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Effect of Fe:ligand ratios on hydroponic conditions and calcareous soil *in Solanum lycopersicum* L. and *Glycine max* L. fertilized with heptagluconate and gluconate

Samira Islas-Valdez, Sandra López-Rayo, Jessica Arcos, Nieves Menéndez, Juan J. Lucena*.

Department of Agricultural Chemistry and Food Science. Universidad Autónoma de Madrid, Av. Francisco Tomás y Valiente 7, 28049 Madrid, Spain. *Corresponding author: juanjose.lucena@uam.es

ABSTRACT

BACKGROUND: The environmental risk of synthetic chelate application promotes the implementation of biodegradables complexes to correct Fe-deficiency in plants. In this paper, the Fe oxidation state, the Fe:ligand ratio and molecular weight distribution for heptagluconate (G7) and gluconate (G6) are consider as key factors for the complexes efficacy as fertilizes. Complexes with different Fe:ligand ratios were prepared and analyzed by gel filtration chromatography (GFC). The ability of Fe:ligand ratios to provide Fe to tomato in hydroponics and soybean in calcareous soil was tested and compared to synthetic chelates (Fe^{3+}:HBED and Fe^{3+}:EDTA).

RESULTS: The G7 presented a higher capacity to complex both Fe(II) and Fe(III) than G6, but the Fe(II) complexes show low stability at pH 9 and oxidation in solution. GFC demonstrated the polynuclear nature of the Fe^{3+}:G7 at various ratios. The effectiveness of the Fe fertilizers depend on the Fe^{3+}:ligand ratio and the ligand type being the Fe^{3+}:G7 (1:1 and 1:2) the most effective. The Fe^{3+}:G7 (1:1) also presented a better response for the uptake of other micronutrients.

CONCLUSION: The Fe^{3+}:G7 molar ratios have shown to be critical for the Fe plant uptake under hydroponic conditions and calcareous soil. Thus, the Fe^{3+}:G7 at equimolar ratio and 1:2 molar ratio can be an environmentally friendly alternative to less degradable synthetic chelates to correct Fe chlorosis in strategy I plants.

Keywords: Fe:ligand ratio, polynuclear complexes, strategy I plants, gluconate, heptagluconate.

1 INTRODUCTION

Iron (Fe) chlorosis is a nutritional disorder characterized by a decrease of chlorophyll content in leaves. This is a common problem of sensitive crops grown in calcareous soils, since under these conditions; the Fe uptake by the plant is prevented[1]. Iron chlorosis harms several physiological processes such as photosynthesis, chlorophyll biosynthesis, respiration and enzymatic activities[2,3]. Dicotyledonous and non-graminaceous monocotyledonous plants have developed a Fe uptake strategy named Strategy I, inducing the rhizosphere acidification followed by the reduction of Fe^{3+} to Fe^{2+} from membrane-bound enzyme ferric-chelate reductase[1]. Tomato (*Solanum lycopersicum* L.) and soybean (*Glycine max*) are widely used as model plants to investigate the Fe deficiency of the Strategy I[2,4]. The strategy I plants growing on calcareous soil usually requires the application of synthetic Fe chelates such as the Fe^{3+}:EDTA (ethylene diamine tetra acetate), Fe^{3+}:EDDHA (ethylene diamine-N,N'-bis(hydroxyl phenil acetate)) or the Fe^{3+}:HBED (N,N-bis(2-hydroxybenzyl) ethylene diamine-N,N-diacetate). While Fe^{3+}:EDTA is mainly used in drip irrigation systems or in crops without severe Fe deficiency under calcareous soil conditions[5], Fe^{3+}:HBED and Fe^{3+}:EDDHA are highly stable[6] and effective fertilizers[7], recommended even in the more adverse conditions for Fe nutrition. Parameters such as the stability of the Fe-chelate and of the chelates formed with the competing ions as Ca^{2+}, the retention on soil surfaces, the plant Fe uptake mechanism[8] and the so called "shuttle effect mechanism"[9] affects the effectiveness of Fe-chelates to correct Fe chlorosis. Despite these benefits, synthetic Fe chelates are expensive and may involve environmental risks related to their mobility in the soil[9]. Complexing agents such as sodium gluconate (G6) or sodium glucoheptonate (G7) have a low environmental impact due to their high biodegradability[10]. They can complex metals through their carboxylic and hydroxylic groups by different binding modes depending on the metal itself and the reaction conditions. Only a few studies have explored the effect of G6 and G7 in plant

