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. For years, the absorption and translocation of Fe in the plant from Fe fertilizers have been studied using different approaches. Many authors suggest the use of radioactive isotopes such as ^{59}Fe (Cesco et al. 2002). However, the use of this isotope requires specific laboratory facilities and trained personnel and does not allow long-term trials, because the isotope activity drops over time. Mössbauer spectroscopy, using the stable isotope ^{57}Fe has also been used in the study of the Fe chemistry in plants (Kovacs et al. 2009), but the concentration of Fe in the plant tissues is usually too low to be detected by this technique. Rodríguez-Castrillón et al. (2008) have developed a new method for studying ^{57}Fe in plant tissues using ICP-MS (Inductively Coupled Plasma Mass Spectrometry) and isotope pattern deconvolution. The main advantage of this technique relies in the high precision and the low detection limits.
The aim of this work is to study the advantages of the use of the stable isotope ^{57}Fe to test the efficacy of synthetic Fe chelates. More specifically, (1) to test whether isotopic exchange occurs between native Fe (mainly ^{56}Fe) from soil and ^{57}Fe from the chelates and (2) to study the Fe absorption by plants treated with Fe chelates enriched with ^{57}Fe.

Materials and Methods

Isotopic exchange between chelates and soil

Prior to the biological experiment, an interaction experiment with ^{57}Fe chelates was performed to test whether isotopic exchange occurs between native Fe (mainly ^{56}Fe) from soil and ^{57}Fe from the chelates. A sandy clay and calcareous soil (pH in H_2O 7.70, O.M. 9.2 g Kg^{-1}, total $CaCO_3$ 380 g Kg^{-1}, active lime 89 g Kg^{-1}) from Picassent (Valencia, Spain) was used as interaction substrate and later in the pot experiments. The $^{57}Fe^{3+}$ chelates prepared as in Rodríguez-Castrillón et al, 2008) with a 2% excess of chelating agent were used. The chelating agents (see figure 1) were: *o,o*EDDHA (ethylenediamine-N, N´bis(*o*-hydroxy-phenylacetic) acid) (Promochem, Barcelona, Spain), *o,p*EDDHA (ethylenediamine- N(*o*-hydroxy-phenylacetic) – N´(*p*-hydroxyphenylacetic) acid) (Syngenta Crop Protection, Basilea, Switzerland); HJB (N,N'-bis(2-hydroxy-5-methylbenzyl) ethylenediamine-N,N'-diacetic acid) (PCC ADOB, Poznan, Poland) and DCHA (2-(2-((2-hydroxy benzyl)amino) ethylamino)-2-(2-hydroxyphenyl)acetic) synthesized by Dr. Sierra group (UCM, Madrid, Spain). Fe was added as ^{57}Fe (Isoflex, San Francisco, USA) (95.38 %) that was dissolved in HNO_3 Suprapur (Merck, Darmstadt, Germany). For the interaction experiment, 2.0 g of soil and 5 mL of each chelate solution containing 4.0 x 10^{-4} M of chelate and 5 mL type I water were used. Chelate (without soil) and soil blanks (without chelate but with 10 mL water) were also prepared. All samples were shaken at 56 cycles min^{-1} at 25ºC in the dark, for 1, 3 and 7 h, and 1, 3, 7 and 30 days. After the incubation time, solutions were filtered through 0.45 µm PVDF Millipore membranes. Samples for each time point and product tested were prepared in duplicate. Soluble Fe concentration was determined by AAS (Atomic Absorption Spectroscopy) and ^{57}Fe by ICP-MS (Inductively coupled plasma mass spectroscopy) using ^{57}Fe standards and correcting Ca and Ar interferences by using a collision cell quadrupole ICP-MS instrument (Varian 820), after acidification of the samples with HNO_3

