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Evaluation of synthetic iron(III)-chelates (EDDHA/Fe^{3+}, EDDHMA/Fe^{3+} and the novel EDDHSA/Fe^{3+}) to correct iron chlorosis

Ana Álvarez-Fernández[1], Sonia García-Marco, Juan J. Lucena*

Agricultural Chemistry Department, Faculty of Sciences, Universidad Autónoma de Madrid, E-28049 Madrid, Spain

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Abstract

Soil application of synthetic Fe(III)-chelates, mainly those derived from ethylendiamine di(*o*-hydroxyphenylacetic) acid (EDDHA) and ethylendiamine di(2-hydroxy-4-methylphenylacetic) acid (EDDHMA), is the most effective, but the most expensive practice used to correct iron deficiency in plants growing on calcareous soils. Previous studies that compared the effectiveness of EDDHA/Fe^{3+} and EDDHMA/Fe^{3+} always used commercial products and their results are contradictory. In this study, the effectiveness of commercial EDDHA/Fe^{3+} and EDDHMA/Fe^{3+} fertilizers to correct iron chlorosis in three different crops (sunflower, peach and pear) was compared using doses calculated with the actual content of chelated iron determined by HPLC. The effectiveness of the Fe(III)-chelate derived from the ethylenediamine di(2-hydroxy-5sulfophenylacetic) acid (EDDHSA), that recently has been marketed as iron fertilizer, was also tested in the sunflower and pear experiments. For the three experiments, several parameters related to the plant iron nutritional status, such as leaf growth, yield, SPAD index (chlorophyll concentration), iron content, Fe/Mn ratio and 50(10P + K)/Fe index were determined. Leaf weight, iron concentration per leaf area, leaf iron content, and K/Ca and 50(10P + K)/Fe ratios were well correlated with the degree of chlorosis, suggesting that these parameters could be used for the diagnosis of the plant iron nutritional status when only iron limited the plant growth. One application of the synthetic Fe(III)-chelates (EDDHA/Fe^{3+}, EDDHMA/Fe^{3+} and EDDHSA/Fe^{3+}) was enough to cause a visible full recovery from iron-deficiency of the three crops. The EDDHSA/Fe^{3+} was as effective as the EDDHA/Fe^{3+} and EDDHMA/Fe^{3+} to correct iron chlorosis in the three different crops, growing either in a soil-less system or in field conditions. However, the doses of EDDHA/Fe^{3+} and EDDHMA/Fe^{3+} were respectively 1.4- and 1.7-times higher than the EDDHSA/Fe^{3+}. Although these results pointed out the EDDHSA/Fe^{3+} as a promising iron fertilizer, further research is needed to know technical details related to the application such as doses, timing and frequencies as well as its mobility, distribution and persistence in the environment. Soil-less experiments could be a good and quick tool to test the effectiveness of these iron fertilizers, since there were no differences in the order of effectiveness found for the Fe(III)-chelates between soil (field experiments) and soil-less experiments.

Keywords: EDDHA; EDDHMA; EDDHSA; Fertilizers; Iron chelates; Iron chlorosis

Abbreviations: EDDHA, ethylendiamine di(*o*-hydroxyphenylacetic) acid; EDDHMA, ethylendiamine di(2-hydroxy-4-methylphenylacetic) acid; EDDHSA, ethylendiamine di(2-hydroxy-5-sulfophenylaetic) acid

* Corresponding author. Tel.: +34-91-3973968; fax: +34-91-3973825.
E-mail address: juanjose.lucena@uam.es (J.J. Lucena).
[1] Present address: Department of Plant Nutrition, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas (CSIC), Apdo. 202, E-50080 Zaragoza, Spain.

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1. Introduction

Iron chlorosis in plants is an old problem occurring in areas of calcareous and/or alkaline soils (Marschner, 1995; Mengel et al., 2001). Diagnosis and correction of iron chlorosis are still being studied (for recent reviews see Tagliavini and Rombolà, 2001; Pestana et al., 2003) as well as many physiological and biochemical aspects of this nutritional disorder. Several fruit crops mainly peach, pear and kiwifruit are among the most susceptible crops to suffer for iron chlorosis (Tagliavini and Rombolà, 2001). In the Ebro river valley, a large agricultural area in northeastern Spain, more than 90% of the peach orchards (23,400 ha) and almost 70% of the pear orchards (13,266 ha) suffer from iron deficiency-chlorosis (Sanz et al., 1992). In fruit trees, soil application of iron compounds is the dominant practice to correct iron chlorosis (Tagliavini et al., 2000; Tagliavini and Rombolà, 2001).

Among all soil-applied iron fertilizers, synthetic Fe(III)-chelates, mainly Fe(III)-chelates of polyamine–carboxylic acids with phenolic groups (see Fig. 1), such as ethylendiamine di(*o*-hydroxyphenylacetic) acid (EDDHA) and ethylendiamine di(2-hydroxy-4-methylphenylacetic) acid (EDDHMA), are the most effective and commonly used. Those molecules together with another homologous molecule, namely ethylendiamine di(2-hydroxy-5-sulfophenylacetic) acid (EDDHSA; see Fig. 1) were synthesized for the first time in the fifties (Dexter and Cranston, 1958) and then the exceptional effectiveness of their iron(III) complex to correct iron deficiencies in plants was claimed. Since then, EDDHA/Fe^{3+} and EDDHMA/Fe^{3+} have been widely studied, marketed and used as fertilizers, whereas little was known about EDDHSA/Fe^{3+}. However, some recent studies dealing with the market availability, characterization and interaction with soils and soil components of EDDHSA/Fe^{3+} molecule have been published (Álvarez-Fernández et al., 2002a,b, 2000; Cantera et al., 2002). These data have confirmed the potential of the EDDHSA/Fe^{3+} as iron-fertilizer claimed in the fifties, which raises the need to research into the effectiveness of EDDHSA/Fe^{3+} to treat iron deficiency in plants.

