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# The Effectiveness of FeEDDHA Chelates in Mending and Preventing Iron Chlorosis in Soil-Grown Soybean Plants

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## 1. Introduction

### 1.1 Iron deficiency – The problem

Iron (Fe) is an essential micronutrient for plants, humans and other animals. An adequate uptake of Fe is needed to ensure proper growth and development, as well as good health of organisms (Marschner, 1995; Vasconcelos and Grusak, 2007). When provided with insufficient quantities of Fe, organisms will suffer from Fe deficiency symptoms.

Fe deficiency is a worldwide problem in crop production, affecting yield both qualitatively and quantitatively (Mortvedt, 1991); plants do not reach their full growth potential, and the nutritional value is compromised, leading to economic losses and limitations in crop selection (Chaney, 1984). In extreme cases, Fe deficiency may result in complete crop failure (Chen and Barak, 1982). The list of plant species affected is vast and includes apple, citrus, grapevine, peanut, dryland rice, sorghum and soybean (Marschner, 1995).

Fe deficiency is typically found in crops grown on calcareous or alkaline soils, in arid and semi-arid regions of the world; these soils cover over 30% of the earth's land surface (Figure 1) (Alvarez-Fernandez, et al., 2006; Chen and Barak, 1982; Hansen, et al., 2006; Mortvedt, 1991). Fe is abundantly present in all soils including calcareous ones; in mineral soils the average Fe content is approximately 2% (20,000 µg/g) (Marschner, 1995; Mengel and Kirkby, 2001). Most agricultural crops require less than 0.5 µg/g in the plough layer (Lindsay, 1974). The occurrence of Fe deficiency in plants grown on calcareous soils, despite the excessive soil-Fe pool, is caused by a limited bioavailability of Fe in such soils.

### 1.2 Symptoms of Fe deficiency

Fe deficiency in plants typically causes chlorosis of leaf tissue because of inadequate chlorophyll synthesis; the leaves become pale green to yellow (Figure 2), often with darker coloured veins. In case of severe chlorosis, leaves can also become necrotic (Figure 2). Due to the reduction in photosynthetic capacity, carbon fixation by plants also becomes reduced, leading to slower growth rates and yield losses (Figure 2) (Alvarez-Fernandez, et al., 2006).

Fe chlorosis develops most strongly in young leaves, because growing plant parts (also fruits, buds and storage organs) have incomplete xylem structures. As a result, Fe is not directly transported from the roots to these sites with the highest demand, but remobilized from older plant parts and secondarily transported through the phloem (Grusak, et al., 1999;

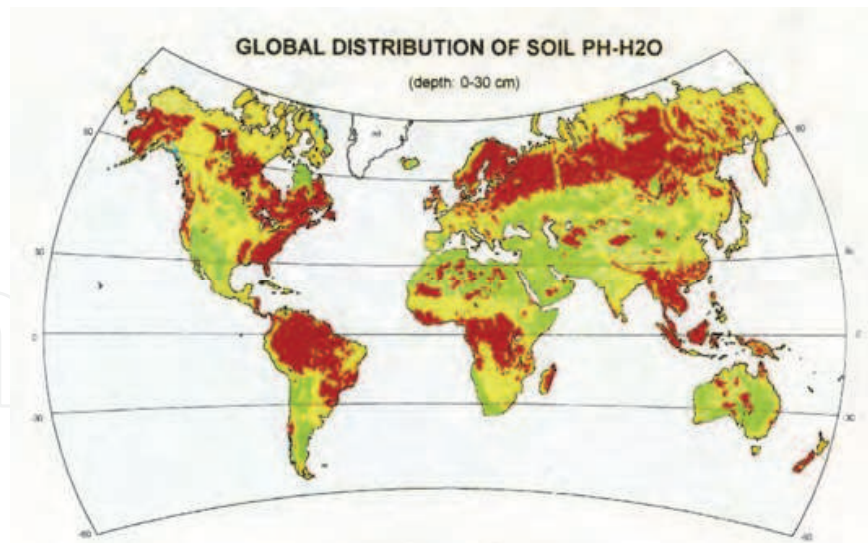


Fig. 1. Global pH-map of the top soil (0-30 cm); red indicates  $\text{pH} < 5.5$ ; yellow indicates  $5.5 < \text{pH} < 7.3$ ; green indicates  $\text{pH} > 7.3$ . Calcareous soils are to be found in the green areas. Source: ISRIC, 1995, derived from the WISE- database.

Zhang, et al., 1995). It has been observed that chlorotic leaves can have comparable or even higher Fe contents than green leaves (the “chlorosis paradox”). This phenomenon has been attributed to impaired expansion growth, leading to diminished dilution of the high Fe concentration in young leaves (Römheld, 2000). Fe deficiency also causes morphological changes in the roots: inhibition of root elongation, increase in diameter of apical rootzone, abundant root hair formation (Römheld and Marschner, 1981) and formation of rhizodermal transfer cells.

### 1.3 Causes of Fe deficiency

Two related soil characteristics are principally responsible for the low Fe availability in calcareous soils: 1) the relatively high pH (7 - 8.5) (Figure 1.1), and 2) the presence of a bicarbonate pH-buffer in soil solution (Boxma, 1972; Chaney, 1984; Lucena, 2000; Marschner, 1995; Mengel, et al., 1984; Mengel and Kirkby, 2001).

In order for soil-Fe to be taken up, it needs to be transported through the soil solution to the root surface. The solubility of soil Fe(hydr)oxides is a function of pH and the type of Fe(hydr)oxide. The concentration of inorganic Fe species in solution reaches a minimum around pH 7.5 - 8.5: in the order of  $10^{-10}$  M (Figure 3); the free  $\text{Fe}^{3+}$  concentration is around  $10^{-21}$  M (Lindsay and Schwab, 1982). For optimal growth, plants require an Fe concentration in soil solution in the order of  $10^{-6}$  to  $10^{-5}$  M (Marschner, 1995). Complexation by dissolved organic substances, like humic acids, fulvic acids and siderophores can increase the total Fe concentrations in soil solution by orders of magnitude in comparison to the inorganic Fe concentration (O'Conner, et al., 1971), but not always sufficiently to prevent Fe deficiency.

The bicarbonate pH-buffer prevents plants from adapting the rhizosphere pH and causes impairment of Fe deficiency stress response mechanisms (except in grasses). Although the pH-buffer capacity of calcareous soils is largely determined by the lime content, the dissolution of carbonate minerals is relatively slow in comparison to bicarbonate diffusion. Therefore, on the short term, the bicarbonate concentration in soil solution is more



Fig. 2. Examples of Fe deficiency symptoms in soybean plants. *Upper*: from left to right - decreasing degree of chlorosis; *Lower left*: necrosis in the leaves; *Lower right*: reduced growth.

important for maintaining a high rhizosphere pH (Lucena, 2000). In addition to the role of bicarbonate as pH-buffer in soil solution, there has been much debate on bicarbonate uptake leading to Fe immobilization inside plants (Gruber and Kosegarten, 2002; Mengel, 1994; Nikolic and Romheld, 2002; Römheld, 2000).

#### 1.4 Prevention and remediation of Fe deficiency

When Fe stress response mechanisms of plants prove inadequate, techniques to prevent or remedy Fe deficiency need to be applied to avoid yield losses. Breeding and genetically modifying plants for a more efficient Fe uptake mechanism is a promising approach. Developing new cultivars should however be done carefully and requires much time. Once crops are in the field, application of Fe fertilizer is the most certain and efficient treatment to ensure that plants do not suffer from Fe deficiency.



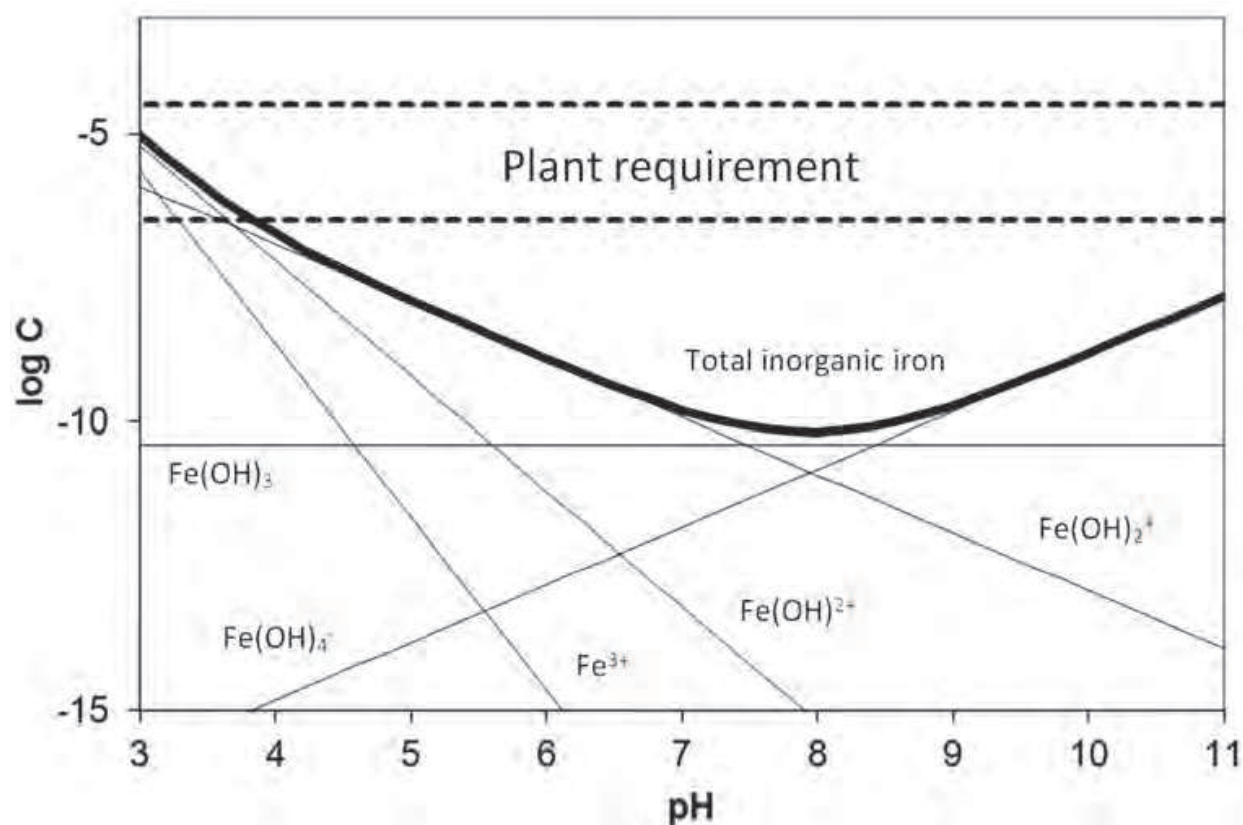


Fig. 3. Hydrolysis species of  $\text{Fe}(3+)$  in equilibrium with soil-Fe ( $\text{pK}_{\text{sol}} = 39.3$ ;  $I = 0.03 \text{ M}$ ), after Lindsay (1979).