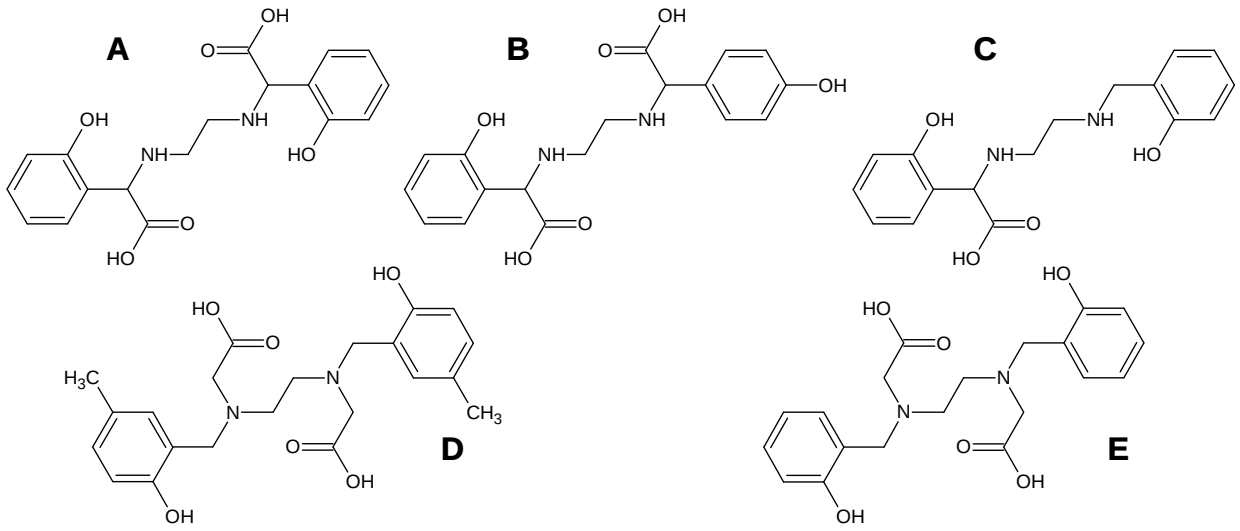

Figure 1. Chelating agents used in the experiments. **A**: *o,o*EDDHA; **B**, *o,p*EDDHA, **C**: DCHA, **D**: HJB and **E**: HBED

Suprapur (Merck).

Biological experiment

For the biological experiments, soybean seeds (*Glycine max* L. cv 'Klaxon') were used. Seedlings were transplanted to one L pots (three plants per pot) filled with the same soil as in the interaction experiment and a 975 g Kg^{-1} $CaCO_3$, 1-3 mm size sand in 2:1 v:v mixture. The experiments were carried out in a growth chamber (day: 16 hours, 30ºC and 50% RH; night: 8 hours, 25ºC and 70% RH). Pots were initially irrigated to 80% field capacity and then daily with the amount of solution necessary, determined by weight loss, to achieve again 80% field capacity. Irrigation was made with a macronutrient nutrient solution (2 times concentrate) with 0.1 g L^{-1} lime and 0.1 g L^{-1} sodium bicarbonate. Trays were put under the pots to avoid leaching. Two experiments were done following the same procedure. The first experiment consisted in 4 treatments: *o,o*EDDHA/$^{57}Fe^{3+}$, *o,p*EDDHA/$^{57}Fe^{3+}$, HJB/$^{57}Fe^{3+}$ and DCHA/$^{57}Fe^{3+}$ and one control without Fe. Five replicate pots per treatment and three sampling times, at 2, 7 and 21 days after the treatment (1 plant per sampling time), were used. The concentration of ^{57}Fe in the treatments was 45 µmol ^{57}Fe Kg^{-1} of soil. In the second assay the concentrations of ^{57}Fe in the treatments were 0, 1.7, 3.4, 8.4, 16.8, 25.1 and 41.9 µmol ^{57}Fe Kg^{-1} of soil in the form of *o,o*EDDHA/$^{57}Fe^{3+}$ or HBED/$^{57}Fe^{3+}$ (chelating agent N,N'-bis(2-hydroxybenzyl)ethylenediamine-N,N'-diacetic acid, see figure 1, Strem Chemicals, Orwell, England). Two and one plants were sampled at 7 and 21 days after the treatment, respectively.

