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REGULAR ARTICLE

FeEDDHA-facilitated Fe uptake in relation to the behaviour of FeEDDHA components in the soil-plant system as a function of time and dosage

Walter D. C. Schenkeveld · Erwin J. M. Temminghoff · Arjen M. Reichwein · Willem H. van Riemsdijk

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Abstract FeEDDHA products are widely used to prevent and remedy Fe chlorosis in crops grown on calcareous soils. These products consist of a mixture of FeEDDHA components: racemic o,o-FeEDDHA, meso o,o-FeEDDHA, o,p-FeEDDHA and rest-FeEDDHA. The FeEDDHA components differ in physical and chemical properties, and as a consequence also in effectiveness as Fe fertilizer. In order to efficiently match dose, frequency and moment of FeEDDHA application with the Fe requirements of plants, it is important to understand the behaviour of the FeEDDHA components in the soil-plant system as a function of time and dosage, and to relate this behaviour to Fe uptake by plants. These issues have been examined in a pot trial study with soybean plants (*Glycine max* (L.) Merr. cv Mycogen 5072) grown on calcareous soil from Santomera, Spain. Four FeEDDHA treatments (two compositions, two dosages) were applied prior to the set in of chlorosis. Leaching of FeEDDHA components was prevented. Plant and soil were sampled every week, for six weeks. From one week onward the Fe concentration in the pore water was largely gouverned by racemic and meso o,o-FeEDDHA. The concentration behaviour of the o,o-FeEDDHA isomers underwent two stages: a strong decline within the first week resulting from linear adsorption, and a gradual decline from one week onward. For meso o,o-FeEDDHA, unlike racemic o,o-FeDDHA, the gradual decline could be mathematically well described with an exponential decay function. Soybean plants mainly took up Fe in the progressed vegetative stage (3rd and 4th week) and in the reproductive stage, when the pods were being filled with seeds (6th week). Fe uptake and removal of racemic o,o-FeEDDHA from the soil system display a similar time-trend, whereas the removal of meso o,o-FeEDDHA had a plant-independent character. This indicates the removal of racemic o,o-FeEDDHA is to a larger extent plant-related.

Keywords EDDHA isomers · FeEDDHA · Iron chelates · Iron chlorosis · Iron uptake · Iron nutrition

Abbreviations

o,o-FeEDDHA	iron (3+) ethylene diamine-N,N'-bis(2-hydroxy phenyl acetic acid) complex
o,p-FeEDDHA	iron (3+) ethylene diamine-N-(2-hydroxy phenyl acetic acid)-N'-(4-hydroxy phenyl acetic acid) complex

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W. D. C. Schenkeveld · E. J. M. Temminghoff (✉) · W. H. van Riemsdijk
Department of Soil Quality, Wageningen University, P.O. Box 47, 6700 AA Wageningen, The Netherlands
e-mail: Erwin.temminghoff@wur.nl

A. M. Reichwein
Department CFC-CMS, AkzoNobel Chemicals BV, P.O. Box 9300, 6800 SB Arnhem, The Netherlands

DOC	Dissolved organic carbon
DTPA	Diethylene triamine penta acetic acid
ICP-MS/AES	Inductively coupled plasma mass spectroscopy/atomic emission spectroscopy
SOC	Soil organic carbon
SSR	Soil-solution ratio

Introduction

Fe deficiency chlorosis is a nutritional disorder characterized by a significant decrease of chlorophyll in the leaves, often observed in plants grown on alkaline and calcareous soils. It decreases crop yield both quantitatively and qualitatively, resulting in economic losses (Chaney 1984; Mortvedt 1991). Elevated bicarbonate concentrations and high pH have been identified as the main soil conditions explaining the incidence of Fe chlorosis (Boxma 1972; Mengel et al. 1984; Shi et al. 1993). Under such conditions, the solubility of Fe(hydr)oxides is low (Lindsay 1979) and Fe uptake mechanisms become impaired (Marschner 1995; Venkatraju and Marschner 1981) or Fe becomes inactivated inside the leaf's apoplast (Mengel 1994).

The application of synthetic Fe chelates is the most common practice to mend or to prevent Fe chlorosis. These chelates increase the solubility of Fe and function as a transporter through solution to the plant. FeEDDHA (iron ethylene diamine-N,N'-bis(hydroxy phenyl)acetic acid) is among the most effective synthetic Fe chelates under neutral and alkaline soil conditions (Lucena et al. 1992; Reed et al. 1988; Wallace et al. 1955). Commercial FeEDDHA formulations consist of a mixture of positional isomers, diastereomers and polycondensates. Such mixtures can be divided into 4 groups of FeEDDHA components: 1) racemic o,o-FeEDDHA, 2) meso o,o-FeEDDHA, 3) o,p-FeEDDHA, and 4) rest-FeEDDHA (largely consisting of polycondensates). The physical and chemical properties of these FeEDDHA components differ strongly (Ahrland et al. 1990; Bannochie and Martell 1989; Frost et al. 1958; Gomez-Gallego et al. 2005, 2006; Yunta et al. 2003a, b), and as a consequence, so does their ability to preserve Fe in solution and deliver it to the plant.

The effectiveness of the individual FeEDDHA components is determined by the following characteristics: 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 chelate Fe from the soil (Lucena 2003).

The first two characteristics have been addressed in a number of interaction studies with soil (Alvarez-Fernandez et al. 1997, 2002; Garcia-Marco et al. 2006; Hernandez-Apaolaza et al. 2006; Schenkeveld et al. 2007). These studies show that racemic o,o-FeEDDHA is superior in maintaining Fe in solution, followed by meso o,o-FeEDDHA, while rest-FeEDDHA and particularly o,p-FeEDDHA are largely removed from solution.

With respect to Fe transfer to the plant, studies with hydroponic systems show that in such systems o,p-FeEDDHA is more effective in mending Fe chlorosis in soybean than o,o-FeEDDHA (Garcia-Marco et al. 2006) and that both are more effective than synthesis by-products (referred to as rest-FeEDDHA in this study) (Hernandez-Apaolaza et al. 2006). Furthermore, meso o,o-FeEDDHA has been claimed to be more effective than racemic o,o-FeEDDHA (Cerdan et al. 2006).

Still little is known about the performance of FeEDDHA components in the soil-plant system. Recently, Schenkeveld et al. (2008) reported a pot trial study with soybean and FeEDDHA administration prior to the set in of chlorosis. The amount of o,o-FeEDDHA (i.e. the sum of racemic an meso o,o-FeEDDHA) largely determined the effectiveness of the treatment in terms of Fe uptake. The superiority of o,o-FeEDDHA over o,p-FeEDDHA in delivering Fe to soil-grown crops was confirmed by Rojas et al. (2008). Furthermore, Schenkeveld et al.(2008) observed that the concentration of the o,o-FeEDDHA isomers decreased considerably throughout the experiment, and that Fe uptake by the plants could not account for the loss of o,o-FeEDDHA.

Thus far, Fe dynamics in the soil-plant system with FeEDDHA addition have not yet been examined. Both FeEDDHA component concentrations in the pore water and Fe requirements of the plant vary over time. An understanding of these time dependencies is essential to determine dose, frequency and moment of FeEDDHA application to soil grown crops, for optimizing yield and crop quality, while minimizing the use of FeEDDHA.

The aim of this study was 1) to examine the pore water concentration of FeEDDHA components in a soil-plant system as a function of time and dosage, and 2) to relate the observed concentration behaviour to FeEDDHA facilitated Fe uptake by plants.

For this purpose a pot trial study was set up, with soybean grown on a calcareous soil from Spain. Four FeEDDHA treatments (two compositions, two Fe dosages) were administered prior to the set in of chlorosis. Leaching of FeEDDHA components from the root-zone, occurring under field conditions as a result of excessive irrigation or atmospheric precipitation (Rombola and Tagliavini 2006), was prevented. Harvesting was done destructively on a weekly basis.

