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Performance of Soil-Applied FeEDDHA Isomers in Delivering Fe to Soybean Plants in Relation to the Moment of Application

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FeEDDHA (iron(3+) ethylenediamine-*N*,*N*′-bis(hydroxyphenylacetic acid) products are commonly applied to mend and prevent Fe deficiency chlorosis in soil-grown crops. Plants mainly take up Fe in the progressed vegetative and in the reproductive stages. This study examined which of the principal constituents of FeEDDHA products (the isomers racemic *o*,*o*-FeEDDHA, meso *o*,*o*-FeEDDHA, and *o*,*p*-FeEDDHA), most effectively meets the Fe requirements of soybean plants (*Glycine max* (L.) Merr.) grown on calcareous soil in the aforementioned growth stages. FeEDDHA isomers were applied once, separately or in mixtures, at $t = 0$, in the progressed vegetative stage or in the reproductive stage. *o*,*p*-FeEDDHA did not significantly contribute to Fe uptake in either growth stage. Both racemic and meso *o*,*o*-FeEDDHA were effective in supplying plants with Fe, approximately to the same extent. The moment of application had a significant effect on yield and FeEDDHA pore water concentrations at harvest, but not on Fe uptake. To optimize yield while minimizing FeEDDHA dosage, FeEDDHA is best applied to soybean plants prior to the onset of chorosis.

KEYWORDS: EDDHA isomers; FeEDDHA; iron chelates; iron chlorosis; iron uptake; iron nutrition

INTRODUCTION

Fe deficiency chlorosis is a common nutrient deficiency, occurring worldwide. It is characterized by a significant decrease in the chlorophyll content of the leaves and results in diminished yield and crop quality (*1*,*2*). Chlorosis is found mainly on alkaline and calcareous soils. The high pH and elevated bicarbonate concentrations in these soils (*3*) result in a limited bioavailability of Fe, due to the low solubility of iron (hydr)oxides (*4*), the impairment of the Fe uptake mechanism (*5*), and the inactivation of Fe in the leaf apoplast (*6*) under such conditions.

The application of synthetic Fe chelates is the most common practice for mending and preventing iron chlorosis. FeEDDHA (iron ethylenediamine-*N*,*N*′-bis(hydroxyphenyl)acetic acid) is among the most effective synthetic Fe chelates under neutral and alkaline soil conditions (*7*,*8*). Commercial FeEDDHA formulations consist of a mixture of positional isomers, diastereomers, and polycondensates. The three quantitatively most important compounds are (1) racemic *o*,*o*-FeEDDHA (the (*R*,*R*) and (*S*,*S*) *o*,*o*-EDDHA enantiomers), (2) meso *o*,*o*-FeEDDHA (the (*R*,*S*) = (*S*,*R*) stereoisomers), and (3) *o*,*p*-FeEDDHA (the four *o*,*p*-EDDHA enantiomers). These compounds will be addressed as FeEDDHA isomers in this study. The physical and chemical properties of the FeEDDHA isomers differ (*9*−*12*) and, as a consequence, so does their ability to preserve Fe in solution and deliver it to the plant. Because the isomeric composition varies strongly among commercial FeEDDHA formulations on the market (*13*), the need for quality assurance arose. This issue has been addressed in the European Fertilizer Law (Regulation (EC) 2003/2003; amendment (EC) 162/2007), through the following parameters: (1) soluble Fe content of the product and (2) percentages of Fe chelated by the *o*,*o*-EDDHA and *o*,*p*-EDDHA isomers.

In recent years, considerable effort has been made to assess the effectiveness of the individual Fe chelate compounds present in FeEDDHA products. The ability of FeEDDHA isomers to deliver Fe to plants has been examined in several studies with hydroponic systems. Garcia-Marco et al. (*14*) concluded that *o*,*p*-FeEDDHA offers a more effective remedy to Fe chlorosis in soybean than *o*,*o*-FeEDDHA. This conclusion is in line with the findings of Lucena and Chaney (*15*,*16*) that less stable Fe chelates are more effective in supplying cucumber plants with Fe, as long as they manage to maintain Fe chelated. Contradicting findings by Rojas et al. (*17*) are probably related to the absence of EDTA in the nutrient solution, resulting in an incremental displacement of Fe from *o*,*p*-FeEDDHA by Cu upon replenishment with nutrient solution. Moreover, both *o*,*o*-FeEDDHA and *o*,*p*-FeEDDHA were found to be more effective than synthesis byproducts (polycondensates) (*18*), and meso *o*,*o*-FeEDDHA has been claimed to be more effective in mending chlorosis in strategy 1 plants than racemic *o*,*o*-FeEDDHA (*19*).

