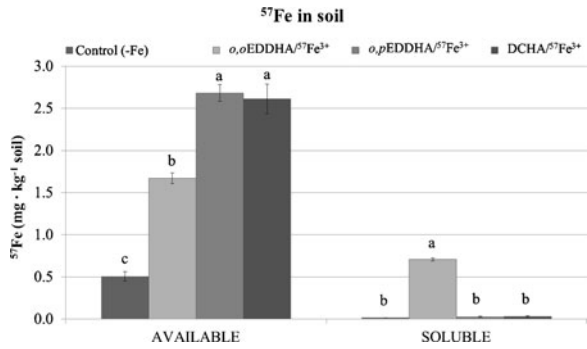

Fig 5 Concentration of ^{57}Fe in the soluble and available fractions of the soils after plant experiment. Different *letters* in the same soil fraction indicate differences between treatments ($p<0.05$)

a Fe(III) reduction rate intermediate between o,pEDDHA/Fe^{3+} and o,oEDDHA/Fe^{3+}. The presence of 2 phenolates in the molecule of DCHA could provide more stability to the Fe chelate than in the case of o,pEDDHA. This fact should increase the stability constant of DCHA/Fe^{3+} in comparison with that of o,pEDDHA/Fe^{3+}, but the Fe stability constant calculated by López-Rayo et al. (2010) for DCHA was only slightly lower than that obtained for o,pEDDHA/Fe^{3+} ($K\approx10^{29}$; Yunta et al. 2003a). From the structure of the molecules, it can be recognized that o,pEDDHA/Fe^{3+} presents a free polar group (the hydroxyl in para-position in one benzene) and then higher polarity than DCHA/Fe^{3+} and o,oEDDHA/Fe^{3+}. This polar group may serve as the contact point between the chelate and the enzyme, favoring the electron transfer and then speeding the reduction processes. This new hypothesis should be demonstrated in further studies and should explain the differences observed between o,pEDDHA/Fe^{3+} and DCHA/Fe^{3+}.

The slight (not significant) differences between o,oEDDHA (6 bonds) and DCHA (5 bonds) in the FCR experiment are also well correlated with the intermediate stability by modeling of DCHA/Fe^{3+} between the o,oEDDHA/Fe^{3+} and o,pEDDHA/Fe^{3+} chelates (López-Rayo et al. 2010), and are in agreement with the findings published by Lucena and Chaney (2006), which concluded that the more stable the chelate, the lower the reduction rates.

Under hydroponic conditions, plants treated with EDTA/Fe^{3+} showed lower recovery (the lowest values of SPAD and the lowest Fe concentration in leaf) than plants treated with phenolic chelates. The chelating agent EDTA may serve as a Fe(II) trapping agent, lowering the amount of Fe that can be taken up by the plants (Lucena and Chaney 2006) after Fe reduction by FCR enzyme. Moreover, Fe can be displaced from EDTA/Fe^{3+} by other cations, reducing the soluble Fe in solution available for the plants (Norvell 1991). At the end of this experiment, plants treated with EDTA showed significantly higher concentrations of Mn, Cu, and Zn than plants treated with the phenolic chelates (see Tables 3 and 4).

In relation with phenolic chelates, the results obtained under hydroponic culture are in good agreement with the

Table 7 Effect of the different Fe chelate treatments on the Mn, Cu, and Zn concentrations (μmol g^{-1} DW) in leaf in each sampling time ± standard error (SE, $n=5$) in the soil experiment

