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Fe Uptake from Meso and *d*,*l*-Racemic Fe(*o*,*o*-EDDHA) Isomers by Strategy I and II Plants

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One of the most efficient fertilizers to correct Fe deficiency in calcareous soils and waters with high bicarbonate content is based on ferric ethylenediamine-*N*,*N*′-bis(*o*-hydroxyphenylacetic) acid [Fe(*o*,*o*-EDDHA)]. Fe(*o*,*o*-EDDHA) forms two groups of geometric isomers known as meso and *d*,*l*-racemic. To determine the Fe uptake from meso and *d*,*l*-racemic Fe(*o*,*o*-EDDHA), four iron-efficient plants, two plants representative of strategy I (tomato and pepper) and two plants representative of strategy II (wheat and oats), were grown in hydroponic culture. Results indicated that strategy II plants took up iron from both Fe(*o*,*o*-EDDHA) isomers equally. However, strategy I plants took mainly the iron associated with the meso form (the lowest stability isomer).

KEYWORDS: Fe(*o*,*o*-EDDHA) isomers; strategy I plants; strategy II plants; iron uptake; *Lycopersicon esculentum* Mill.; *Capsicum annuum* L.; *Triticum aestivum*; *Avena sativa* L.

INTRODUCTION

Iron deficiency is a widespread problem that affects crop yield and quality, mainly in plants grown in calcareous and alkaline soils. Iron-efficient plants are able to develop two different strategies to increase iron availability in soils (*1*−*5*). Strategy I is developed by dicots and nongraminaceous monocot species. This strategy is characterized by the enhancement of the ferric reductase capacity located at the root surface (*4*, *6*, *7*). In some instances, these plants increase the excretion of H^+ (*8*, *9*) and release reductants and/or chelators to the rhizosphere, improving the iron mobility (*3*, *10*). Strategy II is developed by graminaceous species and consists of the release of phytosiderophores (nonproteinogenic amino acids), which mobilize inorganic Fe(III) by the formation of Fe(III)−phytosiderophore complexes (Fe−PS) of high stability (*11*, *12*). The Fe−PS uptake by plants is mediated by a highly specific transport system (*13*).

The high quantity of bicarbonate in calcareous soils neutralizes the activity of strategy I, causing severe iron deficiency in crops and plants that must then be supplied with iron (*5*, *14*−*17*). Nowadays, the use of synthetic iron chelate derivates of *o*,*o*-EDDHA, *o*,*o*-EDDHMA, and *o*,*o*-EDDHSA is the most effective agricultural practice to relieve this problem (*18*). For most authors, *o*,*o*-EDDHA is the most efficient chelating agent (*19*−*22*) because their 1:1 complexes with Fe^{3+} are able to maintain iron in the soil solution over a wide range of pH values.

The chelating agent *o*,*o*-EDDHA is constituted of two geometric isomers, a meso form [(*R*,*S*-*o*,*o*-EDDHA)$^-$] and a racemic mixture [(*R*,*R*-*o*,*o*-EDDHA)$^-$ + (*S*,*S*-*o*,*o*-EDDHA)$^-$] that when linked to iron yield two groups of isomers also known as *d*,*l*-racemic and meso isomers. These isomers show different stability constants, the isomer *d*,*l*-racemic ($K = 10^{35.86}$) being more stable than the meso form ($K = 10^{34.15}$) (*23*). Both isomers can be well-separated and identified by an isocratic HPLC ion-pair chromatographic method (*21*, *24*).

In this paper, we tested if the two isomers of Fe(*o*,*o*-EDDHA) are taken up by plants in different ways depending on the stability constants of these isomers and the strategy developed by plants under Fe deficiency.

MATERIALS AND METHODS

Plant Materials and Nutrient Solution. Four plant species were tested, two representative species of strategy I, tomato plants (*Lycopersicon esculentum* Mill.) cv. Jaguar F_1 and pepper (*Capsicum annuum* L.) cv. Lamuyo F_1, and two representative of strategy II plants, wheat (*Triticum aestivum*) cv. Chamorro and oats (*Avena sativa* L.) cv. Europa.