The effectiveness of FeEDDHA isomers in soil application has received little attention so far. Schenkeveld et al. (*20*) concluded from a pot trial study with soybean that the amount of *o*,*o*-FeEDDHA in FeEDDHA treatments, administered prior to the onset of chlorosis, determines the Fe uptake of plants that would otherwise become chlorotic. Rojas et al. (*17*) confirmed the superiority of *o*,*o*-FeEDDHA over *o*,*p*-FeEDDHA in delivering Fe, in repeated soil application to chlorotic plants. Ryskievich and Boka (*21*) assessed the effectiveness of separated racemic and meso *o*,*o*-FeEDDHA in

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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})	0.30
country	Spain		reactive Al (g kg^{-1})	0.44
soil classification	entisol	DTPA[i]	Fe (mg kg^{-1})	3.5
water-holding capacity (g kg^{-1})	319		Mn (mg kg^{-1})	4.57
pH-$CaCl_2$[a]	8.0		Cu (mg kg^{-1})	4.13
electroconductivity (mS m^{-1})[b]	23		Zn (mg kg^{-1})	0.90
SOC[b] (g kg^{-1})[c]	5.4	HNO_3 (0.43 M)[j]	Fe (mg kg^{-1})	494
clay content (g kg^{-1})[d]	260		Mn (mg kg^{-1})	179
$CaCO_3$ (g kg^{-1})[e]	520		Cu (mg kg^{-1})	10
CEC (cmol kg^{-1})[f]	10.3		Zn (mg kg^{-1})	5

[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. (*34*). [d] Houba et al. (*35*). [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. (*36*). [h] Schwertmann (*37*). [i] Lindsay and Norvell (*38*) and Quevauvillier et al. (*39*). [j] Tipping et al. (*40*) and Fest et al. (*41*).

soil application and concluded both were effective in providing bean plants with Fe. Presumably, the applied FeEDDHA dosage was, however, so high that Fe uptake reached an optimum for both racemic and meso *o*,*o*-FeEDDHA (*20*), preventing a potential difference in effectiveness from being uncovered.

For an efficient use of FeEDDHA fertilizer in soil application, knowledge is required of when plants have need for FeEDDHA and of what FeEDDHA isomers most adequately serve the plant's Fe demand at that particular growth stage. It was recently found that soil-grown soybean mainly takes up Fe from FeEDDHA when the Fe demand is highest: in the progressed vegetative stage and in the reproductive stage (*22*). The efficiency of individual FeEDDHA isomers in supplying soil-grown plants with Fe at these particular growth stages had not previously been addressed and has been examined in this study. For the sake of comparison, FeEDDHA application prior to the onset of chlorosis has also been included. As part of this study, racemic and meso *o*,*o*-FeEDDHA have been separately reassessed with regard to their effectiveness in soil application, at considerably lower dosages in comparison to the study by Ryskievich and Boka (*21*).

A pot trial study was conducted with soybean plants grown on a calcareous soil from Spain, involving six FeEDDHA treatments (separated FeEDDHA isomers, as well as characterized FeEDDHA isomer mixtures) applied at the three aforementioned growth stages.

MATERIALS AND METHODS

Soil. Calcareous clay soil was collected from the top soil layer (0−20 cm) at a site located in Santomera (Murcia, Spain). Lutum fraction (260 g kg^{-1}) and $CaCO_3$ content (520 g kg^{-1}) are 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 (5 g kg^{-1}), and the dissolved organic carbon (DOC) concentration equals 30 mg L^{-1} (0.01 M $CaCl_2$). Fe availability parameters are low: the oxalate extractable ("reactive") Fe content amounts to 0.30 g kg^{-1} Fe, and the diethylenetriaminepentaacetic acid (DTPA) extractable content amounts to 3.5 mg kg^{-1} Fe. Plants grown on this soil became chlorotic, both under field conditions and in previous pot trials (*20*, *22*). Pretreatment consisted of air-drying and sieving (1 cm). Additional relevant soil characteristics are presented in **Table 1**.

FeEDDHA Solutions. FeEDDHA solutions were prepared from racemic *o*,*o*-H_4EDDHA (referring to the purity of the chelating agent) = 100%), meso *o*,*o*-H_4EDDHA (purity = 99.5%), *o*,*p*-H_4EDDHA (purity = 90%), and a mixture of racemic and meso *o*,*o*-H_4EDDHA (in a ratio close to 1; purity = 99%). These chemicals were kindly provided by AkzoNobel. Racemic and meso *o*,*o*-H_4EDDHA were obtained by separation of the *o*,*o*-H_4EDDHA mixture, as described in Bannochie and Martell (*11*) and Bailey et al. (*23*).

Solid H_4EDDHA was dissolved by adding sufficient 1 M NaOH. Fe was added as $FeCl_3 \cdot 6H_2O$ in a 2% excess based on a 1:1 stoichiometry between metal and EDDHA ligand (chelating capacity of impurities was corrected for). The pH was raised to 7 (± 0.5), and the solutions were left overnight in the dark to allow excess Fe to precipitate as hydroxides. The following day, the solutions were filtered through a 0.45 μm nitrocellulose micropore filter (Schleicher & Schuell, ref. no. 10401114) and further diluted for application in the pot trial. The composition of the experimental solutions was analyzed through combined ICP and HPLC analysis at time $t = 0$.

Pot Trial. A pot trial with a runtime of 8 weeks was done from mid-August until mid-October 2006.

