RESPONSE OF SOYBEAN PLANTS TO THE APPLICATION OF SYNTHETIC AND BIODEGRADABLE Fe CHELATES AND Fe COMPLEXES

Short running tittle: Response of soybeans to Fe chelates and complexes.

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ABBREVIATIONS

EDDHA/Fe^{3+}: Ethylenediamine-N,N'-bis(hydroxyphenylacetic) acid

*o,o*EDDHA: Ethylenediamine-N,N′-bis(*o*-hydroxyphenylacetic) acid

EDTA: Ethylenediaminetetraacetic acid

EDDS: Ethylenediaminedisuccinic acid

IDHA: N-(1,2-dicarboxyethyl)-D,L-aspartic acid

LS: Lignosulfonate

LN: Leonardite

GA: Gluconate

FMM: FlouroMeter Modul

SPAD: Soil and Plant Analyzer Development

FCR: Ferric chelate reductase

ICP-MS: Inductively coupled plasma mass spectrometry

BPDS: Bathophenanthroline disulfonic acid

DTPA: Diethylenetriaminepentaacetic acid

NS: Nutrient solution

DAT: Days after treatments

ABSTRACT

The growing concern over the environmental risk of synthetic chelate application promotes the search for alternatives in Fe fertilization, such as biodegradable chelating agents and natural complexing agents. In this work, plant responses to the application of several Fe treatments (chelates and complexes) was analyzed to study their potential use in Fe fertilization under calcareous conditions. Thus, the root ferric chelate reductase (FCR) activity of soybean (*Glycine max* cv. Klaxon) plants was determined, and the effectiveness of the Fe chelates and complexes assessed in a pot experiment, by SPAD and fluorescence induction measurements, and the determination of Fe distribution in plant and soil. Additionally, ^{57}Fe Mössbauer spectroscopy was conducted to identify the Fe forms present in the soybean roots. The highest FCR activity was observed for the chelates EDDS/Fe^{3+} and IDHA/Fe^{3+}; while no activity was observed when using complexes as Fe substrates. In contrast to the FCR data, the pot experiment confirmed that the *o,o*EDDHA/Fe^{3+} is the most effective treatment, and the complexes LS/Fe^{3+} and GA/Fe^{3+} are able to alleviate Fe chlorosis, also indicated by SPAD data and the maximal quantum efficiency of photosystem II reaction centers as vitality parameters, and the enhanced plant uptake of Fe from natural sources.

1. INTRODUCTION

Iron (Fe) chlorosis is a widespread agricultural concern, especially in crops grown in calcareous soils, where calcium carbonate buffers soil solution pH in a range of 7.5-8.5 (Lindsay and Schwab, 1982) and high bicarbonate concentration is present (Lucena, 2000). It is presented by a yellowing of the leaves, due to the decrease of the concentration of photosynthetic pigments, especially chlorophylls (Chl; Abadía and Abadía, 1993). Plants are differentiated in Strategy I (dicotyledonous and non-grass monocotyledonous) and Strategy II (grasses) depending on their mechanism for Fe uptake (Abadía et al., 2011). Strategy I involves the activation of a ferric chelate reductase (FCR) enzyme in roots to reduce Fe(III) to Fe(II). This reductase activity of plants is affected by the stability constant of the chelate and the number of functional groups biding Fe (Lucena, 2006) as well as the polarity of the complexing molecule (Escudero et al., 2012).

Fe deficiency is a problem that affects the productivity and quality of crops. Several types of fertilizers are used with diverse success in correcting iron chlorosis. Inorganic salts, such as $FeSO_4$ are normally of scarce effectivity. The ferrous phosphate Vivianite (Rosado et al, 2002) or the stabilized polyphosphates (Chandra et al., 2009) have a slow release behavior. Recently other nanoparticle materials such as the nonosponges are being studied (Vercelli et al., 2015). However, for strategy I plants the Fe chelates and complexes are the fertilizers that provide a fast recovery.

Studies about the efficacy of Fe chelates and complexes should focus not only in economics but also in environmental costs.

Traditionally, EDDHA/Fe^{3+} (ethylenediamine-N,N'-bis(hydroxyphenylacetic) acid) (Figure 1) based products have been used to control and solve this problem (Álvarez-Fernández et al., 2005; Lucena, 2006). These chelates have a high stability in calcareous soils, being able to maintain Fe in the soil solution and transport it to the plant roots (López-Rayo et al., 2009). However, there has been growing concern about the environmental risk of synthetic chelate application (Hyvönen et al, 2003); thus, other chelating agents, such as EDDS (ethylenediaminedisuccinic acid) or IDHA (N-(1,2-dicarboxyethyl)-D,L-aspartic acid) (Figure 1), have been tested as a biodegradable alternative to the use of recalcitrant products (Villén et al., 2007; Lucena et al., 2008; Rodríguez-Lucena et al., 2010). Both EDDS/Fe^{3+} and IDHA/Fe^{3+} are non-phenolic chelates structurally similar to EDTA/Fe^{3+} (ethylenediamine tetraacetic acid) (Figure 1).

Other Fe complexes include a large number of substances from different origins, such as industry byproducts (e.g., lignosulfonates, LS), and are considered as a sustainable alternative in Fe fertilization. Despite those Fe complexes have ~~having~~ lower efficacies than the synthetic chelates (Goos et al., 2001; Rodríguez-Lucena et al., 2010), they are cheaper and biodegradable, and can be used in a larger number of crops. EU Directive 2003/2003 includes the list of chelating agents authorized as Fe fertilizers, being amended by the 223/2012 to include lignosulfonates, and recently by the 1618/2016 to include heptagluconate as complexing agents. Other complexing agents, such as humic and fulvic acids, exagluconate (GA), amino acids and citrate are recognized by Spanish legislation (RD 824/2005; Orden APA 863/2008).

When assessing the ability of chelates or complexes as Fe fertilizers, the measurement of the root FCR activity provides an approximation about the plant response to the different Fe products; but the analysis of Fe in plant tissues is essential to determine the actual Fe uptake. However, the Fe concentration in leaves does not

always correspond to the Fe nutritional status of the plant, due to the "chlorosis paradox" (Morales et al., 1998), and therefore to the effectiveness of Fe fertilizers. A good way to complement the information given by the leaf Fe analysis is to follow the evolution of leaf Chl after Fe treatments application (El-Jendoubi et al, 2011)

The SPAD (Soil and Plant Analyzer Development) hand-held devices measure the transmittance through the leaf at two wavelengths (650 and 940 nm), making an estimation of the Chl *a* + *b* content of the leaf. However, the relationship between SPAD and Chl content is non-linear (Uddling et al., 2007). Additionally, the use of Chl *a* fluorescence measurements in algae and plants is widespread in physiological and ecophysiological studies (Baker, 2008). In the present paper, the instrument developed by Barócsi et al. (2009), a Chl *a* fluorometer (FlouroMeter Modul, FMM), was used. The FMM allows the measurement of traditional Kautsky induction kinetic curves detected simultaneously at the two maxima of the Chl *a* fluorescence in leaves (at the 690 nm red and the 735 nm far-red bands).

The aim of this work is to test sustainable alternatives to the use of *o,o*EDDHA/Fe^{3+} in Fe fertilization. Thus, the root FCR activity of soybean (*Glycine max* cv. Klaxon) plants was determined, and ^{57}Fe Mössbauer spectroscopy was conducted to identify the Fe forms present in the soybean roots. Moreover, the efficacy of several synthetic and biodegradable Fe chelates and Fe complexes was assessed in a pot experiment with calcareous soil, by measuring physiological parameters that correspond to the vitality of the plants and the determination of Fe distribution in plant tissues and soil fractions.