SPAD index was determined in all the leaf levels every two days. Leaves and stems were separated and washed, first with Tween 80 in 0.1 M HCl for 30 s (Álvarez-Fernández et al, 2001), and then with abundant distilled water, weighted and dried. Total Fe and ^{57}Fe were determined in leaves and roots after dry digestion by AAS and ICP-MS, respectively.

Results and Discussion

Isotopic exchange between chelates and soil

Table 1 shows the percentage of ^{57}Fe with respect to the total soluble Fe (mol/mol) in solution after different periods in the interaction assay. It can be observed that isotopic exchange in these soil conditions was quite slow. Only 2-4 % of the Fe was exchanged in 7 d for the strong chelates HJB/$^{57}Fe^{3+}$ and *o,o*EDDHA/$^{57}Fe^{3+}$, while approximately 10% was exchanged for *o,p*EDDHA/$^{57}Fe^{3+}$ and DCHA/$^{57}Fe^{3+}$. After 30 days ^{57}Fe was still the isotope that contributed most to the total Fe in soil solution. Hill-Cottingham and Lloyd-Jones (1958) also observed a minimal isotopic exchange when o,oEDDHA/Fe^{3+}chelate were prepared using radioactive ^{59}Fe isotope and interaction was made for 15 days with a 28% lime soil. From these results, it can be inferred that when the isotope ^{57}Fe is applied in biological experiments using calcareous soils, its quantification in the plant may be used to confirm the origin of the Fe source.

		Incubation Time			
	Initial	**1 d**	**3 d**	**7 d**	**30 d**
HJB/$^{57}Fe^{3+}$	87.6	85.1	85.1	83.7	79.9
***o,o*EDDHA/$^{57}Fe^{3+}$**	87.4	87.5	85.7	85.0	80.5
***o,p*EDDHA/$^{57}Fe^{3+}$**	87.0	83.1	81.0	77.0	64.9
DCHA/$^{57}Fe^{3+}$	86.8	81.6	81.0	76.1	72.1

Table 1: Percentage of ^{57}Fe with respect to the total amount of Fe in solution after the interaction of the chelates with the calcareous soil in the interaction experiment. "Initial" stands for the average ratio in the chelate solutions used.

The results of the interaction experiment also gives information of the reactivity of the chelates in the soil conditions. The percentage of ^{57}Fe remaining in solution after 30 days of interaction was very high for *o,o*EDDHA/$^{57}Fe^{3+}$ (similar to the observations of Hill-Cottingham and Lloyd-Jones, 1958) and HJB/$^{57}Fe^{3+}$ (95-99 % of that added) indicating that there was no or very little displacement of the Fe from the chelate or sorption reactions. However, the percentage of ^{57}Fe remaining in solution after 30 days of interaction was approximately 25% and 50% of the initial values for *o,p*EDDHA/$^{57}Fe^{3+}$ and DCHA/$^{57}Fe^{3+}$, respectively, indicating that, besides the slight isotope exchange, some displacement or sorption had occurred.

Biological experiments

All treated plants except the control showed a good recovery from chlorosis according to the visual observations and SPAD readings (see figure 2). The time course of the changes in the ratios (Fe in treated plants/untreated Fe control plants) for the total Fe and ^{57}Fe in leaves at the three sampling times in the first experiment are shown in Table 2.

The use of the isotope ^{57}Fe allows studying the absorption pattern from the chelate, being larger in the early stages (first sampling time). Besides, the increases in the absorption with respect to the control plants are more marked using ^{57}Fe measurements than when using total Fe.