nutrition, with contradictory results. An adequate capacity of the Fe^{3+}:G6 (1:2) to correct chlorotic soybeans grown in calcareous soil was observed by Martín-Fernández et al.[11] similar to Fe^{3+}:IDHA (Iminodisuccinate), but not comparable to Fe^{3+}:EDDHA when applied at similar dose. A similar result was obtained by Rodriguez-Lucena et al.[12] in hydroponics, where the G6 was able to provide Fe to deficient soybean plants in a similar concentration than the Fe^{3+}:EDTA and the Fe^{3+}:IDHA, but lower than for Fe^{3+}:EDDHA. Lucena et al.[13] also confirmed that the percentage of Fe remaining in solution for G6 in the pH range 5-7.5 was around 20%. In contrast, Clemens et al.[14] suggested that the G7 would be a better complexing agent for Fe^{3+} than EDTA and G6 in alkaline soils. Also, Fuentes et al.[15] in a gene expression study in Fe-deficient cucumber plants found that the foliar application of Fe^{3+}:G7 was effective providing Fe, but its delivering was slow or scarce. On the contrary, Shaddox et al.[16] reported a decrease of soluble Fe after one-day application of the Fe^{2+}:G7 in incubated soils similar to ferrous sulfate. Similar results were obtained by Goos and Germain[17] when Fe^{3+}:G7 and Fe^{3+}:G6 were applied to soil in batch incubation experiments in comparison with EDTA, DTPA (diethylen triamine penta acetic), EDDHA and EDDHSA (ethylen diamino-N'N-bis (2-hydroxy-5-sulfo) phenyl acetic acid). Thus, the effect of Fe complexes of G6 and G7 on plant nutrition is limited or not conclusive.

Most of these studies were conducted by using a 1:1 and 1:2 (Fe:ligand) molar ratio. However, the metal complexes can form a wider variety of chemical species, depending on the ligand type, pH, the Fe:ligand molar ratio and synthesis temperature, affecting to the effectiveness of the complexes. These effects have been observed for complexes of several natures. For instance, Fe^{3+} forms very stable complexes in solution with ligands such as sugars and citrate due to the formation of polymers, preventing the precipitation of the Fe(III)-hydroxy-polymer[18,19]. Studies conducted by Silva et al.[20] with Fe citrate complexes, demonstrated that at pH 9, a mononuclear Fe complexe is predominant with a low Fe^{3+}:citrate molar ratio, whereas a high Fe^{3+}:citrate molar ratio at neutral pH lead to

the formation of oligomeric complexes. Stevenson[21], showed that humic metal complexes with high metal:humic acid ratios presented a lower stability than those with low metal:humic acid ratios. Experiments conducted with lignosulfonates and humic acid from leonardite with different iron sources [Fe (III) and Fe (II)] confirmed the effect of the Fe:ligand ratios in the binding sites and the complexes stability. The Fe source was also determinant in the formation of weak or strong complexes[22,23], indicating that strong complexes are prepared from the Fe^{3+}. It is also remarkable that an excess of chelating agents in nutrient solutions can retard, and even inhibit, the uptake of metals by plants[24,25]. Then, a better knowledge of the effect of the Fe:ligand ratio with G6 and G7 complexes plays an important role to study their efficacy as Fe fertilizers and their possible "shuttle effect" for the turnover of micronutrients naturally present in the soil solution, similar to that already demonstrated for synthetic chelating agents.

Currently, several spectroscopic techniques such as ^{13}C nuclear magnetic resonance (^{13}C-NMR), Fourier transformed infrared (FTIR) and Mössbauer spectroscopy are considered to determine some physical characteristics of the metal complexes such as the purity, geometry and structure, the bonding sites and the Fe oxidation states. Besides, gel filtration chromatography (GFC) on Sephadex® has demonstrated to be a useful tool for the characterization of metal-complexes and metal:chelates. The GFC permits the fractionation based on size[18,26] and also the identification of the free and complexed metal by comparison between the retention time and the quantification of the soluble Fe in the obtained fractions[27,28]. By the application of this technique, the presence of polynuclear compounds in Fe^{3+}:G6 (1:1) complexes[11], and low molecular compounds in Fe^{2+}:G6 (1:2)[29] could be determined. To understand, the discrepancies obtained in previous studies for the molecular weight distribution of Fe complexes, which may be related to the Fe:ligand ratio and the Fe source used.

Although a few studies have proved the efficacy of the G6 and G7 Fe complexes to correct Fe chlorosis, their effectiveness related to parameters such as Fe:ligand ratio, molecular weight distribution, and the Fe complexing capacity with different Fe sources has not been explored. Therefore, it can be hypothesized that the ligand type (G6 or G7) and the Fe:ligand molar ratio are important parameters for their effectiveness as Fe fertilizers in plants under hydroponics and calcareous soil. For that purpose, firstly, the complexing capacity of the complexing agents G6 and G7 with a different Fe source [Fe (III) and Fe (II)] was evaluated to improve the stability of the complexes and the amount of Fe provided to the plants. Secondly, the molecular weight distribution of the Fe:ligand molar ratios obtained were chemically characterized. Finally, the effectiveness of the complexes to supply Fe to strategy I plants under hydroponic (different Fe:G7 ratios) and calcareous soil conditions (G6 and G7, and different Fe:G7 ratios) was evaluated.