Tomato and pepper seeds were germinated on quartz sand in a controlled chamber (Sanyo MLR-350H) under a temperature of 25 °C and 70% relative humidity in darkness. Seeds were moistened with saturated $CaSO_4$ solution to prevent the appearance of fungi. After the cotyledons emerged, the seedlings were watered with nutrient solution (**Table 1**) (*25*) diluted with water (1:1) and left stand with a day/night regimen of 16/8 h, temperature of 25/15 °C, and relative humidity of 70%. When the plants reached 7 cm of total length, they were transferred to 3 L black plastic pots that contained continuously aerated nutrient solution (**Table 1**). Pots were placed in a greenhouse, located on the campus of the University of Alicante (Spain), under controlled environmental conditions: 17/25 °C (night/day) and natural day/night regimen of light intensity. The plants were grown for 74 days. Every 3 or 4 days, the losses of volume were replaced with distilled water, and samples of 50 mL were taken.

For wheat and oats, several holes were made in plastic dishes. The bottom of the dishes was covered with a mesh and a thin layer of quartz

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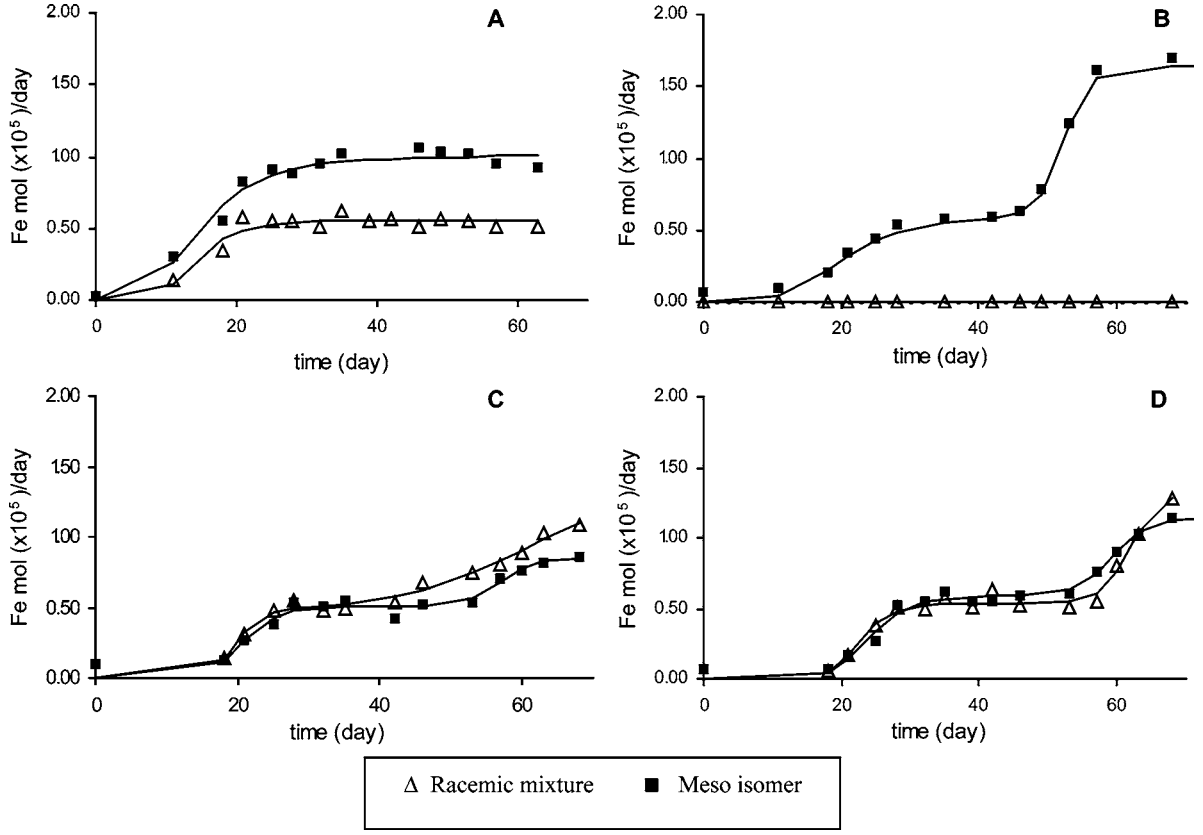

Figure 1. Fe uptake by strategy I and II plants: (**A**) tomato; (**B**) pepper; (**C**) wheat; (**D**) oats.