Fe fertilizers can be administered through trunk injection, foliar application, and soil application. Trunk injection is expensive and only suitable for trees. Foliar application does not provide full control of Fe chlorosis, but can be useful as complementary technique next to soil application (Alvarez-Fernandez, et al., 2004). Soil application is the most common technique to manage Fe deficiency in soil grown crops (Lucena, 2006). The technique is based on increasing the Fe concentration in soil solution. On calcareous soils, soil application of Fe fertilizers based on organic Fe salts, Fe complexes of lignosulfonates, citrates, gluconates, and synthetic Fe chelates of limited stability (e.g. FeEDTA, FeDTPA and FeHEDTA) has limited or no result, because these fertilizers are not able to maintain Fe in soil solution. Only Fe chelates of higher stability (FeEDDHA and derivatives, with phenolic functional groups) are effective and provide the most efficient treatment to control Fe deficiency (Lucena, 2006).

### 1.5 Fe deficiency in soybean

Fe deficiency chlorosis is a persistent, yield-limiting condition for soybean (*Glycine max* (L.) Merr.) production in regions with calcareous soils (Inskeep and Bloom, 1986). In the North Central U.S., Fe deficiency is responsible for an estimated loss in soybean grain production of \$120 million per year (Hansen et al., 2004). Foliar Fe treatments and soil application of Fe chelates can be efficient in alleviating Fe deficiency chlorosis in soybean. However, in agricultural practice, these methods are only economically feasible for high-value crops and not for soybean (Fairbanks 2000).

Although soybean is not a target species for application of synthetic Fe chelates, it is an attractive test species due to the availability of soybean cultivars with a high susceptibility to Fe deficiency, the ease in handling of the plants, and the relatively short growth cycle in comparison to many of the target species (e.g. citrus trees and grape vines). There is much experience with soybean in Fe chlorosis research; in nutrient solutions, in pot cultures and in the field (e.g. Garcia-Marco et al. 2006; Goos et al. 2004; Goos and Johnson 2000; Heitholt et al. 2003; Wallace and Cha 1986).

### 1.6 FeEDDHA based fertilizers

FeEDDHA is the iron(3+) complex of the chelating agent EDDHA, which is an acronym for ethylene diamine di(hydroxy phenyl acetic acid). EDDHA is also referred to as EHPG (ethylenebis-(hydroxy phenyl glycine)). This chelating agent was first synthesized by Kroll, introduced in 1955, but only fully described in 1957 (Kroll, 1957; Kroll, et al., 1957; Wallace, 1966). FeEDDHA was quickly recognized as very effective in correcting Fe chlorosis under soil conditions, also in comparison to other chelating agents (Wallace, et al., 1955; Wallace, 1962). The  $\text{Fe}^{3+}$  ion is bound by 2 carboxylate groups, 2 phenolate groups and 2 secondary amine groups in an octahedral complex of high stability with an intense red colour at neutral pH. The FeEDDHA complex owes its high stability in comparison to FeEDTA or FeDTPA complexes to the Fe-O (phenolate) bonds.

The current synthesis pathway for manufacturing EDDHA on an industrial scale is a Mannich-like reaction between phenol, ethylenediamine and glyoxylic acid. This reaction produces a mixture of 1) positional isomers, 2) diastereomers and 3) polycondensates, because 1) the reaction pathway allows for aromatic substitution in (o) ortho and (p) para position, 2) two chiral centers are introduced into the molecule leading to (R,R); (R,S); (S,R) and (S,S) configurations, and 3) undesired addition reactions take place between reactants and half products. The composition of the mixture of reaction products can be steered. After the reaction is terminated, an Fe salt is added to the reaction products to form Fe chelates.

Commercial FeEDDHA formulations can be operationally divided into 4 groups of compounds:

1. racemic o,o-FeEDDHA (Figure 4a); referring to the (R,R) and (S,S) configurations of o,o-FeEDDHA (iron (3+) ethylene diamine-N,N'-bis(2-hydroxy phenyl acetic acid) complex). These configurations are mirror images, but identical in most physical and chemical properties, including binding strength.
2. meso o,o-FeEDDHA (Figure 4b); referring to the (S,R) = (R,S) configuration of o,o-FeEDDHA. Due to the internal mirror plane of the chelate, the (S,R) and (R,S) configurations are identical.
3. o,p-FeEDDHA (Figure 4c); referring to the 4 configurations of o,p-FeEDDHA (iron (3+) ethylene diamine-N-(2-hydroxy phenyl acetic acid)-N'-(4-hydroxy phenyl acetic acid) complex). The o,p-FeEDDHA configurations are not identical in physical and chemical properties.
4. rest-FeEDDHA; referring to the 3 configurations of p,p-FeEDDHA (iron (3+) ethylene diamine-N,N'-bis(4-hydroxy phenyl acetic acid) and a variety of polycondensates and half products. An example of a polycondensate is depicted in Figure 4d.

In this chapter, these 4 groups will be referred to as the FeEDDHA components. In commercial FeEDDHA formulations, the sum of the racemic and meso o,o-FeEDDHA content is referred to as the o,o-FeEDDHA content of the product. Generally racemic and meso o,o-FeEDDHA are synthesized in a ratio close to 1.

Racemic and meso *o,o*-FeEDDHA are diastereomers; the chelated Fe is bound by the same functional groups, but the geometry of the chelate differs: in racemic *o,o*-FeEDDHA, both phenolic rings are in equatorial position, while in meso *o,o*-FeEDDHA one phenolic ring is in equatorial and the other in axial position (Figure 4a and 4b). Due to the difference in geometry the amount of strain on the bonds with Fe differs, which is reflected in a higher complexation constant for racemic *o,o*-FeEDDHA.

The position of the hydroxyl group on the phenolic ring affects the complexation constant of FeEDDHA components more strongly than strain: in para-position the hydroxyl group is sterically inhibited from contributing to binding Fe. As a consequence, *o,o*-EDDHA binds Fe more strongly than *o,p*-EDDHA (see Table 1.1), which in turn binds Fe more strongly than *p,p*-EDDHA. Rest-FeEDDHA is a very heterogeneous group, comprising of compounds that vary in molecular weight, number of functional groups, etc, and hence also in complexation constant.