2 MATERIALS AND METHODS

Pure reagents of sodium gluconate (Sigma Aldrich, ≥99%) assigned as G6, and sodium glucoheptonate dihydrate (G7) kindly provided by DABEER (99%, Barcelona, Spain) were used. Synthetic chelating agents of ethylenediaminetetraacetic and disodium salt [Na_2EDTA, tritriplex III (Merck, 99%)] and *N-N´*-bis (2-hydroxybenzyl) ethylenediamine-*N-N´*-diacetic acid (HBED, 93.72%) provided by ADOB PPC; Poznan, Poland were used for comparison. Iron complexes were prepared with $FeCl_3 \cdot 6H_2O$ (Merck, 99%), or $FeSO_4 \cdot 7H_2O$ (Merck, 99%) of analytical grade and the water used was grade I[30], free of organic contaminants.

2.1 Complexing capacity of gluconate and heptagluconate and Fe complexes preparation

The maximum complexing capacity (MCC) of G6 and G7 with Fe (III)/Fe (II) was determined by the maximum Fe content remaining in solution at pH 9 after one day in the dark according to the method previously described by Villén et al.[31].

After that, Fe complexes were prepared at room temperature with the aim to prepare several Fe:ligand molar ratios according the dry weights of the ligands: 1:0.5 for G7 with Fe^{3+}, and 1:1, 1:2 and 1:3 (Fe:ligand molar ratios) for both G6 and G7 with Fe^{3+}, and 1:2 for G6 with Fe^{2+}, and 1:1 and 1:2 (Fe:ligand molar ratios) for G7 with Fe^{2+}. These complexes were selected based on the stability observed during the determination of the MCC, being the Fe^{3+} complexes the most stable and the Fe^{2+} the less. The complexes were prepared at pH 6-7 and then freeze-dried. The total soluble Fe in the freeze-dried samples was determined following the methods 9.2, 9.3 and 9.4[32] by extraction with water and filtration through a 0.45 µm filter. The total complexed Fe in the samples was analyzed according to EN 15962:2011[33], following the same preparation then that for the soluble element but rising the pH to 9 before the filtration. In addition, the complexed fraction (expressed as percentage of complexed Fe with respect to the soluble Fe) was determined as an index of the complexe stability and its effectiveness as fertilizer[12]. A flame atomic absorption spectrometer (AAS, Perkin-Elmer AAnalyst 800; Shelton, CT, USA) was used for all the Fe determinations.

2.2 Structural characterization of the complexing agents and the Fe complexes

The structural changes of G6 and G7 after complexation with Fe^{3+} in the different ratios G6 (1:1) and G7 (1:0.5, 1:1, 1:2 and 1:3) were analyzed by FTIR spectra on a Bruker IFS66vd spectrometer (Germany) using KBr pellet method in the 3800-600 cm^{-1} region at a resolution of 4 cm^{-1} in the transmittance mode. ^{13}C-NMR spectra of G6 and G7 dissolved in deuterated water (D_2O) were recorded on a Bruker DRX 500 MHz (Germany) to check their chemical structure. ^{57}Fe Mössbauer spectroscopy was used to analyze the oxidation

155 state of Fe of G7 complexes prepared with Fe^{2+} or Fe^{3+} (at 1:2 molar ratio). The analysis
156 was conducted at T= 298 K in triangle mode with a ^{57}Co (Rh) source with and activity of 50
157 mCi and calibrated with 6 μm α-iron at room temperature. The principal parameters
158 obtained from the spectra were isomer shift (δ, mm s^{-1}), quadrupole splitting (Δ, mm s^{-1}),
159 and relative content area (A, %), which can provide information about the oxidation state,
160 the coordination number of the resonant nucleus and the semiquantitative information for
161 the species obtained.

162 **2.3 Gel filtration chromatographic**

163 The molecular weight distribution of the Fe^{3+}:G7 complexes were analyzed on a glass
164 column (1.0 x 30 cm) packed with Sephadex® G-10 (molecular weight cut-off, MWCO
165 >700 Da; 40-120 μm particle size distribution from Sigma Aldrich). The Fe^{3+}:G7 (1:3) was
166 also analysed by a Sephadex® G-15 (MWCO >1500 Da; 40-120 μm particle size
167 distribution). The Fe^{3+}:G7 complexes were additionally eluted in a Sephadex® G-25
168 (MWCO >5000 Da; 50-150 μm particle size distribution). The samples were dissolved in
169 0.6 % (w/v) of 0.1 M NaCl at pH 6, filtrated by 0.22 μm filter, and the Fe content measured
170 by AAS prior to the fractionation under gravity of 125 μL in the columns at room
171 temperature. The fractions were monitored at 220 nm in a Spectrostar nano microplate
172 reader (BMG Labtech, Ortenberg, Germany) and the Fe concentration analysed by AAS
173 after gel filtration chromatography on Sephadex® G-10. The exclusion volume (V_o) was
174 determined with blue dextran 2000 (MW~2000 kDa) and the total volume (V_p) with
175 Fe^{3+}:HBED (MW~440 Da) from 0.1 M NaCl at pH 6. In agreement with a previous study in
176 which the molecular weight distribution of Fe:lignosulfonate complexes were evaluated[34].