2. MATERIALS AND METHODS

2.1. Preparation of Fe chelates and complexes.

The synthetic Fe(III) chelates containing *o,o*EDDHA, IDHA, EDTA and EDDS, and the Fe(III) complexes Gluconate (GA), Eucalyptus or Spruce Lignosulfonate (LS) and Leonardite (LN) were used in the following experiments.

The general procedure for the preparation of the chelates and complexes was a modification of that described by Lucena et al 1996, to assure the fast and complete chelate or complex formation in solution.

In brief, for the preparation of the Fe chelate solutions, ligands calculated to be 2% in excess of the molar amount of Fe were dissolved, when needed, in sufficient NaOH (1:3 molar ratio). For the pot experiment, an amount of ^{57}Fe (96.66%; Isoflex, Moscow, Russian Federation), previously dissolved in HNO_3 Suprapur (Merck, Germany), was slowly added. In the case of the FCR assay, $Fe(NO_3)_3$ (Merck, Germany) was used. During the chelation process, pH was maintained between 6 and 8, and adjusted to 7 at the end. Solutions were left to stand overnight to allow excess Fe to precipitate. After that period, solutions were filtered through a 0.45 µm Millipore membrane, and made up to volume with type I grade water free of organic contaminants (Millipore, Mildfor, USA). Light exposure was avoided during the preparation and storage of the chelate solutions because of the potential photodecomposition of chelates (Hill-Cottingham, 1955, Hernández-Apaolaza et al., 2011).

For the preparation of the Fe complex solutions, firstly the complexing agent calculated to be 10% in excess of the molar amount of Fe was dissolved in water and pH adjusted to 7 with NaOH. Then, an amount of ^{57}Fe or $Fe(NO_3)_3$ (as for the preparation of chelates) was added and the solution was stirred for 30 minutes. After that, pH was readjusted to 7. The final solution was left to stand overnight and the pH of the solutions was readjusted to 7 and made up to volume with type I grade water.

2.2. Root FCR activity

2.2.1. Plant material

For testing the FCR activity soybean (*Glycine max* cv. Klaxon) seeds were germinated for 3 days at 30°C. Seedlings of similar development were transferred individually to 500 mL plastic pots filled with 1/5 diluted nutrient solution (NS) for 4 days, and then, 7 more days in full-strength. Plants were grown in Fe deficiency with 5 μM of EDTA/Fe^{3+}. The composition of full-strength NS was: macronutrients (mM) 1 $Ca(NO_3)_2$, 0.9 KNO_3, 0.3 $MgSO_4$, 0.1 KH_2PO_4; micronutrients (μM) 35 NaCl, 10 H_3BO_3, 0.05 Na_2MoO_4, 2.5 $MnSO_4$, 1 $CuSO_4$, 10 $ZnSO_4$, 1 $NiCl_2$, 1 $CoSO_4$, 115.5 ~~μM~~ Na_2EDTA. In order to simulate calcareous conditions, 0.1 g/L $CaCO_3$ was added to the NS, and pH was buffered with HEPES 0.1 mM.

2.2.2. Enzyme assay

For the FCR activity test, the roots were washed in distilled water and macronutrient solution also containing 37.5 μM of Na_2BPDS. Plants with intact roots were individually placed in 120 mL macronutrients solution with 300 μM of Na_2BPDS and MES 2 mM. According to Lucena and Chaney (2006), the FCR measurement was performed at 2 hours of daylight cycle and pH 6. At time zero, the Fe treatments (Fe chelates: *o,o*EDDHA/Fe^{3+}, EDTA/Fe^{3+}, IDHA/Fe^{3+}, and EDDS/Fe^{3+}; and Fe complexes: Spruce and Eucalyptus LS/Fe^{3+}, LN/Fe^{3+} and GA/Fe^{3+}) were added to achieve a final Fe concentration of 100 μM. Samples were taken at 0, 10, 20 and 60 minutes, in 6 replicates for each chelate or complex. Two blanks per chelate or complex without plants were performed. The fresh weight (FW) of roots was determined at the end of the experiment.

Reduction of Fe(III) chelate was measured spectrophotometrically with the ferrous color reagent BPDS at 535 nm, and also 480 and 600 nm to consider the absorbance contribution of the Fe fertilizers used (see equation for EDDHA/Fe^{3+} in Lucena and Chaney, 2006). The reductase activity (Fe(III) reduction rate) is expressed in terms of Fe(II) nmol g^{-1} root FW min^{-1} units.

2.3. ^{57}Fe Mössbauer spectroscopy of the roots

2.3.1. Plant material

To identify the Fe forms present in the soybean roots, plants were grown in hydroponics in Fe deficient (calcareous) conditions as in 2.2.1. Then the roots were washed in distilled water and macronutrient solution also containing 37.5 µM of Na_2BPDS. Plants with intact roots were individually placed in 120 mL macronutrients solution with MES 2 mM. After two hours, they were supplied with 100 µM ^{57}Fe-spruce LS and ^{57}Fe-GA for 30 min. Then roots were cut and immediately frozen in liquid nitrogen.

2.3.2. ^{57}Fe Mössbauer spectroscopy

The roots of soybean plants supplied with GA/$^{57}Fe^{3+}$ and Spruce LS/$^{57}Fe^{3+}$ were freeze-dried, then measured with Mössbauer spectroscopy at 80 K. The ^{57}Fe Mössbauer measurements were recorded with a conventional Mössbauer spectrometer (WISSEL) operating in the constant acceleration mode and equipped with a 3×10^{9} Bq ^{57}Co/Rh source. Samples were kept in a helium cryostat (JANIS SVT-400-MOSS) filled with liquid nitrogen. The ^{57}Fe isomer shifts are given relative to α-iron at room temperature.

The Mössbauer spectra were evaluated by standard computer-based statistical analysis methods that included fitting the experimental data to a sum of Lorentzians

using a least-squares minimisation procedure for χ^2 with the help of the MOSSWINN program (Klencsár et al. 1996).

2.4. Pot experiment.

2.4.1. Plant material, nutrient solution and treatment application.

The pot experiment was conducted in a research growth chamber model CCKF 0/16985, with a daily growth cycle of day: 16h, 28°C and 40% relative humidity (RH); and night: 8h, 20°C and 60% RH. Soybean seeds, initially washed for 30 minutes in distilled water and 2% of H_2O_2, were germinated for 4 days at 28 °C in darkness and placed in trays between cellulose paper sheets soaked with distilled water. Seedlings of similar development were placed on a holed plate floating over containers filled with 5 L of 1/5 diluted NS, for 4 days containing 5 μM EDTA/Fe^{3+}, and then 5 more days under Fe starvation, to induce Fe chlorosis in plants. The composition of full-strength NS was the same as in the FCR experiment. The pH was buffered with HEPES 0.1 mM and adjusted to 7.5 with KOH 1.0 M.

After the pregrowth period in hydroponics, seedlings were individually transplanted to single pots (9 cm diameter, 28 cm high methacrylate cylinders) containing 2 Kg of a mixture 70:30 soil:sand (w:w). The soil was a sandy loam soil from Picassent (Valencia, Spain; described in Table 1), and mixed with calcareous sand (97.5% $CaCO_3$; 2-4 mm). Pots were covered with aluminum foil and black plastic on top to avoid algae development and iron chelates photodegradation (Hill-Cottingham, 1955; Hernández-Apaolaza et al., 2011). Two days prior to transplanting, pots were irrigated to achieve the 80% of the soil water holding capacity (WHC), and then, daily irrigated to keep the 80% WHC by weighting control with a macronutrient full NS with

221 0.1 g L^{-1} of $CaCO_3$ (pH 7.5-8), to simulate carbonate irrigation waters as those used in

222 agronomic conditions.