Comparative studies of the efficacy of the EDDHA/Fe^{3+} and EDDHMA/Fe^{3+} to correct iron deficiency-chlorosis in plants have presented contradictory results. For example, soil applications of EDDHMA/Fe^{3+} were almost as effective as EDDHA/Fe^{3+} to correct iron chlorosis in peach trees, resulting slightly in less intense re-greening in grapes (Reed et al., 1989). Other studies used chelate trunk-injections in peach and olive trees (Fernández-Escobar et al., 1993) and chelate hydroponical crops of corn, sunflower and tomato plants (Hernández-Apaolaza et al., 1995; Álvarez-Fernández et al., 1996). Interestingly, the recovery of iron-deficient plants treated with EDDHA/Fe^{3+} and EDDHMA/Fe^{3+} commercial products, was not significantly affected by the type of chelating agent, whereas significant differences in the Fe nutritional status of plants treated with commercial products containing the same active component and the same chelated iron content declared on the label were found (Hernández-Apaolaza et al., 1995). Those contradictory results could be explained by lack of agreement between the declared and the actual chelated iron content of EDDHA/Fe^{3+} and EDDHMA/Fe^{3+} commercial products (recently reported by Hernández-Apaolaza et al. (1997) and Álvarez-Fernández (2000)), since this fact implies that in the experiments published until now, the doses of active component applied were probably different to the actual ones.

On the other hand, a proper evaluation of the efficacy of the iron treatments to correct iron chlorosis requires the assessment of the iron nutrition status of the plants during the treatment period. The most straightforward approach to detect nutrient deficiencies in plants is to analyze the mineral content of the leaves.

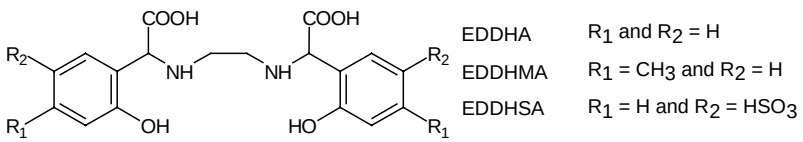

Fig. 1. Molecular structure of Fe(III) synthetic chelating agents used as fertilizers.

However, it is well known that in the case of the iron deficiency, sometimes, the total iron content might not reflect the iron nutritional status of the plant (Pestana et al., 2003). It has been established that when plants are grown under iron deficiency in field conditions, the total leaf iron concentration is generally the same or even higher than in iron-sufficient plants. Many alternative diagnostic methods have been proposed to evaluate ferric nutrition in plants (Abadía et al., 1989; Köseoglu, 1995; Morales et al., 1998) that use different plant characteristics, mainly leaf morphological and chemical characteristics such as leaf area, Fe concentration per unit area and some nutrient ratios such as K/Ca, P/Fe, Fe/Mn and 50(10P + K)/Fe. Biomass and chlorophyll content in leaves are also used as iron nutritional indices but their utilization has the disadvantage that is affected by other nutrients and some plant stresses.

In this work, we compare the effectiveness of EDDHA/Fe^{3+} and EDDHMA/Fe^{3+}, at the same rate of chelated iron, to recover iron-deficient plants of three different species (sunflower, peach and pear) using a soil-less crop system and field conditions. A second aim of this study was to test the EDDHSA/Fe^{3+} as iron fertilizer. Also, different plant characteristics for evaluating their Fe nutritional status were compared.

2. Materials and methods

2.1. Synthetic iron chelates

Three different commercial synthetic Fe(III)-chelates containing EDDHA/Fe^{3+}, EDDHMA/Fe^{3+} and EDDHSA/Fe^{3+}, chosen among the market leaders in Spain were used in this study. The content of soluble and chelated iron were assessed in all batches of each product used.

The content of chelated iron in the fertilizers was determined by HPLC. For the EDDHA/Fe^{3+} and EDDHMA/Fe^{3+}, the Lucena et al. (1996) HPLC method was used, whereas this was modified in order to determine EDDHSA/Fe^{3+} and their condensation products. For the EDDHSA/Fe^{3+} fertilizer, the elution was carried out with aqueous solutions of acetonitrile containing 5 mM tetrabutylammonium hydroxide, at a pH value of 6.0, with the following acetonitrile gradient: 0 min 35% (by volume); 5 min 35%; 6 min 75% and 11 min 75%. For all HPLC analysis, a Waters Symmetry C_{18} 150 mm × 3.9 mm column, and an HPLC with a Waters 2690 Separation Module (Alliance), a Waters 996 photodiode array detector and a Millenium 2010 chromatography data system were used. The flow was always 1.5 ml/min and 20 μl of samples and standards were injected. Solutions of the fertilizers were prepared by dissolving the formulations in deionized water. Solutions were left to stand overnight, filtered and made up to volume. In order to quantify the chelated iron, peak areas at 280 nm were compared with those of standard solutions of EDDHA/Fe^{3+}, EDDHMA/Fe^{3+} and EDDHSA/Fe^{3+}. For the standard preparation, EDDHA was obtained from Sigma (lot no. 117F50221) and EDDHMA was synthesized by using the new synthesis pathway developed by Sierra et al. (2002). NAC Química S.A. (Spain) synthesized and purified sodium salt of EDDHSA. An assay of the EDDHMA and EDDHSA ligands by iron(III) automatic photometric ($\lambda = 480$ nm) titration analysis showed that the EDDHMA was 92.3 ± 0.5% pure and the EDDHSA was 55.5 ± 0.7% pure. Primary standards of each iron chelate were prepared by dissolving the chelating agent in NaOH (1:3 molar ratio). Then an amount of $Fe(NO_3)_3$ that was calculated to be 5% in excess of the molar amount of ligand was added, the pH was adjusted to 7.0 with NaOH, and the solution was left to stand overnight to allow excess iron to precipitate as oxides. The final solution, with an iron concentration of 100 mg/l, was filtered and made to volume with water.