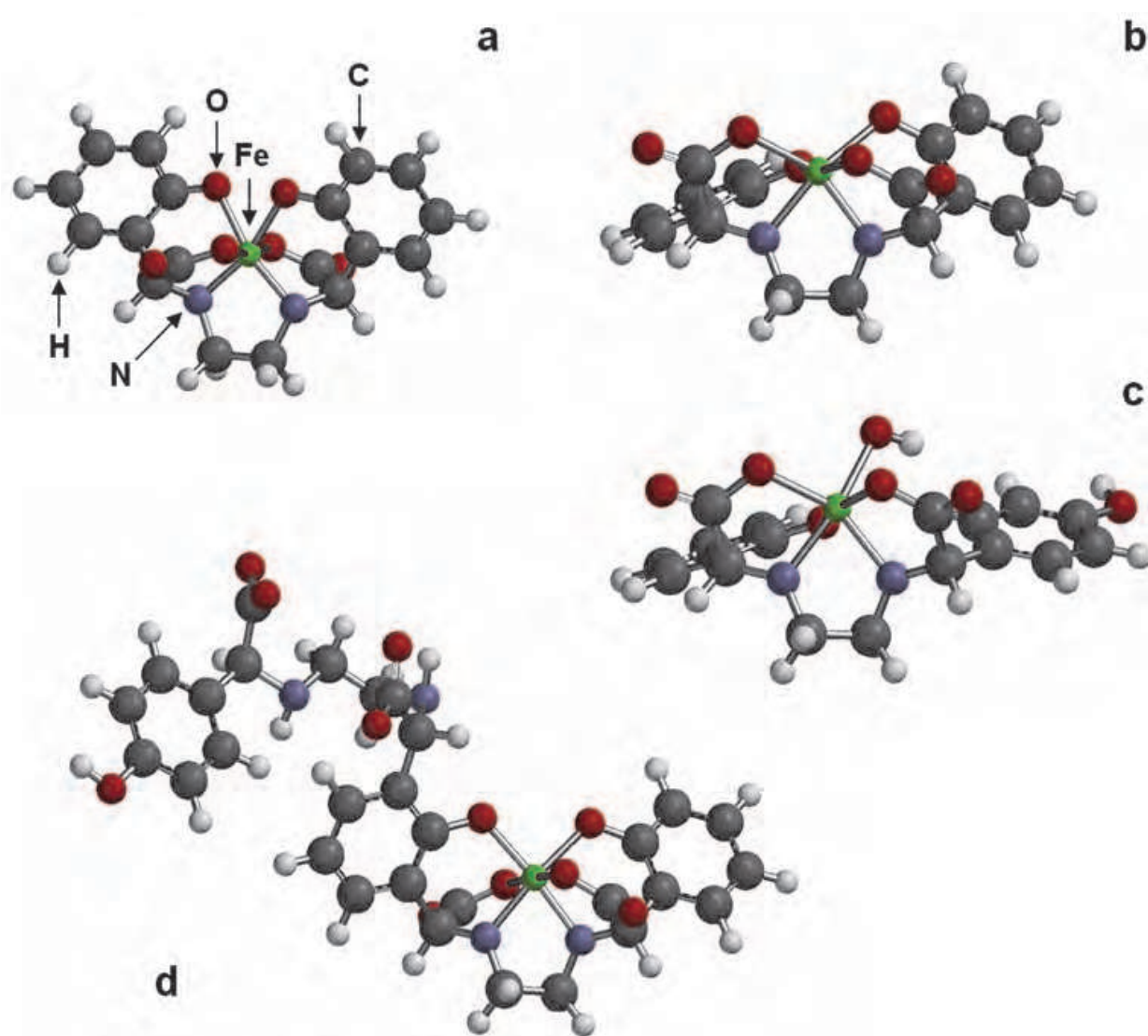


Fig. 4. Spatial structures of the FeEDDHA components **a)** racemic *o,o*-FeEDDHA; **b)** meso *o,o*-FeEDDHA; **c)** *o,p*-FeEDDHA with OH<sup>-</sup> on the coordination complex; and **d)** rest-FeEDDHA (one possible polycondensate) (Schenkeveld et al., 2007).

Component	Log K (I = 0.1 M (NaCl))	
racemic o,o-FeEDDHA	35.86	Yunta et al. (2003a)
meso o,o-FeEDDHA	34.15	Yunta et al. (2003a)
o,p-FeEDDHA	28.72	Yunta et al. (2003b);

Table 1. Complexation constants of FeEDDHA components.

1.7 The market and regulation of FeEDDHA products

The market size for products based on FeEDDHA or related phenolic aminocarboxylate Fe chelates (e.g. FeEDDHMA, FeEDDHSA), is approximately 10 thousand tonnes per year, corresponding with a market value of around 60 million Euros. It is linked to areas of high soil-pH, in particular the Mediterranean area and the Middle East.

From the variability in composition of FeEDDHA products, and the difference in fertilizer value of the FeEDDHA components arose the need to ensure the quality of commercial FeEDDHA formulations. Several tests and methodologies have been developed to assess the quality of FeEDDHA products (Cantera, et al., 2002; Garcia-Marco, et al., 2003; Lucena, et al., 1992a; b). At present the quality of FeEDDHA products is guarded in the European Fertilizer Law (Regulation (EC) No. 2003/2003; amendment (EC) No. 162/2007) through the following parameters: (1) soluble Fe content of the product, (2) percentage of Fe chelated, and (3) percentages of Fe chelated by respectively o,o-EDDHA and o,p-EDDHA. Data on these parameters have to be indicated on the product label. FeEDDHA products should comprise at least 5 weight percent of water-soluble Fe, of which at least 80 percent should be chelated, and at least 50 percent should be chelated to either o,o- or o,p-EDDHA. To be included on the product label, there is a threshold value for both o,o- and o,p-EDDHA of 1 weight percent of chelated Fe.

In order to quantify the composition of FeEDDHA products, both for product information and law enforcement purposes, suitable protocols for analysis had to be developed. The method that is currently used for quantitative analysis is the high performance liquid chromatography (HPLC) method laid down by the European Committee for Standardization (CEN. EN 13368-2:2007). This method is almost identical to the ion-pair HPLC method developed by Lucena et al (1996).

1.8 The effectiveness of FeEDDHA components as Fe fertilizer

The efficacy of FeEDDHA as Fe fertilizer relies on its ability to increase the solubility of Fe, thereby enhancing its bioavailability through an increase in diffusive flux of Fe to the root. The effectiveness of individual FeEDDHA components is determined by: 1) their ability to remain in solution, 2) their susceptibility to cation competition and biodegradation, 3) their ability to transfer Fe to the plant, and 4) the ability of the corresponding EDDHA component to selectively mobilize Fe (Lucena 2003). Considerable effort has been invested to improve the understanding of these characteristics. The interaction between FeEDDHA components and soil and soil constituents has been examined by Alvarez-Fernandez, et al., 2002; Cantera, et al., 2002; Garcia-Marco, et al., 2006a; Hernandez-Apaolaza, et al., 2006; Hernandez-Apaolaza and Lucena, 2001 and Schenkeveld et al., 2007; Fe uptake from FeEDDHA components in hydroponic systems has been examined by Cerdan, et al., 2006; Garcia-Marco, et al., 2006a; Hernandez-Apaolaza, et al., 2006; Lucena and Chaney, 2006;



2007; Rojas, et al., 2008; and mobilization of Fe from Fe oxides by EDDHA ligands has been studied by Perez-Sanz and Lucena, 1995.

Still, the question of how much individual FeEDDHA components actually contribute to supplying soil-grown plants with Fe had remained unaddressed up until recently. An understanding of this issue is however particularly relevant for agricultural practice, since nowadays the composition of FeEDDHA products in terms of FeEDDHA components varies greatly. An efficient use of FeEDDHA fertilizer, implying maximizing the benefits in terms of crop yield and Fe uptake by plants, while minimizing the applied FeEDDHA dosage, is desirable both for the applier in view of cost efficiency, and from an environmental perspective to minimize the input of synthetic chemicals into the environment. In practical terms efficient FeEDDHA application translates into applying the right fertilizer (right composition) at the right moment in the right quantity. This requires a profound understanding of the effectiveness of individual FeEDDHA components in soil application. This chapter aims to inform on recent advances made in understanding the performance of FeEDDHA components in soil application (Schenkeveld et al. 2008; 2010a; 2010b). In a series of pot trial studies with soybean, FeEDDHA-facilitated Fe uptake was examined in relation to 1) the composition of the FeEDDHA treatments, 2) the soil solution concentrations of the FeEDDHA components as a function of time, and 3) the moment of FeEDDHA application.

## 2. Materials and methods

### 2.1 Soil

The calcareous soil used for the pot experiments was collected at a site located in Santomera (Murcia, Spain), from the top soil layer (0 – 20 cm). Plants grown on Santomera soil became chlorotic under field conditions. Pre-treatment of the soil consisted of air drying and sieving (1 cm). Santomera soil is a clay soil with a lutum fraction of 260 g kg<sup>-1</sup> and a CaCO<sub>3</sub> content of 520 g kg<sup>-1</sup>. The soil has a high pH: 8.0 (pH-CaCl<sub>2</sub>) and a low soil organic carbon (SOC) content: 5 g kg<sup>-1</sup>. The dissolved organic carbon (DOC) concentration amounts 30 mg l<sup>-1</sup> (0.01 M CaCl<sub>2</sub>), and Fe availability parameters are low: oxalate extractable Fe content: 0.30 g kg<sup>-1</sup> Fe, and diethylene triamine penta acetic acid (DTPA) extractable Fe content: 3.5 mg kg<sup>-1</sup> Fe. A more complete overview of soil characteristics of Santomera soil is presented in Schenkeveld et al., 2010a.

### 2.2 FeEDDHA solutions

Depending on the pot trial experiment, FeEDDHA solutions were prepared from EDDHA stock solutions varying in EDDHA component composition, from a solid o,o-H<sub>4</sub>EDDHA mixture (purity: 99%), or from separated solid racemic o,o-EDDHA (purity: 100%), meso o,o-EDDHA (purity: 99.5%) and o,p-EDDHA (purity: 90%). Racemic and meso o,o-H<sub>4</sub>EDDHA were obtained by separation of the o,o-H<sub>4</sub>EDDHA mixture, as described in Bannochie and Martell (1989) and Bailey et al. (1981). Solid H<sub>4</sub>EDDHA was first dissolved in sufficient 1 M NaOH solution. An FeCl<sub>3</sub> solution was added to the EDDHA solution in a 2-5% excess, based on a 1:1 stoichiometry between Fe and ethylene diamine (incorporated in the EDDHA ligands). pH was raised to 7 ± 1, and the solution was stored overnight in the dark to allow excess Fe to precipitate as Fe(hydr)oxides. Subsequently the FeEDDHA solutions were filtered over a 0.45 µm nitro cellulose filter (Schleicher & Schuell, refno: 10401114) and further diluted for application in the pot trial. The composition of FeEDDHA solutions was examined at t=0 and at the end of the experiment by ICP and HPLC analysis.

### 2.3 Pot trial studies

The effectiveness of FeEDDHA components in providing soybean plants from the chlorosis susceptible cultivar Mycogen 5072 with Fe was examined in three pot trial studies.

#### *Effect of FeEDDHA treatment composition on Fe uptake- pot trial 1*

In pot trial 1, soybean plants were given FeEDDHA treatments similar in Fe dose ( $\approx 7 \text{ mg l}^{-1}$  Fe in the pore water; 0.13 mM), but differing in FeEDDHA component composition. Four FeEDDHA treatments (16%o,o; 34%o,o; 49%o,o and 99%o,o) and a blank treatment were included in the experiment; the composition of the treatments is presented in Table 2. The treatments are named after the combined percentage of the Fe chelated by racemic and meso o,o-EDDHA and were given at  $t=0$ . The pot trial experiment had a run time of eight weeks. A more elaborate description of the experiment is provided in Schenkeveld et al., 2008.