223

When plants showed chlorosis symptoms (visual observation and SPAD values

224 under 23 at the second leaf stage, 5 days after transplanting), treatments were applied in

225 a single dose to pots in 5 replicates per sampling time (in total, 10 pots per treatment).

226 Two kinds of Control plants were considered: plants without Fe addition (C –Fe), and

227 plants with a high dose of *o,o*EDDHA/$^{57}Fe^{3+}$ (C +Fe). Doses applied were 10 µmol Fe^{3+}

228 Kg^{-1} soil for *o,o*EDDHA/$^{57}Fe^{3+}$, IDHA/$^{57}Fe^{3+}$, LS/$^{57}Fe^{3+}$ and GA/$^{57}Fe^{3+}$; and 46 µmol

229 Fe^{3+} Kg^{-1} soil for C +Fe plants. The Fe treatment dose of C +Fe plants was applied

230 twice, at 2 and 5 days after transplanting, to keep the plants in a good Fe nutritional

231 status.

232 ***2.4.2.*** *Recovery tests*

233 To follow the recovery of Fe chlorosis, SPAD and Chl *a* fluorescence

234 induction measurements were done. SPAD index was determined twice a week in all

235 leaf stages (5 replicates per treatment and sampling time) using a SPAD Chlorophyll

236 meter Minolta 502 (Osaka, Japan). The adaxial side of the leaves was always placed

237 toward the emitting window of the instrument and major veins were avoided. The Chl *a*

238 fluorescence induction measurements of the 3rd leaf stage were performed 4 days after

239 treatment applications (DAT), using a FMM Chl *a* fluorometer (Budapest University of

240 Technology and Economics, Department of Atomic Physics, Barócsi et al. 2009). The

241 internal light source is a 635 nm laser diode (QL63H5SA, Roithner Lasertechnik

242 GmbH, Wien, Austria) with 20 mW maximum optical power. Kautsky kinetics was

243 monitored following a 5 min dark adaptation under 5 min excitation time at 690 nm and

244 735 nm by selective filters, where the minimal (F_0'), maximal (F_m) and steady-state (F_s)

fluorescence values were recognized at both wavelengths. Maximal quantum efficiency of the photosystem II (PSII) reaction centers was calculated as $F_v/F_m=(F_m-F_0')/F_m$ at both emission maxima. Red to far-red Chl *a* fluorescence ratios (F690/F735) were calculated from F_s values.

2.4.3. *Plant and soil Fe analysis*

Plant and soil material were sampled 7 and 21 DAT. At each sampling time, five pots per treatment, including both C +Fe and C –Fe plants, were taken. Leaves, stems, roots and flowers were separated and washed with Tween 80 in 0.1 M HCl for 30 seconds (Álvarez-Fernández et al., 2001), and then with water and distilled water. After drying for 3 days at 65°C, dry weight (DW) was determined. Plant samples were crushed using a titanium mill (Retsch ZM200) and ashed in a muffle furnace at 480 °C for 2 hours. Mineral concentration was analyzed after acid digestion with HNO_3 for ash solubilization (Jones, 2001).

After root removal, the concentration of soluble and plant available Fe remaining in soil was also determined. The pot content was separated in two portions, in relation to the height of the pot. Each portion was weighted and mixed with 1 L of distilled water, and shaken at 90 cycles min^{-1} for 10 minutes in an orbital shaker. Then, 40 mL of the substrate-water mix was centrifuged at 6000 cycles min^{-1} for 5 minutes (Rotofix 32 Hettich) and the supernatant filtrated (Millipore, cellulose, 0.45 µm) to obtain the Fe soluble fraction. Then, HNO_3 (65%, Suprapur) was added to the supernatant to obtain a concentration of 10 g L^{-1} in the soluble extracts before Fe determination. The remaining solid was extracted with 25 mL of Soltanpour and Schwab extractant (1977) (0.005 M DTPA and 1 M NH_4HCO_3) and then filtered. The extraction was repeated three times and, after filtration, the three extracts were merged

(Fe available fraction). Before Fe determination, HNO_3 (65%, Suprapur) was added to neutralize excess bicarbonate and to obtain a concentration of 10 g L^{-1} in the samples, and finally volume was made up to 100 mL.

The stable isotope ^{57}Fe was used as a tracer to analyze the distribution of Fe provided with the treatments. Therefore, Fe isotopes (^{54}Fe, ^{56}Fe, ^{57}Fe and ^{58}Fe) were analyzed in plant and soil samples by Inductively Coupled Plasma Mass Spectrometry (IPC-MS NexION 300XX; Perkin-Elmer, Waltham, MA, USA) in order to determine the concentration and distribution of Fe in plant tissues and fractions of soil. The total Fe is expressed in terms of the Fe from fertilizer (Fe_{Fert}) and from natural sources (Fe_{Nat}) using the isotope pattern deconvolution (Rodríguez-Castrillón et al, 2008).

2.5. Statistical analysis

All results were statistically analyzed using SPSS 22.0 for Windows, by Analysis of Variance (ANOVA) and Duncan's test, to discuss the significant differences among treatments.

3. RESULTS

3.1. Root FCR activity

The reductase activity of soybean roots was determined using the chelates (Figure 1): *o,o*EDDHA/Fe^{3+}, EDTA/Fe^{3+}, IDHA/Fe^{3+}, and EDDS/Fe^{3+}; and the Fe complexes: Spruce and Eucalyptus LS/Fe^{3+}, LN/Fe^{3+} and GA/Fe^{3+}.

The FCR measured with Fe chelates for 60 minutes is presented in Figure 2. Several differences were observed relative to the chemical structure of the chelating agents. The highest activity was observed for the biodegradable chelates EDDS/Fe^{3+} and IDHA/Fe^{3+}. The FCR activity for EDTA/Fe^{3+} was found to be higher than

292 *o,o*EDDHA/Fe^{3+} but no statistical differences were found between them because of the
293 high variability of data. No reductase activity was observed when using complexes (LS,
294 LN or GA) as Fe substrates at any period of time analyzed.

295 3.2. Mössbauer spectroscopy

296 The Mössbauer spectra of the freeze-dried soybean roots supplied with 100 μM
297 Spruce LS/$^{57}Fe^{3+}$ and GA/$^{57}Fe^{3+}$ are shown in Figure 3, the corresponding Mössbauer
298 parameters are listed in Table 2. The spectra show a wide symmetrical quadrupole
299 doublet which cannot be fitted with one single Lorentzian doublet component. Thus, in
300 the applied fit model we assumed a quadrupole splitting distribution having Gaussian
301 shape and applied the VBF method (Rancourt & Ping, 1991) as implemented in the
302 MOSSWIN software (Klencsár, 2015). The isomer shift (~0.5 mms^{-1}) and quadruploe
303 splitting (~0.8 mms^{-1}) values are typical for high spin Fe^{3+} in distorted octahedral O_6
304 environments thus no reduced Fe^{2+} species can be observed. Although the origin of the
305 quadrupole splitting distribution cannot be unambiguously determined on the basis of
306 our data, we suggest the presence of different geometrical arrangements around the Fe^{3+}
307 ions. This is typical of hydrolyzed Fe^{3+} compounds resulting in the presence of
308 polynuclear Fe-OH-Fe moieties (Cornell and Schwertmann, 2003) and/or of hydrous
309 ferric oxide/hydroxide species of poor crystallinity as already observed in iron sufficient
310 Fe-citrate supplied cucumber roots (Kovács et al, 2016).