177 **2.4 Plant experiments**

178 Two experiments were conducted by using two different Strategy I plants sensitive to Fe
179 chlorosis. Tomato was grown under hydroponics and soybean was grown in calcareous

soil conditions. Both experiments were done in a growth chamber (Dycometal type CCK) with a photoperiod of 16 h to 23ºC and 40% relative humidity during the light period, and 8 h to 19ºC and 60% relative humidity during the dark period. The composition of the full-strength nutrient solution (NS) was: macronutrient (mM) 1.0 Ca $(NO_3)_2 \cdot 4H_2O$, 0.9 KNO_3, 0.3 $MgSO_4 \cdot 7H_2O$, 0.1 KH_2PO_4; micronutrients (μM) 2.5 $MnSO_4 \cdot H_2O$, 1.0 $CuSO_4 \cdot 5H_2O$, 10.0 $ZnSO_4 \cdot 7H_2O$, 1.0 $NiCl_2 \cdot 6H_2O$, 1.0 $CoSO_4 \cdot 7H_2O$, 115.5 Na_2EDTA, 35.0 NaCl, 10.0 H_3BO_3, 0.05 $Na_2MoO_4 \cdot 2H_2O$.

2.4.1 Growth conditions in the hydroponic experiment

Tomato (*Solanum lycopersicum* L., cv. Marmade) seeds were germinated for 12 days in vermiculite (1-4 mm grain; Projar, Spain) moistened with 1mM $CaSO_4$. Uniform seedlings were transferred to 1.8 L vessels filled with 1/4 diluted NS for three days containing 5 μM Fe^{3+}:HBED at pH 6, and then, 11 more days in full strength NS at pH 7.5. After that, they grow for eight days in a Fe-free full-strength NS. The plants were individually transferred to 250 mL vessels containing the NS and the Fe treatments. In this experiment, the Fe^{3+}:G7 complexes at several molar ratios (1:0.5, 1:1, 1:2 and 1:3) were studied and compared to the synthetic chelate Fe^{3+}:HBED as the positive control, assayed in a concentration of 10 μM of Fe^{3+}. This low Fe concentration permits a better differentiation of the effect in the Fe nutrition between the treatments[35]. Likewise, a Fe-free negative control (-Fe) was also assayed for comparison. The nutrient solution was continuously aerated and buffered at pH 7.5 with 1.0 x 10^{-4} M HEPES and 0.1g L^{-1} of $CaCO_3$ to simulate calcareous soil conditions. The sampling was done 15 days after the treatment (DAT). The NS was renewed every seven days. Six replicates (one vessel with one plant each) per treatment were assayed.

2.4.2 Growth conditions in the soil experiment

204 Soybean *(Glycine max* L., cv. RGT Speeda) seeds were germinated in perlite (1-5 mm
205 grain; Projar, Spain) moistened with distilled water for 12 days. Uniform seedlings were
206 transferred to 4 L vessels filled with a 1/5 diluted NS containing 10 μM Fe^{3+}:HBED at pH 6
207 for seven days and for seven more days in a Fe-free full strength NS. After this pre-growth
208 period under hydroponic conditions, two seedlings per pot were transplanted to
209 polystyrene pots covered with aluminium foil to avoid photodegradation of Fe complexes[36],
210 filled with 180 g calcareous sand (975 g Kg^{-1} $CaCO_3$; 2-4 mm) mixed with 420 g of a sandy
211 loam soil (pH 7.9; 435 g Kg^{-1} sand, 80 g Kg^{-1} silt, 485 g Kg^{-1} clay; 9.2 g Kg^{-1} organic
212 matter; 380 g Kg^{-1} total $CaCO_3$, 89 g Kg^{-1} active lime; and Soltanpour and Schwab[37]
213 extractable micronutrients: 5.3 g Kg^{-1} Fe, 4.5 g Kg^{-1} Mn, 1.0 g Kg^{-1} Cu and 3.0 g Kg^{-1} Zn)
214 from Picassent (Valencia, Spain). This soil has been previously described[11].