The soluble iron content in all fertilizers was measured after digestion with the following procedure, similar to the one indicated by the 93/1CE European directive. Aliquots of 2.5 ml of 0.5 M HCl and 2.5 ml 30% H_2O_2 were added to 10 ml of the fertilizer solutions containing approximately 100 mg l^{-1} of iron. After one hour, the solution was boiled for 30 min. When the solution reached room temperature, 10 ml of a solution containing 0.5% La as $La(NO_3)_3$, 0.02% Cs as CsCl and 5% HCl were added and then the final solution was transferred to a 50 ml volumetric flask and made to volume with water. This digestion avoids the large molecular interference observed for EDDHSA/Fe^{3+} products. Iron was assessed by atomic absorption spectrophotometry (AAS) using a Perkin-Elmer 4000.

Table 1
Percentage of chelated and water-soluble iron of the commercial Fe(III)-chelates used in each experiment

Experiment	Active component	Chelated iron[a] (%)	Water-soluble iron
Peach	EDDHA/Fe^{3+}	3.67 (6)	7.45
Sunflower, pear	EDDHA/Fe^{3+}	3.61 (6)	7.44
Peach	EDDHMA/Fe^{3+}	3.70 (6.5)	6.61
Sunflower, pear	EDDHMA/Fe^{3+}	3.54 (6)	6.46
Sunflower, pear	EDDHSA/Fe^{3+}	3.24 (6)	5.81

[a] Numbers in parentheses next to the percentage of chelated iron indicate the percentage of chelated iron declared by the manufacturer.

Table 1 shows the results obtained for the analysis of the Fe(III)-fertilizers. As described in the introduction section, the values of the percentage of chelated iron were much lower than those declared on the label. The doses of each product applied in each experiment were then calculated using the values of chelated iron content shown in Table 1.

2.2. Plant material, experimental design and treatments

2.2.1. Sunflower

Sunflowers seeds (*Helianthus annuus* L. c.v. Sirio G-100) previously washed for 10 min in 45 g l^{-1} active Cl_2 and then washed with distilled water for 30 min were germinated and grown in vermiculite in a growth cabinet (Conviron E-15) for 4 days at 25 °C during the day/15 °C at night, with a 16 h photoperiod and relative humidity 60–80%. After germination, the nursery bed was placed in an experimental greenhouse where the seedlings were grown with a nutrient solution without iron, and with temperatures of between 15 and 35 °C. From the 4th day, the solution was renewed every 2 days with nutrient solution increasing in strength, following the sequence 1/10, 1/5, 1/2 and 1/1. Nutrient solution was prepared using analytical reagent grade products as follows: 4.0 mM KNO_3, 3.0 mM $Ca(NO_3)_2$, 1.0 mM KH_2PO_4, 1.0 mM $MgSO_4$, 0.2 mM NaCl, 18.2 μM $MnSO_4{\cdot}H_2O$, 7.9 μM $CuSO_4{\cdot}5H_2O$, 7.6 μM $ZnSO_4{\cdot}7H_2O$, 1.0 μM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 46.2 μM H_3BO_3. The pH of the nutrient solution was raised to approximately 7.7 with 1 mM NaOH and 1 g of solid $CaCO_3$ per litre to simulate conditions usually found in the field, that leads to iron deficiency (Susín et al., 1996). On the 11th day, the plants were transferred to 2.5 l plastic pots (20 plants per pot) where they were supported by a solid plastic plate with holes, and the roots were submerged in the aerated nutrient solution described above. Plants were grown under these conditions until the severe symptoms of iron deficiency were observed (10 days), and then the different treatments were started. There was one control (no iron chelate added) and three iron treatments: 7.2 μM EDDHA/Fe^{3+}, 7.2 μM EDDHMA/Fe^{3+}, 5.0 μM EDDHSA/Fe^{3}. The control and treatments were replicated three times in a completely randomized design layout. To permit chelate turnover from the chelating agent once the plant has taken up iron from the chelate, dialysis bags with 0.05 g synthetic ferrihydrite were placed inside the pots for all treatments and control. Pots were refilled with water as needed throughout the experiment. During the following 7 days SPAD index was measured daily on 10 fully expanded young leaves from each pot as is described below. On the 7th day, each plant was harvested, and then the leaves, stems, and roots were separated and processed as is indicated below.