311 3.3. Pot experiment

312 The efficacy as Fe fertilizers in calcareous soil of the Fe complexes Eucalyptus
313 LS and GA, and the biodegradable chelate IDHA/Fe^{3+} was studied, in comparison to the
314 traditionally used *o,o*EDDHA/Fe^{3+}. Two types of Control plants were used: C +Fe (high

dose of *o,o*EDDHA/Fe^{3+}; positive control) and C –Fe (without Fe addition; negative control).

3.3.1. *Photosynthetic pigment content and photosynthetic activity*

In order to monitor the chlorosis symptoms in the leaves after the treatment applications, SPAD index was determined twice a week starting at 0 DAT, and Chl *a* fluorescence induction measurements were performed at 4 DAT.

Figure 4 represents the maximal quantum efficiency of PSII reaction centers at 690 nm (Fig. 4A), F690/F735 ratio (Fig. 4B), and the SPAD index (Fig. 4C). The maximal quantum efficiency of PSII reaction centers at F690 correlates the vitality of plants (Baker, 2008). The reciprocal ratio of 690 and 735 nm steady-state Chl a fluorescence correlates to Chl content (linear correlation) (Hák et al., 1990). SPAD index is related with the chlorophyll content in leaves and is normally used to assess the efficacy of Fe fertilizers (El-Jendoubi et al, 2011).

The C –Fe treatment caused a strong decrease in the PSII activity but all recovery treatments regenerated it. All recovery treatments induced a significantly higher PSII activity than the Fe deficiency one. Nevertheless, no significant differences were found among treatments and between treatments and C +Fe plants.

Fe deficiency in C –Fe plants decreased the Chl content (F690/F735 ratio) which has been elevated by the recovery treatments, except for the GA/Fe^{3+} treatment that also has the lowest SPAD index at 4 DAT. SPAD index was significantly higher for C +Fe plants than for the other treatments. Plants treated with the complexes or IDHA/Fe^{3+} did not differ from C –Fe plants. However, F690/F735 ratios indicated that LS/Fe^{3+} treated plants have the highest Chl content, but not significantly, in a similar

amount that C +Fe plants, not corresponding to the SPAD index. It must be noted that variability of the fluorescence data (error bars in figure 4B) was higher than the SPAD data (error bars in fig 4C).

The evolution of SPAD index and its increment along time (ΔSPAD) for the 2^{nd} and 4^{th} leaf stages are presented in Table 3. The ΔSPAD is calculated by the difference in measurements of 7 and 21 DAT for each Fe treatment or Controls. Attending to the 2^{nd} leaf stage, C +Fe plants significantly had the highest values because they were grown in sufficient Fe concentration since the beginning of the experiment. At the end of the experiment, SPAD index did not reach differences between Fe treatments and C –Fe plants. SPAD index decreased in all treatments and C –Fe plants, due to the senescence of the leaves, and this is observed in a greater extent for the LS/Fe^{3+} treated plants.

In the case of the 4^{th} leaf stage, SPAD index increased over time in all treatments and Controls. Plants treated with *o,o*EDDHA/Fe^{3+} reached the highest SPAD index values. At 7 DAT, SPAD index of plants treated with LS/Fe^{3+} and GA/Fe^{3+} did not differ from C –Fe plants, but it increased to be comparable to *o,o*EDDHA/Fe^{3+} plants at the end of the experiment.

3.3.2. *Fe content in soybean and soil fractions.*

Soybean plants and soil were sampled at 7 and 21 DAT, determining the DW and the Fe content in plant tissues and soil fractions. Thus, the efficacy of the different Fe treatments providing Fe to plants is evaluated.

No differences in shoot DW were observed between treatments (data not shown). Higher root DW was recorded only at 7 DAT for the IDHA/Fe^{3+} and C –Fe

plants. The data consisted of (g): 0.35 (a) for C –Fe plants, 0.16 (b) for C +Fe plants, 0.28 (ab) for *o,o*EDDHA/Fe^{3+}, 0.40 (a) for IDHA/ Fe^{3+}, 0.30 (ab) for LS/ Fe^{3+}, and 0.29 (ab) for GA/ Fe^{3+} (letters in brackets indicate statistical differences according to Duncan test, α=0.05).

Using the isotope pattern deconvolution of the data (Rodríguez-Castrillón et al., 2008), Fe sources (natural or fertilizers) can be differentiated in the samples. Figure 5 shows the results of leaf analysis at 7 and 21 DAT. The Fe_{Nat} content was the lowest when plants were treated with a high dose of *o,o*EDDHA/Fe^{3+} (C +Fe) at 7 and 21 DAT; and also at 21 DAT when a regular dose of this chelate was applied. At the end of the experiment, the highest Fe_{Nat} content corresponded to GA/Fe^{3+} treated plants. Moreover, the Fe_{Nat} increased statistically between 7 and 21 DAT in the case of GA/Fe^{3+}, IDHA/Fe^{3+} and the two types of Controls.

Both treatments based on *o,o*EDDHA/Fe^{3+} provided more Fe_{Fert} to leaves than IDHA/Fe^{3+}, LS/Fe^{3+} or GA/Fe^{3+}; however, more Fe_{Fert} was found in leaves of plants treated with *o,o*EDDHA/Fe^{3+} than C +Fe plants. If only data of IDHA/Fe^{3+}, LS/Fe^{3+} and GA/Fe^{3+} are statistically compared, IDHA/Fe^{3+} had a faster action supplying Fe than the complexes. However, at the end of the experiment Fe from GA/Fe^{3+} increased greatly and no difference in the Fe_{Fert} content was observed compared to IDHA/Fe^{3+}. Only Fe_{Fert} of leaves in GA/Fe^{3+} and C +Fe plants increased statistically between sampling times.

The soluble and plant available Fe fractions in the soil at the end of the experiment (21 DAT) is presented in the Figure 6, differentiating Fe_{Nat} and Fe_{Fert}. Pots in which GA/Fe^{3+} and C +Fe plants were cultivated had the highest Fe_{Nat} in the soluble fraction at both sampling times (data of 7 DAT not shown). However, increments in

385 Fe_{Nat} over time were not observed for Fe treatments or Control plants. Higher amounts
386 of Fe_{Fert} are found for *o,o*EDDHA/Fe^{3+} and C +Fe plants, while Fe_{Fert} for the other
387 treatments is lower than Fe_{Nat}. Moreover, the Fe_{Fert} was almost 4 times greater for C +Fe
388 plants than for those treated with a regular dose of *o,o*EDDHA/Fe^{3+}. In both cases, this
389 amount decreased throughout the experiment.

390 The highest plant available Fe_{Nat} content was found after the LS/Fe^{3+} and
391 GA/Fe^{3+} treatments at the end of the experiment. Higher available Fe_{Fert} was observed
392 in the soil of C +Fe plants, followed by LS/Fe^{3+} and GA/Fe^{3+}; while the lowest content
393 was found after *o,o*EDDHA/Fe^{3+} treatment.

394 ***3.3.3.*** *^{57}Fe distribution in soybean tissues and soil.*

395 The use of ^{57}Fe as a tracer facilitates the study of Fe distribution given by each
396 treatment. Table 4 shows the distribution (%^{57}Fe) in plant tissues and soil fractions. The
397 ^{57}Fe analyzed was mostly found in soil samples for all treatments applied. In the case of
398 the Fe complexes and IDHA/Fe^{3+}, it is located in the plant available fraction of the soil;
399 while the ^{57}Fe from the two doses applied of *o,o*EDDHA/Fe^{3+} was located mainly in the
400 soluble fraction.