Table 1. Composition of Nutrient Solution

compound	concn (M)	compound	concn (M)
Fe(*o,o*-EDDHA)	3.58×10^{-5}	NH_4NO_3	5.0×10^{-4}
$Ca(NO_3)_2 \cdot 4H_2O$	3.5×10^{-3}	$CuSO_4 \cdot 5H_2O$	3.14×10^{-7}
$MgSO_4 \cdot 7H_2O$	1.25×10^{-3}	$ZnSO_4 \cdot 7H_2O$	1.36×10^{-6}
KNO_3	4.5×10^{-3}	$MnSO_4 \cdot H_2O$	1.27×10^{-5}
K_2SO_4	7.5×10^{-4}	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	5.95×10^{-8}
KH_2PO_4	1.5×10^{-3}	H_3BO_4	4.63×10^{-5}

sand, in which wheat and oat seeds were planted. The dishes covered the 3 L pots that contained aerated nutrient solution (**Table 1**), which ascended by capillarity and moistened the quartz sand. The wheat and oat plants were placed in the greenhouse under the same experimental conditions as the tomato and pepper plants. After 18 days, a first sampling was made. Pots containing the aerated nutrient solution and no plant were used as controls, to check the loss of iron chelate owing to the environmental and microbial conditions. All assays were made in triplicate.

The source of iron used in the experiment was Fe(*o,o*-EDDHA), which was synthesized in the laboratory (*26*). To prepare the Fe(*o,o*-EDDHA) solution, H_4EDDHA (Sigma, E4135) was dissolved in NaOH (Panreac, analytical grade) (1:3 molar ratio). A 5% excess (in moles) of $Fe(NO_3)_3 \cdot 9H_2O$ was added, the pH was adjusted with HCl (Panreac, analytical grade) to 7.0, and the solution was left to stand for 24 h in darkness to allow the excess Fe to precipitate as oxides. The solution

Table 2. Kinetic Parameters for Fe Uptake

plant		isomer	$(\text{mol of Fe})_{eq} \times 10^6$	*b*	$\tau_{1/2}$ (days)	$V\tau_{1/2}$ (mol of Fe/day) $\times 10^7$	R^2
tomato	total	racemic	5.5 ± 0.2	5 ± 1	14 ± 1	5 ± 2	0.9306
		meso	10.1 ± 0.1	3 ± 1	15.0 ± 0.8	6 ± 2	0.9711
pepper	stage 1	racemic					
		meso	7 ± 1	4 ± 1	21 ± 3	3 ± 2	0.9675
	stage 2	racemic					
		meso	10.8 ± 0.2	26 ± 2	52.1 ± 0.2	14 ± 1	0.9981
	total	racemic					
		meso	17 ± 1				0.9971
wheat	stage 1	racemic	5.2 ± 0.3	10 ± 3	20.1 ± 0.5	7 ± 2	0.9869
		meso	5.6 ± 0.7	7 ± 3	21.1 ± 0.5	5 ± 3	0.9361
	stage 2	racemic	10 ± 6	6 ± 2	64 ± 11	2 ± 2	0.9685
		meso	5 ± 1	10 ± 4	57 ± 3	2 ± 1	0.9579
	total	racemic	15 ± 6				0.9885
		meso	11 ± 2				0.9622
oat	stage 1	racemic	5.5 ± 0.3	20 ± 9	24.1 ± 0.6	11 ± 5	0.9689
		meso	6.1 ± 0.6	8 ± 2	24 ± 1	5 ± 2	0.9539
	stage 2	racemic	8 ± 3	29 ± 3	61.6 ± 0.3	10 ± 1	0.9960
		meso	6.0 ± 0.2	20 ± 2	59.0 ± 0.4	5.1 ± 0.7	0.9945
	total	racemic	13 ± 3				0.9901
		meso	12.1 ± 0.8				0.9883

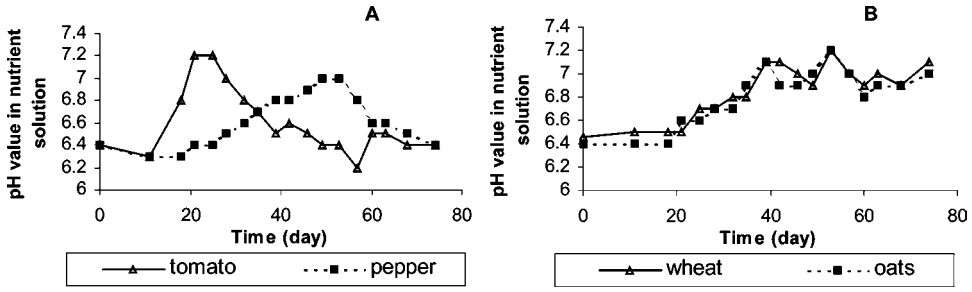

Figure 2. pH value in nutrient solution: (**A**) strategy I plants; (**B**) strategy II plants.

was filtered through 0.45 μm nylon filters (Millipore) to eliminate the iron oxides and made up to volume with distilled water.