Material and methods

Soil

Calcareous soil was collected from the top soil layer (0–20 cm) at a site located in Santomera (Murcia, Spain). Relevant soil characteristics are presented in Table 1. Santomera soil is a clay soil with a lutum fraction of 260 g kg^{-1} and a $CaCO_3$ content of 520 g kg^{-1}, common for calcareous soils from that area. The pH of the soil is 8.0 (pH $CaCl_2$). The soil organic carbon (SOC) content is low (0.5%), and the dissolved organic carbon (DOC) concentration equals 30 mg l^{-1} ($CaCl_2$). Fe availability parameters are low: the oxalate extractable ('reactive') Fe content amounts 0.05 g kg^{-1} Fe, and the diethylene triamine penta acetic acid (DTPA) extractable content amounts 3.5 mg kg^{-1} Fe. Plants grown on Santomera soil became chlorotic, both under field conditions and in a previous pot trial (Schenkeveld et al. 2008). Pretreatment consisted of air drying and sieving (1 cm).

FeEDDHA solutions

FeEDDHA solutions were prepared from a sodium-EDDHA stock solution and solid o,o-H_4EDDHA[1] (99% pure). The sodium-EDDHA stock solution was synthesized through a Mannich-like reaction (patent - Petree et al. 1978). Prior to Fe addition, the solid o,o-H_4EDDHA was dissolved by adding sufficient 1 M NaOH. Fe was added as $FeCl_3$*$6H_2O$ in a 5% excess based on a 1:1 stoichiometry between Fe and ethylene diamine.[2] The pH was raised to 7 (±0.5) and the solutions were left over-night in the dark to allow excess Fe to precipitate as hydroxides. The following day, the solutions were filtered over a 0.45 μm nitro cellulose micro pore filter (Schleicher & Schuell, ref-no: 10401114) and further diluted for application in the pot trial. The composition of the FeEDDHA solutions was analysed through combined ICP and HPLC analysis at t=0.

Pot trial

A pot trial with a runtime of six weeks was done from late August until mid October 2005. The main experiment consisted of five treatments with plants: a blank and four FeEDDHA treatments. The FeEDDHA treatments varied in amount of chelated Fe and in FeEDDHA component composition (Table 2). In this way the effect of dosage of the FeEDDHA components on their concentration behaviour in soil solution, and on Fe uptake by plants could be examined. Labels of the treatments indicate the combined percentage of Fe chelated by racemic and meso o,o-EDDHA (30 or 100%), and the Fe dose applied (L or H). The L(ow) dose corresponds to ≈ 4 mg l^{-1} Fe (0.07 mM) and the H(igh) dose to ≈ 40 mg l^{-1} Fe (0.7 mM) in the pore water at t=0 (see Table 2). FeEDDHA was applied once, at the start of the trail. The blank and L-treatments were harvested (destructively) every week, the H-treatments every second week. The L-treatments were harvested more frequently, because at 4 mg l^{-1} Fe, the composition of the FeEDDHA treatment was certain to affect Fe uptake; at 40 mg/l Fe this was questionable (Schenkeveld et al. 2008). The experiment was carried out in triplicates, comprising 72 pots in total.

To examine the influence of plants on the concentration of the FeEDDHA components in soil solution, a second experiment was carried out with the blank and the L-treatments, both with and without plants. Harvest was after 6 weeks only. This experiment was done in duplicates.

The pot experiment was executed in a greenhouse with 7 L Mitscherlich pots. Pots, bottom plates and related materials were cleaned with 0.01 M HCl prior

[1] These chemicals were kindly provided by AkzoNobel.

[2] Refers to the ethylene diamine incorporated in the synthesized EDDHA-components.

Table 1 Soil characteristics

		Extraction		
Origin/Name	Santomera	$CaCl_2$[g]	DOC (mg l^{-1})	30
Region	Murcia	Oxalate[h]	Reactive Fe (g kg^{-1})	0.05
Country	Spain	DTPA[i]	Fe (mg kg^{-1})	3.5
Soil classification	entisol		Mn (mg kg^{-1})	4.57
Water holding capacity (g kg^{-1})	319		Cu (mg kg^{-1})	4.13
pH-$CaCl_2$[a]	8.0		Zn (mg kg^{-1})	0.90
Electro conductivity (mS m^{-1})[b]	23	HNO_3 (0.43 M)[j]	Fe (mg kg^{-1})	494
SOC (g kg^{-1})[c]	5.4		Mn (mg kg^{-1})	179
Clay content (g kg^{-1})[d]	260		Cu (mg kg^{-1})	10
$CaCO_3$ (g kg^{-1})[e]	520		Zn (mg kg^{-1})	5
CEC (cmol kg^{-1})[f]	10.3			

[a] ISO/DIS 10390 Soil Quality–Determination of pH

[b] ISO/DIS 11265 Soil Quality–Determination of the specific electric conductivity

[c] Walinga et al. (1992)

[d] Houba et al. (1997)

[e] ISO 10693, Soil Quality–Determination of carbonate content, volumetric method

[f] ISO/DIS 11260 Soil Quality–Determination of cation exchange capacity and base saturation–method using barium chloride solution

[g] Houba et al. (2000)

[h] Schwertmann (1964)

[i] Lindsay and Norvell (1978) and Quevauviller et al. (1996)

[j] Tipping et al. (2003). Fest et al. (2005)

to usage. The inside of the pots were covered with polyethylene sacks with tiny holes allowing for aeration. The pots contained six kg of soil, thoroughly and successively mixed with a number of nutrient solutions. Per pot 40 mmol NH_4NO_3, 25 mmol K_2HPO_4, 20 mmol $CaCl_2$, 10 mmol $MgSO_4$, 0.5 mmol H_3BO_3 and 3.75 μmol $(NH_4)_6Mo_7O_{24}$ were added. Additionally, the L- and H-treatments received respectively 0.069 and 0.69 mmol pot^{-1} Fe as FeEDDHA solution. Based on previous trials with Santomera soil, no micronutrient deficiencies other than Fe deficiency were expected in the blank. Therefore Cu, Mn and Zn fertilization was omitted. The moisture content was made up to 50% of the water holding capacity with demineralized water.

Seeds of the Fe chlorosis susceptible soybean (*Glycine max* (L.) Merr.) cultivar Mycogen 5072[3] 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. The pots from the main experiment were rotated on a daily basis. Every day the pots received an amount of demineralized water equal to the weight loss due to evapo-transpiration. At later growth stages the plant weight was compensated for in this respect. Evapo-transpiration did not exceed 260 ml pot^{-1} day^{-1} (i.e. 27% of the soil water content). The temperature in the greenhouse was kept above 20°C. The time-span between sunrise and sunset decreased from 14 to 11 h, approximately. No supplementary light was provided to assist plant-growth.

Sampling and measurement

SPAD-measurement

SPAD-measurements were done three times per week with a Minolta-502 SPAD-meter to compare the chlorophyll content of leaves among treatments.

[3] Soybean seeds were kindly provided by dr. R. J. Goos from the Department of Soil Science of the North Dakota State University.