401 Data of the different plant tissues point out that plants took up more ^{57}Fe from
402 *o,o*EDDHA/Fe^{3+} applied in a regular dose than in a high dose (C +Fe plants), increasing
403 along the experiment for both doses specially in leaves and roots. In the case of
404 IDHA/Fe^{3+}, LS/Fe^{3+} and GA/Fe^{3+}, the %^{57}Fe in plants is quite low compared to
405 *o,o*EDDHA/Fe^{3+}, and increments were not observed over time, except for GA/Fe^{3+}.
406 Plants treated with GA/Fe^{3+} increased ^{57}Fe not only in leaves but also in roots in a
407 higher extent; at the end of the experiment %^{57}Fe in roots was two times greater than in

leaves. Also a higher $\%^{57}Fe$ was observed in flowers of plants treated with *o,o*EDDHA/Fe^{3+} than for the other treatments at the end of the experiment.

The $\%^{57}Fe$ not determined in plant and soil samples with respect to the total applied is also calculated. Almost 45% of ^{57}Fe added as IDHA chelate treatment was lost both at 7 and 21 DAT. In the case of the other treatments, losses of ^{57}Fe increased over time, except for the C +Fe plants.

At each sampling time, the total amount of soil of each pot was divided in two portions, according to the height of the pot (28 cm). Thus, the distribution of Fe_{Nat} and Fe_{Fert} along the pot was determined (data not shown). The Fe_{Nat} was equally distributed in both soluble and plant available fractions of soil along the pot for all treatments assayed. Nevertheless, the Fe_{Fert} was mainly found in the upper side of the pot (14 cm) for all treatments in the available fraction, and also in the soluble fraction for the treatments based on *o,o*EDDHA/Fe^{3+}.

4. DISCUSSION

In this work, the soybean response to the application of Fe chelates and complexes from different sources is studied through several techniques. Firstly, the reductase activity of roots was determined, finding that the Fe complexes did not activate this response in soybean plants. However, in the subsequent pot experiment the ability of these products to provide Fe to plants and alleviate Fe chlorosis is evaluated.

The determination of the root FCR activity is used to explore the ability of different molecules to act as Fe fertilizers. In this work, no FCR activity up to 60 minutes is observed when the Fe complexes are added as Fe substrates. However, they cause a positive response in plants, as seen on the analysis of the pot experiment. Thus,

FCR activity measurement is not a valid method to understand the mechanism which enables these fertilizers to work in plants.

In the case of Fe chelates, the FCR activity is determined by the stability constant and the number of functional groups able to bind Fe (Lucena, 2006), and the polarity of the molecule (Escudero et al., 2012). As expected, the most stable chelate, *o,o*EDDHA/Fe^{3+}, causes a lower FCR activity compared to the pentadentated and less stable chelates. However, when applied to calcareous soil, the *o,o*EDDHA/Fe^{3+} is more effective than IDHA/Fe^{3+} providing Fe to plants, as seen on the pot experiment. Villén et al. (2007) found a high retention of IDHA/Fe^{3+} after 3 days of interaction with several soil materials, compared to EDTA/Fe^{3+} and *o,o*EDDHA/Fe^{3+}. The Fe(III) reduction in roots is an important physiological step for Fe uptake, but not determinant to explain the ability of a chelating agent to act as Fe fertilizer in soil culture.

The Mössbauer spectra clearly show that there is no accumulation of Fe^{2+} in the roots which is in good agreement with the FCR data. The partial hydrolysis of Fe^{3+} in the presence of natural complexing agents (LS and humic substances) has been already shown (Carrasco et al. 2012, Kovács et al. 2013) which can result in the accumulation of hydrous ferric oxide/hydroxide species in the apoplast. This may lower the utilization of Fe in the plant especially at calcareous conditions where the pH of the nutrient solution was slightly alkaline. However, slow dissolution and mobilization of Fe may occur resulting in a continuous, low-dose Fe supply to the plants.

PSII activity proved to be more sensitive to follow the recovery of the plants, since increases in its value was not reflected in the Chl content or by SPAD data. PSII maximal quantum efficiency is a robust parameter, the decrease of which indicates inhibitory effects in the photosynthetic electron transport chain, among others processes

around the PSII reaction centres (Baker, 2008). Nevertheless, most of the abiotic stresses are known to decrease its value, thus it is often used as an indicator of the vitality (Kalaji et al., 2012). By the resupply of Fe to Fe deficient plants, a small amount of Fe taken up by the chloroplasts induces the restoration of the photosynthetic apparatus, connected with the synthesis of photosystem I complexes that leads to the recovery of the photosynthetic electron transport chain and thus the elimination of acceptor side inhibition of PSII. Nevertheless, the accumulation of Chls requires a large-scale synthesis of light harvesting complexes that is a slow process (Solti et al., 2016). Buschmann et al. (2013) point out that the photosynthetic activity of barley was achieved after 8 hours of greening, but SPAD data and extracted Chl after 48 hours showed that the accumulation of pigments in leaves at that time is not yet accomplished.

At 4 DAT, the increases in PSII maximal quantum efficiency and the SPAD index of the treated plants indicates that the Fe uptake and accumulation effectively restored the photosynthetic functions in all treatments. Nevertheless, the time-scale revision of data is necessary to conclude how the Fe fertilizers were utilized in the Fe nutrition of the plants.

Considering the nutrient allocation among the different organs, except that of the C +Fe plants, SPAD index of the 2nd leaves of treated and C –Fe plants decreased through all the experimental time as a result of senescence. This trend is in good agreement with the SPAD data observed in a similar recovery experiment with high efficient chelates for soybean plants (Martín-Fernández et al, 2017). Nevertheless, C +Fe plants had been in a good Fe nutritional status since the beginning of the experiment, showing the highest SPAD index, but not corresponding to the Fe content of leaves. The total Fe content in leaves was higher when a regular dose of *o,o*EDDHA/Fe^{3+} was applied to plants compared to the application of a higher dose.

SPAD index, measured separately on each leaf stage, also indicates the distribution of Fe among the leaf stories. In C +Fe plants, SPAD index was higher in the 2nd than in the younger 4th leaves, contrary to the plants treated with a regular dose of *o,o*EDDHA/Fe^{3+} or the other Fe fertilizers applied. Thus, Fe was mobilized towards the younger leaves when *o,o*EDDHA/Fe^{3+} was applied in a regular dose, and when IDHA/Fe^{3+}, LS/Fe^{3+} or GA/Fe^{3+} were applied as treatments. In contrast, SPAD index of 4th leaves increased between sampling times for all treatments, including both of the C +Fe and C –Fe plants. Nevertheless, this increment was more intensive under GA/Fe^{3+} treatment, followed by the LS/Fe^{3+} and *o,o*EDDHA/Fe^{3+} treatments. Despite its slow effect, GA/Fe^{3+} was able to recover Fe chlorosis, also indicated by a similar SPAD index to the *o,o*EDDHA/Fe^{3+} and LS/Fe^{3+} treatments at the end of the experimental period. Rodríguez-Lucena et al, 2010, applied both foliarly and hydroponically several Fe chelates and complexes, and concluded that chelates presented better SPAD increments than complexes, mainly when applied in hydroponics. In our current work, also chelates present some advantages with respect to the complexes when applied to the soil.

Whereas SPAD and Chl *a* fluorescence induction measurements were performed on the different leaves separately, Fe analysis were done by mixing all the leaves of the individuals. Thus, by comparing Fe analysis results to SPAD data at 7 and 21 DAT, the trends for changing were found to be similar, but differences for Fe content data were large between the treatments. Despite SPAD indexes of 4th leaves showed no differences at the end of the experimental period, Fe content was higher in leaves of plants treated by a regular dose of *o,o*EDDHA/Fe^{3+} than in leaves of C +Fe and of any other treated plants. Root Fe chelate reductase activity is known to be higher when plants are grown under Fe deficiency (Lucena and Chaney, 2006). Since all plants were grown under limiting Fe nutrition before the Fe utilization treatments, it is expected that

*o,o*EDDHA/Fe^{3+} plants took up more Fe than the C +Fe plants, which were grown under Fe sufficient conditions during the total experimental period.