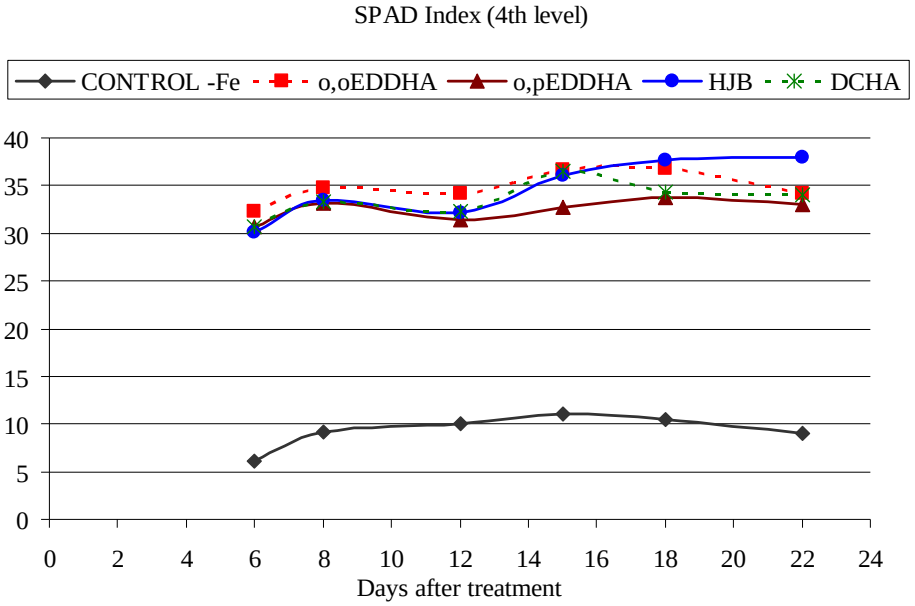

Figure 2: SPAD index in the forth leaf level along the 1st experiment.

	Total Fe			^{57}Fe		
	3 d	**7 d**	**21 d**	**3 d**	**7 d**	**21 d**
HJB/$^{57}Fe^{3+}$	3	2	3	23	20	14
***o,o*EDDHA/$^{57}Fe^{3+}$**	8	6	5	101	68	29
***o,p*EDDHA/$^{57}Fe^{3+}$**	4	3	2	41	26	8
DCHA/$^{57}Fe^{3+}$	5	3	2	79	31	10

Table 2: Time course of the changes in the ratios (Fe in treated plants/Fe control plants) for the total Fe and ^{57}Fe in leaves in the 1st experiment.

Therefore, the use of the stable isotope ^{57}Fe and the growing technique described in this work has the advantage that allows better observing differences among treatments.

Clear differences between the Fe supply with any of the chelates and the control were observed. García-Marco et al. (2006) observed a fast effect of *o,p*EDDHA/Fe^{3+} to provide Fe to plants. However, Rojas et al (2008) concluded that *o,p*EDDHA/$^{57}Fe^{3+}$ was inefficient to provide ^{57}Fe to tomato and peach seedlings grown on a calcareous soils. Our results, both the measurement of Fe in leaves (data not shown) as the measurement of the SPAD index in the leaves (Figure 2) clearly indicate that Fe-deficient soybean plants treated with *o,p*EDDHA/$^{57}Fe^{3+}$ absorbed an adequate amount of Fe, although it was less than that taken up by plants treated with *o,o*EDDHA/$^{57}Fe^{3+}$. Rojas et al. (2008) did not test isotopic exchange processes in the soil that could affect their results. Recently, Orera et al (2009) have shown that isotopic exchange processes may occur within the plant. DCHA/$^{57}Fe^{3+}$ is a new chelate (Sierra et al, 2008) with intermediate structural features between *o,o*EDDHA/$^{57}Fe^{3+}$ and *o,p*EDDHA/$^{57}Fe^{3+}$, containing 5 bonds between the Fe and the chelating agent as in *o,p*EDDHA/$^{57}Fe^{3+}$, two of them between phenolates and Fe as in *o,o*EDDHA/$^{57}Fe^{3+}$. This intermediate behavior was clearly observed in the ^{57}Fe results. HJB/$^{57}Fe^{3+}$ showed a intermediate stability between those of *meso* and *racemic* regiosisomers of