Table 2 Composition of the FeEDDHA treatments expressed as pore water concentrations at t=0

	Racemic o,o-FeEDDHA (mg l^{-1} Fe)	Meso o,o-FeEDDHA (mg l^{-1} Fe)	o,p-FeEDDHA (mg l^{-1} Fe)	rest-FeEDDHA (mg l^{-1} Fe)	Totaal Fe (mg l^{-1} Fe)
Blank	0	0	0	0	0
30%o,oL	0.60 (14%)	0.68 (16%)	0.79 (19%)	2.18 (51%)	4.25
100%o,oL	1.93 (48%)	2.00 (50%)	0	0.05 (1%)	3.98
30%o,oH	5.97 (14%)	6.75 (16%)	7.94 (19%)	21.8 (51%)	42.5
100%o,oH	19.3 (48%)	20.0 (50%)	0	0.55 (1%)	39.8

Measurements started 6 days after the transfer of the seedlings to the pots, when the leaves had grown sufficiently large. Per pot, SPAD-indices were measured for two youngest leaves and two leaves from the second youngest trifoliate of every second plant. Measurement was done at the middle section of the leaf, midway between the central vein and the leaf edge. If a leaflet was necrotic or too small to analyze, no value was recorded. SPAD-indices for the youngest and second youngest trifoliate were averaged separately per pot. The plants harvested after 6 weeks were monitored throughout the experiment. SPAD-measurements on plants harvested earlier, started respectively one and two weeks before harvest for the L- and H-treatments. To ascertain representitativeness, the SPAD-values of plants harvested after 6 weeks were compared, per treatment, to the SPAD-values of plants harvested earlier. Chlorosis was operationally established as a significant difference (α=0.05) in SPAD-indices of the youngest leaves between the blank and the treatment with the highest SPAD-indices. The size of the difference in SPAD-value has been interpreted as a measure for the severity of chlorosis.

Harvest

At each harvesting round, 16 of the youngest trifoliate leaves were separately collected per pot, with exception of the first harvesting round when plants were still too small. The (remaining) shoots were cut off right above the soil surface. Prior to harvest, the moisture content of the soil had been restored to 50% of the water holding capacity. After a few hours equilibration time, a 1 kg mixed subsample was taken from the soil, from which roots were collected manually. The soil subsample was stored overnight at 4°C. The remaining roots were collected by rinsing out the soil over a 1 mm sieve. Plant parts (youngest leaves, shoot and roots) were washed with demineralized water and dried at 70°C. After 48 h, the plant parts were weighed (dry weight).

Mineral analysis of plant tissue

The mineral contents of the plant parts were determined through microwave digestion with nitric acid, fluoric acid and hydrogen peroxide (Novozamsky et al. 1996). Al, Co, Cu, Fe, Mn, Ni and Zn concentrations were measured by ICP-AES (Varian, Vista Pro).

Pore water collection and analysis

A fraction of the pore water was collected from the soil subsamples by centrifugation at $7443g$ (7,000 rpm) for 15 min (Sorvall RC 5C plus) in Delrin (polyacetal) cylindrical 2 compartment containers the day after harvest. The centrifugate was led from the soil containing compartment (approximately 150 cm^3) over a 0.45 μm nitro cellulose micro pore filter (Schleicher & Schuell, ref-no: 10401114) into a soil solution collection compartment (approximately 40 cm^3). Per unit soil, the amount of water collected did not exceed evapotranspiration; therefore the collected pore water was assumed available to plants.

pH was measured directly after pore water 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-measurement. FeEDDHA component concentrations were determined after separation through high-performance liquid chromatography (HPLC) as described by Schenkeveld et al. (2007). The Fe concentration chelated by rest-EDDHA was

calculated by subtracting the Fe concentrations related to the other FeEDDHA components and the Fe concentration in the blank treatment from the total Fe concentration measured by ICP-AES. To avoid contamination, the preparation of the experimental solutions and dilution of samples for measurement were done with analytical grade chemicals and ultra pure water.

Statistical analysis

Statistical analyses were performed with the program SPSS 12.0. Differences between variables were determined by applying the one-way ANOVA procedure with a Tukey post-hoc test (α=0.05). The growth rates of the shoot were compared by analysis of the slopes of linear regression lines (α=0.05).

Results

Fe and FeEDDHA component concentrations in soil solution

The Fe concentration in soil solution of the blank treatment was below detection limit throughout the experiment (data not shown). This implies that the Fe concentrations in the treatments with FeEDDHA addition entirely resulted from FeEDDHA components. In Fig. 1, the Fe and FeEDDHA component concentrations are presented as a function of time for the 30%o,oL treatment. During the first week, o,p-FeEDDHA and rest-FeEDDHA were removed from solution practically entirely, resulting in a drop in Fe concentration from 4.25 mg l^{-1} Fe at t=0 to 0.81 mg l^{-1} Fe after 1 week (Fig. 1a). From week 1 onward, the Fe concentration was largely determined by the sum of the racemic and the meso o,o-FeEDDHA concentration (>92%) (Fig. 1b).

The concentration behaviour of the o,o-FeEDDHA isomers can be subdivided into two stages: a rapid decline within the first week, and a gradual decline from one week onward. Within the first week, the meso o,o-FeEDDHA concentration decreased more strongly (≈ 54%) than the racemic o,o-FeEDDHA concentration (≈ 28%). From one week onward, the decline in meso o,o-FeEDDHA concentration remained faster, resulting in an increasing relative contribution of racemic o,o-FeEDDHA to the total Fe concentration in soil solution.

Adsorption is proposed as the process causing the strong decline in concentration within the first week; FeEDDHA components are known to adsorb to soil reactive surfaces (Hernandez-Apaolaza and Lucena 2001; Schenkeveld et al. 2007), and adsorption (pseudo)equilibrium of ionic compounds interacting

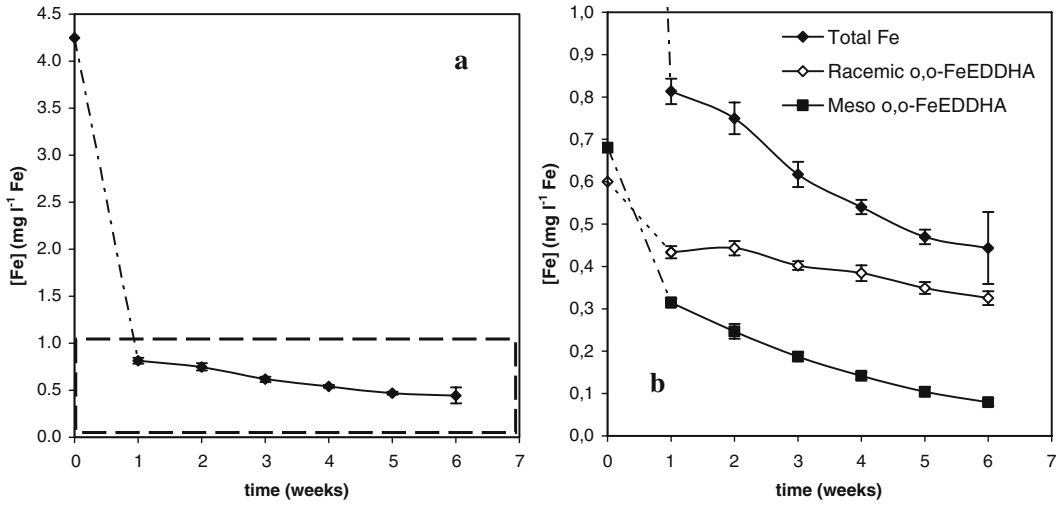

Fig. 1 **a** Fe concentration in the pore water of Santomera soil as a function of time for the 30%o,oL treatment. Error bars indicate standard deviations. **b** Enlargement of the indicated area from Fig. 1a; Total Fe, racemic o,o-FeEDDHA and meso o,o-FeEDDHA concentrations in the pore water of Santomera soil as a function of time for the 30%o,oL treatment. Error bars indicate standard deviations

with soil is generally reached within hours to days rather than weeks. Moreover, the larger decline in concentration of meso o,o-FeEDDHA compared to racemic o,o-FeEDDHA corresponds with the stronger tendency of meso o,o-FeEDDHA to adsorb (Alvarez-Fernandez et al. 1997, 2002; Hernandez-Apaolaza and Lucena 2001; Schenkeveld et al. 2007).