The source of Fe (Fe_{Nat} or Fe_{Fert}) is determined in plant and soil samples to evaluate the efficiency of the different treatments assayed. Both sources have an influence in alleviating the Fe chlorosis in leaves; while the main Fe source of plant treated with *o,o*EDDHA/Fe^{3+} is the Fe given by the chelate, Fe_{Nat} is the major contributor to Fe nutrition in plants treated with IDHA/Fe^{3+} and the Fe complexes.

Although plants treated with *o,o*EDDHA/Fe^{3+} took up more Fe_{Fert}, the Fe complexes provide more Fe_{Nat} to the plants over time, revealing a shuttle effect of the complexes. The higher amount of Fe_{Fert} found in leaves of IDHA/Fe^{3+} than for the Fe complexes at 7 DAT point out a faster action of this chelate, previously seen in hydroponics when compared to EDTA (Villén et al., 2007), but does not disclose a rise of Fe over time. Attending to the complexes, LS/Fe^{3+} has a faster action that GA/Fe^{3+}, but a long lasting effect is observed for the last one, as well observed in SPAD data.

The different treatments applied influence the mobility of Fe inside the plant. A low mobility of ^{57}Fe from roots to shoots is observed in C +Fe plants and in those treated with LS/Fe^{3+} and GA/Fe^{3+}, since they present similar %^{57}Fe in leaves and roots, two times greater in roots than in leaves for GA/Fe^{3+} plants. So, the translocation of Fe to flowers and fruits may be compromised. An experiment longer in time could show if Fe is remobilized from roots to shoots when these treatments are used. Translocation of ^{57}Fe to the shoots when *o,o*EDDHA/Fe^{3+} is applied in a regular dose is observed at the two sampling times, in contrast to C +Fe plants, similarly as it was observed in hydroponics by Rodríguez-Lucena, 2010, and in soil experiments by Nadal et al, 2012 and Martínez-Fernández et al. 2017.

Nadal et al, 2012, studied the dose-response curves for two chelates in a similar soil pot experiment as the one here described. They concluded that increasing the doses, both the soluble and the available fractions of the Fe_{Fert} increased; however the relative amount of Fe_{Fert} in leaves decreased as the doses increased. Similar results are observed in this work: the analysis of soil samples shows that the excess of Fe added with *o,o*EDDHA to C +Fe plants is mostly remained in the soil, mainly in the soluble fraction. The application of a higher dose of *o,o*EDDHA/Fe^{3+} does not denote a higher uptake of Fe_{Fert} by the plant and, additionally, the uptake of Fe_{Nat} is being negatively affected by the use of this chelating agent. The tested fertilizers are distributed in a different way in the soil. While Fe from *o,o*EDDHA/Fe^{3+} is mainly found in the soluble fraction, Fe from IDHA/Fe^{3+}, LS/Fe^{3+} and GA/Fe^{3+} is almost totally localized in the available fraction (DTPA extractable). Thus, Fe from *o,o*EDDHA/Fe^{3+} can be rapidly used by the plant, and the Fe from the complexes and IDHA/Fe^{3+} is available long term. The %^{57}Fe when added as GA/Fe^{3+} increases along the experiment in plant tissues while decreases in the available fraction of soil, revealing a long lasting effect of this complex. Despite the Fe complexes are not directly reduced in roots, they may slowly release Fe in a plant available form.

High losses of ^{57}Fe are found both at 7 and 21 DAT when IDHA/Fe^{3+} is applied, due to its low stability in calcareous conditions and its biodegradability (Villén et al., 2007). Almost 16% and 22% of ^{57}Fe are lost at the end of the experimental period when added as LS/Fe^{3+} and GA/Fe^{3+}, respectively. A possible Fe displacement in the complexes by Cu, Mn or Zn is not observed in soil samples (data not shown). Leaching is excluded as a reason for ^{57}Fe losses because is not observed in the results of Fe distribution in pots and plants are irrigated by weighting control of pots to keep the 80% WHC. The high pH of the calcareous soil can induce the sorption or degradation of the

complexes (Lucena et al., 2010) and, consequently, the precipitation of Fe in unavailable forms for the plant.

5. CONCLUSIONS

Despite the FCR activity measurements indicates that the *o,o*EDDHA/Fe^{3+} is less effective as Fe fertilizer and the Fe complexes are not reduced directly, the pot experiment confirmed the high effectiveness of the *o,o*EDDHA/Fe^{3+} and the potential use of LS/Fe^{3+} and GA/Fe^{3+} as Fe fertilizers. The Fe complexes (LS and GA) and IDHA/Fe^{3+} provide a smaller amount of Fe to plants than *o,o*EDDHA/Fe^{3+}, but they enhance the uptake of Fe_{Nat} and thus they have a positive effect on SPAD indexes and the maximal quantum efficiency of the PSII centers. Therefore, they are able to alleviate the physiological symptoms of the Fe deficiency, but their effect is not comparable to that of *o,o*EDDHA/Fe^{3+} when applied at similar doses, because of their lower stability under soil conditions. Nevertheless, longer-term experiments may expose if plants can utilize the high content of available Fe in soil accumulated when LS/Fe^{3+} and GA/Fe^{3+} are applied.

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TABLES

Table 1. Chemical characteristics of the soil used.

Parameter	Picassent soil
Texture	Sandy loam
pH (H_2O)	7.9
pH (KCl)	7.4
EC (1:5 extract) ($dS \cdot m^{-1}$)	0.2
OM ($g \cdot Kg^{-1}$)	9.2
N Kjeldahl ($g \cdot Kg^{-1}$)	0.3
$CaCO_3$ total ($g \cdot Kg^{-1}$)	380
$CaCO_3$ active ($g \cdot Kg^{-1}$)	89
Micronutrients ($mg \cdot Kg^{-1}$) (Soltanpour and Schwab, 1977)	
Fe	5.3
Mn	4.5
Cu	1.0
Zn	3.0

Table 2. ^{57}Fe Mössbauer parameters of Fe components found soybean roots treated with 100 µM ^{57}Fe-spruce Lignosulfonate (LS) or ^{57}Fe-Gluconate (GA) for 30 minutes. Before the Mössbauer readings, roots were freeze-dried. The numbers between parentheses give the statistical uncertainty (1× standard deviation) in the last digit.

	^{57}Fe-spruce LS	^{57}Fe-GA
δ[a] (mms-1)	0.469(1)	0.469(1)
Δ0[b] (mms-1)	0.788(1)	0.863(1)
σ (Δ)[c] (mms-1)	0.320(6)	0.348(2)
Γ[d] (mms-1)	0.326(8)	0.361(3)

[a]Isomer shift, relative to α-Fe.

[b]Center of the Gaussian quadrupole splitting distribution

[c]Standard deviation of the Gaussian quadruploe splitting distribution.

[d]Line width at half maximum

Table 3. SPAD data of the 2nd and 4th leaf stage of plants grown in a calcareous soil at 7 and 21 DAT, and the variation (ΔSPAD) between those days. Different letters indicate significant differences between treatments according to Duncan test (α=0.05).