Treatments	Mn			Cu			Zn		
	2 DAT	7 DAT	21 DAT	2 DAT	7 DAT	21 DAT	2 DAT	7 DAT	21 DAT
Control (-Fe)	1.48±0.06 ns	1.23±0.07 a	1.07±0.08 a	0.024±0.001 a	0.63±0.06 a	1.04±0.09 a	0.39±0.02 b	0.62±0.07 a	1.00±0.08 a
DCHA/Fe^{3+}	1.41±0.08	0.67±0.08 b	0.21±0.02 b	0.020±0.001 b	0.21±0.05 b	0.62±0.08 b	0.37±0.03 b	0.28±0.02 b	0.54±0.07 b
o,oEDDHA/Fe^{3+}	1.45±0.13	0.71±0.05 b	0.25±0.02 b	0.021±0.001 ab	0.26±0.04 b	0.32±0.00 c	0.45±0.02 ab	0.33±0.03 b	0.33±0.01 b
o,pEDDHA/Fe^{3+}	1.43±0.12	0.60±0.06 b	0.21±0.01 b	0.022±0.001 ab	0.15±0.03 b	0.38±0.03 c	0.50±0.04 a	0.34±0.03 b	0.36±0.03 b

Different letters in the same column indicate significant differences among treatments ($p<0.05$); *ns* not significant

hypothesis of a faster activity when open molecules are the substrate instead of closed ones. It was expected that o,pEDDHA/Fe^{3+} and DCHA/Fe^{3+} provided a faster recovery of the chlorosis symptoms than o,oEDDHA/Fe^{3+}. This hypothesis has been corroborated with the results of SPAD measurements and Fe concentrations in leaf at the beginning of the experiment. However, no significant differences could be observed between phenolic treatments at the end of the experiment. This fact could be explained by considering that most of the Fe reduced could be re-oxidized, and this reaction should occur faster for the more stable Fe chelates, such as o,oEDDHA/Fe^{3+}, impeding or slowing down Fe(II) absorption (Lucena and Chaney 2007).

Iron uptake can be estimated by determining the fraction acquired by the plant of a Fe stable isotope (or isotopes) given as a tracer (Álvarez-Fernández 2006). In the soil culture experiment, where the effectiveness of Fe chelates may be affected by the reaction of these compounds with soil materials (Schenkeveld et al. 2007), the use of treatments prepared with the stable isotope ^{57}Fe provide a good tool to study the plant uptake from synthetic Fe chelates and the shoot translocation rate of the Fe supplied by these compounds.

In the soil interaction experiment, o,pEDDHA/$^{57}Fe^{3+}$ and DCHA/$^{57}Fe^{3+}$ showed lower perdurability in solution (≈55 and 40% ^{57}Fe in solution for DCHA/Fe^{3+} and o,pEDDHA/Fe^{3+}, respectively, after 21 days of interaction with soil) than o,oEDDHA/$^{57}Fe^{3+}$ (≈98% ^{57}Fe in solution), and plants treated with these chelates presented a lower ^{57}Fe and total Fe (data not shown) concentration in leaf than the ones treated with o,oEDDHA/$^{57}Fe^{3+}$ during all the experiments. In spite of this fact, no significant differences in the SPAD and leaf biomass at 21 DAT were found.

Results obtained in the hydroponic and soil experiments showed that Mn, Cu, and Zn uptake by plants was affected when phenolic chelates are applied. While in the hydroponic experiment, the low weight of the Control (−Fe) plants could lead to a concentration effect for Mn, Cu, and Zn, the high differences among treatments and Control (−Fe) in the soil experiments (Table 7) may not be completely explained by that concentration effect, and should be attributed to the antagonism effect between Fe and Mn and also with Zn and Cu.

Finally, the study of the solubility and availability of ^{57}Fe in soil at the end of the experiment gave us information about their expected long-term behavior in field conditions. The amount of soluble ^{57}Fe that remained in the soil at 21 DAT, adding o,oEDDHA/$^{57}Fe^{3+}$, was higher than that obtained with the addition of o,pEDDHA/$^{57}Fe^{3+}$ and DCHA/$^{57}Fe^{3+}$, whereas the amount of available ^{57}Fe obtained from o,pEDDHA/$^{57}Fe^{3+}$ and DCHA/$^{57}Fe^{3+}$ was higher than that obtained from o,oEDDHA/$^{57}Fe^{3+}$, and this fact entails a Fe reservoir in the soil for the plants.