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. In the first four FeEDDHA treatments, an Fe dose corresponding to a pore water concentration of around 0.6 mg L^{-1} Fe (i.e., 11 μM) was applied; the latter two treatments comprised an Fe dose corresponding to around 1.8 mg L^{-1} Fe (i.e., 32 μM). The treatments with 1.8 mg L^{-1} Fe were included to ascertain that Fe uptake had not yet reached its maximum in the treatments with 0.6 mg L^{-1} Fe. This is a precondition for a sound comparison of the effectiveness of the FeEDDHA isomers. The composition of the treatments is presented in **Table 2**. The mixed treatments have been included to examine potential synergetic effects. Except for the blank treatments, all pots received an FeEDDHA treatment once, either at $t = 0$, at the start of the experiment, a day after the transfer of the seedlings to the pots; at $t = 3$ weeks, in the middle of the vegetative stage; or at $t = 6$ weeks, at the beginning of the reproductive stage when the pods started to fill. Not all FeEDDHA treatments were administered at all three moments; moments of application are also indicated per treatment in **Table 2**. The *o*,*p* treatment has been omitted at $t = 0$ because of the short lifetime of *o*,*p*-FeEDDHA in a soil environment and the lack of Fe deficiency at this growth stage. Treatments are named after the FeEDDHA treatment administered and the moment of application. The experiment was carried out in triplicates, which were divided over three tables in accordance with a randomized complete block design.

The pot experiment was executed in a greenhouse, with 7 L Mitscherlich pots containing 5 kg of soil at 50% of the water-holding capacity. Each pot received 35 mmol of NH_4NO_3, 20 mmol of K_2HPO_4, 17.5 mmol of $CaCl_2$, 10 mmol of $MgSO_4$, 0.5 mmol of H_3BO_3, and 5 μmol of $(NH_4)_6Mo_7O_{24}$. All FeEDDHA treatments were applied through a sand column with a diameter of around 6 cm, which was positioned in the center of the soil surface and went about 10 cm deep into the soil. After FeEDDHA addition, the column was flushed with demineralized water.

After 5 days of germination, eight soybean (*Glycine max* (L.) Merr.) seedlings of the Fe chlorosis susceptible cultivar Mycogen 5072 (soybean seeds were kindly provided by Prof. Dr. R. J. Goos from the Department of Soil Science of the North Dakota State University) were transferred to each pot.

Preparation of the pot trial, germination of the seeds, foliar fertilization with micronutrients other than Fe, and plant care were performed as described in Schenkeveld et al. (*20*).

Sampling and Measurement. SPAD measurements were done three times per week on leaves from the youngest and the second youngest trifoliate with a Minolta 502 SPAD-meter, as described in Schenkeveld et al. (*20*).

At harvest, the shoots were cut off right above the soil surface. A 1 kg mixed subsample was taken from the soil, from which roots were collected manually. The soil subsample was stored at 4 °C until further use. The shoots were washed with demineralized water and dried at 70 °C. After 48 h, the shoots were weighed (dry weight).

The mineral contents of the shoots were determined through microwave digestion with nitric acid, fluoric acid, and hydrogen peroxide (*24*). Cu, Fe, Mn, and Ni concentrations were measured by ICP-AES (Varian, Vista Pro).

Pore water was collected by centrifugation of the soil subsample at 7443*g* (7000 rpm) for 15 min as described in Schenkeveld et al. pH 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 measurement.

FeEDDHA isomer concentrations were determined after separation through high-performance liquid chromatography (HPLC) as described in Schenkeveld et al. (*25*).

Table 2. Treatment Overview

	composition[a]			moment of application		
treatment	racemic *o,o*-FeEDDHA (mg L^{-1} Fe)	meso *o,o*-FeEDDHA (mg L^{-1} Fe)	*o,p*-FeEDDHA (mg L^{-1} Fe)	$t = 0$	$t = 3$ weeks	$t = 6$ weeks
blank						
o,p			0.53		x	x
meso *o,o*		0.56		x	x	x
racemic *o,o*	0.58			x	x	x
o,o-mix low	0.29	0.31			x	
o,o-mix low + *o,p*	0.29	0.31	1.06		x	
o,o-mix high	0.87	0.93		x	x	

[a] Composition of treatments is expressed in terms of pore water concentration prior to interaction with soil.

The limits of quantification (LOQ) were, respectively, 2 μg L^{-1} Fe for racemic *o,o*-FeEDDHA, 5 μg L^{-1} F for meso *o,o*-FeEDDHA, and 40 μg L^{-1} Fe for *o,p*-FeEDDHA (20 μg L^{-1} Fe for each *o,p*-FeEDDHA peak). To avoid contamination, the preparation of the experimental solutions and dilution of samples for measurement were done with analytical grade chemicals and ultrapure water.

Statistical Analysis. Statistical analyses were done with the program SPSS 12.0. Homogeneity of the data was tested with Levene's test (α = 0.05). A data transformation was executed in case data proved to be nonhomogeneous. Differences among treatments were determined by applying the univariate general linear model (GLM) procedure with a Tukey post hoc test (α = 0.05). Block effects from the tables were accounted for by including table as a random factor.

RESULTS AND DISCUSSION

Chlorosis and SPAD Indices. SPAD measurements were done to monitor and compare the treatments with regard to Fe status of the plants. In **Figure 1**, the SPAD indices of the youngest leaves are presented as a function of time for all treatments. The treatments have been clustered per moment of FeEDDHA application. The moments of application are indicated by the dashed vertical lines. The plants receiving treatment at t = 3 and t = 6 weeks were chlorotic at the moment of FeEDDHA application, with SPAD indices ranging from 16.7 to 20.4. These SPAD indices were significantly lower than the SPAD indices of plants that had received an (*o,o*-)FeEDDHA application at t = 0.