Potential causes for the gradual decline in concentration of racemic and meso o,o-FeEDDHA after the first week are discussed further on in relation to Fe uptake by plants.

In both L-treatments, the gradual decline in meso o,o-FeEDDHA concentration was continuous from one week onward, while for racemic o,o-FeEDDHA there was no decline during the second week, and the gradual decline only set in after two weeks. In Fig. 2a, the meso o,o-FeEDDHA concentration data are presented on a logarithmic scale as a function of time for all FeEDDHA treatments. The gradual decline in meso o,o-FeEDDHA concentration can be accurately described with an exponential decay function[4]:

$$C_t = A_0 \cdot e^{-\lambda t} \quad (1)$$

in which C_t is the meso o,o-FeEDDHA concentration in the pore water at t = t; A_0 is a fitted meso o,o-FeEDDHA concentration at t=0, disregarding the concentration drop caused by adsorption and the moment the gradual decline set in; λ is the decay constant; and t is the time passed since FeEDDHA application. For all treatments R^2 of the exponential fit was 0.99 or higher.

A similar procedure was followed for the racemic o,o-FeEDDHA concentration data from 2 weeks onward (Fig. 2b). The data from the L-treatments could also be fitted reasonably well with an exponential equation (R^2=0.99); for the H-treatments, the fits were poor (R^2=0.83 and 0.23).

Under the assumption that adsorption equilibrium was reached before the impact of the process causing the gradual decline became substantial, the amounts of racemic and meso o,o-FeEDDHA adsorbed in the first stage (Q_0) can be determined through extrapolation of the concentration trends to t=0. Q_0 can then be calculated from:

$$Q_0 = \frac{\left(C_0 - C_0^*\right)}{SSR} \quad (2)$$

in which C_0 is the initial pore water concentration applied with the treatment; C_0^* is the pore water concentration at adsorption equilibrium, determined through extrapolation to t=0; and SSR is the soil-solution ratio. For meso o,o-FeEDDHA, the intercepts (C_0^*) were determined through extrapolation of the exponential fits (as indicated by the dashed lines in Fig. 2a), and equal A_0 (Eq. (1)). For racemic o,o-FeEDDHA, C_0^* was assumed to equal the concentration after 1 and 2 weeks, since the gradual decline only set in after 2 weeks (indicated in Fig. 2b). C_0 and C_0^* are indicated on the Y-axis of Fig. 2a and b for all treatments. For meso o,o-FeEDDHA, C_0 is consequently a factor 1.6–1.9 higher than C_0^*; for racemic o,o-FeEDDHA consequently a factor 1.3–1.4.

With the extrapolated pore water concentrations at adsorption equilibrium (C_0^*) and the corresponding adsorbed amounts (Q_0) at t ≈ 0, adsorption isotherms for racemic and meso o,o-FeEDDHA to Santomera soil were derived (Fig. 3a). The adsorption isotherms are linear in shape and can be described with:

$$Q = K \cdot C \quad (3)$$

in which Q is the adsorbed amount by the soil, K is an effective affinity parameter of Santomera soil for the o,o-FeEDDHA isomers, and C is the equilibrium concentration in soil solution. The slope of the meso o,o-FeEDDHA isotherm (0.139) is a factor 2.5 steeper than the slope of the racemic o,o-FeEDDHA isotherm (0.055). Because the isotherms only comprise 4 data points, a double logarithmic transformation of the data was carried out to check for over-representation of the data point with the highest concentration (see SI-Figure 1 of Supporting Information). Also the transformed data could be fitted linearly with R^2=1.00 and tangents of 1.10 and 0.95 for meso and racemic o,o-FeEDDHA respectively, implying the assumption of linear adsorption isotherms is reasonable.

The decay constant in Eq. 1 increases with decreasing meso o,o-FeEDDHA concentration applied with the treatment (Fig. 3b). This dependency is remarkable because it implies that neither the pore water concentration, nor the linearly dependent amount adsorbed

[4] The terms "exponential decay" and "decay constant" are used in a mathematical sense as opposed to "exponential growth" and "growth constant"; as such these terms do not address the cause for the decline and bear no direct reference to "decomposition" or "biodegradation".

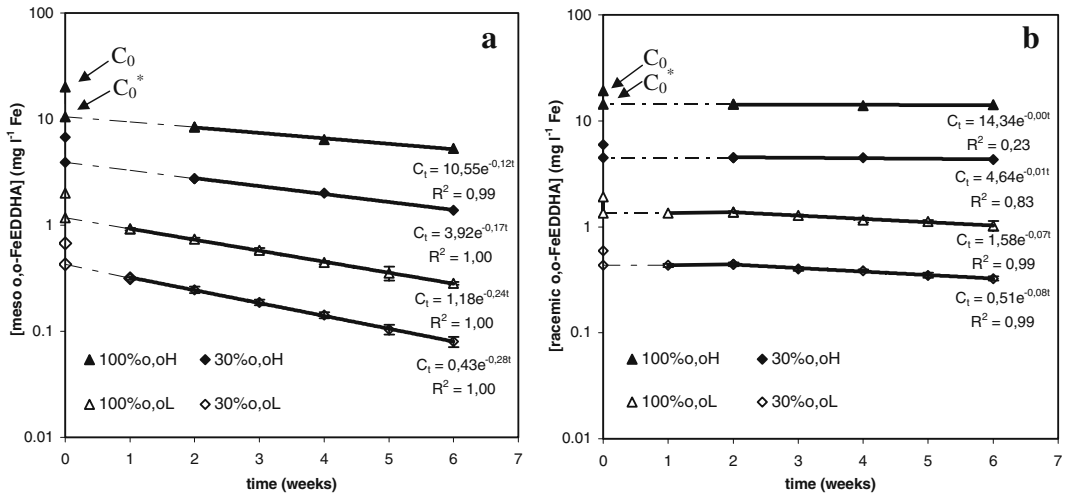

Fig. 2 Concentration of **a** meso and **b** racemic o,o-FeEDDHA in the pore water of Santomera soil as a function of time for all FeEDDHA treatments. The concentrations are presented on a logarithmic scale. Error bars indicate standard deviations. The interrupted lines represent trend extrapolations in between the start of the experiment and the first moment of harvest. C_0 indicates the concentration applied with the treatment, C_0^* indicates the extrapolated concentration corresponding to adsorption equilibrium at $t \approx 0$

determines the rate of decline, like in standard first order reaction kinetics, at which the exponential fits hint (Fig. 2a). The relation between decay constant and concentration applied can be accurately described (R^2= 0.99) with the following equation:

$$\lambda = b + c \cdot \ln C_0 \qquad (4)$$

in which b and c are fitting constants.

With the linear adsorption isotherm (Eq. (3)) and the relation between the decay constant and the applied concentration (Eq. (4)), the parameters needed to describe the exponential decrease in meso o,o-FeEDDHA concentration in the Santomera soil as a function of time from one week onward (Eq. (1)), can be calculated for any applied meso o,o-FeEDDHA concentration in between 0.68 and 20 mg l^{-1} Fe.