In conclusion, according to the results obtained, DCHA/Fe^{3+} has adequate fertilizer properties since it is able to correct the Fe chlorosis and to maintain good nutritional status of plants over time, both in hydroponic and soil cultures. This is related to its ability to serve as substrate for the FCR and its good stability in solution and in soil conditions observed in this and previous studies (López-Rayo et al. 2010).

Acknowledgements Financial support has been provided by TRADECORP and by the project AGL2010-18048 of the Spanish Ministry of Science and Innovation. Paloma Nadal is the recipient of a fellowship from the FPI program of Spanish Ministry of Science and Innovation. We thank Edgar Ropero for technical support in conducting the hydroponic experiment.

References

Álvarez-Fernández A (2006) Application of Stable Isotopes in Plant Iron Research. In: Barton LL, Abadía J (eds) Iron Nutrition in Plants and Rhizospheric Microorganisms. Springer, Dordrecht, pp 437–448

Álvarez-Fernández A, Pérez-Sanz A, Lucena JJ (2001) Evaluation of effect of washing procedures on minerals analysis of orange and peach leaves sprayed with seaweed extracts enriched with iron. Commun Soil Sci Plant Anal 32:157–170

Álvarez-Fernández A, Abadía J, Abadía A (2006) Iron Deficiency, Fruit Yield and Fruit Quality. In: Barton LL, Abadía J (eds) Iron Nutrition in Plants and Rhizospheric Microorganisms. Springer, Dordrecht, pp 85–101

Benton J Jr (2001) Laboratory Guide for Conducting Soil Tests and Plant Analysis. CRC Press, Florida

Chaney RL, Brown JC, Tiffin LO (1972) Obligatory reduction of ferric chelates in iron uptake by soybeans. Plant Physiol 50:208–213

Chen Y, Barak P (1982) Iron nutrition of plants in calcareous soils. Adv Agron 35:217–240

Degryse F, Smolders E, Parker DR (2006) Metal complexes increase uptake of Zn and Cu by plants: implications for uptake and deficiency studies in chelator-buffered solutions. Plant Soil 289:171–185

García-Marco S, Martínez N, Yunta F, Hernández-Apaolaza L, Lucena JJ (2006) Effectiveness of ethylenediamine-N(o-hydroxyphenylacetic)-N' (p-hydroxy- phenylacetic) acid (o, p-EDDHA) to supply iron to plants. Plant Soil 279:31–40

Gómez-Gallego M, Sierra MA, Alcázar R, Ramírez P, Piñar C, Mancheño MJ, García-Marco S, Yunta F, Lucena JJ (2002) Synthesis of o, p-EDDHA and its detection as the main impurity in o, o-EDDHA commercial iron chelates. J Agric Food Chem 50:6395–6399

Gómez-Gallego M, Pellico D, Ramírez-López P, Mancheño MJ, Romano S, de la Torre MC, Sierra MA (2005) Understanding of the mode of action of Fe^{III}-EDDHA as iron chlorosis corrector based on its photochemical and redox behavior. Chem Eur J 11:1–10

Hansen NC, Hopkins BG, Ellsworth JW, Jolley VD (2006) Iron Nutrition in Field Crops. In: Barton LL, Abadía J (eds) Iron Nutrition in Plants and Rhizospheric Microorganisms. Springer, Dordrecht, pp 23–59

Hill-Cottingham DG (1955) Photosensitivity of iron chelates. Nature 175:347–348

Inskeep WP, Bloom PR (1984) A comparative study of soil solution chemistry associated with chlorotic and nonchlorotic soybeans in western Minnesota. J Plant Nutr 7:513–531

López-Rayo S, Hernández D, Escudero R, M Gómez-Gallego, Sierra MS, Lucena JJ (2010) The synthesis and chemical characterization of the novel agronomically relevant pentadentate chelate 2-(2-((2-Hydroxybenzyl)amino)ethylamino)-2-(2-hydroxyphenyl)acetic acid (DCHA). J Agric Food Chem 58:7908–7914