Throughout the experiment, the SPAD indices of neither *o,p* treatment significantly exceeded those of the blank treatment. Market claims of a fast regreening effect of *o,p*-FeEDDHA were not confirmed in either *o,p* treatment. From 3 weeks onward, the SPAD indices of the blank and the *o,p* treatments remained more or less constant, whereas in previous pot trials, SPAD indices of the blank had gradually increased or chlorosis had even entirely been overgrown (*20*, *22*).

All treatments involving *o,o*-FeEDDHA isomers, either separated or in a mixture, resulted in an increase in SPAD indices relative to the blank. In general, the time trends in SPAD indices of *o,o* treatments, which were applied at the same moment, were similar. For both application at t = 0 and t = 3 weeks, SPAD indices were highest for the *o,o*-mix high treatment.

For all corresponding treatments applied at t = 0 and t = 3 weeks, the SPAD indices of the treatment applied at t = 3 weeks eventually surpassed those of the treatment applied at t = 0 and remained higher for the rest of the experiment. SPAD indices were analyzed by means of the GLM procedure, from the moment the SPAD index of the treatment applied at t = 3 weeks exceeded the value of the corresponding treatment applied at t = 0 until the end of the experiment (approximately the final 4 weeks). Although differences in SPAD index did not exceed 4 SPAD units, the effect of moment of application proved to be significant (α = 0.05) for all corresponding treatments. *o,o*-FeEDDHA application at t = 6 weeks did lead to an increase in SPAD indices, but to a lesser extent than *o,o*-FeEDDHA application at t = 0 or t = 3 weeks. Although at t = 6 weeks the reproductive stage was already progressing and the pods were filling, the plants apparently did not exclusively invest in securing the Fe demand of their offspring, but also still allocated Fe to the leaves for chlorophyll synthesis.

Fe and FeEDDHA Isomer Concentrations in Pore Water. The Fe and FeEDDHA isomer concentrations in the pore water at harvest are presented in **Table 3**, grouped per treatment composition. *o,p*-FeEDDHA was not detected in any of the samples and has been omitted from **Table 3**. In none of the treatments, including those receiving FeEDDHA application only 2 weeks before harvest, the remaining FeEDDHA concentration accounted for >24% of the dose applied. For each moment of application separately, racemic *o,o*-FeEDDHA remained in soil solution to a larger extent than meso *o,o*-FeEDDHA. This corresponds with previous observations (*20*, *22*).

For corresponding FeEDDHA treatments administered at t = 0 and t = 3 weeks, the FeEDDHA isomer concentrations were consequently lower for the treatments applied at t = 3 weeks (**Table 3**). This is remarkable, for despite the shorter residence time in the soil−plant system, a larger portion of the FeEDDHA has been removed from soil solution. The principal difference in conditions between FeEDDHA application at t = 0 and t = 3 weeks is that the plants receiving treatment at t = 3 weeks were chlorotic at that stage, whereas the plants receiving treatment at t = 0 never became Fe deficient to this extent. This suggests that the enhanced FeEDDHA consumption in treatments administered at t = 3 weeks is related to an Fe deficiency stress response mechanism of the plant. When strategy I plants, for example, soybean, become Fe deficient, the enzymatic ferric chelate reductase (FCR) system at the root surface is up-regulated (*26*, *27*). Thus, the efficiency with which chelated Fe is reduced, detached from the chelating agent, and taken up by the plant increases. 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 decrease more swiftly and strongly in the presence of Fe-deficient plants than with plants that are not Fe-deficient. Other stress response mechanisms such as the excretion of protons and phenolic compounds (*26*) alter rhizosphere conditions such as pH, availability of competing cations, and bacterial activity and may lead to enhanced cation competition or biodegradation. Contrary to an increase in reduction capacity, such processes result in only a decreased FeEDDHA concentration in soil solution, and not in increased Fe uptake, relative to the blank treatment. To discriminate, these findings will be further discussed in relation to Fe uptake.

Comparison of corresponding treatments applied at t = 0 and t = 6 weeks (**Table 3**) shows that racemic *o,o*-FeEDDHA concentrations at harvest (133 and 145 μg L^{-1} Fe, respectively) were equal, whereas meso *o,o*-FeEDDHA concentrations (20 and