#### *FeEDDHA-facilitated Fe uptake as a function of time - pot trial 2*

In pot trial 2, the relation between FeEDDHA component concentrations in the pore water and Fe uptake by plants was examined as a function of time. Soybean plants were offered two different FeEDDHA treatments, (30%o,o and 100%o,o) and a blank treatment. The treatments were equal in Fe dose ( $\approx 4 \text{ mg l}^{-1}$  Fe in the pore water; 0.07 mM), but differed in the percentage of Fe chelated by racemic and meso o,o-EDDHA. The composition of the treatments is presented in Table 2. FeEDDHA was applied once, at the start of the experiment. The pot trial with had a runtime of six weeks. Plants were harvested and soil solution was sampled every week. The experiment is described more elaborately in Schenkeveld et al., 2010a.

#### *Effect of moment of application on Fe uptake from FeEDDHA components- pot trial 3*

In pot trial 3, the influence of the moment of application on the effectiveness of individual FeEDDHA components in proving soybean plants with Fe was examined. The experiment involved a blank treatment and six FeEDDHA treatments: o,p; meso o,o; racemic o,o; o,o-mix low; o,o mix-low + o,p; and o,o-mix high. Two levels of FeEDDHA application were distinguished; a low level in the first four treatments, corresponding to a pore water concentration of around  $0.6 \text{ mg l}^{-1}$  Fe (i.e.  $11 \mu\text{M}$ ), and a high level in the latter two treatment, corresponding to a pore water concentration of around  $1.8 \text{ mg l}^{-1}$  Fe (i.e.  $32 \mu\text{M}$ ). The high level FeEDDHA application was included to ensure that Fe uptake had not yet reached a maximum in the low level application, which is prerequisite for comparing the effectiveness of the FeEDDHA components. The mixed treatments were included to examine potential synergetic effects. The composition of the treatments is presented in Table 2. With exception of the blank treatments, all pots received one FeEDDHA treatment, either at  $t=0$  after transfer of the seedlings, after 3 weeks in the progressed vegetative stage, or after 6 weeks in the reproductive stage. Which FeEDDHA treatment was applied in which growth stage is indicated in Table 2. Treatments are named after the FeEDDHA treatment administered and the moment of application. The pot trial had a runtime of 8 weeks. Schenkeveld et al., 2010b describes the pot trial more elaborately.

All pot trial experiments were carried out in a greenhouse with 7 liter Mitscherlich pots containing either 6 kg (pot trial 1 and 2) or 5 kg (pot trial 3) of soil at 50% of the waterholding capacity. The experiments were done in triplicates. In pot trial 1 and 2, FeEDDHA solutions were mixed through the soil prior to filling the pots; in pot trial 3, the FeEDDHA treatments were applied through a sand column in the middle of the pot, which went up to a depth of approximately 10 cm into the soil. After FeEDDHA addition, the sand column was flushed with demineralized water.

Seeds of the Fe chlorosis susceptible soybean (*Glycine max* (L.) Merr.) cultivar Mycogen 5072 were germinated on quartz sand with demineralised water. After five days eight seedlings were transferred to each pot, which had been filled with soil one day prior to the transfer. Preparation of the pot trial, soil fertilization with macronutrients, foliar fertilization with micronutrients other than Fe, and plant care were performed as described in Schenkeveld et al. (2008). In pot trial 2, foliar fertilization was omitted. In pot trial 3 the amounts of macronutrients added to the soil were lowered, in proportion to the smaller quantity of soil used per pot.

Treatment	racemic o,o- FeEDDHA (mg l <sup>-1</sup> Fe)	meso o,o- FeEDDHA (mg l <sup>-1</sup> Fe)	o,p- FeEDDHA (mg l <sup>-1</sup> Fe)	rest- FeEDDHA (mg l <sup>-1</sup> Fe)	total Fe (mg l <sup>-1</sup> Fe)	Moment of application
<i>Pot trial 1</i>						
blank	-	-	-	-	-	-
16%o,o	0.58 (8%)	0.61 (8%)	1.15 (16%)	5.02 (68%)	7.36	t = 0
34%o,o	1.07 (16%)	1.24 (18%)	1.26 (19%)	3.14 (47%)	6.71	t = 0
49%o,o	1.69 (22%)	2.03 (27%)	1.31 (18%)	2.50 (33%)	7.53	t = 0
99%o,o	3.44 (48%)	3.64 (51%)	-	0.10 (1%)	7.18	t = 0
<i>Pot trial 2</i>						
blank	-	-	-	-	-	-
30%o,o	0.60 (14%)	0.68 (16%)	0.79 (19%)	2.18 (51%)	4.25	t = 0
100%o,o	1.93 (48%)	2.00 (50%)	-	0.05 (1%)	3.98	t = 0
<i>Pot trial 3</i>						
blank	-	-	-	-	-	-
o,p	*	*	0.53	0.05	0,58	t = 3 and 6 weeks
meso o,o	-	0.56	-	-	0.56	t = 0, 3 and 6 weeks
racemic o,o	0.58	-	-	-	0,58	t = 0, 3 and 6 weeks
o,o-mix low	0.29	0.31	-	-	0.60	t = 3 weeks
o,o-mix low + o,p	0.29	0.31	1.06	0,10	1.76	t = 3 weeks
o,o-mix high	0.87	0.93	-	-	1.80	t = 0 and 3 weeks

Table 2. Treatment overview of the pot trials; \* o,p-EDDHA standard contains traces of racemic and meso o,o-EDDHA

2.4 Sampling and measurement

SPAD measurements were done, as described in Schenkeveld et al., 2008, on the youngest leaves throughout the pot trials, to monitor chlorosis. Chlorosis was established based on a

significant difference ( $\alpha = 0.05$ ) in SPAD-indices between the blank and the treatment with the highest SPAD-index.

At harvest, the shoots were cut off directly above the soil surface. A 1 kg mixed subsample was taken from the soil. Roots were collected manually from the soil subsample, which was stored at 4 °C until further use. The shoots were washed with demineralized water and dried at 70 °C. After 48 hours, the shoots were weighed (dry weight). The mineral contents of the shoots were determined by microwave digestion with nitric acid, fluoric acid and hydrogen peroxide (Novozamsky, et al., 1996). Cu, Fe, Mn and Ni concentrations were measured by ICP-AES (Varian, Vista Pro). Fe uptake was calculated as the product of shoot dry weight yield and Fe content of the shoot. Roots were left out of consideration, due to contamination with soil material.

Pore water was collected from the soil subsample by centrifugation at 7,000 rpm for 15 minutes as described in Schenkeveld et al 2008. The pH of the pore water was measured directly after collection. Fe, Ca and Mg concentrations were measured by ICP-AES (Varian, Vista Pro); Cu, Al, Mn, Zn, Ni and Co concentrations were measured by ICP-MS (Perkin Elmer, ELAN 6000). The samples were acidified with nitric acid before ICP-analysis. FeEDDHA isomer concentrations were determined after separation by high-performance liquid chromatography (HPLC) as described in Schenkeveld et al. (2007). Preparation of experimental solutions and dilution of samples was done with analytical grade chemicals and ultra pure water.

## 2.5 Statistical analysis

Statistical analysis of the data was performed using SPSS 12.0. Homogeneity of variance was tested with the Levene's test ( $\alpha = 0.05$ ). A log transformation of the data was executed in case the variance proved non-homogenous. Differences among treatments were determined by applying the multivariate general linear model procedure with a Tukey post-hoc test ( $\alpha = 0.05$ ). Block effects from the tables were accounted for by including table as a random factor.

## 3. Results and discussion

### *Chlorosis*

Inducing Fe deficiency chlorosis is a prerequisite for testing the effectiveness of the FeEDDHA components. In all three pot trials, the plants in the blank treatment became chlorotic, approximately a week after transfer of the seedlings to the pots. The development of chlorosis differed per pot trial; in pot trial 1 and 2, the degree of chlorosis reached a maximum after around three weeks, after which the difference in SPAD-index started to decrease. In pot trial 1, chlorosis in the youngest leaves of the blank treatment was actually entirely over-grown by the time the plants were harvested (Schenkeveld et al., 2008). Possibly, the decrease in degree of chlorosis is related to an increased root density in the pots as a result of an ongoing development of the root system. This high root density leads to increased rhizosphere effects and an enhanced ability of the plants to acquire Fe. In pot trial 3, the degree of chlorosis stabilized and remained more or less constant towards the end of the experiment.

### 3.1 Effect of FeEDDHA treatment composition on Fe uptake

#### *Pore water concentrations*

The Fe concentration in the pore water of the blank treatment was below detection limit, both in this and the other two pot trial experiments, indicating that FeEDDHA components



were responsible for all Fe in solution in the FeEDDHA treatments. At harvest of pot trial 1, the total Fe concentration in the pore water proved linearly related to the o,o-FeEDDHA content of the FeEDDHA treatments (Figure 5). Racemic o,o-FeEDDHA accounted for approximately 80% of the Fe in solution, and meso o,o-FeEDDHA for the remaining 20%. o,p-FeEDDHA and rest-FeEDDHA had been removed from soil solution practically completely. These components have a tendency to adsorb due to a relatively high affinity for soil reactive surfaces. Moreover, upon interaction with soil, Cu may rapidly displace Fe from o,p-FeEDDHA resulting in solubilization of o,p-CuEDDHA (Garcia-Marco et al., 2006; Hernandez-Apaolaza et al., 2006; Schenkeveld et al., 2007). Hence, removal of FeEDDHA components from soil solution is to a large extent unrelated to plant processes. From the amount of Fe added with the FeEDDHA treatment only in between 4 and 20 % was retrieved at harvest. The recovery of racemic o,o-FeEDDHA and meso o,o-FeEDDHA was around 30% and 7%, respectively. The recovery of o,p-FeEDDHA and rest-FeEDDHA was below 1%.