2.2.2. Peach

The experiment was carried out in a peach orchard in Sudanell, Lleida, in north-eastern Spain (latitude 41.6N, longitude 0.6E, altitude 152 m). The peach trees (*Prunus persica* L. Batsch, cv Sudanell) were 12-year-old and grafted on plum rootstock. The soil has a sandy–clay–loamy texture (46:28:26/sand:silt:clay), with 179 g kg^{-1} of total lime, 52 g kg^{-1} of active lime, 24 g kg^{-1} of organic matter, 1.4 g kg^{-1} of Kjeldahl N, pH in water 7.8 and micronutrients extracted by using the Soltanpour and Schwab (1977) method (mg kg^{-1}): Fe 26.7, Mn 5.38, Cu 47.1 and Zn 4.3. The orchard was fertilized, except for iron, and irrigated as needed, to prevent any nutrient disorder and water stress. The treatments, other than the control (no iron added), were soil application of the following synthetic iron chelates:

EDDHA/Fe^{3+} and EDDHMA/Fe^{3+} at 2 g of chelated iron per tree. The control and treatments were replicated four times in a completely randomized design layout. The control and each treatment consisted of 10 trees per block, 6 of them were used for leaf and fruit sampling and fruit yield measurement. The experiment was carried out over 1 year. Iron treatments were applied on 31 March 1995. Fully expanded young leaves were sampled on 16th May, 22nd June and 20th July. For each sampling date, a composite sample was made up of 72 leaves selected from six trees (12 leaves per tree from set points around the tree) for each treatment and block. For each leaf of the composite sample, the SPAD index was assessed prior to the mineral analysis. Peach harvesting began on 10th August. The yield per tree and the calibre of 10 fruits per tree as a quality parameter were obtained.

2.2.3. Pear

The pear (*Pyrus communis*, cv. Conference) orchard was located in Tarazona, Zaragoza, in north-eastern Spain (latitude 41.9N, longitude 1.7W, altitude 480 m). The soil was clay loam (23:42:35/sand:silt:clay; pH (H_2O) 7.75; total lime (g kg^{-1}) 430; active lime (g kg^{-1}) 140; O.M. (g kg^{-1}) 15; Kjeldahl N (g kg^{-1}) 1.2 and micronutrients extracted using the Soltanpour and Schwab (1977) method (mg kg^{-1}): Fe 12.1, Mn 4.11, Cu 6.86 and Zn 4.45. Two rows of 50 trees, 5 m apart, 2 m between trees in the row, were used. All trees were drip irrigated and fertilized (except iron) as needed. The experiment took place over 2 years (1998 and 1999). According to a randomized complete block experimental design, one control (no iron added) and three treatments with two blocks were soil-applied on 1st June 1998 and on 22nd April 1999. Each treatment consisted of nine sampled trees per block. The treatments, other than control (no iron added), included three synthetic iron chelates: 1 g of Fe as EDDHA/Fe^{3+} per tree, 1 g of Fe as EDDHMA/Fe^{3+} per tree and 0.6 g of Fe as EDDHSA/Fe^{3+} per tree. Leaves were sampled four times each year by the procedure described above for peach trees but in this case the composite sample was made up of 90 leaves selected from nine trees (10 leaves per tree). In 1998, leaves were sampled on 1st June, 2nd July, 28th July and 28th August. The following year the leaf sampling occurred on 22nd April, 20 May, 21st June and 21st July. The SPAD index measurements were made on twelve fully expanded young leaves per usable tree on the following dates: 1st June, 17th June, 2nd July, 13rd July, 28th July, 28th August in 1998 and 22nd April, 20 May, 7th June, 21st June, 8th July, 21st July, 10th August and 23rd August in 1999. Pear harvest started on 26th August 1998 and 23rd August 1999. The yield of pear fruits was determined in both years. Ten fruits per tree were randomly sampled on the harvesting date to determine the maximum diameter.

2.3. SPAD index determination

The most visible effect of iron chlorosis in higher plants is the decrease of photosynthetic pigment, especially chlorophylls (Abadía et al., 1989). The green colour of the leaf is often positively related to the concentration of chlorophyll (Yadava, 1986). Peryea and Kammereck (1997) proposed to use the green colour of the leaf, assessed with a SPAD chlorophyll meter, as an unbiased quantitative measure of the severity of leaf chlorosis associated with iron deficiency and of the relative effectiveness of iron fertilization treatments. The SPAD index was measured using a Minolta SPAD-502 chlorophyll meter. The colour was measured at the middle section of the leaf midway between the central vein and the leaf edge.

2.4. Mineral analysis

The leaves were kept cold and sent to the laboratory. For the peach and pear experiments, the area of the leaves was measured with an automatic area meter (model AAM-7 Hayashi Denko Co. Ltd., Tokyo, Japan). Afterwards, the leaves were washed following the procedure of Sonneveld and van Dijk (1982) as discussed by Álvarez-Fernández et al. (2001) and then dried at 65–75 °C for 24 h. The dry weight was obtained, then samples were mill ground and after dry digestion in a muffle furnace (480 °C) the ashes were digested using HCl, according to Gárate et al. (1984). The elements K, Ca, Mg, Na, Fe, Mn, Cu and Zn were determined using an atomic absorption Perkin-Elmer 4000 spectrophotometer. Phosphorous was analyzed by automated colorimetry in a Technicon Acta CIII auto-analyzer. The total nitrogen was measured by automated colorimetry after a Kjeldahl digestion.

2.5. Statistical analysis

In order to compare the efficacy of the iron chelates in correcting iron chlorosis, the averages of different parameters related to iron plant status for each experiment, were subjected to analysis of variance for randomized block design, and to Duncan's multiple range test using the computer program SAS (SAS Institute, 1985).