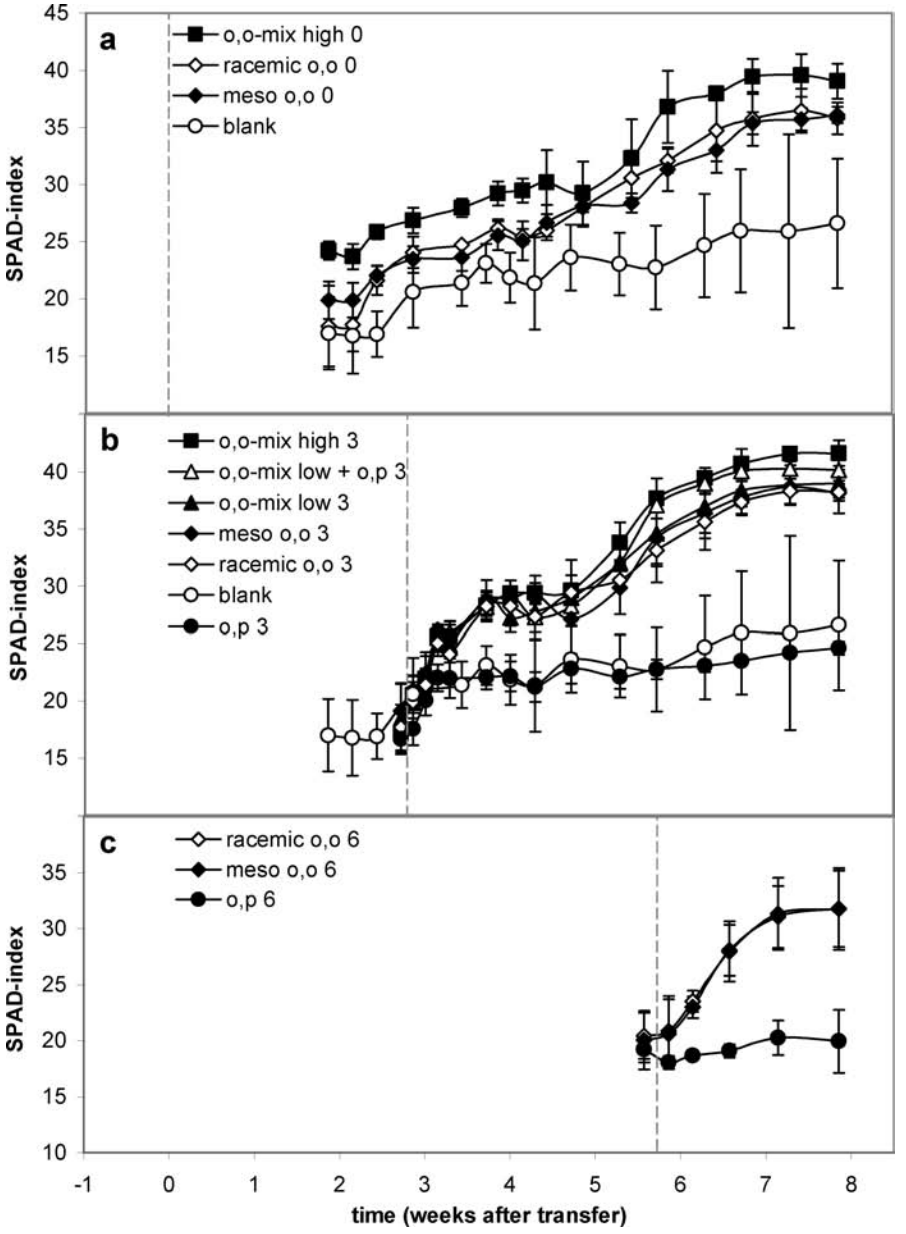

Figure 1. SPAD indices of the youngest leaves of soybean plants grown on Santomera soils as a function of time for all treatments. FeEDDHA treatments were applied at (approximately) $t = 0$ (**a**), $t = 3$ weeks (**b**), and $t = 6$ weeks (**c**). Dashed lines indicate the moment of FeEDDHA application. Error bars indicate standard deviations. The blank treatment has been omitted as a reference in the third cluster (**c**) because the large standard deviations in the blank treatment obscure the trends in the other treatments.

47 μg L^{-1} Fe, respectively) differed only a little, relative to the concentration applied (560 μg L^{-1} Fe; **Table 2**). In this study, the impact of stress response mechanisms on FeEDDHA concentrations exceeded the impact of 3 weeks of residence time in the soil−plant system for meso *o*,*o*-FeEDDHA and equaled the impact of 6 weeks of residence time for racemic *o*,*o*-FeEDDHA (**Table 3**).

Comparison of the concentrations of both *o*,*o*-FeEDDHA isomers between the *o*,*o*-mix low 3 and the *o*,*o*-mix low + *o*,*p* 3 treatment showed that for pots from the same table, both racemic and meso *o*,*o*-FeEDDHA concentrations were always higher in the *o*,*o*-mix low + *o*,*p* 3 treatment. As indicated in **Table 3**, the concentrations were, however, not identified as different in the overall Tukey post hoc test. Because the treatments were identical, except for the additional *o*,*p*-FeEDDHA in the *o*,*o*-mix low + *o*,*p* 3 treatment, the concentrations of both *o*,*o*-FeEDDHA isomers could also be compared with a simple *t* test. Then the concentration of meso *o*,*o*-FeEDDHA proved to be significantly higher in the *o*,*o*-mix low + *o*,*p* 3 treatment ($p = 0.04$), whereas the concentration of the racemic *o*,*o*-FeEDDHA isomer was not ($p = 0.20$). Despite the fact that the effect is small, the observation implies that *o*,*p*-FeEDDHA is involved in a "guarding mechanism", which somewhat diminishes the decrease in meso *o*,*o*-FeEDDHA concentration. Nonetheless, comparison of the *o*,*o*-mix low + *o*,*p* 3 and the *o*,*o*-mix high 3 treatment with regard to the remaining Fe concentration in soil solution shows that the beneficial effect from *o*,*p*-FeEDDHA on the meso *o*,*o*-FeEDDHA concentration cannot compete with simply applying more meso *o*,*o*-FeEDDHA instead.