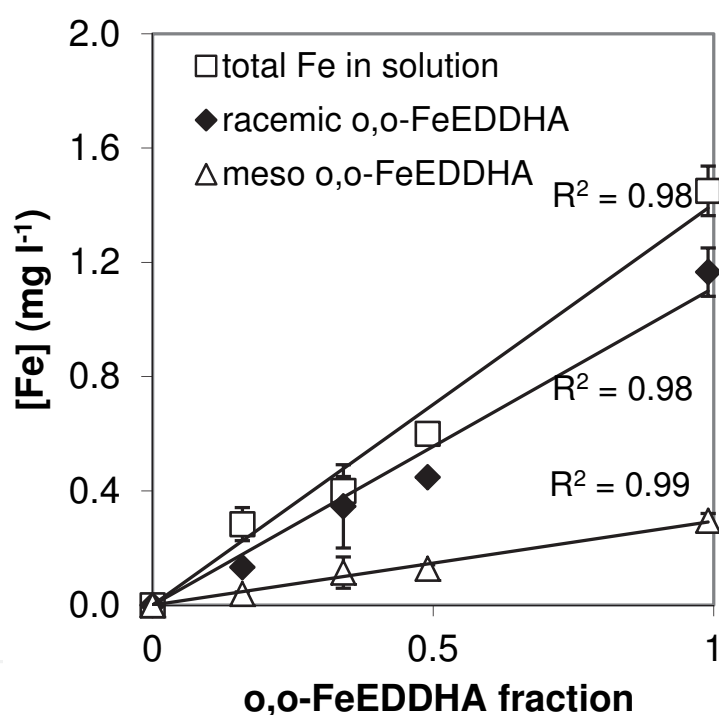


Fig. 5. Fe and FeEDDHA component concentrations in soil solution of Santomera soil at harvest as a function of the o,o-FeEDDHA content of the FeEDDHA treatment. Error bars indicate standard deviations. (based on Schenkeveld et al., 2008)

#### Fe uptake

Fe uptake by soybean plants increased with increasing o,o-FeEDDHA content of the FeEDDHA treatment (Figure 6a). At low o,o-FeEDDHA content, the increase in Fe uptake is relatively strong, but the slope of the curve flattens with increasing o,o-FeEDDHA content, and eventually an optimum is reached (Schenkeveld et al., 2010a).

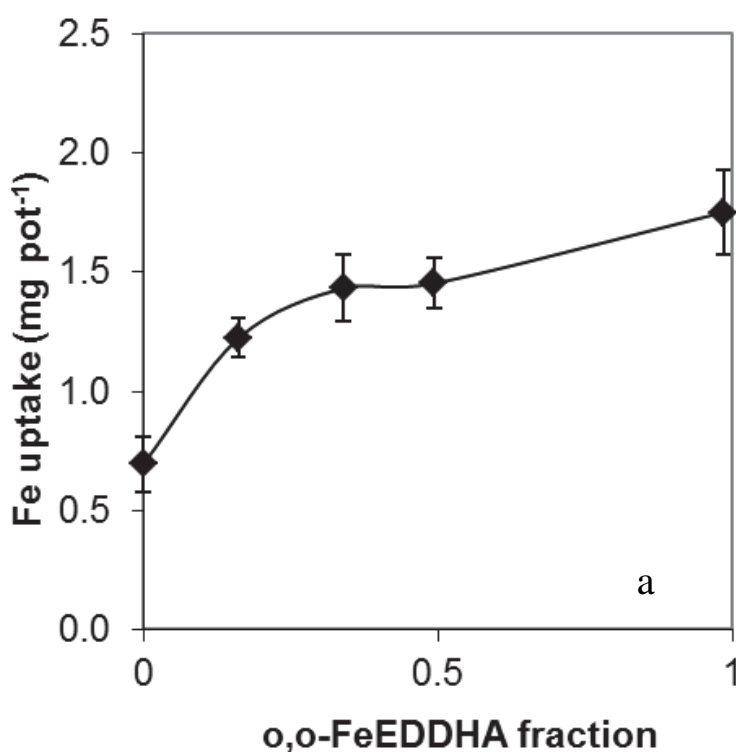
The increase in Fe uptake with increasing o,o-FeEDDHA content suggests that Fe uptake is related to the Fe concentration in soil solution (Figure 5). This makes sense, since the limited solubility of Fe in calcareous soil is one of the primal causes for Fe chlorosis. The fact that Fe

uptake, unlike Fe concentration in soil solution, is not linearly related to the o,o-FeEDDHA content suggests a saturation effect, commonly observed with micronutrient uptake in relation to bioavailability (Marschner, 1995).

As a result of the FeEDDHA treatments, Fe uptake increased from 0.70 mg pot<sup>-1</sup> in the blank to 1.75 mg pot<sup>-1</sup> in the 99% o,o-FeEDDHA treatment; a 150% increase. The 16%o,o FeEDDHA treatment already increased Fe uptake by approximately 75%, to 1.22 mg pot<sup>-1</sup>. The additional Fe uptake in the FeEDDHA treatments in comparison to the blank only accounted for 7 to 15% of the Fe provided as FeEDDHA, and for 15 to 44% of the Fe added as o,o-FeEDDHA.

The increased Fe uptake manifested both in an increased Fe content of the shoot (Figure 6b), and in an increased dry weight yield (Figure 6c). The trends in Fe content and dry weight yield as a function of o,o-FeEDDHA content are similar as for Fe uptake. The relative effect on Fe content of the shoot: an increase from 31 to 60 mg kg(dw)<sup>-1</sup> ( $\approx 100\%$  increase), was larger than the relative effect on dry weight yield; an increase from 22.1 to 29.0 g(dw) pot<sup>-1</sup> ( $\approx 30\%$  increase). Comparable results were also obtained with soybean grown on another calcareous soil (Schenkvelde et al., 2008; results not shown).

An important practical implication of these results for FeEDDHA application prior to the onset of chlorosis is, that for obtaining similar results in terms of crop yield and crop quality, a smaller dosage of FeEDDHA products with a higher o,o-FeEDDHA content is required in comparison to products with a lower o,o-FeEDDHA content.



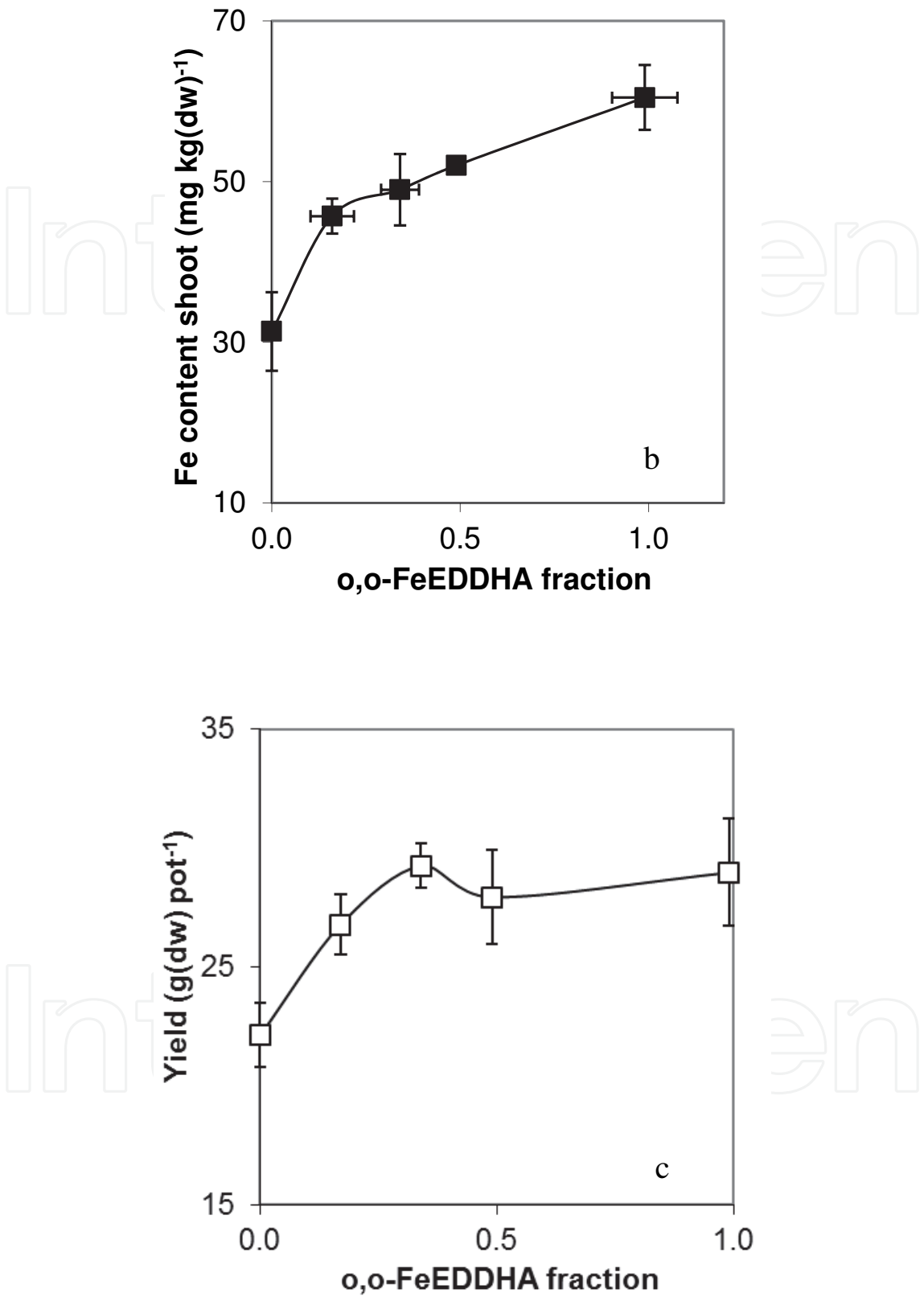


Fig. 6. a) Fe uptake; b) Fe content of the shoot; and c) dry weight yield (shoot) of soybean plants grown on Santomera soil as a function of the *o,o*-FeEDDHA content of the FeEDDHA treatment. Error bars indicate standard deviations. (based on Schenkeveld et al., 2008)