215 Two days before transplanting, pots were irrigated until 100% of the soil-sand mixture
216 water holding capacity (SWHC). Two days after transplanting, the treatments were
217 initiated: the Fe^{3+}:G6 (1:1), the molar ratios of Fe^{3+}:G7 (1:0.5, 1:1, 1:2 and 1:3) and, the
218 Fe^{3+}:EDTA (positive control). In all cases there were 6 replicate pots, two plants each, per
219 treatment. The solutions of the Fe complexes and the Fe^{3+}:EDTA were split over the
220 experiment: 0, 7 and 14 after the first treatment application at an Fe dose of 4.2, 2.1, and
221 2.1 μmol Fe^{3+} per pot, respectively. In addition, a Fe-free negative control (-Fe) was
222 assayed. During the experiment, pots were irrigated until 80% SWHC every two or three
223 days with a macronutrient NS in 0.1g L^{-1} of $CaCO_3$. One plant shoot per pot was sampled
224 at 7 DAT and grouped in three pairs per treatment (three analytical replicates). At 21 DAT,
225 the remaining plants shoots in each pot and the roots were sampled and kept separated in
226 six replicates per treatment. On completion of the experiments, the soluble and available
227 Fe, Mn, Zn and Cu fractions in soil were determined in all the pots (six replications) by the
228 extraction method proposed by Nadal et al.[38] with water and DTPA solutions[37] followed by
229 the acidification with HNO_3 (65%, Merck) to 1% and analyzed by AAS.

2.5 Physiological parameters

Leaf chlorophyll index was measured every two or three days after the beginning of the treatments on the youngest and fully expanded leaf (three readings per level), by using a portable chlorophyll meter Dualex 4 Scientific (FORCE-A, Orsay, France). Shoot and root lengths were measured after each sampling. Leaves, stems and roots were separated and washed with 0.1% non-ionic detergent (Tween 80) and 0.1M HCl followed by tap-water and distilled water[39], and finally wiped and weighed to obtain the fresh weight. Plant tissues were dried in a forced air oven at 65 ºC for three days until constant weight to obtain dry weight (DW) and ground with a porcelain mortar and pestle. Samples were mineralized by dry digestion in a muffle furnace at 480 ºC for 4 h followed by the acid digestion with HCl suprapur (1:1) for the ash solubilization at 80 ºC for 30 min[40]. Total Fe, Mn, Cu and Zn concentration in the plant tissues extracts was determined by AAS.

2.6 Statistical analysis

Data were analysed by using the IBM SPSS statistical software (version 23.0; SPSS Inc., Chicago, IL, USA). Differences among treatments were tested by one-way analysis of variance (ANOVA), with a Duncan post hoc test at *p-value* < 0.05.

3. RESULTS

3.1 Fe complexing capacity of gluconate and heptagluconate

The Fe MCC complexing capacity of Fe^{3+}:G7, Fe^{2+}:G7, Fe^{3+}:G6 and Fe^{2+}:G6 (Fig. 1), was determined by the intersection point between the complexing segment and the coagulation segment, as a consequence of the excess of metal. Here, the highest values of the MCC for Fe^{3+} and Fe^{2+} were obtained by G7 (2.65 and 1.25 mol Fe mol^{-1} ligand, respectively), and the lowest for G6 (1.85 and 0.47 mol Fe mol^{-1} ligand, respectively).

3.2 Soluble and complexed Fe of the complexes at the different Fe:ligand ratios

Because of the highest complexing capacity presented by G7 with Fe^{3+}, a wider range of molar ratios was prepared for this complexe (1:0.5, 1:1, 1:2 and 1:3) for further analysis. In addition, the equimolar ratio of Fe^{3+}:G6 was studied since it has been previously evaluated in other studies. As a result of the low complexing capacity presented by G7 and G6 with the divalent Fe^{2+}, only the 1:1 and 1:2 molar ratios for Fe^{2+}:G7, as well as the 1:2 molar ratio for Fe^{2+}:G6 were further explored. The freeze-dried samples of the Fe^{2+} complexes presented a pale green color while the Fe^{3+} complexes a yellow color. However, after dissolution of the solid samples, both Fe^{2+} and Fe^{3+}complexes presented a yellow color, which suggest the oxidation of the Fe^{2+} complexes.

The results obtained for the analysis of the soluble Fe in the above mentioned prepared complexes were checked with the European official method for fertilizers[32]. Accordingly, the complexes of Fe^{3+}:G7 (1:0.5, 1:1, 1:2 y 1:3), Fe^{3+}:G6 (1:1), Fe^{2+}:G7 (1:1 y 1:2) and Fe^{2+}:G6 (1:2) presented 14, 11, 9.1, 5.8, 11, 9.9, 6.7 and 6.2%, respectively. Then, all of them complied with the minimum percentage of the soluble Fe required by the regulation[32] (5% for solid samples). Attending to the fraction of Fe complexed[33], a minimum of 80% is required[32]. The Fe^{3+}:G7 (1:1), Fe^{3+}:G7 (1:3), Fe^{2+}:G7 (1:1) and Fe^{2+}:G6 (1:2) products presented higher values (86, 100, 90 and 89%, respectively), but Fe^{3+}:G7 (1:0.5), Fe^{3+}:G7 (1:2), Fe^{3+}:G6 (1:1) and the Fe^{2+}:G7 (1:2) (2.0, 74, 77 and 74%, respectively) lower than the 80% required.