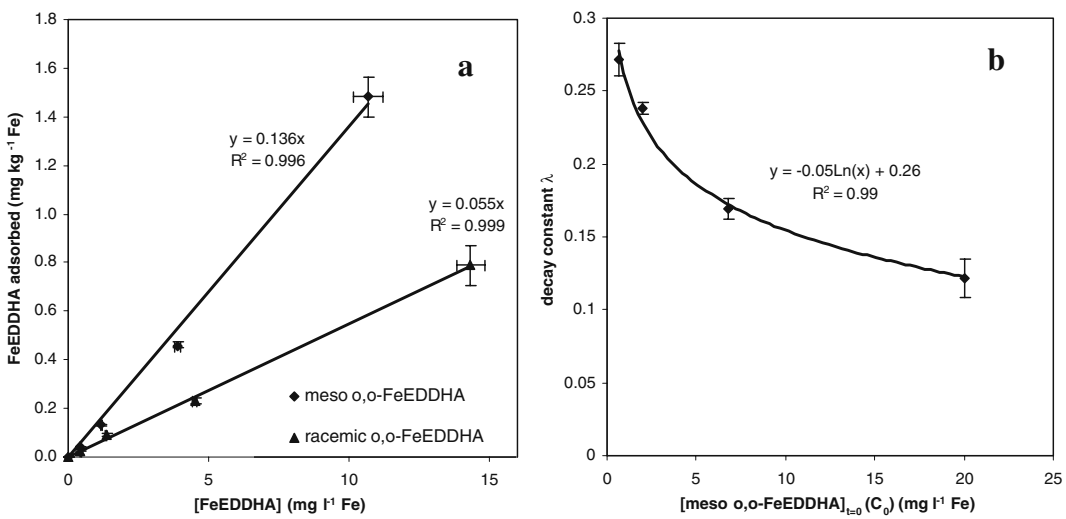

Fig. 3 **a** Derived adsorption isotherms for racemic and meso o,o-FeEDDHA to Santomera soil. Error bars indicate standard deviations. **b** The decay constant (from Eq. (1)), as a function of the meso o,o-FeEDDHA concentration applied with the treatment. Error bars indicate standard deviations

The results from the second experiment show that, after 6 weeks, the racemic as well as the meso o,o-FeEDDHA concentration are significantly higher in the treatment without plants than in the corresponding treatment with plants (Table 3). However, in agreement with the results from Schenkeveld et al. (2008), the effect of plants on the concentration is relatively small. Furthermore, the concentration profiles as a function of time were similar in shape in systems with and without plants (see Fig. 1, and SI-Figure 2 of Supporting Information), in particular for meso o,o-FeEDDHA. Hence, non-plant related processes, made a large contribution to the gradual decline in concentration of the o,o-FeEDDHA isomers. The amount of racemic and meso o,o-FeEDDHA removed from soil solution as a result of Fe uptake cannot be calculated from the difference in concentration between the treatments with and without plants, because the kinetics of Fe uptake and other processes affecting the o,o-FeEDDHA isomer concentrations are mutually affected; they have at least one reactant (the o,o-FeEDDHA isomer) and possibly also reaction products in common.

Chlorosis and SPAD-indices

SPAD-indices of the youngest leaves of plants harvested after 6 weeks are presented as a function of time in Fig. 4a for the blank, the 30%o,oL and the 30%o,oH treatment. The general trend shows a strong decline in SPAD-index after 8 days and a more or less gradual increase from 2 weeks onward. The plants of the blank treatment became chlorotic after 8 days and remained chlorotic until the end of the experiment. It required 17 days before the SPAD-indices of the youngest leaves of the 30%o,oL treatment differed from those of the blank treatment (α=0.05). In Fig. 4b the differences in SPAD-indices relative to the blank are presented for all FeEDDHA treatments as a function of time. Because this figure merely serves to illustrate the trends, error bars have been omitted. Chlorosis was most severe after 3 weeks (up to a difference of 11 SPAD-units), corresponding with observations from a previous pot trial (Schenkeveld et al. 2008). Initially, the youngest leaves of the H-treatments had higher SPAD-indices than those of the L-treatments, while the SPAD-indices of the L-treatments hardly differed from those of the blank. After 2 weeks, the difference in SPAD-indices between the blank and the L-treatments rapidly increased. SPAD-indices of the 100%o,oL-treatment reached the same level as those of the H-treatments after about 3 weeks and remained similar until the end of the experiment. So, after 3 weeks no additional cosmetic effects were obtained from increasing the o,o-FeEDDHA dose beyond the level of the 100%o,oL treatment. The SPAD-indices of 30%o,oL treatment remained lower than those of the H-treatments, but higher than those of the blank.

Fe content

The influence of FeEDDHA treatments on the Fe content was examined separately for the youngest leaves and the shoot (see Fig. 5). The Fe content of the roots was not further considered, due to its overestimation resulting from contamination of the roots with soil material - as confirmed by the linear relation between the Fe and Al content of the roots (R^2=0.93).

The Fe content of the youngest leaves (53–143 mg kg^{-1} Fe) was at all times higher than the corresponding Fe content of the shoot (40–114 mg kg^{-1} Fe) and in 75% of the cases significantly

Table 3 Comparison of FeEDDHA component concentrations after 6 weeks for the 30%o,oL and 100%o,oL treatment with and without plants. Standard deviations are indicated between parentheses

Treatment	Plants	Racemic o,o-FeEDDHA (mg l^{-1} Fe)	Meso o,o-FeEDDHA (mg l^{-1} Fe)	o,p-FeEDDHA (mg l^{-1} Fe)	Rest-FeEDDHA (mg l^{-1} Fe)
30%o,oL	–	0.35 (0.01)	0.07 (0.00)	b.d.*	b.d.
	+	0.30 (0.00)	0.05 (0.00)	b.d.	b.d.
100%o,oL	–	1.18 (0.00)	0.25 (0.01)	b.d.	b.d.
	+	0.97 (0.00)	0.17 (0.02)	b.d.	b.d.

* *b.d.* below determination limit

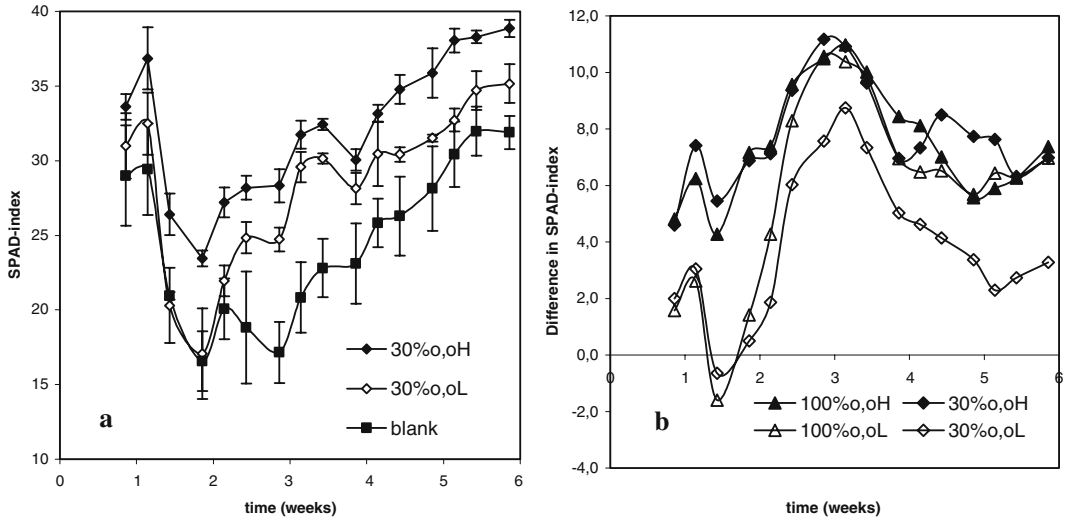

Fig. 4 **a** SPAD-indices of the youngest leaves of soybean plants grown on Santomera soil as a function of time for the blank, 30%o,oL and 30%o,oH treatments. Error bars indicate standard deviations **b** Difference in SPAD-index of the youngest leaves of soybean plants grown on Santomera soil between the blank treatment and the FeEDDHA treatments as a function of time. Error bars have been omitted

different (α=0.05). For the blank treatment, differences were only significant for 2 out of 5 sampling moments. No lasting significant increase in Fe content of the youngest leaves was observed, except in the 100%o,oH treatment (from 112 to 133 mg kg^{-1} Fe).