3. Results

One of the main results of this study is that there were no significant differences among Fe (III)-chelate treatments in most of plant parameters studied. The average values for the three chelate treatments are normally presented in the following paragraphs and compared with the values obtained for the control (non-treated) plants.

3.1. Sunflower experiment

The rate of iron chlorosis and of the recovery after the treatment applications were estimated by means of the SPAD index values (related to chlorophyll concentration) for the youngest leaves during the treatment week (Table 2). The control plants showed severe iron chlorosis symptoms at the end of the experiment, whereas chelate-treated plants presented completely green leaves. The control plants showed a linear decrease in SPAD index whereas a linear increase with time was found for each chelate treatment. The slope of the linear regression were 0.70 and 2.74 SPAD units per day, (R^2 0.930 and 0.941) for control and chelate treatments, respectively.

In Table 3, the dry weight of roots, shoots, the youngest but completely developed leaves and the whole plant are presented. These biometric data were higher in chelated-treated than in control sunflower plants and were unaffected by the type of chelate including the dry weight of the youngest leaves that are the most susceptible to suffer damage by an insufficient iron supply (individual data not shown).

Iron nutrition status was also assessed by means of the iron concentration per unit weight, and Fe/Mn and 50(10P + K)/Fe indices in the youngest but completely developed leaves (Table 3). Control plants had lower leaf iron concentration and Fe/Mn ratio as well as higher 50(10P + K)/Fe index than

Table 2
Effect of Fe(III)-chelate treatments (average (avg.) of the different chelate treatments) on the evolution of the SPAD index (related to chlorophyll concentration) of the youngest and completely developed sunflower leaves

	Date[a]							
	1/7	2/7	3/7	4/7	5/7	6/7	7/7	8/7
SPAD index								
Control	8.2	7.9	6.2	6.4	4.8	4.7	4.7	2.9
Chelates (avg.)	9.3	10.8[b]	13.7[b]	17.9[b]	20.4[b]	22.2[b]	25.7[b]	27.1[b]

[a] Treatments started on the 1st July 1999.
[b] Significantly different from the control value of the same column at $P > 0.05$ level.

Table 3
Effect of Fe(III)-chelate treatments (average (avg.) of the different chelate treatments) on dry weight (g per plant) of roots, shoots, the whole plant as well as dry weight (g per plant), iron concentration and nutrient ratios (Fe/Mn and Fe index) in the youngest and completely developed leaves

	Roots	Shoots	Whole plant	Youngest leaves
Control	0.121	0.398	0.519	0.073
Chelates (avg.)	0.184[a]	0.534[a]	0.718[a]	0.169[a]
	Fe (μg/g D.W.)	Fe/Mn	50(10P + K)/Fe index	
Control	24.1	0.051	41.3	
Chelates (avg.)	115.7[a]	0.543[a]	6.4[a]	

[a] Significantly different from the control value of the same column at $P > 0.05$ level.

Table 4
Effect of Fe(III)-chelate treatments (average (avg.) of the different chelate treatments) on SPAD Index in Sudanell peach leaves

Treatment	Date[a]			
	16/5	22/6	20/7	Avg.[b]
SPAD index				
Control	31.2	27.9	27.4	28.8
Chelates (avg.)	37.4[c]	35.4[c]	35.6[c]	36.1[c]

[a] Treatments started on the 31st March 1995.
[b] Avg. indicates the average of the three sampling dates.
[c] Significantly different from the control value of the same column at $P > 0.05$ level.

chelate-treated plants. The Fe(III)-chelate used did not affect the iron concentration and Fe/Mn ratio in leaves. However, plants treated with EDDHSA/Fe^{3+} had significantly higher 50(10P + K)/Fe index values than those of EDDHA/Fe^{3+}-treated plants (data not shown).

3.2. Peach experiment

Table 4 shows the time course of SPAD Index in chelate-treated trees and in control trees. Treated trees exhibited significantly higher values of leaf SPAD index than the control ones at the three sampling dates. The type of iron chelate (EDDHA/Fe^{3+} or EDDHMA/Fe^{3+}) applied did not significantly change the leaf SPAD Index during the experimental period.

Dry weight per leaf and leaf area were assessed in our field experiments to follow the chlorosis recovery. Table 5 shows the results of dry weight per leaf and leaf area at the three sampling dates and the average of these values for control and treated trees. Since the statistical analysis showed no interaction between treatment and sampling times for those parameters, the average values of the three sampling times indicate differences between control and chelate-treated trees. Treatments increased dry weight per leaf but there were no major differences in leaf area between control and chelate-treated trees.

For all trees, the iron concentration per unit weight and Fe/Mn ratio (Table 5) in leaves decreased from 45 to 82 days after treatments and there was a general increase thereafter. Treated trees always had a significantly higher leaf iron concentration and Fe/Mn ratio than the control trees, whereas 50(10P + K)/Fe index values were higher in control than in chelate-treated trees. The K/Ca ratio was unaffected by iron chlorosis. Chelate treatment increased peach yield and fruit diameter (Table 6).