Lucena JJ (2000) Effect of bicarbonate, nitrate and other environmental factors on iron deficiency chlorosis. A review. J Plant Nutr 23:1591–1606

Lucena JJ (2006) Synthetic Iron Chelates to Correct Iron Deficiency in Plants. In: Barton LL, Abadía J (eds) Iron Nutrition in Plants and Rhizospheric Microorganisms. Springer, Dordrecht, pp 103–128

Lucena JJ, Chaney R (2006) Synthetic iron chelates as substrates of root ferric chelate reductase (FCR) in green stressed cucumber plants. J Plant Nutr 29:423–439

Lucena JJ, Chaney R (2007) Response of Cucumber Plants to Low Doses of different Synthetic Iron Chelates in Hydroponics. J Plant Nutr 30:795–809

Mckenzie D, Lucena JJ and Jackson DA (2005) Plant nutrient based on o,p-Ethylene(bis)hydroxyphenyl glycines. WO 2005/095305.

Nadal P, Hernandez-Apaolaza L, Lucena JJ (2009) Effectiveness of N, N'-Bis(2-hydroxy-5-methylbenzyl) ethylenediamine-N, N'-diacetic acid (HJB) to supply iron to dicot plants. Plant Soil 325:65–77

Norvell WA (1991) Reactions of metal chelates in soils and nutrient solutions. In: Morvedt JJ, Cox SR, Shuman LM, Welch RM (eds) Micronutrients in Agriculture, 2nd edn, SSSA Book series no. 4. Soil Science Society of America, Madison, pp 187–223

Rombolá AD, Tagliavini M (2006) Iron nutrition of fruit tree crops. In: Barton LL, Abadía J (eds) Iron Nutrition in Plants and Rhizospheric Microorganisms. Springer, Dordrecht, pp 61–83

Rojas CL, Romera FJ, Alcántara E, Pérez-Vicente R, Sariego C, García-Alonso JI, Boned J, Martí G (2008) Efficacy of Fe (o, o-EDDHA) and Fe(o, p-EDDHA) isomers in supplying Fe to strategy I plants differs in nutrient solution and calcareous soil. J Agric Food Chem 56:10774–10778

Sanz M, Cavero J, Abadía J (1992) Iron chlorosis in the Ebro river basin, Spain. J Plant Nutr 15:1971–1981

Schenkeveld WDC, Reichwein AM, Temminghoff EJM, Riemsdijk WHV (2007) The behaviour of EDDHA isomers in soils as influenced by soil properties. Plant Soil 290:85–102

Sierra MA, Gómez-Gallego M, Escudero R, Lucena JJ, García-Marco S (2008) New non-symmetrical ethylene diamino hydroxyphenyl acetic acid products for the treatment of the iron chlorosis. WO 2008/077897

Soltanpour PN, Schwab AP (1977) A new soil test for simultaneous extraction of macro- and micro-nutrients in alkaline soils. Commun Soil Sci Plant Anal 8:195–207

Susín S, Abadía A, González-Reyes JA, Lucena JJ, Abadía J (1996) The pH requirement for in vivo activity of the iron-deficiency-induced "Turbo" ferric chelate reductase. Plant Physiol 110(1):111–123

Yunta F, García-Marco S, Lucena JJ (2003a) Theoretical speciation of ethylenediamine-N-(o-hydroxyphenylacetic)-N'-(p-hydroxyphenylacetic) acid (o, p-EDDHA) in agronomic conditions. J Agric Food Chem 51:5391–5399

Yunta F, García-Marco S, Lucena JJ, Gómez-Gallego M, Alcazar R, Sierra MA (2003b) Chelating agents related to ethylenediamine bis(2-hydroxyphenyl)acetic acid (EDDHA): synthesis, characterization, and equilibrium studies of the free ligands and their Mg^{2+}, Ca^{2+}, Cu^{2+}, and Fe^{3+} chelates. Inorg Chem 42:5412–5421