The Fe content of the shoot declined over time in both the blank and the 30%o,oL treatment. This decrease indicates a dilution effect resulting from a higher relative increase in biomass dry weight than in accumulated Fe in the shoot. In the 100%o,oL treatment the Fe content of the shoot was only significantly lower after 5 weeks, while in the H-treatments no differences were observed among sampling moments. So, a minimum amount of o,o-FeEDDHA needed to be applied to maintain the Fe content of the shoot.

Throughout the trial, the Fe content of the youngest leaves and the shoot of the blank were

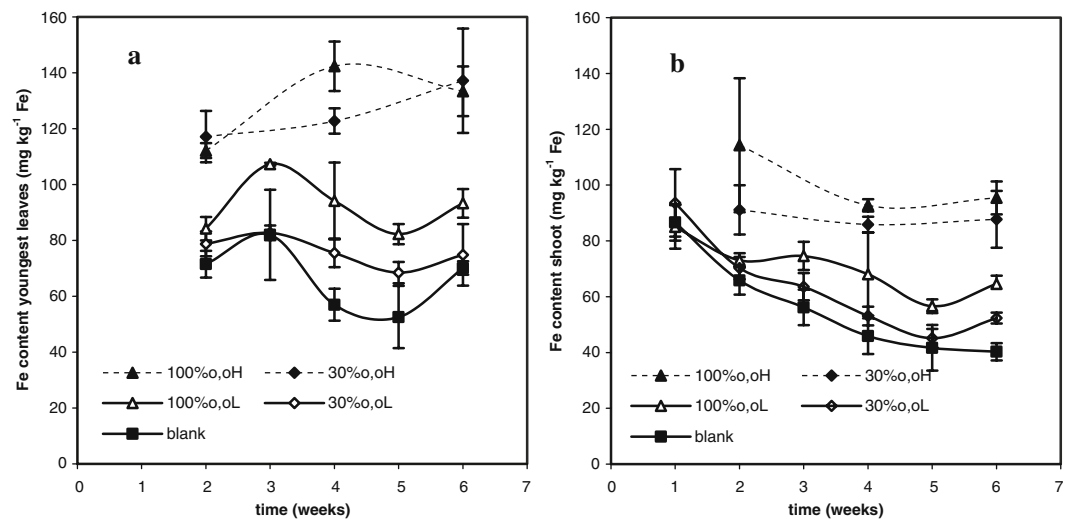

Fig. 5 **a** Fe content of the youngest leaves, and **b** Fe content of the shoot of soybean plants grown on Santomera soil as a function time for all treatments. Error bars indicate standard deviations

lower than in all other treatments. Compared to the 30%o,oL treatment the differences were at no stage significant, compared to the 100%o,oL treatment the differences were significant from 3 weeks onward with exception of the shoot after 4 weeks. The H-treatments had significantly higher Fe contents than both the blank and the 30%o,oL treatment throughout the trial, with exception of the shoot of the 30%o,oH treatment after 2 weeks. No significant differences in Fe content were found between the H-treatments, despite the substantially higher Fe concentration in the pore water of the 100%o,oH treatment; apparently a plateau had been reached.

Yield

The biomass of the shoot (including youngest leaves) increased linearly from 2 weeks onward for all treatments (R^2=1.00), as illustrated in Fig. 6a through the blank, the 30%o,oL and the 30%o,oH treatment. The total dry weight yield displayed a similar trend (data not shown). After 6 weeks the difference in shoot biomass between the blank (lowest yield) and the 30%o,oH treatment (highest yield) amounted 3.1 g; an increase of 27% due to FeEDDHA application. The growth rates (corresponding to the slopes of the regression lines in Fig. 6a) increased from 2.32 to 3.06 g (dw) pot^{-1} week^{-1} (an increase of 32%) and depended on the amount o,o-FeEDDHA applied with the treatment, as shown in Fig. 6b. With increasing o,o-FeEDDHA dosage, the growth rate initially increased strongly but the slope flattened. A plateau was reached and the growth rate corresponding to the 100%o,oH treatment, although not statistically different from that of that 30%o,oH treatment, may hint at the set-in of a decline in yield as a result of excessive FeEDDHA application. This is supported by the observation that, in particular at harvest after 6 weeks, parts of the roots of the 100%o,oH treatment were coloured black and seemed necrotic.

Fe uptake

Cumulative Fe uptake was calculated as the product of shoot dry weight yield and Fe content of the shoot (including youngest leaves). The Fe initially present in the soybean seeds (0.14±0.02 mg Fe per pot) was not corrected for. The combination of a linear time-trend for yield and a non linear one for Fe content results in a non linear cumulative Fe uptake function, as illustrated by the blank and the L-treatments in Fig. 7a. All cumulative Fe-uptake curves continuously increase throughout the experiment; implying that decreases in Fe content were always overcompensated by increases in yield.

From 2 weeks onward, cumulative Fe uptake increased with increasing o,o-FeEDDHA concentration applied with the treatment, as illustrated in

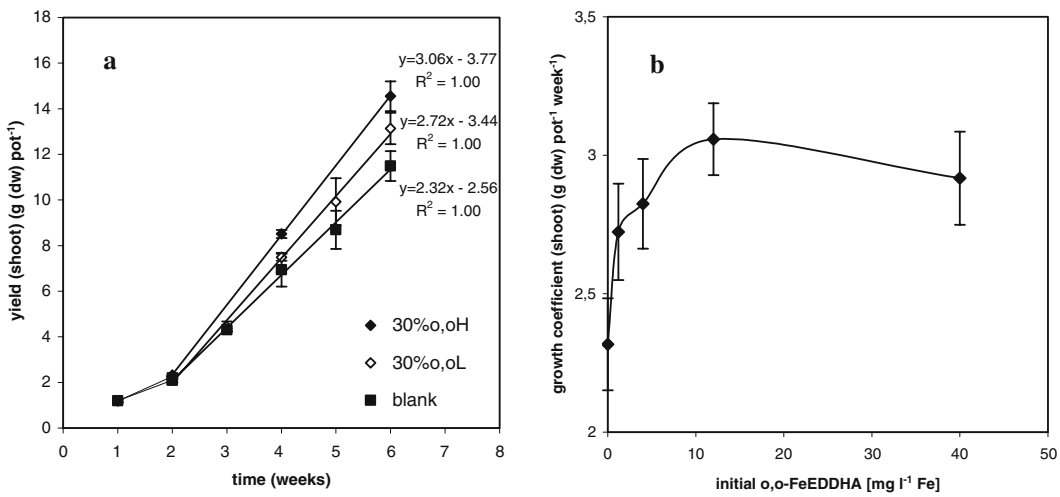

Fig. 6 **a** Shoot dry weight yield per pot of soybean plants grown on Santomera soil as a function of time for the blank, the 30%o,oL and the 30%o,oH treatment. Error bars indicate standard errors **b** Relation between the growth coefficient of the shoot of soybean plants grown on Santomera soil and the initial o,o-FeEDDHA concentration of the treatment. Error bars indicate standard errors