Fe(*o,o*-EDDHA) Isomer Analysis. Samples were filtered through 0.20 μm syringe filters (Osmonics) and meso Fe(*o,o*-EDDHA) and *d,l*-racemic Fe(*o,o*-EDDHA) were analyzed by high-pressure liquid chromatography (HPLC), according to the method described by Lucena et al. (*24*). HPLC separation and analysis were carried out in a Shimadzu chromatographic system, with an LC-7A pump, an SIL-10A autosampler, an SPD-M6A photodiode array detector, and Windows 98 chromatographic software CLASS-LC10 V.1.6. For Fe(*o,o*-EDDHA) the column used was a Lichrospher 100RP-18 (5 μm) (Hp), 250 mm $\times$ 4 mm, with a flow rate of 1.5 mL/min, an oven temperature of 25 °C, a detection wavelength of 300 nm, and an injection volume of 100 μL. The mobile phase was constituted of tetrabutylammonium hydroxide 2% (v/v) (Sigma) and acetonitrile 30% (v/v) (HPLC Scharlau FEROSA). The content of meso Fe(*o,o*-EDDHA) and racemic Fe(*o,o*-EDDHA) was quantified on the basis on the pick areas. Each pick was identified by its retention time and by the UV−vis spectra carried out between 200 and 600 nm [maxima of absorbance: *d,l*-racemic Fe(*o,o*-EDDHA), 477.7 nm; meso Fe(*o,o*-EDDHA), 489.9 nm] (*21*, *24*, *26*). Fe(*o,o*-EDDHA) solutions were used as calibration standards.

The iron uptake by plants was calculated as the difference between the loss of iron in nutrient solution of the samples and the no-plant control.

pH Analysis. pH values of the nutrient solution samples were measured with a pH-meter (Crison micro pH 2000) with a standard pH electrode.

Statistical Analysis. Data of iron uptake by plants were fitted to eq a by using the SPSS statistical software to establish the Fe uptake process by plants:

$$\text{mol of Fe} = (\text{mol of Fe})_{eq}\left(\frac{t^b}{(\tau_{1/2})^b + t^b}\right) \qquad \text{(a)}$$

The parameter mol of Fe is the Fe uptake by plants (mol), t is the time of culture in days, $(\text{mol of Fe})_{eq}$ is the Fe uptake (mol) by plants at equilibrium ($t = \infty$), $\tau_{1/2}$ is the time (days) that the reaction takes to reach half of the $(\text{mol of Fe})_{eq}$ value, and b is an undimensional constant that allowed us to optimize the iron uptake evolution.

To establish the rate of Fe uptake by plants, the derivative of eq a was obtained and the rate of Fe uptake at $t = \tau_{1/2}$ calculated using the equation

$$V_{\tau_{1/2}} = \frac{(\text{mol of Fe})_{eq} b}{4\tau_{1/2}} \qquad \text{(b)}$$

In some instances, two different stages in the process of Fe uptake were observed and the sum of two equations of type a better fit the experimental data.

RESULTS AND DISCUSSION

Strategy I Plants. The quantity of Fe taken up by the strategy I plants is plotted in **Figure 1**. These experimental data were fitted to the appropriate equation as was stated under Materials and Methods to calculate the kinetic parameters that described the Fe uptake process by strategy I plants (**Table 2**).

Tomato and pepper plants took up preferably the iron from the meso form (the less stable isomer) (**Figure 1A**,**B** and **Table 2**). Tomato plants were able to take Fe from both isomeric forms in a unique stage (**Figure 1A**). Although the Fe uptake from the meso isomer was 2-fold the Fe uptake from the racemic isomer (**Table 2**), the values of $V_{\tau_{1/2}}$ and $\tau_{1/2}$ were similar for both isomers (**Table 2**). After 20 days of cultivation, the pH values decrease sharply (**Figure 2A**). It shows the activation of the proton pump as a result of the Fe deficiency; however, no further Fe uptake was observed. Although the meso Fe(*o,o*-EDDHA) form is the less stable isomer and it is especially sensitive to low pH values (*27*), this isomer was not affected after day 20 because the pH varied only between 6 and 7 (*27*).

Unlike tomato plants, pepper plants took up only the less stable isomer (the meso form) because the uptake of the racemic form was undetectable (**Figure 1B**). The iron uptake by pepper took place in two stages. The first stage lasts 42 days. After that, a sharp increase in the iron uptake occurred (**Figure 1B**). The rate of Fe uptake in the second stage was higher than in the first one (**Table 2**). It shows a higher requirement of Fe during the second stage of the cultivation. Probably, the starting of flowering and ripening period promoted these two stages in iron uptake (*28*). Like for tomato plants, a decrease in the pH value can be observed during the second stage (**Figure 2A**) because of the activation of the proton pump under iron deficiency situation. This deficiency results from the decrease of iron in the nutrient solution according to the higher iron requirements during the ripening.