### 3.2 FeEDDHA-facilitated Fe uptake as a function of time

#### *Pore water concentrations*

In Figure 7, the Fe and FeEDDHA component concentrations are presented as a function of time for the 30%o,oL treatment from pot trial 2. Within the first week of the experiment, the Fe concentration underwent a strong drop, from 4.25 to 0.81 mg l<sup>-1</sup> Fe, after which it gradually declined further (Figure 7a). This drop was largely caused by the practically complete removal of o,p-FeEDDHA and rest-FeEDDHA from soil solution. From 1 week onward, the Fe concentration was largely (> 92%) governed by racemic and meso o,o-FeEDDHA (Figure 7b). The concentration of racemic and meso o,o-FeEDDHA underwent two stages: 1) a rapid, strong decline within the first week, and 2) a gradual decline from one week onward. The initial decrease in racemic o,o-FeEDDHA concentration (≈28%) was smaller than for meso o,o-FeEDDHA (≈54%). This fast decline has been attributed to adsorption, which can be described with linear adsorption isotherms (Schenkeveld et al 2010a). The rate of the gradual decline was higher for meso o,o-FeEDDHA than for racemic o,o-FeEDDHA, resulting in a continuous increase in relative contribution of racemic o,o-FeEDDHA to the total Fe in solution. The nature of the gradual decline differed for racemic and meso o,o-FeEDDHA: for meso o,o-FeEDDHA it could be accurately described with an exponential decay function, whereas for racemic o,o-FeEDDHA no decline was observed in the second week of the experiment and from 2 weeks onward, the rate of decline was less consistent (Figure 7b). The decay constant in the exponential function describing the gradual decline in meso o,o-FeEDDHA concentration proved dependent on the applied amount of meso o,o-FeEDDHA (Schenkeveld et al., 2010a).

#### *Fe uptake*

Fe uptake as a function of time is presented in Figure 8 and was calculated by subtracting total Fe uptake of two consecutive harvesting rounds for a corresponding treatment. Fe uptake at 2 weeks actually represent the Fe taken up during the second week, and so on. During the 2<sup>nd</sup> week, in the early vegetative stage, Fe requirements were still low. Chlorosis had just developed in the soybean plants and possibly utilization of Fe which had been present in the seeds, still covered part of the Fe requirements. In the 3<sup>rd</sup> and the 4<sup>th</sup> week, during the progressed vegetative stage, Fe demand strongly increased and in the blank treatment chlorosis was most severe. In the course of the 4<sup>th</sup> and during the 5<sup>th</sup> week, the transfer from the vegetative to the reproductive stage took place; the plants flowered and started to grow pods. In the 6<sup>th</sup> week, the seed formation inside the pods progressed and Fe requirements were even larger than during the vegetative stage, in order to provide the seeds with sufficient Fe (Grusak, 1995). Throughout the experiment, the sequence in Fe uptake was: blank < 30%o,o < 100%o,o. The difference in Fe uptake among the treatments was largest in growth stages in which Fe requirements were largest. The large differences in Fe uptake during the reproductive stage did not show in an increased difference in SPAD-indices (Schenkeveld et al., 2010a).

#### *Relation between FeEDDHA removal and Fe uptake*

The amount of FeEDDHA components removed from the soil system (solid and solution phase combined) per week was calculated from the decrease in soil solution concentration (Figure 8b), assuming linear adsorption (Schenkeveld et al., 2010a), and is presented as a function of time for the 100%o,o treatment in Figure 9a. The removal of meso o,o-FeEDDHA was larger than the removal of racemic o,o-FeEDDHA throughout the experiment. Still, racemic o,o-FeEDDHA, seems to have a more pronounced influence on the shape of the total o,o-FeEDDHA removal-curve (Figure 9a).



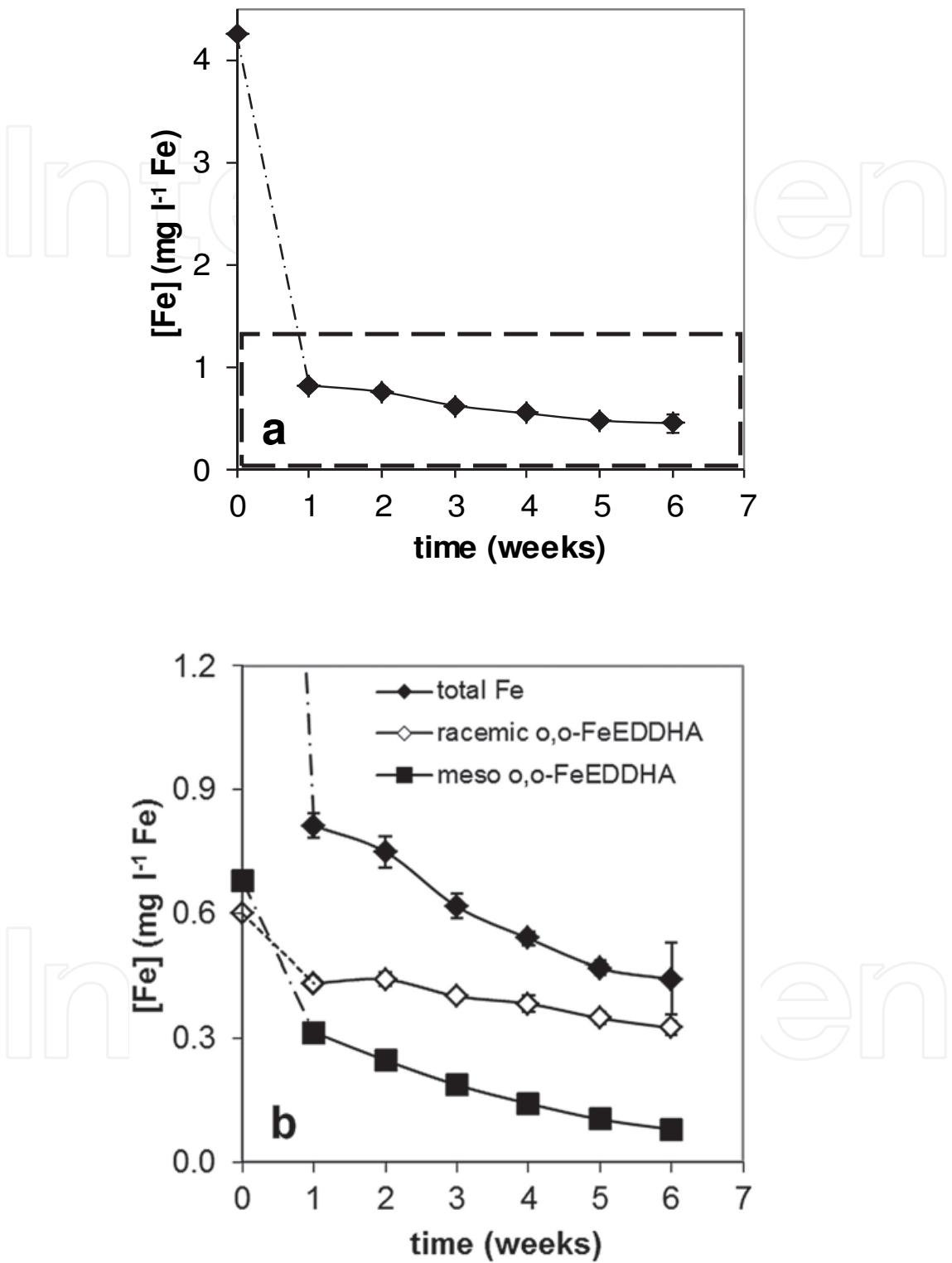


Fig. 7. Fe and FeEDDHA component concentrations in the pore water of Santomera soil as a function of time for the 30%o,o treatment. Error bars indicate standard deviations. (based on Schenkeveld et al., 2010a)