Yield. The dry weight yield ranged from 16.7 to 22.3 g pot^{-1} (see **Table 4**). The Tukey post hoc test indicated that differences in yield were significant neither between different FeEDDHA treatments applied at the same moment nor between corresponding FeEDDHA treatments applied at different moments. An overall effect of moment of application was examined by comparing the yields from different moments of application collectively

Table 3. Fe, Racemic *o,o*-FeEDDHA, and Meso *o,o*-FeEDDHA Concentrations in the Pore Water of Santomera Soil at Harvest for All Treatments[a]

	total Fe (μg L^{-1} Fe)	racemic *o,o*-FeEDDHA (μg L^{-1} Fe)	meso *o,o*-FeEDDHA (μg L^{-1} Fe)
blank	7 (3) a		
o,p 3	17 (8) a		
o,p 6	20 (13) a		
meso *o,o* 0	62 (33) a		20 (3) bc
meso *o,o* 3	28 (3) a		12 (1) ab
meso *o,o* 6	55 (5) a		47 (4) d
racemic *o,o* 0	150 (13) b	133 (16) b	
racemic *o,o* 3	63 (6) a	54 (7) a	
racemic *o,o* 6	147 (25) b	145 (26) b	
o,o-mix low 3	40 (15) a	27 (5) a	6 (3) a
o,o-mix low + *o,p* 3	57 (6) a	33 (5) a	11 (1) ab
o,o-mix high 0	313 (86) c	212 (17) c	42 (7) d
o,o-mix high 3	168 (15) b	133 (9) b	29 (5) c

[a] Standard deviations are indicated between parentheses. Letters indicate significantly different groups as identified by the Tukey post hoc test including all FeEDDHA treatments and all moments of application.

Table 4. Dry Weight Yield and Fe Content of the Shoot of Soybean Plants Grown on Santomera Soil for All Treatments[a]

treatment	dry weight yield (g pot^{-1})	Fe content (mg kg^{-1})
blank	19.0 (1.4) abc	38.6 (5.7) a
meso *o,o* 0	21.1 (0.9) bc	59.2 (6.5) cd
racemic *o,o* 0	20.0 (1.7) abc	57.6 (5.6) c
o,o-mix high 0	21.7 (1.6) bc	62.2 (2.8) cd
o,p 3	19.3 (1.2) abc	41.4 (1.4) ab
meso *o,o* 3	21.5 (2.1) bc	51.6 (4.0) bc
racemic *o,o* 3	22.2 (0.4) c	57.5 (1.8) c
o,o-mix low 3	22.1 (1.7) bc	50.1 (2.7) abc
o,o-mix low + *o,p* 3	22.2 (1.7) bc	52.7 (2.7) bc
o,o-mix high 3	21.6 (1.0) bc	70.8 (3.8) d
o,p 6	16.7 (0.8) a	37.9 (2.4) a
meso *o,o* 6	18.9 (3.2) ab	58.8 (6.7) cd
racemic *o,o* 6	19.8 (0.7) abc	55.2 (2.3) c

[a] Standard deviations are indicated between parentheses. Letters indicate the significantly different groups as identified by the Tukey posthoc test including all FeEDDHA treatments and all moments of application.

instead of per treatment. Only corresponding FeEDDHA treatments were included in the comparison. The overall effect of moment of application was significant ($p = 0.022$) and, for corresponding treatments, application at $t = 6$ weeks yielded less biomass than application at $t = 3$ weeks ($p = 0.013$).

Fe Content of the Shoot. After 8 weeks of growth, the Fe content of the shoot ranged from 37.9 to 70.8 mg kg^{-1} Fe (see **Table 4**). The Fe contents of the blank (38.6 mg kg^{-1} Fe) and the *o,p* treatments (37.9 and 41.4 mg kg^{-1} Fe) did not significantly differ, but were all significantly lower than the Fe contents of all treatments involving *o,o*-FeEDDHA with exception of the *o,o*-mix low 3 treatment (50.1 mg kg^{-1} Fe). Plants grown with the *o,o*-mix high treatments had the highest Fe contents (62.2−70.8 mg kg^{-1} Fe). For none of the moments of application were significant differences in Fe content between the racemic *o,o* and the meso *o,o* treatment found. Only at $t = 3$ weeks were significant differences between the treatments involving *o,o*-FeEDDHA found: the *o,o*-mix high treatment had a significantly higher Fe content than the other treatments. For corresponding FeEDDHA treatments, the Tukey post hoc test did not identify significant differences in Fe content between the moments of application.

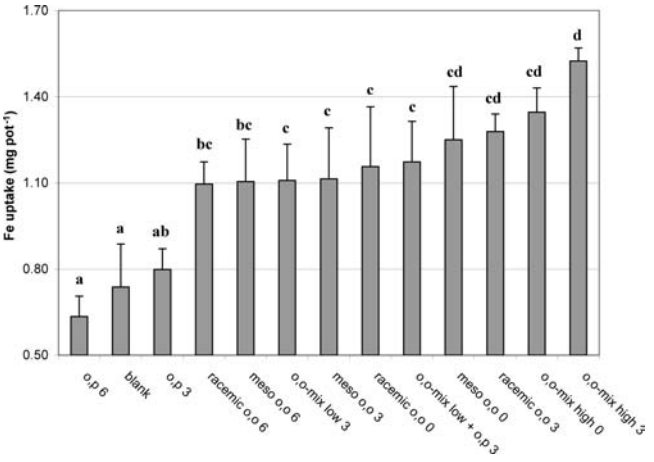

Figure 2. Fe uptake by soybean plants grown on Santomera soil for all FeEDDHA treatments. Error bars indicate standard deviations. Letters indicate the significantly different groups as identified by the Tukey post hoc test including all FeEDDHA treatments and all moments of application.