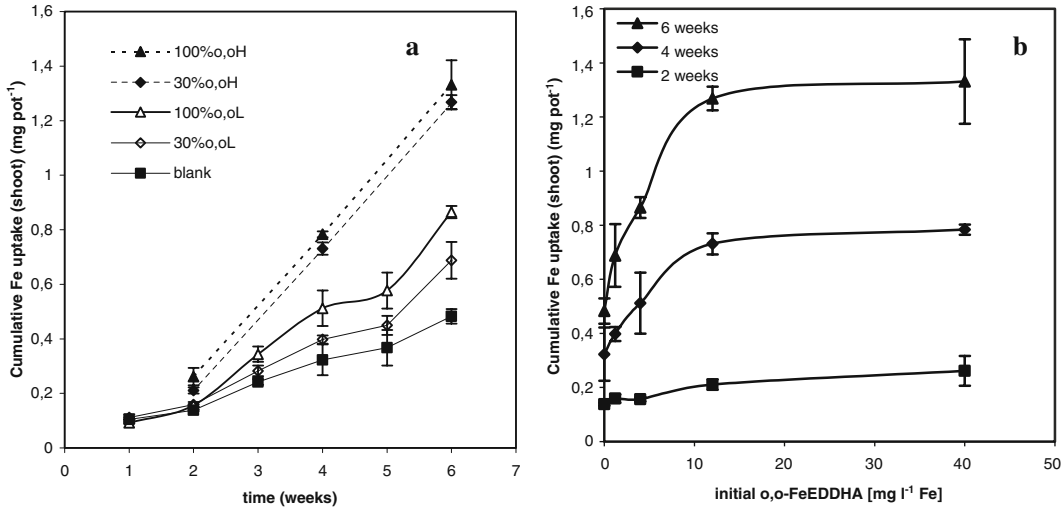

Fig. 7 **a** Cumulative Fe uptake (shoot) per pot as a function of time for all treatments. Error bars indicate standard errors. **b** Cumulative Fe uptake (shoot) per pot after 2, 4 and 6 weeks as a function of the initial o,o-FeEDDHA concentration. Error bars indicate standard deviations

Fig. 7b for 2, 4 and 6 weeks. After 2 weeks, cumulative Fe uptake ranged from 0.14 to 0.26 mg pot^{-1} Fe, after 4 weeks from 0.32 to 0.78 mg pot^{-1} Fe and after 6 weeks from 0.48 to 1.33 mg pot^{-1} Fe. The slope at the lower-end of the curve grows steeper with time, indicating FeEDDHA continues to enhance Fe uptake throughout the experiment. Cumulative Fe uptake does not differ significantly between the 30% o,oH and the 100%o,oH treatment at any sampling moment. This implies Fe uptake is maximized in the 30%o,oH treatment and application of additional o,o-FeEDDHA is superfluous.

The (additional) Fe uptake per week has been calculated as the difference in cumulative Fe uptake between two consecutive data points of the same treatment and is presented as a function of time in Fig. 8 for the blank and the L-treatments. The Fe uptake indicated at 2 weeks is in fact the Fe uptake during the 2nd week, and so on. Because the figure only serves to illustrate the trends, error bars have been omitted.

For all sampling moments the sequence in Fe uptake per week was identical: blank <30%o,oL <100%o,oL. Fe uptake per week displayed the same time trend in all three treatments. During the 2nd week Fe uptake was relatively low. The small Fe requirements at this stage are probably related to the small size of the plants during the early vegetative stage and the utilization of Fe present in the seeds. Fe uptake increased during the 3rd and the 4th week. As the vegetative stage progressed, the plants grew bigger and Fe from the seeds became increasingly insufficient, resulting in an increased Fe demand. In the blank treatment chlorosis was most

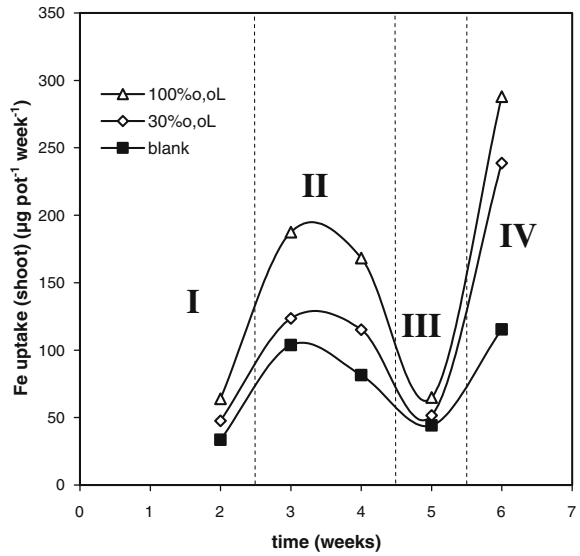

Fig. 8 Fe uptake (shoot) per pot, per week by soybean plants grown on Santomera soil as a function of time, for the blank, the 30%o,oL and the 100%o,oL treatment. Error bars have been omitted. I = Early vegetative stage (initial chlorosis); II = Progressed vegetative stage (maximum chlorosis); III = Transfer from vegetative to reproductive stage (flowering and pod formation); IV = Progressed reproductive stage (pod filling)

severe at this stage (Fig. 4b). In the course of the 4th and during the 5th week, the plants flowered and pods were formed, indicating the shift from the vegetative to the reproductive stage. The plants hardly grew in size anymore, leading to small Fe requirements during the 5th week. During the 6th week the seed formation inside the pods progressed and Fe uptake increased strongly again, in order to provide the seeds with sufficient Fe (Grusak 1995). The Fe requirements in the reproductive stage were larger than in any preceding week.

Fe uptake in relation to removal of o,o-FeEDDHA isomers from the soil system

Finally, the relation between FeEDDHA-facilitated Fe uptake to the shoot, and the amount of racemic and meso o,o-FeEDDHA removed from the soil system (solid and solution phase combined) was examined as a function of time. The removed amounts of racemic and meso o,o-FeEDDHA were calculated from the decrease in soil solution concentration under the assumption adsorption equilibrium was preserved and could be described by the derived adsorption isotherms (Fig. 3a). In Fig. 9a the amounts of racemic, meso and total o,o-FeEDDHA removed from the soil system per week, are presented for the 100%o,oL treatment. The amount of meso o,o-FeEDDHA removed per week was larger than the amount of racemic o,o-FeEDDHA, throughout the experiment. Racemic o,o-FeEDDHA however, appears to have a more pronounced influence on the shape of the total o,o-FeEDDHA removal-curve.

In Fig. 9b FeEDDHA-facilitated Fe uptake is presented as a function of time for the 100%o,oL treatment. Two uptake scenarios have been included: 1) a maximum scenario, in which all Fe uptake was assumed FeEDDHA-facilitated, and 2) a minimum scenario, in which only the difference in Fe uptake between the 100%o,oL and the blank treatment was assumed FeEDDHA-facilitated. In both scenarios, FeEDDHA-facilitated Fe uptake was highest in the growth stages that Fe requirements were largest (3rd, 4th and 6th week). The curve representing the amount of racemic o,o-FeEDDHA removed from the soil system per week shows a strong similarity in shape to the Fe uptake curves, and up until the 6th week, the amount of racemic o,o-FeEDDHA removed was in range with Fe uptake. This indicates that the removal of racemic o,o-FeEDDHA from the soil-system was to a larger extent plant-determined than the removal of meso o,o-FeEDDHA. 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.