Treatment	2nd leaf			4th leaf		
	7d	21d	ΔSPAD	7d	21d	ΔSPAD
C -Fe	36.3 c	32.6 b	-3.3 a	24.6 c	26.7 b	2.3 b
C +Fe	46.1 a	48.9 a	1.0 a	30.3 ab	34.7 ab	3.2 b
o,oEDDHA	40.8 b	35.6 b	-5.1 a	31.8 a	39.2 a	8.2 ab
IDHA	37.6 bc	32.6 b	-5.3 a	25.3 c	30.2 b	5.4 b
LS	39.0 bc	26.2 b	-13.7 b	26.0 bc	31.1 ab	6.0 ab
GA	36.9 bc	34.8 b	-3.8 a	22.7 c	35.3 ab	12.4 a

Table 4. Distribution of ^{57}Fe (%) applied in the soil pot experiment among plant tissues, soluble and available fractions of soil, and $\%^{57}Fe$ not determined (n. d.). Different letters indicate significant differences between treatments according to Duncan test (α=0.05).

Treatment	Sampling (DAT)	Leaf	Stem	Root	Flower	Soluble Fr.	Available Fr.	$\%^{57}Fe$ n.d.
C+Fe	**7**	0.86 b	0.20 b	0.99 b	-	69.2 a	31.8 d	0
o,oEDDHA		6.95 a	0.80 a	1.91 a	-	62.2 b	33.1 d	0
IDHA		0.23 b	0.03 b	0.19 c	-	0.19 c	54.0 c	45.4
LS		0.11 b	0.02 b	0.15 c	-	0.20 c	88.1 b	11.4
GA		0.08 b	0.02 b	0.05 c	-	0.27 c	101.5 a	0
C+Fe	**21**	1.76 b	0.33 b	1.76 b	0.16	62.2 a	36.3 c	0
o,oEDDHA		8.34 a	1.04 a	3.13 a	0.67	44.7 b	31.4 c	10.6
IDHA		0.23 b	0.04 c	0.05 c	0.02	0.22 c	53.7 b	45.7
LS		0.13 b	0.03 c	0.17 c	0.01	0.20 c	83.6 a	15.9
GA		0.26 b	0.04 c	0.52 c	0.04	0.23 c	77.1 a	21.8

FIGURES

Figure 1. Structures of the chelating agents used in the experiments.

Figure 2. Root FCR activity (nmol Fe g^{-1} root min^{-1}) of soybean plants grown hydroponically and treated with 100 μM Fe chelates. Readings were taken at 0, 10, 20 and 60 minutes. Error bars represent standard error (n=6). Different letters indicate differences between treatments at each measurement time, according to Duncan test (α=0.05).

Figure 3. Mössbauer spectra taken at *T*=80K of freeze-dried Fe deficient soybean roots after treatment for 30 minutes with 100 μM (A) ^{57}Fe-spruce lignosulfonate and (B) ^{57}Fe-gluconate supply

Figure 4. PSII activity (Fv/Fp at 690 nm) (A), Chl content (1/(R at F=Fs)) (B), and SPAD index (C) at 4 DAT, of the 3rd leaf stage of plants grown on a calcareous soil with different chelate and complexes treatments. Error bars represent standard error (n=10). Different letters indicate differences between treatments according to Duncan test (α=0.05).

atural sources (Fe_{Nat}) or from Fe

759 treatments (Fe_{Fert}) at 7 and 21 DAT of plants grown on a calcareous soil with different

760 chelate and complexes treatments. Error bars represent standard error (n=5). Different

761 letters indicate significant differences between treatments according to Duncan test

762 (α=0.05). Capital letters correspond to Fe_{Fert} data.

763 **Figure 6.** Fe content (µmol pot $^{-1}$) in the soluble and available fractions of soil from

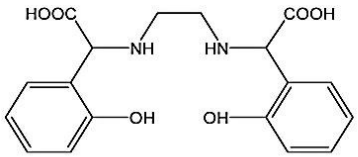
764 natural sources (Fe_{Nat}) or Fe treatments (Fe_{Fert}) at the end of the soil pot experiment.

765 Error bars represent standard error (n=5). Different letters indicate significant differences between treatments according to Duncan test (α=0.05). Capital letters correspond to Fe_{Fert} data.

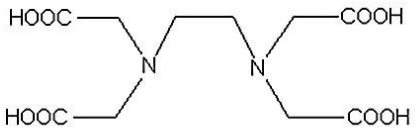
766

767

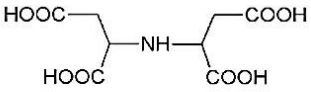
Figure 1.



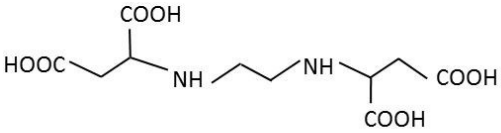
*o,o*EDDHA



EDTA



IDHA



EDDS

Figure 2.

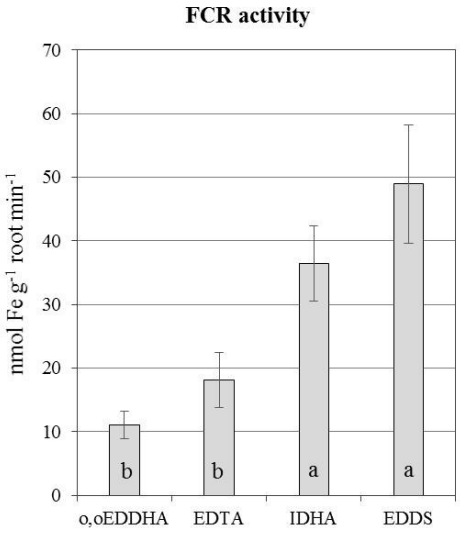

FCR activity
nmol Fe g-1 root min-1
70
60
50
40
30
20
10
0
b
b
a
a
o,oEDDHA
EDTA
IDHA
EDDS

Figure 3.

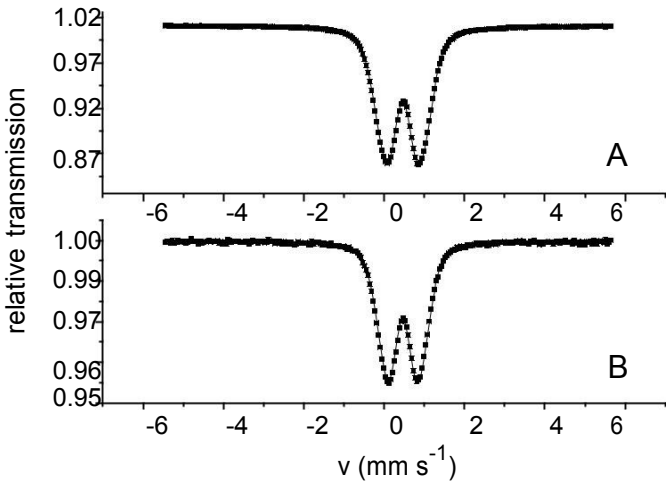

relative transmission
1.02
0.97
0.92
0.87
A
-6
-4
-2
0
2
4
6
1.00
0.99
0.97
0.96
0.95
B
v (mm s-1)

Figure 4.