Fe Uptake. Fe uptake was calculated as the product of shoot dry weight and Fe content of the shoot. Due to previous experience with contamination of the roots with soil material, the roots were left out of consideration. As shown in **Figure 2**, Fe uptake ranges from 0.63 to 1.52 mg of Fe pot^{-1}.

No significant differences in Fe uptake were observed between the blank, the *o,p* 3 and the *o,p* 6 treatment. Therefore, the plants did not benefit from *o,p*-FeEDDHA, even under conditions in which the Fe uptake efficiency had increased as a result of Fe deficiency stress mechanisms (*26*), neither in the vegetative stage nor in the reproductive stage. With regard to the vegetative stage, this finding is in agreement with findings from Rojas et al. (*17*). *o,p*-FeEDDHA is known to largely adsorb to soil reactive surfaces and exchange Fe for Cu (*25*). Apparently, fast adsorption and cation competition kinetics heavily outweighed the preferential Fe transfer by *o,p*-FeEDDHA compared to *o,o*-FeEDDHA, demonstrated in hydroponics systems (*14*).

By comparing Fe uptake in the *o,o*-mix low 3 and the *o,o*-mix low + *o,p* 3 treatments it was examined if the presence of *o,p*-FeEDDHA might enhance the performance of *o,o*-FeEDDHA, through the aforementioned "guarding mechanism". However, no significant difference in Fe uptake was found, although the *o,p*-FeEDDHA dose in the *o,o*-mix low + *o,p* 3 treatment was twice as high as in the *o,p* 3 and *o,p* 6 treatments. The higher Fe uptake in the *o,o* mix high 3 treatment confirms that distinction between the *o,o*-mix low and *o,o*-mix low + *o,p* 3 treatments is not compromised by Fe saturation of the plants and indicates that substituting the *o,p*-FeEDDHA from the *o,o*-mix low + *o,p* 3 treatment for *o,o*-FeEDDHA significantly increases the effectiveness of the treatment.

The Fe uptake was highest in the *o,o*-mix high treatments (1.35 ($t = 0$) and 1.52 ($t = 3$ weeks) mg of Fe), but not significantly different from all other treatments. An overall effect of FeEDDHA treatment was examined by comparing the Fe uptake from different FeEDDHA treatments for all corresponding moments of application combined. Overall, the *o,o*-mix high treatments led to a significantly higher Fe uptake than the racemic *o,o* treatments ($p = 0.030$) and the meso *o,o* treatments ($p = 0.012$). This is in agreement with the finding by Schenkeveld et al. (*20*) that a higher dose of *o,o*-FeEDDHA results in a higher Fe uptake.

Fe uptake in racemic *o,o* and meso *o,o* treatments was in all cases significantly higher than in the blank. This testifies that both

racemic and meso *o*,*o*-FeEDDHA are effective in soil-applied Fe fertilization, in accordance with the study by Ryskievich and Boka (*21*). Fe uptake in the racemic *o*,*o* treatments was not significantly different from Fe uptake in the meso *o*,*o* treatments, neither overall ($p = 0.73$) nor for any of the moments of application separately. The lack of significant difference in Fe uptake, while Fe uptake was not maximal, demonstrates that the effectiveness of racemic and meso *o*,*o*-FeEDDHA in soil application is comparable. This does not correspond with the conclusions from hydroponics studies (*15*, *16*, *19*), in which meso *o*,*o*-FeEDDHA was found to be more effective than racemic *o*,*o*-FeEDDHA. In studies in which racemic and meso *o*,*o*-FeEDDHA were applied as a mixture to Fe-deficient plants, the decrease in meso *o*,*o*-FeEDDHA concentration was considerably stronger than in racemic *o*,*o*-FeEDDHA concentration (*19*, *28*). This stronger decrease may, however, in part be explained by a redistribution of Fe over the available *o*,*o*-EDDHA ligands, upon Fe transfer from racemic *o*,*o*-FeEDDHA to the plant; the greater stability of the racemic *o*,*o*-FeEDDHA complex favors Fe complexation by racemic *o*,*o*-EDDHA (*11*).

A possible explanation of why, in the soil−plant system, contrary to hydroponics systems (*15*, *16*), Fe uptake was not larger in meso *o*,*o* treatments than in racemic *o*,*o* treatments involves the lower Fe concentration in soil solution in the meso *o*,*o* treatments, due to the larger degree of meso *o*,*o*-FeEDDHA adsorption to soil reactive surfaces and the faster non-plant-related decline in concentration (*22*). The effect of preferential Fe uptake from meso *o*,*o*-FeEDDHA may thus have been undone by the lower soil solution concentration. Schenkeveld et al. (*22*) even suggested that racemic *o*,*o*-FeEDDHA might be more effective in supplying soil-grown plants with Fe than meso *o*,*o*-FeEDDHA, because the removal of racemic *o*,*o*-FeEDDHA from the soil−plant system proved to a larger extent to be plant-related. The present study does, however, not support this suggestion.