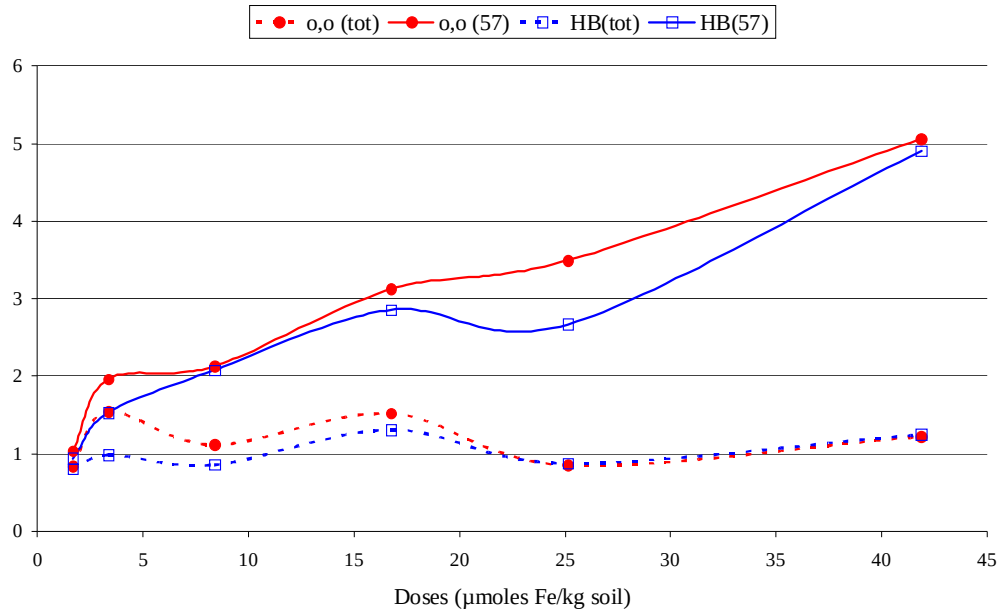

Figure 3: Changes in the ratios (Fe in treated plants/Fe control plants) for the total Fe and ^{57}Fe in roots, with respect to Fe fertilizer doses, at the end of the second experiment.

*o,o*EDDHA/$^{57}Fe^{3+}$, but its behavior was different. At the beginning, it was the chelate that provided less ^{57}Fe, but plants maintained a more constant usage of the chelate with time.

A second biological experiment, using soybean plants grown in calcareous soils and treated with different doses of *o,o*EDDHA/$^{57}Fe^{3+}$ and HBED/$^{57}Fe^{3+}$, was carried out. In leaves the amount of ^{57}Fe in the control was negligible, but all the treated plants contained significant amounts of ^{57}Fe, related to the fertilizer doses and similar for *o,o*EDDHA/$^{57}Fe^{3+}$ and HBED/$^{57}Fe^{3+}$. However, in these conditions the interpretation of the data using total Fe was similar to that using ^{57}Fe data, especially at high doses. Generally, total Fe content in roots is not a good index of the Fe nutrition, since apoplastic precipitation occurs and high Fe concentrations are usually observed. In Figure 3 the variation of the ratios (Fe in treated plants/Fe control plants) for the total Fe and ^{57}Fe in roots with respect to Fe fertilizer doses at the end of the second experiment is shown. As in the previous experiment, the increase in Fe concentration (including apoplastic) from the chelate with respect to the control plants was observed with ^{57}Fe, whereas it was not possible, as expected, to observe differences considering the total Fe concentrations. HBED/Fe^{3+} has been considered as a chelate too stable to be used in agriculture. However, the results here presented indicate a good potential use of this chelate in agreement with Chaney (1988) who concluded that in hydroponic cultures HBED/Fe^{3+} supplied an adequate Fe for growth of non-graminaceae species at pH 7.5

Conclusions

With the results obtained in this work, we can conclude that the use of the stable isotope ^{57}Fe in biological experiments using calcareous soils can be useful to assess the efficacy of new Fe

chelates, since the Fe nutrition can be ascribed to the Fe source. Moreover, the use of this technique will allow more information to be obtained on Fe mobility in the soil, the use of Fe by roots, the chelate capacity to take Fe from the soil and root apoplastic Fe pool (shuttle effect), the translocation of Fe in the plants, etc.

Acknowledgements

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