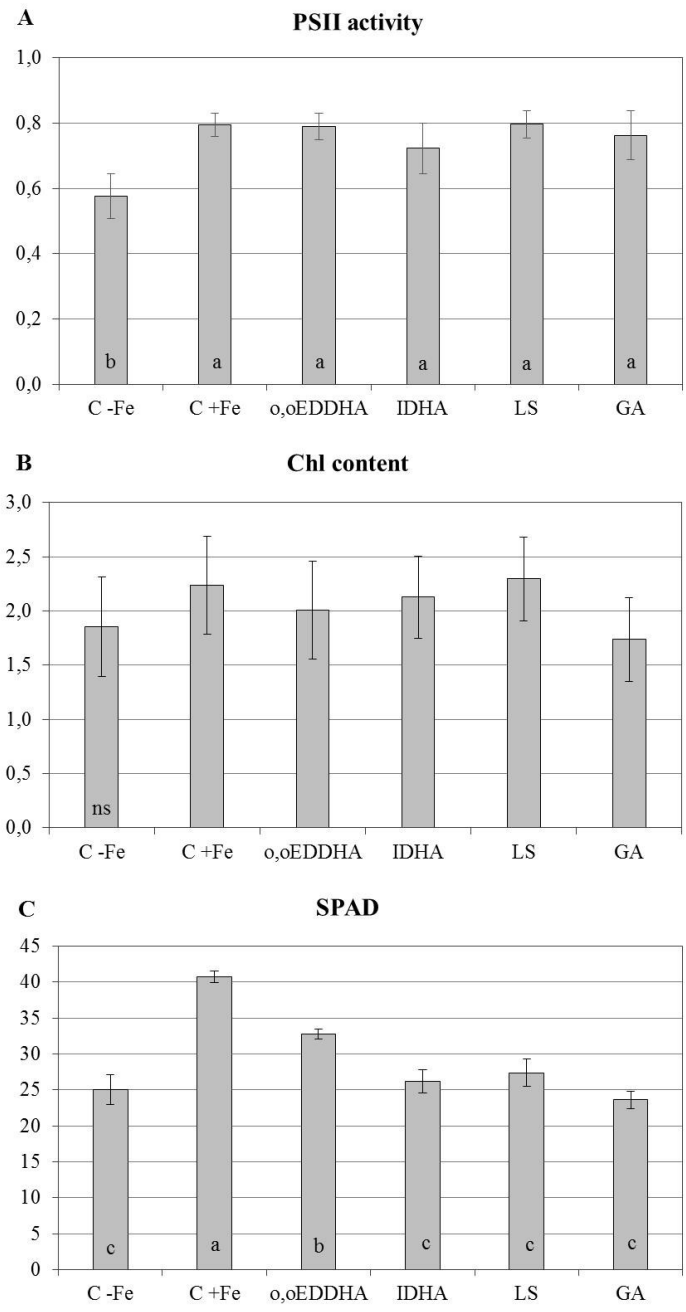
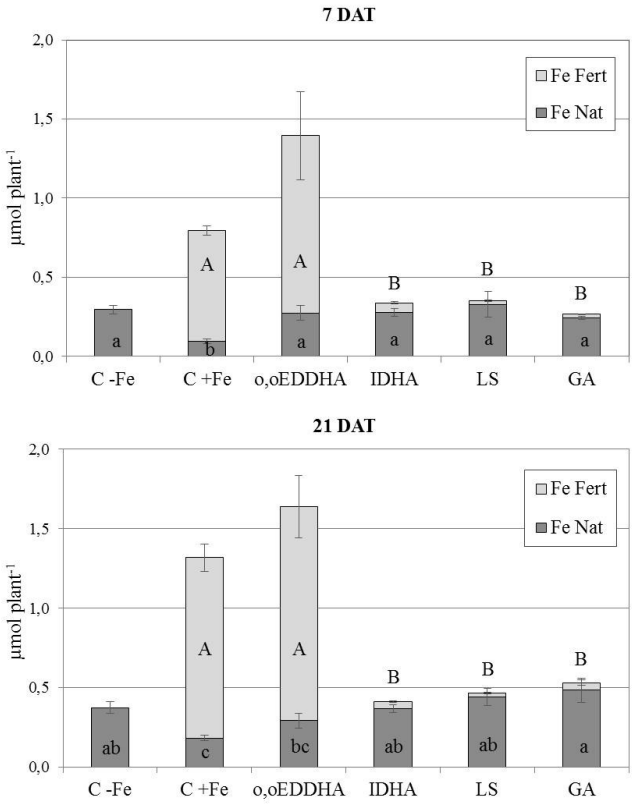
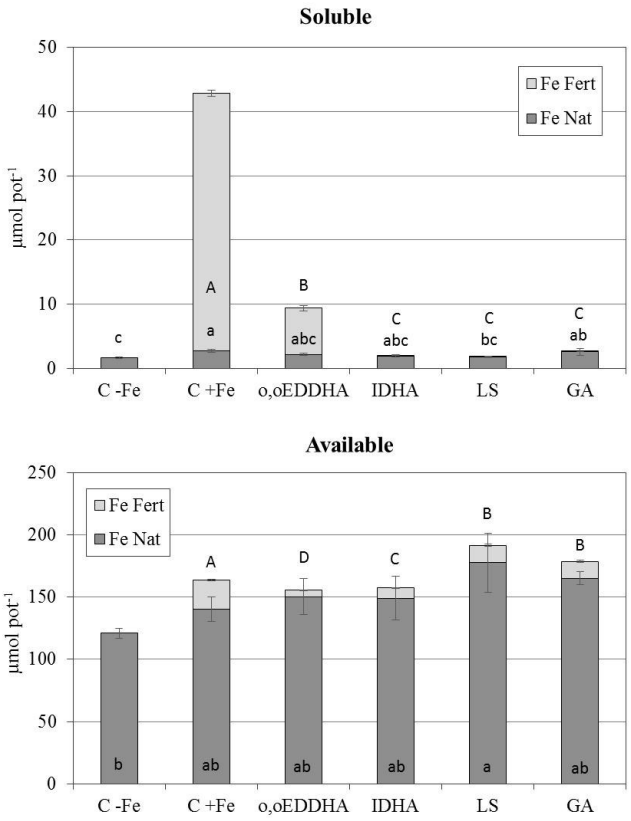

A
PSII activity
1,0
0,8
0,6
0,4
0,2
0,0
b
a
a
a
a
a
C -Fe
C +Fe
o,oEDDHA
IDHA
LS
GA
B
Chl content
3,0
2,5
2,0
1,5
1,0
0,5
0,0
ns
C -Fe
C +Fe
o,oEDDHA
IDHA
LS
GA
C
SPAD
45
40
35
30
25
20
15
10
5
0
c
a
b
c
c
c
C -Fe
C +Fe
o,oEDDHA
IDHA
LS
GA

Figure 5.


7 DAT
Fe Fert
Fe Nat
µmol plant-1
2,0
1,5
1,0
0,5
0,0
A
A
B
B
B
a
b
a
a
a
a
C -Fe
C +Fe
o,oEDDHA
IDHA
LS
GA
21 DAT
Fe Fert
Fe Nat
µmol plant-1
2,0
1,5
1,0
0,5
0,0
A
A
B
B
B
ab
c
bc
ab
ab
a
C -Fe
C +Fe
o,oEDDHA
IDHA
LS
GA

781 **Figure 6.**


Soluble
Fe Fert
Fe Nat
µmol pot-1
50
40
30
20
10
0
A
a
B
abc
C
abc
C
bc
C
ab
c
C -Fe
C +Fe
o,oEDDHA
IDHA
LS
GA
Available
Fe Fert
Fe Nat
µmol pot-1
250
200
150
100
50
0
A
D
C
B
B
b
ab
ab
ab
a
ab
C -Fe
C +Fe
o,oEDDHA
IDHA
LS
GA

*Contribution

AUTHOR CONTRIBUTIONS

CMF carried out the plant experiments and FCR activity measurements. Furthermore, CMF analyzed the plant and soil material, did the mathematical deconvolution and performed the statistical study. AS helped with the recovery tests, performed the fluorescence induction experiments and made the interpretation. VC helped with the update of the references and discussion on the project. KK: performed the Mossbauer analysis and interpretation. FF, LHA and JJL conceived the study. AG worked on the design of experiments and FCR activity measurements. CMF wrote the manuscript together with the revision of JJL. JJL designed the manuscript and with LHA and FF supervised all the experimental work presented. All the authors read and approved the final manuscript.