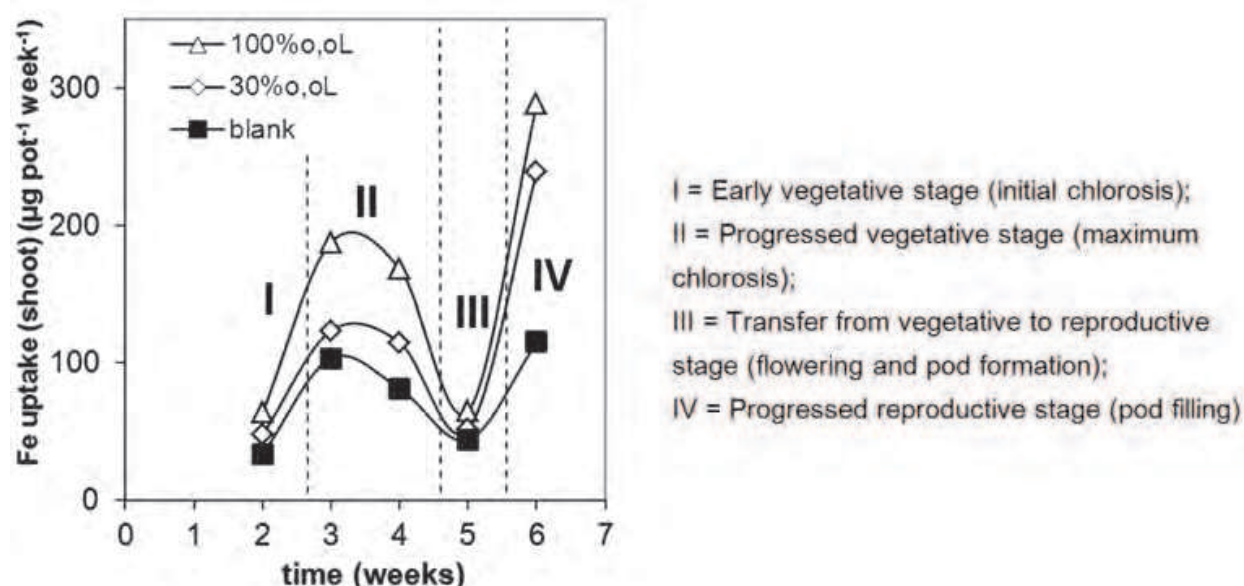


Fig. 8. Fe uptake (shoot) by soybean plants grown on Santomera soil as a function of time. Error bars have been omitted. (based on Schenkeveld et al., 2010a)

In Figure 9b, two scenarios for FeEDDHA-facilitated Fe uptake are presented as a function of time for the 100%o,o treatment. In the maximum FeEDDHA-facilitated uptake scenario, all Fe uptake by the soybean plants is assumed FeEDDHA-facilitated; in the minimum FeEDDHA-facilitated uptake scenario, only the Fe uptake in addition to Fe uptake in the blank treatment is assumed FeEDDHA-facilitated. The shape of the racemic o,o-FeEDDHA removal curve strongly resembles the shape of the FeEDDHA-facilitated Fe uptake curves (Figure 9b). This suggests that the removal of racemic o,o-FeEDDHA from the soil system is to a large extent plant-related. The fact that the gradual decline in racemic o,o-FeEDDHA concentration only started after 2 weeks, when the plants developed a strong need for Fe, further supports this reasoning. The shape of the meso o,o-FeEDDHA removal curve (Figure 9a) does not show a similar resemblance, which suggests that the removal of meso o,o-FeEDDHA from the soil system is to a large extent non-plant related. The nature of the plant-independent process causing a decline in meso o,o-FeEDDHA concentration remains unclear.

### 3.3 Effect of moment of application on Fe uptake from FeEDDHA components

#### Pore water concentrations

The FeEDDHA component concentrations in the pore water at harvest of pot trial 3 are presented in Figure 10. o,p-FeEDDHA was not detected in any of the samples and has not been included in the figure. In agreement with the results from the other two pot trails, for each of the moments of application separately, racemic o,o-FeEDDHA remained in solution to a larger extent than meso o,o-FeEDDHA. The recovered concentrations only accounted for up to 25% of the racemic o,o-FeEDDHA and up to 8% of the meso o,o-FeEDDHA applied. In particular for the treatment applied at t=6 weeks these low recoveries are remarkable; there was only 2 weeks of residence in the soil-plant system.

For corresponding treatments applied at t=0 and t=3 weeks, the recovery of the treatment applied at t=3 weeks was consequently lower than for the treatment applied at the start of the experiment. This seems counter-intuitive, because the residence time in the soil-plant

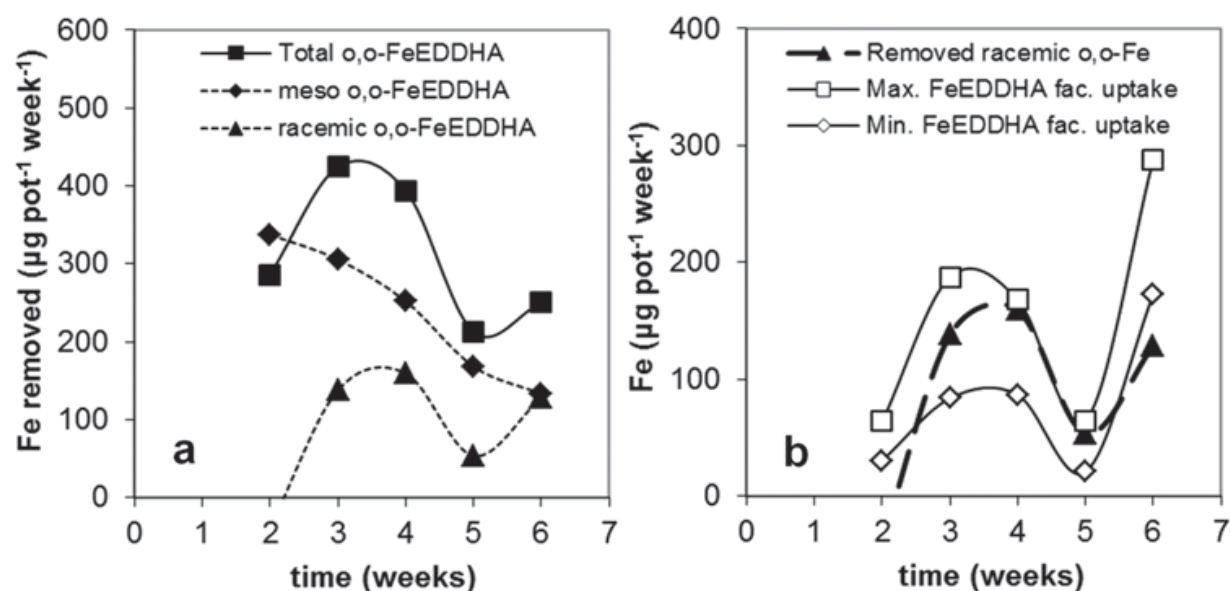


Fig. 9. a) Amount of total, racemic and meso o,o-FeEDDHA removed from the soil system per week for the 100%o,o treatment; b) Minimum and maximum FeEDDHA-facilitated Fe uptake (shoot) per week by soybean plants grown on Santomera soil as a function of time, and the amount of racemic o,o-FeEDDHA removed from the soil system per pot per week, both for the 100%o,oL treatment. Error bars have been omitted. (based on Schenkeveld et al., 2010a)

system of the treatment applied at  $t=0$  is 3 weeks longer. An essential difference regarding the system to which the FeEDDHA treatments were applied, is that with application at  $t=3$  weeks, the soybean plants had grown chlorotic and Fe deficiency stress mechanisms had been activated by the time the treatment was applied, whereas plants receiving FeEDDHA treatment at  $t=0$  never grew Fe deficient to this extent in the first place. For strategy I plants like soybean, one of the stress response mechanisms involves up-regulation of the ferric chelates reductase (FCR) system at the root surface (Robinson et al., 1999; Marschner, 1995), enabling plants to more efficiently reduce and take up chelated Fe. Provided that the efficiency of the corresponding EDDHA ligand in complexing and solubilizing Fe from the soil is limited, the FeEDDHA isomer concentration in soil solution will hence decrease more swiftly and strongly in the presence of Fe deficient plants than with plants that are not Fe deficient.

Comparison of corresponding treatments applied at  $t=0$  and  $t=6$  weeks shows that the racemic o,o-FeEDDHA concentrations are comparable ( $133$  and  $145 \mu\text{g l}^{-1}$ ), and the meso o,o-FeEDDHA concentrations are approximately twice as high in the  $t=6$  weeks treatment ( $20$  and  $47 \mu\text{g l}^{-1}$ ); still these differences in meso o,o-FeEDDHA concentration are small in comparison to the dosage applied ( $560 \mu\text{g l}^{-1}$ ). The effect of stress response mechanisms on o,o-FeEDDHA concentrations equaled six weeks of residence time in the soil-plant system for racemic o,o-FeEDDHA, and over 3 weeks for meso o,o-FeEDDHA.

#### Fe uptake

The Fe uptake data presented in Figure 11 demonstrate that, in agreement with Rojas et al., 2008, o,p-FeEDDHA did not significantly increase Fe uptake in any of the treatments; neither applied as a single substance (o,p 3 and o,p 6 treatment) nor in a mixture through a

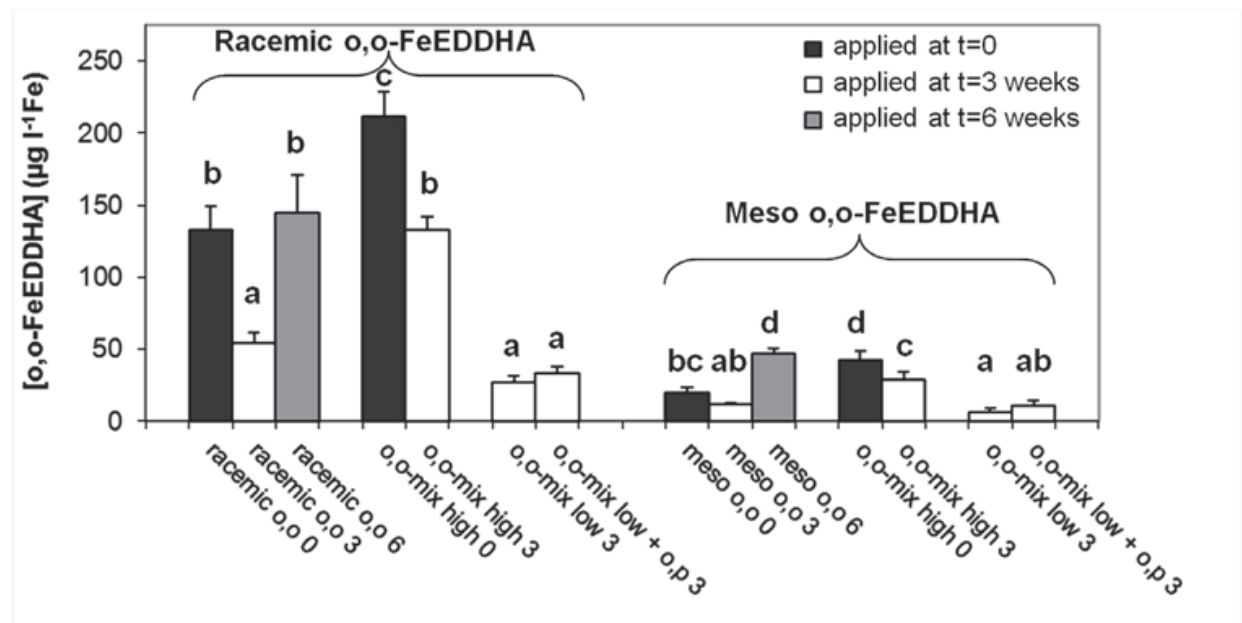


Fig. 10. Racemic and meso o,o-FeEDDHA concentration in the pore water of Santomera soil at harvest. Error bars indicate standard deviations. Letters indicate the significantly different groups as identified by the Tukey post-hoc test, for both FeEDDHA components separately. (based on Schenkeveld et al., 2010b)