Table 5
Effect of Fe(III)-chelate treatments (average (avg.) of the different chelate treatments) on dry weight, leaf area, iron concentration per unit weight and per unit area, Fe/Mn ratio, K/Ca ratio and 50(10P + K)/Fe index in Sudanell peach leaves

Treatment	Date[a]			
	16/5	22/6	20/7	Avg.[b]
Dry weight (g D.W. per leaf)				
Control	0.176	0.185	0.179	0.180
Chelates (avg.)	0.196[c]	0.198	0.196[c]	0.196[c]
Leaf area (cm^2 per leaf)				
Control	36.4	28.7	35.1	33.4
Chelates (avg.)	36.6	29.8	36.3	34.2
Fe (μg/g D.W.)				
Control	44.5	30.9	45.5	40.3
Chelates (avg.)	51.3[c]	42.1[c]	61.0[c]	51.5[c]
Fe (μg/cm^2)				
Control	0.222	0.206	0.225	0.218
Chelates (avg.)	0.266[c]	0.286[c]	0.340[c]	0.297[c]
Fe/Mn				
Control	0.519	0.230	0.266	0.338
Chelates (avg.)	0.742[c]	0.464[c]	0.603[c]	0.603[c]
K/Ca				
Control	1.65	1.22	0.78	1.22
Chelates (avg.)	1.37	1.12	0.84	1.11
50(10P + K)/Fe index				
Control	6.21	7.80	4.63	6.21
Chelates (avg.)	5.00[c]	5.00[c]	3.54[c]	4.51[c]

[a] Treatments started on the 31st March 1995.
[b] Avg. indicates the average of the three sampling dates.
[c] Significantly different from the control value of the same column at $P > 0.05$ level.

Table 6
Effect of various Fe(III)-chelate treatments (average (avg.) of the different chelate treatments) on yield and fruit maximum diameter in Sudanell peach trees

	Yield (kg per tree)	Diameter (mm)
Control	63.1	77.2
Chelates (avg.)	77.9[a]	80.2[a]

[a] Significantly different from the control value of the same column at $P > 0.05$ level.

Table 7
Effect of various Fe(III)-chelate treatments (average (avg.) of the different chelate treatments) on SPAD index in conference pear leaves in 1998 and 1999 at different sampling dates[a]

	1998						1999							
	1/6	17/6	2/7	13/7	28/7	28/8	22/4	20/5	7/6	21/6	8/7	21/7	10/8	23/8
SPAD index														
Control	27.4	27.2	22.1	19.6	17.7	18.2	18.6	21.3	11.6	9.5	–	–	–	–
Chelates (avg.)	29.2	37.4[b]	41.1[b]	42.4[b]	44.1[b]	45.8[b]	24.5[b]	37.3[b]	40.6[b]	45.5[b]	46.3	49.5	49.7	49.1

[a] Treatments started on the 1st June 1998 and on the 22nd April 1999, respectively.
[b] Significantly different from the control value of the same column at $P > 0.05$ level.

3.3. Pear experiment

The green colour (SPAD index) was the parameter most affected by treatments (Table 7). It should be noted that in June 1999 (60 days after treatments) control trees were severely affected by iron chlorosis. Therefore, those trees were iron-treated in order to prevent their death and then for control treatment no data are presented after June 1999 excluding yield and fruit caliber. Control leaves had the lowest SPAD index values in 1998, and 1999, except for the first sampling of 1998. Control trees showed a continuous decrease of the leaf SPAD index with time in both years, whereas chelate-treated trees showed a continuous increase.

Table 8 shows the effect of treatments on leaf morphological characteristics of pear trees in 1998 and 1999. Larger increases of dry weight per leaf and leaf area with time were observed in 1998 than 1999 because in 1999, the experimental period included the leaf development stage. Dry leaf weight in control trees was lower than in chelate-treated trees at 57 and 88 days after treatments in 1998 and at 60 days after treatments in 1999. At 90 days after application in 1998, dry weight per leaf was affected by the different chelate treatments, since leaves from EDDHMA/Fe^{3+}-treated trees presented lower values of this parameter than those of trees treated with EDDHA/Fe^{3+} and EDDHSA/Fe^{3+} (data not shown) However, this effect was not observed in 1999. Leaf area was not significantly affected by iron chlorosis in either season.

The iron concentration per unit weight and per unit area of leaf (Table 8) increased with time for all trees in both years. The control trees had lower leaf iron concentrations than treated trees from 31 days after treatment application to the end of the experiment period in 1998, and thereafter in 1999. Leaf iron concentration was also largely affected by the type of chelate applied. Although chelate treatments had similar iron concentration until 60 days after treatments in 1998 (data not shown), in the last sampling of 1998, EDDHA/Fe^{3+}-treated trees had larger leaf iron concentration (75.2 μg g per D.W.) than those of EDDHMA/Fe^{3+} (68.6 μg g per D.W.) and EDDHSA/Fe^{3+} (62.3 μg g per D.W.) treated trees. In 1999, in the first sampling chelate-treated trees had higher leaf iron concentration than control trees (Table 8). Sixty days after treatments, EDDHSA/Fe^{3+}-treated trees had a lower leaf iron concentration (65.4 μg g per D.W.) than EDDHA/Fe^{3+} and EDDHMA/Fe^{3+} treated trees (94.1 μg Fe g per D.W. for EDDHA/Fe^{3+} and 74.0 μg Fe g per D.W. for EDDHMA/Fe^{3+}). On that date and thereafter the highest iron concentrations were presented by trees treated with EDDHA/Fe^{3+} (data not shown). As in 1998, there were no major differences in iron concentration between EDDHMA/Fe^{3+} and EDDHSA/Fe^{3+} treatments at the last sampling date (90 days after treatments).