Strategy II Plants. Figure 1 and **Table 2** show the evolution of the Fe uptake and the kinetic parameters that describe the Fe uptake process by strategy II plants, respectively. There were no statistically significant differences in the iron uptake from both isomers (**Figure 1C**,**D** and **Table 2**). The iron absorption also took place in two stages (**Figure 1C**,**D** and **Table 2**). Unlike pepper, a small increase in the pH value can be observed (**Figure 2B**) because Fe deficiency mechanisms developed by this kind of plant do not include H^+ release. **Table 2** shows that the quantities of iron consumed by wheat and oat plants in the first and second stages were similar, so the iron requirement was no different between both stages. Moreover, the rate of Fe uptake in the second stage was also statistically similar to the rate in the first stage (**Table 2**).

The observed differences between strategy I and II plants can be explained on the basis of the different mechanisms of iron uptake of each plant species. The Fe deficiency mechanism developed by strategy I plants [Fe(III) reduction and H^+ release] (*5*), the lesser stability of the meso isomer (*23*), and the higher sensibility to low pH values of this isomer (*27*) would implicate a lower energy consumption in the Fe uptake for the meso Fe(*o,o*-EDDHA) than for the *d,l*-racemic Fe(*o,o*-EDDHA). As a result, there is a preferential uptake of Fe from the meso isomer by these plants. However, the behavior of strategy II plants

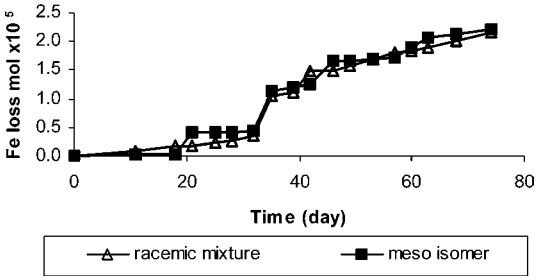

Figure 3. Loss of iron in the nutrient solution of no-plant control.

showed that phytosiderophores could compete with both *o*,*o*-EDDHA isomeric forms for iron in the nutrient solution, so there were no differences in Fe uptake from Fe(*o*,*o*-EDDHA) isomers for strategy II plants.

Solutions of *o*,*o*-EDDHA contain ~50% of each isomer and remain unaltered for a long time (*29*). Losses of Fe(*o*,*o*-EDDHA) in the nutrient solution in the absence of plants were observed along the time of the experiment, and the relative quantities of each isomer were constant and near 50% (**Figure 3**). If the meso Fe(*o*,*o*-EDDHA) isomer is preferably decomposed or taken up by plants, there would be free meso (*o*,*o*-EDDHA) chelating agent in solution, and the only possibility would be the transformation of the racemic form into the meso form to restore equilibria. The same is valid if the racemic form is preferably decomposed. If the transformation is quick enough, 50% of racemic and meso forms will be always observed and it will be difficult to say if either isomer is equally decomposed (or equally taken up by plants as occurs in strategy II plants) or if one of them is preferred and the system quickly evolves to solutions with equivalent quantities of both isomers. However, the behavior of the chelate in the solutions containing strategy I plants seems to indicate that the transformation of racemic into meso forms is slow enough to allow an imbalance between the two forms, which can be explained only by a direct effect of the plant on the Fe uptake; in conclusion, the only explanation of the observed behavior is that strategy I plants took preferably the meso form and that strategy II plants took Fe from both isomers.

The results suggest that the use of Fe(*o*,*o*-EDDHA) products with a higher percentage of meso isomer could be more efficient, at least in hydroponics for strategy I plants, than the use of the present products containing 50% of each isomer.

ABBREVIATIONS USED

o,*o*-EDDHA, ethylenediamine-*N*,*N*′-bis(*o*-hydroxyphenylacetic) acid; *o*,*o*-EDDHMA, *N*,*N*′-ethylenediaminedi(*o*-hydroxy-*p*-methilphenylacetic) acid; *o*,*o*-EDDHSA, *N*,*N*′-ethylenediaminedi(*o*-hydroxy-*p*-sulfoxyphenylacetic) acid; Fe(*o*,*o*-EDDHA), ferric *o*,*o*-EDDHA chelate; HPLC, high-performance liquid chromatography.

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