synergistic effect (*o,o-mix low + o,p*). Due to its interaction with soil constituents, the residence time of *o,p*-FeEDDHA in soil solution can be short (Schenkeveld et al., 2007). Therefore *o,p*-FeEDDHA had only been applied to soybean plants that were already Fe deficient at the moment of application. However, even when applied in the growth stages that Fe requirements were highest and Fe stress response mechanisms were activated, facilitating a more efficient Fe uptake, *o,p*-FeEDDHA still did not significantly increase the Fe uptake of soybean plants.

Both racemic *o,o*-FeEDDHA and meso *o,o*-FeEDDHA did contribute to Fe uptake (Figure 11), as shown from the fact that in all treatments with *o,o*-FeEDDHA, Fe uptake was significantly higher than in the blank treatment. This is in agreement with the conclusion from the study by Ryskiewicz and Boku (1962). For none of the moments of application, significant differences in Fe uptake were found between the *racemic o,o* and *meso o,o* treatment. Because overall Fe uptake in the *o,o-mix high* treatments was higher than in the *racemic o,o* ( $p = 0.030$ ) and the *meso o,o* ( $p = 0.012$ ) treatments, Fe uptake was not yet maximal in the *racemic o,o* and the *meso o,o* treatments. Therefore it can be concluded that racemic and meso *o,o*-FeEDDHA were approximately equally effective in facilitating Fe uptake. Lucena and Chaney (2006) reported that meso *o,o*-FeEDDHA was more effective in delivering Fe to hydroponically grown cucumber plants than racemic *o,o*-FeEDDHA, as a result of a lower stability favouring Fe reduction at the root surface. Possibly in soil, a preferential Fe uptake from meso *o,o*-FeEDDHA was balanced by a higher affinity for the solid phase and a faster decline in soil solution concentration.

Moreover, for both the *racemic o,o* and *meso o,o* treatments, no significant difference in Fe uptake was observed between the different moments of application. This is remarkable, because the plants receiving treatment after 6 weeks had much less time to benefit from the



applied *o,o*-FeEDDHA. Apparently, as a result of Fe deficiency stress response mechanisms and development of the root system, the soybean plants had grown much more efficient with regard to Fe uptake. In only two weeks time, the soybean plants from the *racemic o,o* 6 and *meso o,o* 6 treatments took up an additional 0.36 mg of Fe per pot, which corresponds with 50% of the total Fe uptake in the blank treatment.

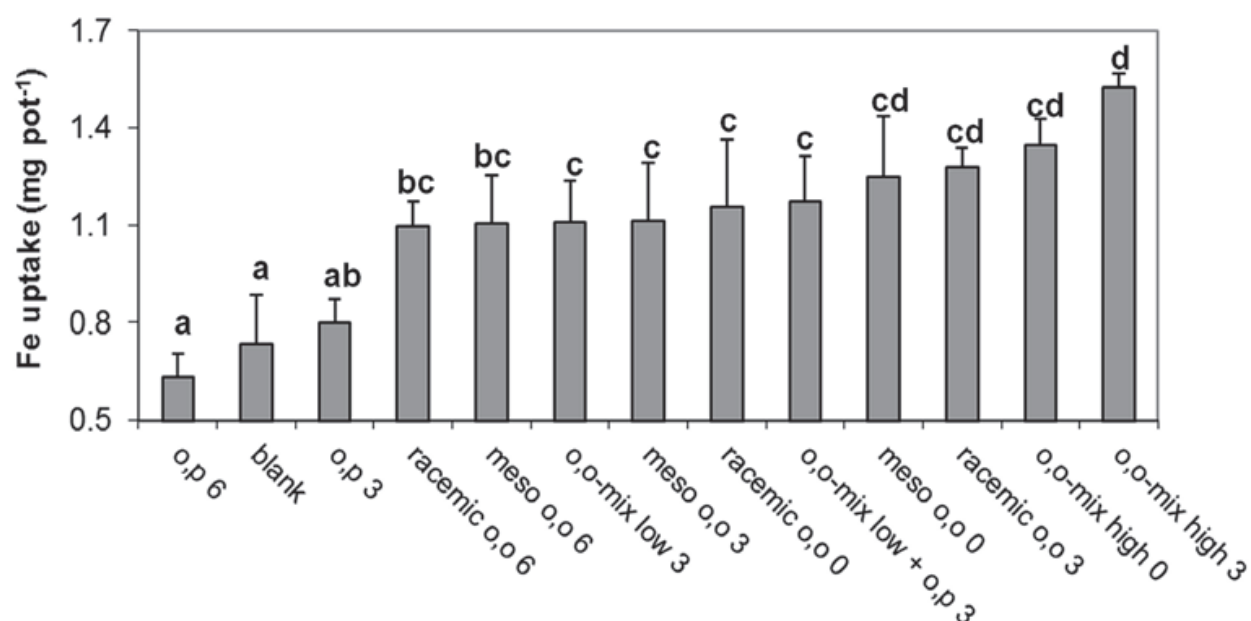


Fig. 11. Fe uptake by soybean plants grown on Santomera soil for all FeEDDHA treatments. Error bars indicate standard deviations. Letters indicate the significantly different groups as identified by the Tukey post-hoc test including all FeEDDHA treatments and all moments of application. (based on Schenkeveld et al., 2010b)

#### 4. Conclusions, limitations and challenges for future research

In the pot trial experiments conducted, it was found that the effectiveness of FeEDDHA components in delivering Fe to soil-grown plants is largely determined by their ability to remain in solution. The residence time of *o,p*-FeEDDHA in soil solution proved too short to significantly contribute to facilitating Fe uptake. The residence time of both *racemic* and *meso o,o*-FeEDDHA was much longer and both isomers did contribute to Fe uptake, approximately to the same extent on the time scale considered. *o,o*-FeEDDHA facilitated Fe uptake increased both the Fe content and the dry weight yield of the soybean plants. Contrary to *racemic o,o*-FeEDDHA, the residence time of *meso o,o*-FeEDDHA in soil solution was substantially compromised by plant-independent processes. Due to its longer residence time, *racemic o,o*-FeEDDHA is likely to remain effective for a longer time-span than *meso o,o*-FeEDDHA. The effectiveness of *rest*-FeEDDHA has not been separately assessed in the pot trials. In the study examining the effect of FeEDDHA treatment composition, it was concluded that *o,o*-FeEDDHA governed Fe uptake; the contribution of *rest*-FeEDDHA was marginal, at most.

The findings from the presented pot trial studies may serve applicers of FeEDDHA fertilizer to make a better selection out of the available products and help them to optimize the dosage and frequency of application. Furthermore, they may provide producers of

FeEDDHA fertilizers with leads for optimizing the compositions of their formulations and for effectively marketing their products.

Although the processes examined in these pot trials also take place in a field situation, a translation of the results to a field situation should be treated with caution, because plant care and growth conditions differ strongly between the field and a conditioned greenhouse, not all processes affecting FeEDDHA concentration in the field have been considered, and the relative impact of the individual process may well be different in the field than in a controlled environment.

The presented studies pursued insights on a level, transcending an individual soil or crop. Still, for practical reasons, only one plant species (soybean) and one soil (Santomera) have been used. This inevitably holds a risk of over-representation of soil-, species- or even cultivar specific peculiarities. Challenges for future research would therefore include carrying out comparative studies with different soils and crops, and conducting field trials to examine how the results from the pot trials relate to agricultural practice.

Another focal point for further research concerns the fate of FeEDDHA components in the soil-plant system. The results from the pot trial studies show that for most of the FeEDDHA components, the fate is determined by plant-independent processes. A better understanding of the soil processes affecting the effectiveness of FeEDDHA components, or in a more general sense, of Fe chelates applied as fertilizer, would enable a more efficient and soil specific application of Fe fertilizer products. Processes to examine more closely than reported so far would for instance include biodegradation, adsorption, cation competition and leaching.

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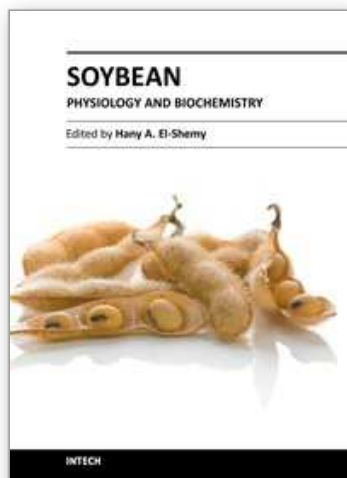
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