The 50(10P + K)/Fe index in leaves decreased with time in control and treated trees (Table 8). Generally, control leaves had the largest 50(10P + K)/Fe index values corresponding with the larger iron chlorosis status. Even in 1999, prior to the treatment application, control trees presented the highest index, indicating a lower iron download from the tree reserves.

In 1998, treatments affected fruit size, whereas fruit yield was not affected (Table 9). Control trees had smaller fruits than treated trees. In 1999, extreme climatic conditions (hail storms) during pear development caused pear fall, thus the yield was reduced, and then was not representative of the chelate treatments. No differences in fruit yield and size were found between control and treated trees.

Table 8
Effect of various Fe(III)-chelate treatments (average (avg.) of the different chelate treatments) on dry weight per leaf, leaf area, iron concentration per unit weight and per unit leaf area, Fe/Mn ratio, K/Ca ratio and 50(10P + K)/Fe index in conference pear leaves in 1998 and 1999 at different sampling dates[a]

	1998				1999			
	1/6	21/7	28/7	28/8	22/4	20/5	21/6	21/7
Dry weight (g D.W. per leaf)								
Control	0.214	0.221	0.223	0.216	0.073	0.173	0.154	–
Chelates (avg.)	0.231	0.242	0.269[b]	0.320[b]	0.081	0.202	0.204[b]	0.231
Leaf area (cm^2 per leaf)								
Control	22.2	17.6	20.1	18.9	15.4	21.2	16.3	–
Chelates (avg.)	23.2	18.3	20.3	20.9	15.9	21.9	16.9	17.1
Fe (μg/g D.W.)								
Control	27.7	40.5	37.7	57.8	33.5	62.4	64.7	–
Chelates (avg.)	31.4	52.6[b]	50.0[b]	68.7	52.3[b]	87.4[b]	77.7[b]	80.0
Fe (μg/cm^2)								
Control	0.269	0.519	0.418	0.660	0.159	0.519	0.636	–
Chelates (avg.)	0.315	0.702[b]	0.674[b]	1.056[b]	0.280[b]	0.819[b]	0.964[b]	1.099
Fe/Mn								
Control	0.93	1.34	1.36	2.45	1.32	2.24	2.37	–
Chelates (avg.)	0.81	1.16	1.05	1.43[b]	1.69	2.75	2.30	1.86
K/Ca								
Control	1.07	0.95	0.62	0.48	2.68	1.17	1.27	–
Chelates (avg.)	1.29	0.92	0.59	0.46	2.79	1.24	0.88[b]	0.59
50(10P + K)/Fe index								
Control	7.22	4.37	3.96	2.26	9.95	3.45	3.84	–
Chelates (avg.)	6.15	3.08	2.51[b]	1.62	6.28[b]	2.04[b]	2.18[b]	1.69

[a] Treatments started on the 1st June 1998 and on the 22nd April 1999, respectively.
[b] Significantly different from the control value of the same column at $P > 0.05$ level.

Table 9
Effect of various Fe(III)-chelate treatments (average (avg.) of the different chelate treatments) on yield and fruit maximum diameter in Conference pear in 1998 and 1999

	1998		1999	
	Yield (kg per tree)	Diameter (mm)	Yield (kg per tree)	Diameter (mm)
Control	34.1	59.6	11.4	53.7
Chelates (avg.)	30.7	61.0[a]	11.3	51.9

[a] Significantly different from the control value of the same column at $P > 0.05$ level.

4. Discussion

4.1. Evaluation of the plant iron nutritional status

Different plant characteristics were used to evaluate the plant iron nutritional status and then to compare the effectiveness of the iron treatments. The plant characteristic most affected by iron chlorosis was the leaf SPAD index that markedly increased in iron-treated plants and decreased in control plants. Since in the three experiments other nutrient disorders or plant stresses that also affected the leaf green colour did not occur, the leaf green colour was the better tool to determine the iron nutritional status according to Pestana et al. (2003). Although the other

Table 10
Correlation coefficients between leaf SPAD index and different plant characteristics considering all data and data corresponding to each experiment

SPAD	Leaf weight	Leaf area	Fe			Fe/Mn	K/Ca	50(10P + K)/Fe	Yield	Fruit diameter[a]
			μg g D.W.$^{-1}$	μg cm^{-2}	μg per leaf					
Total	0.605**	−0.024	0.296	0.671**	0.754**	0.226	−0.498**	−0.602**	0.128	−0.531
Sunflower	0.989**	–	0.781	–	0.891	0.929	0.244	−0.984*	0.998**	–
Peach	0.823**	0.284	0.550	0.679*	0.646	0.598	0.101	−0.514	0.899	0.891
Pear	0.605**	0.044	0.516**	0.763**	0.741**	0.010	−0.573**	−0.666**	−0.492	−0.347

[a] Fruit diameter.
* $P > 0.05$.
** $P > 0.01$.

plant characteristics were significantly less affected than the leaf green colour, significant correlations were found between some of them (leaf weight, iron concentration per leaf area, leaf iron content, and K/Ca and 50(10P + K)/Fe ratios) and the SPAD index (Table 10). This suggests that those plant characteristics also indicated the iron nutritional status but they were less sensitivity than leaf green colour. Leaf SPAD index was negative correlated with K/Ca and 50(10P + K)/Fe ratios, and positively with leaf weight, iron concentration per leaf area and leaf iron content.