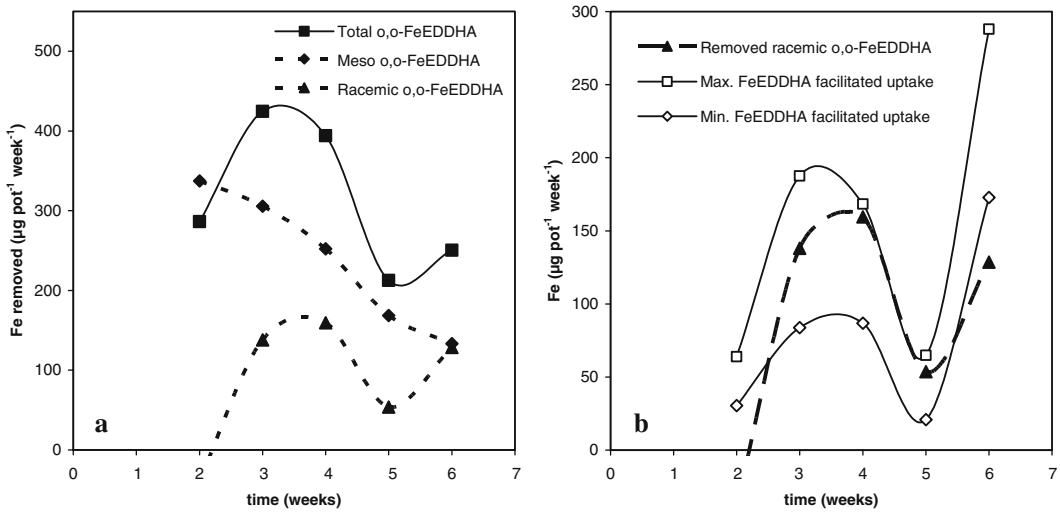

Fig. 9 **a** Amounts of total, racemic and meso o,o-FeEDDHA removed from the soil system per pot, per week for the 100%o,oL treatment. Error bars have been omitted. **b** Minimum and maximum FeEDDHA-facilitated Fe uptake (shoot) per pot, per week by soybean plants grown on Santomera soil as a function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

Discussion

For an efficient use of FeEDDHA fertilizer in soil application, dosage and moment of FeEDDHA application should be matched with the Fe requirements of the plant. In order to do so, the fate of FeEDDHA components in the soil-plant system and the plant's demand for (FeEDDHA-facilitated) Fe need to be understood as a function of time. This study presents important first insights in both these issues.

The concentration behaviour of the o,o-FeEDDHA isomers, which almost completely gouverned the Fe concentration in the pore water from one week onward, could be subdivided into two stages: a rapid decline in concentration within the first week, and a gradual decline from one week onward. The rapid decline has been attributed to adsorption, the extent of which could be described with linear adsorption isotherms, for both o,o-FeEDDHA isomers. For meso o,o-FeEDDHA, the gradual decline was accurately described with an exponential decay function, in which the decay constant is a logarithmic function of the concentration applied; for racemic o,o-FeEDDHA a similar description was inadequate.

The linear shape of the adsorption isotherms does not correspond with the shapes of adsorption isotherms determined by Hernandez-Apaolaza and Lucena (2001) for racemic and meso o,o-FeEDDHA to several soil constituents. For this reason, the adsorption behaviour of racemic and meso o,o-FeEDDHA to Santomera soil was further examined in a separate batch experiment, presented in the Supporting Information. This experiment also resulted in linear adsorption isotherms (see SI-Figure 3). However, the slopes of corresponding isotherms from batch and pot experiment differ considerably; in the pot experiment the slopes are approximately a factor 4 and 6 higher for racemic and meso o,o-FeEDDHA respectively. This difference in adsorption behaviour may result from the difference in soil-solution ratio or the difference in ionic strength of the solution phase. Results from an incubation experiment at field capacity by Cantera et al. (2002) support this. FeEDDHA was recovered from a calcareous soil comparable to Santomera soil through extraction with distilled water instead of through direct centrifugation. This lowered the soil-solution ratio (SSR=0.83) and ionic strength. The FeEDDHA recovery and corresponding adsorbed fraction strongly differed from those in the pot experiment (SSR=6), but were approximately equally large as in the batch experiment with Santomera soil (SSR=1). The effect of ionic strength and soil-solution ratio on FeEDDHA adsorption need to be further examined.

Evapo-transpiration caused daily fluctuations in the pore water concentration of the FeEDDHA components, on top of the trends described. Near the end of the trial, the relative increase may have amounted 35% at maximum, before replenishment with demineralized water. The actual fluctuations were probably smaller due to the mitigating effect of adsorption.

Preventing leaching may have caused the FeEDDHA component concentrations to decline slower than under field conditions. Studies with soil columns have demonstrated that FeEDDHA components are susceptible to leaching (Cesco et al. 2000; Lucena et al. 2005). The actual impact of leaching will however depend on the water balance in the field. The effects of leaching on FeEDDHA component concentrations and Fe uptake in the soil-plant system need to be further examined.

The impact of plants on the racemic and meso o,o-FeEDDHA concentration after 6 weeks was relatively small, but significant. Plants may enhance the gradual decline through several processes. First, uptake of the FeEDDHA complex as a whole (Bienfait et al. 2004) may play a role. Secondly, after Fe reduction and chelate splitting at the root surface, and subsequent Fe uptake (Chaney et al. 1972), the chelating agent o,o-EDDHA may move back into soil solution and be degraded or form a complex with a strongly binding competing cation like Cu or Al instead of with Fe (Schenkeveld et al. 2007). Thirdly, plants enhance evapo-transpiration which may increase FeEDDHA surface precipitation resulting from water transport from lower parts of the pot to the soil surface.

The gradual decline of in particular meso o,o-FeEDDHA, is however mostly caused by non-plant related processes. Leaching is excluded as a sink, because the pots were closed at the bottom end. Precipitation at the soil surface may decrease the o,o-FeEDDHA isomer concentrations, but if this process were dominant, the most mobile isomer i.e. racemic o,o-FeEDDHA would be most affected, which was not the case. FeEDDHA consumption by microorganisms (e.g. through biodegradation of the chelating agent), chemical degradation (e.g. related to local anaerobic

conditions in the soil), or slow adsorption onto, or absorption into soil particles may (partly) account for the loss of o,o-FeEDDHA. Furthermore, a slow displacement of Fe from FeEDDHA complexes by a competing cation could decrease the o,o-FeEDDHA concentrations. However, no corresponding increases in concentration of competing cations like Cu, Al or Co were observed (data not shown). Further research is needed to clear up the processes underlying the plant-independent decrease in concentration of the o,o-FeEDDHA isomers.

Time trends in SPAD-index and Fe content of the plants did not correspond: SPAD-indices more or less continuously increased from two weeks onward in all treatments (Fig. 4a), while the Fe content of the leaves only significantly increased in the 100%o,oH treatment (Fig. 5a); the Fe content of the shoot even decreased unless a certain dose of o,o-FeEDDHA was applied (Fig. 5b). This confirms the notion that SPAD-indices are useful for comparing the Fe status of plants among treatments, but give no absolute indication of the Fe content of a leaf. The plant's stage of development and growth conditions also affect SPAD-indices through parameters like e.g. leaf-thickness.

The soybean plants mainly took up Fe from FeEDDHA in the progressed vegetative stage (3rd and 4th week) and in the reproductive stage, when the pods were being filled with seeds (6th week). Fe deficiency in the reproductive stage may be overlooked, because Fe storage in seeds does not reflect in a visible parameter like leaf colour; SPAD-indices did not change much during the 6th week (Fig. 4a). A good allocation of Fe to the seeds is however vital for a high Fe nutritional value in the plant parts suitable for consumption, and for the viability of the next generation of plants; Fe deficient plants are likely to generate offspring that will be more susceptible to Fe deficiency than non Fe-deficient plants (Grusak 1994).

Fe uptake and removal of racemic o,o-FeEDDHA from the soil system display a similar time-trend, whereas the removal of meso o,o-FeEDDHA had a plant-independent character. This indicates the removal of racemic o,o-FeEDDHA is to a larger extent plant-related and suggests that racemic o,o-FeEDDHA might be more effective in supplying soil-grown plants with Fe than meso o,o-FeEDDHA. The effectiveness of the individual isomers in soil application needs to be further examined.

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