3.3 Structural characterization of the complexing agents and Fe complexes

The ^{13}C NMR spectra of the G6 and G7 free ligands presented six and seven, respectively, well-defined peaks in the range of 62.61-178.59 ppm (Fig. 2), suggesting that both products contained the pure complexing agent. This fact was confirmed by the FTIR spectra of G6 and G7, presenting the G6 a similar spectrum to the sodium gluconate previously reported[29]. The FTIR spectra of Fe^{3+}:G6 (1:1) and the different molar ratios of

279 Fe^{3+}:G7 were also analyzed. In all of them, a broad band at 3400 cm^{-1} due to the vibration
280 of hydroxyl groups, bands at 1625 and 1380 cm^{-1} assigned to asymmetric and symmetric
281 carboxylate anion were observed. The C-O vibration of the primary and secondary
282 hydroxyl groups appeared at 1057 cm^{-1} and 1090 cm^{-1}, respectively[29]. In addition, bands
283 at 2943-2893 cm^{-1} were associated with C-H stretching vibration[18] and the vibrations of
284 Fe-O were presented in the region 1000-600 cm^{-1}. A weak band was additionally
285 presented by Fe^{3+}:G7 (1:2) at 1778 cm^{-1}, which indicated the dissociation of the carboxylic
286 group[18] involved in the Fe complexation.

287 To confirm the oxidation state presented in the final complexes, Fe^{2+}:G7 (1:2) and Fe^{3+}:G7
288 (1:2) were selected and further analyzed by Mössbauer spectroscopy. The Fe^{3+}:G7 (1:2)
289 spectrum reflects two symmetrical doublets (Fig. 3a). The main component denoted by
290 Fe^{3+}_A with δ= 0.37 mm s^{-1}, Δ= 0.75mm s^{-1} and *a*= 56% represents a high spin Fe^{3+} that can
291 be associated with ferrihydrite. The Fe^{3+}_B has δ= 0.38 mm s^{-1}, Δ= 1.22 mm s^{-1} and *a*= 44%
292 compatible with Fe^{3+} polynuclear structures[41]. Attending to Fe^{2+}:G7 (1:2) spectrum (Fig.
293 3b) reflects an asymmetric doublet by the superposition of the Fe^{2+} (δ=1.25 mm s^{-1}, Δ=2.51
294 mm s^{-1} and a=76%) and Fe^{3+} (δ= 0.26 mm s^{-1}, Δ= 1.10 mm s^{-1} and a=24%) phases,
295 respectively. The Fe^{2+} phase is in good agreement with those of high-spin Fe^{2+} in a
296 distorted octahedral O^6 coordination[23], which may indicate bind of the Fe^{2+} with carboxylic
297 and hydroxylic groups of the G7. The Fe^{3+} phase is also related to iron polynuclear
298 structures.

299 As mentioned above, the Fe^{2+} complexes oxidized after dissolution and the G6 complexes
300 presented lower complexing capacity, thus the different molar ratios were only prepared
301 for the Fe^{3+}:G7 complexes. These complexes were analyzed by UV absorption
302 spectroscopy (Fig. 3c), showing a strong band about 350 nm due to the oxo-metal charge
303 transfer absorption band. This band is characteristic of the Fe^{3+} complexes in a high spin

304 state octahedrally chelated by oxygen, as it has been reported for Fe^{3+}:gluconate with an
305 approximate molecular weight 11.6 kDa[42].

306 Concerning the molecular weight distribution of the Fe^{3+}:G7 complexes with different molar
307 ratios on Sephadex® G-10, all eluted in the exclusion volume similar to the blue dextran
308 2000 eluting at 7.2 mL, suggesting the formation of complexes with a molecular weight
309 higher than 700 Da (Fig. 3d). This fact was confirmed by the high percentage of Fe
310 recovered in the peak exclusion with values about 88%, 75%, 70% and 85% for Fe^{3+}:G7
311 (1:0.5, 1:1, 1:2 and 1:3 molar ratios, respectively) of the total content of Fe eluted. In
312 addition, Fe^{3+}:G7 (1:3) was also passed through the Sephadex® G-15 (Fig. 3e) and eluted
313 in the exclusion volume (8.1 mL), indicating the formation of a complexe with a molecular
314 weight higher than 1500 Da. Finally, all samples with the different molar ratios were also
315 passed through the Sephadex® G-25 (Fig. 3f), as well as those which also eluted in the
316 exclusion volume (8.4 mL), indicating a molecular weight higher than 5000 Da. These
317 results confirmed the tendency of the Fe^{3+} complexes to form polynuclear compounds.