Nutrient ratios present the advantage that use an internal reference for the iron content in the plant. Since iron accumulation and organ weight may be affected differently by several factors, nutrient ratios seems to be a more reliable index to quantify iron status in the plant than iron content. On the other hand, biometric parameters such as leaf or roots weights are normally useful to determine plant response in greenhouse pot experiments, but in field experiments yield and fruit quality may also be used. In the greenhouse experiment, biometric data has been adequate to distinguish between control and chelate-treated sunflower plants, mainly when the youngest part of the plant is considered. However, in the field experiments, differences between control and treated trees were observed only for leaf weight, but not for leaf area. This is not in agreement with Morales et al. (1998) who reported that iron chlorosis decreases leaf area. The reason could be that our trees had not been affected by iron chlorosis in the last 2 or 3 years, whereas the orchards used by Morales et al. (1998) had been. This could also be related to the fact that yield and fruit size were not good parameters to determine the tree iron nutritional status.

4.2. EDDHA/Fe^{3+} versus EDDHMA/Fe^{3+} fertilizers to correct iron chlorosis

For the first time, the effectiveness of commercial EDDHA/Fe^{3+} and EDDHMA/Fe^{3+} fertilizers at the same doses of active component has been compared. Both of them showed similar effectiveness to correct iron chlorosis in a soil-less crop as well as in field conditions and for three different crops (sunflower, peach and pear). However, in previous works (Reed et al., 1988; Fernández-Escobar et al., 1993; Álvarez-Fernández et al., 1996) comparison among EDDHA/Fe^{3+} and EDDHMA/Fe^{3+} was made using doses based on the soluble Fe content declared on the label instead of the chelated one and significant differences in the effectiveness to correct iron chlorosis were found. In fact in a previous work on hydroponically grown tomato plants (Álvarez-Fernández et al., 1996) we concluded that a commercial product containing EDDHMA/Fe^{3+} (6% soluble Fe) was more effective correcting iron chlorosis than another one containing EDDHA/Fe^{3+} (6% soluble Fe), but in a recent analysis we found a higher chelated iron content in the EDDHMA/Fe^{3+} product (3.10% (w/w)) compared to the EDDHA/Fe^{3+} product (2.60% (w/w)), that could cause the higher effectiveness of the EDDHMA/Fe^{3+} product. These facts strongly suggest that it is necessary to determine the chelated iron content of the commercial Fe(III)-chelate products to properly compare the effectiveness of their active ingredients.

4.3. Effectiveness of EDDHSA/Fe^{3+} to correct iron chlorosis

As far as we know, for the first time the effectiveness of the EDDHSA/Fe^{3+} to correct iron chlorosis

in plants is compared with that of EDDHA/Fe^{3+} and EDDHMA/Fe^{3+}. Although the EDDHA/Fe^{3+} and EDDHMA/Fe^{3+} doses employed in the experiments were between 1.4- and 1.7-fold higher than the EDDHSA/Fe^{3+} one, their effectiveness to re-green iron chlorotic plants was similar both in a soil-less system and in field conditions. These results indicate that the EDDHSA/Fe^{3+} is a promising iron fertilizer. However, further research is needed to know the technical details related to the utilization of EDDHSA/Fe^{3+} as fertilizer. Since its solubility is 3.4-fold higher than that of products containing EDDHA/Fe^{3+} (Álvarez-Fernández, 2000) and its retention in soils is lower (Álvarez-Fernández et al., 2002a), due to the high negative charge (−3) of the EDDHSA/Fe^{3+} as compared to EDDHA/Fe^{3+} or EDDHMA/Fe^{3+} (−1), doses, timing and frequencies should be studied as well as their mobility, distribution and persistence in the environment.

4.4. Effect of the type of experiment on the evaluation of the effectiveness of the Fe(III)-chelates (EDDHA/Fe^{3+}, EDDHMA/Fe^{3+} and EDDHSA/Fe^{3+})

Soil and soil-less crops have been studied. In soil-less crops, the effectiveness of Fe(III)-chelates is not affected by the reaction between chelate and soil. However, the soil-less experiment with sunflower produced similar results to those experiments carried out under field conditions with peach and pear trees. This fact is attributed to the low reaction between this type of Fe(III)-chelates (EDDHA/Fe^{3+}, EDDHMA/Fe^{3+} and EDDHSA/Fe^{3+}) and soils, reported by Álvarez-Fernández et al. (2002a). Therefore, almost the total applied amount of iron supplied by the different Fe(III)-chelates could have been available to the plant in both type of experiments (soil and soil-less). Moreover, differences among chelate treatments should be related to the ability of the plant to take iron from these chelates. Dicotyledonous stressed plants may increase the root iron reduction capacity using the iron chelate as a substrate, then the differences in the inorganic source of iron between both types of experiments do not seem to be relevant. Soil-less experiments with dicotyledonous plants carried out, as described in this work, could be a good and quick tool to test the effectiveness of those iron fertilizers that react slightly with soils.

5. Conclusion

Leaf weight, iron concentration per leaf area, leaf iron content, and K/Ca and 50(10P + K)/Fe ratios were well correlated with the degree of chlorosis, suggesting that these parameters could be used for the diagnosis of the plant iron nutritional status when only iron limited plant growth. The EDDHSA/Fe^{3+} was as effective at re-greening iron chlorotic plants growing both in a soil-less system and in field conditions as the well-known Fe(III)-chelates (EDDHA/Fe^{3+} and EDDHMA/Fe^{3+}), that had similar efficacy.

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