The effect of moment of application on Fe uptake was not significant, neither for FeEDDHA treatments individually nor for corresponding treatments collectively. This seems counterintuitive, because plants that receive a treatment containing *o*,*o*-FeEDDHA at a later stage have less time to benefit from it until harvest. Still, the difference in Fe uptake between the blank treatment, on the one hand, and the racemic *o*,*o* 6 and meso *o*,*o* 6 treatments, on the other, amounted to 0.36 mg pot^{-1} Fe and was built up in merely 2 weeks. The amount of 0.36 mg pot^{-1} Fe corresponds with 50% of the total Fe uptake in the blank treatment after 8 weeks. Two factors may help to explain the lack of significant difference in Fe uptake between the moments of application. First, as mentioned earlier, when FeEDDHA treatments were applied after 3 or 6 weeks, the plants were Fe deficient and stress response mechanisms facilitated a faster, more efficient Fe uptake (*26*). Second, by applying the same FeEDDHA dose at a later stage, losses related to residence in the soil were smaller and the FeEDDHA concentrations in soil solution were highest when plants were actually Fe -deficient. Although no quantitative effect on Fe uptake was found, it seems likely the moment of application will affect the Fe distribution over the plant (leaves versus seeds) (*29*).

The mechanism of Fe uptake has not been specifically considered in this study. Although Fe reduction and chelate splitting are considered to comprise the prevailing Fe uptake mechanism (*27*,*30*), uptake of the racemic and meso *o*,*o*-FeEDDHA complex as a whole has been demonstrated in several plant studies on substrate and in plain nutrient solution (*31*, *32*). In these studies racemic and meso *o*,*o*-FeEDDHA were taken up to approximately to the same extent. Recently, also uptake of the *o*,*p*-FeEDDHA complex was demonstrated (*32*). Whether uptake of the FeEDDHA complex as a whole also occurs in soil-grown plants, and if so, to what extent it accounts for overall Fe uptake, needs to be further examined.

Practical Implications. In this study *o*,*p*-FeEDDHA did not significantly contribute to Fe uptake of Fe-deficient, soil-grown plants, regardless of whether it was applied in the vegetative or in the reproductive stage, as a single substance or in a mixture. The acclaimed short-term effectiveness in soil application (*14*) was rejected. Soil contamination with Cu had been identified to cause limited stability of *o*,*p*-FeEDDHA, thereby reducing its effectiveness (*9*, *14*, *33*). The Santomera soil is, however, not Cu-contaminated (the Cu content of Santomera soil is below the Dutch soil specific background value "AW-2000" (35 mg kg^{-1} Cu), indicating that, with respect to Cu, the soil is not contaminated and suitable for any application in accordance with current regulation (Besluit Bodemkwaliteit 2008/Administrative Order on Soil Quality 2008)), so the lack of Cu contamination is no guarantee for *o*,*p*-FeEDDHA functionality. In general, it seems questionable if *o*,*p*-FeEDDHA in commercial FeEDDHA formulations has any added value as Fe fertilizer in soil application; chances for positive results seem highest in sandy soils with a low Cu content (*25*).

Both racemic and meso *o*,*o*-FeEDDHA proved to be effective in supplying soil-grown plants with Fe, approximately to the same extent. The outcomes of this study offer no incentive for altering the ratio between racemic and meso *o*,*o*-FeEDDHA in commercial FeEDDHA formulations for soil application or for discriminating between the *o*,*o*-FeEDDHA isomers on product labels.

The moment of application had no significant effect on Fe uptake by soybean plants, but did have a significant effect on yield and on the FeEDDHA isomer concentrations in the pore water at harvest. *o*,*o*-FeEDDHA application in the reproductive stage resulted in diminished yield in comparison to earlier application. *o*,*o*-FeEDDHA application in the vegetative stage, after the onset of chlorosis, resulted in lower FeEDDHA isomer concentrations in comparison to corresponding treatments applied earlier or later. Thus, in view of an efficient use of Fe fertilizer, FeEDDHA application prior to the onset of chlorosis is recommended; to obtain similar results, less FeEDDHA is required than in application after the onset of chlorosis, and yield loss related to FeEDDHA application in the reproductive stage is avoided. The distribution of Fe over the plant has not been examined in this study; FeEDDHA application in a later growth stage might be favorable for the allocation of Fe to edible plant parts, for example, the fruits or seeds.

In conclusion, because the amount of *o*,*o*-FeEDDHA applied determined the effectiveness of the treatment, regardless of the moment of application, the conclusion that FeEDDHA formulations with a higher *o*,*o* content can be applied at a lower dose (*20*) is not limited to application prior to the onset of chlorosis but remains valid throughout the growing season.

ABBREVIATIONS USED

o,*o*-FeEDDHA, iron(3+) ethylenediamine-*N*,*N*′-bis(2-hydroxyphenylacetic acid) complex; *o*,*p*-FeEDDHA, iron(3+) ethylenediamine-*N*-(2-hydroxyphenylacetic acid)-*N*′-(4-hydroxyphenylacetic acid) complex; DOC, dissolved organic carbon; DTPA, diethylenetriaminepentaacetic acid; ICP-MS/AES, inductively coupled plasma mass spectrometry/atomic emission spectrometry; SOC, soil organic carbon.

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