318 **3.4 Effect of the Fe complexes on tomato seedlings under hydroponic conditions**

319 The evolution of the chlorophyll index of the youngest, fully expanded leaf (fifth leaf level)
320 by Dualex is presented in (Fig. 4). The –Fe treatment suffered a detriment of the
321 chlorophyll index over the experiment, showing visible symptoms of Fe deficiency such as
322 the yellowing of the fully expanded leaves and the proliferation of lateral roots. In contrast,
323 all the Fe treatments showed a recovery of the chlorophyll index from 6 DAT with a leaf re-
324 greening. All the molar ratios of the Fe^{3+}:G7 complexes presented similar chlorophyll
325 indices (29.5-30.5) than the positive control Fe^{3+}:HBED (31.9) at 15 DAT.

326 No differences in the shoot length among treatments were observed at the end of the
327 experiment (15 DAT) (Table 1). The application of the Fe^{3+}:G7 (1:1) and the Fe^{3+}:G7 (1:2)
328 increased significantly the DW (by 0.3- and 0.2-fold, respectively) and the elongation of the

329 root (by 0.5- and 0.6-fold, respectively) compared to the Fe^{3+}:HBED. The lowest values
330 were obtained for the –Fe treatment with a 0.8-fold lower than the Fe^{3+}:HBED. A similar
331 effect of the different molar ratios of Fe^{3+}:G7 to Fe^{3+}:HBED on the Fe concentration in leaf
332 was obtained. They presented an Fe concentration 1.5-fold higher than the –Fe treatment,
333 indicating a clear Fe deficiency at 15 DAT. Although, the Fe treatments increased the Fe
334 concentration in stem compared to the Fe-deficient plants, the highest Fe concentration
335 was obtained by Fe^{3+}:HBED with a 2-fold increase. In roots, the Fe concentration was
336 clearly lower in the -Fe treatment. Here, the Fe^{3+}:HBED presented the lower value of the
337 Fe treated plants; similar Fe concentrations were obtained by the Fe complexes at
338 different ratios except for the 1:0.5 ratio which presented the highest, raising 1.3-fold as
339 compared to the Fe^{3+}:HBED treatment. Furthermore, the Fe translocation (TR) was
340 calculated as the percentage of Fe in leaf per Fe in the root. The highest Fe TR was
341 presented by the Fe^{3+}:G7 (1:1), similar to the Fe^{3+}:HBED, but the lowest was obtained by
342 the Fe^{3+}:G7 (1:0.5) with a 1.2-fold decrease compared to the Fe^{3+}:HBED.

343 In addition, the influence of the treatments in other metal micronutrient concentrations was
344 studied (Table 2). The –Fe treatment presented the highest values for Mn, Zn and Cu in
345 some tissues as compared to the Fe treatments. A similar increase was obtained for the
346 Mn concentration in leaf and stem by Fe^{3+}:G7 (1:1) and Fe^{3+}:G7 (1:2), respectively. The
347 Fe^{3+}:HBED treatment showed a negative effect on the Cu concentration in leaf as
348 compared to the rest of the treatments. Also, the Fe^{3+}:G7 (1:3) treatment decreased the
349 Cu and Zn concentrations in root and leaf, respectively. The Fe:Mn molar ratio was also
350 evaluated as an index to evaluate an adequate Fe nutrition, which should be within the
351 optimal range 1.5-2.5 in healthy plants according to Adriano[43]. With the exception of the
352 Fe^{3+}:G7 (1:0.5), the Fe:Mn molar ratio was within the range of 1.5-2.5 for the Fe
353 treatments while the –Fe treatment presented a low value.

3.5 Effect of the Fe complexes on soybean seedlings under calcareous soil conditions

No significant differences among treatments were found in the plant growth, nor for the shoot and root DW (average data ± standard error: 1.09 ± 0.10 and 0.28 ± 0.02 g $plant^{-1}$, respectively) or shoot and root length (average data ± standard error: 45.9 ± 8.24 and 20.0 ± 1.41 cm $plant^{-1}$, respectively). The chlorophyll indices by Dualex readings were recorded for all the leaf levels during the experiment but the evolution of the third and fifth leaf levels (from 15 DAT) were used to describe the changes induced by the Fe treatments (Fig. 5a and 5b, respectively). Attending to the third leaf level, the Fe^{3+}:EDTA reached the highest chlorophyll index at 7 DAT, and no differences were presented among the other treatments with respect to the –Fe treatment at that time. At the end of the experiment, all the Fe treatments except of the Fe^{3+}:G7 (1:0.5) were significantly different from the –Fe treatments, showing a recovery in the chlorophyll index with leaf re-greening. For the fifth leaf level, Fe^{3+}:G7 (1:1, 1:2 and 1:0.5) corrected the Fe chlorosis similar to Fe^{3+}:EDTA at 21 DAT, whereas Fe^{3+}:G7 (1:3) and Fe^{3+}:G6 (1:1) along with the –Fe treatment presented the lowest values.

The highest Fe concentration in leaf was presented by the Fe^{3+}:G7 (1:2), which did not present significant differences in comparison with Fe^{3+}:EDTA at 7 DAT (Table 3). For the stem, the Fe^{3+}:EDTA presented the highest Fe concentration, whereas Fe^{3+}:G7 (1:2), Fe^{3+}:G6 (1:1) and the –Fe treatment presented the lowest. At 21 DAT, the Fe^{3+}:G7 (1:0.5, 1:1 and 1:2) presented the highest Fe concentration in leaf similarly to the Fe^{3+}:EDTA, but Fe^{3+}:G7 (1:3) and Fe^{3+}:G6 (1:1) presented the lowest values, similarly to the –Fe treatment at both 7 and 21 DAT. Similar Fe concentrations were found in the root for all the treatments with the exception of the Fe^{3+}:G7 (1:1) presenting the highest Fe concentration, but this data must be taken with care since they could be affected by the precipitation of Fe which may be not completely removed by washing, considering the high values

obtained in all the cases. The soluble and the available Fe concentration in soil were also analyzed, but the quantification of the soluble Fe was not possible because of the instrument detection limit. The highest value in the available fraction was obtained by the Fe^{3+}:EDTA (1.8-fold) followed by the Fe^{3+}:G7 (1:1) (1.2-fold) as compared to the –Fe treatment.

No differences among treatments were shown in the Mn concentration in leaf or stem at 7 DAT, whereas Fe^{3+}:G7 (1:1) presented the highest concentration at 21 DAT in all the soybean tissues analysed as compared to the rest of Fe^{3+}:HBED (Table 4). The Fe^{3+}:G7 (1:3) and Fe^{3+}:G6 (1:1) presented the lowest concentrations similar to the -Fe at 21 DAT in all the soybean tissues. Regarding to the Fe:Mn molar ratio in leaf , the highest values were obtained by the Fe^{3+}:G7 (1:2) and the Fe^{3+}:EDTA at 7 DAT while at 21 DAT they corresponded to the Fe^{3+}:G7 (1:0.5) and the Fe^{3+}:EDTA. For the Zn concentration, the highest value was presented by the Fe^{3+}:EDTA at both 7 and 21 DAT in all the soybean tissues, whereas the Fe^{3+}:G7 (1:3) and the Fe^{3+}:G6 (1:1) presented the lowest at both 7 and 21 DAT in leaf and root. The Fe^{3+}:G7 (1:1) increased the Zn concentration at 7 DAT in leaf and at 21 DAT in stem similar to the Fe^{3+}:HBED. For the Cu concentration in leaf, no differences were observed among the Fe treatments with G6 or G7 at 7 DAT, whereas both control treatments showed the lowest Cu concentration. A similar result was observed in leaf at 21 DAT for both control treatments along with Fe^{3+}:G7 (1:3) and the Fe^{3+}:G6 (1:1). With the exception of the Fe^{3+}:EDTA, these treatments also presented the lowest Cu concentration in the stem. In contrast, the Fe^{3+}:G7 (1:1) presented the highest Cu concentration at 21 DAT in all soybean tissues as compared to the Fe^{3+}:HBED. The soluble fraction of the micronutrient concentrations (Table 3) was restrained by the instrument detection limits, whereas the available fraction showed an increase of 1.5-2.0-fold by the Fe^{3+}:EDTA and the Fe^{3+}:G7 (1:1) for all the micronutrients as compared to the rest of the treatments.

4 Discussion

4.1 Complexing capacity of the gluconate and heptagluconate and characterization in function of the Fe:ligand ratios

For both G6 and G7 the MCC with Fe^{3+} was higher than that with Fe^{2+} inorganic salts, indicating a higher stability of the Fe^{3+} complexes than for the Fe^{2+} complexes at high pH. This parameter has been confirmed by the low complexed fraction by Fe^{2+}:G7 (1:2) at pH 9 mentioned above, as a consequence of the formation of Fe oxides and hydroxides and, thus, decreasing the solubility of the Fe[22,23]. The presence of a Fe^{3+} phase in the Mössbauer spectrum of the Fe^{2+}: G7 (1:2) (see Fig. 3b) also confirms the oxidation of these complexes. The higher complexing capacity with Fe^{3+} as compared to the Fe^{2+} salts, have been also described for other complexes such as the lignosulfonates[22]. These complexing agents present a wide variety of functional groups such as carboxylic and hydroxyl groups, which are also present in G7 and G6. The presence of other complexing agents in the complexing agents G6 and G7 used was discarded by the ^{13}C NMR and FTIR spectra (Fig. 2), thus confirming that the higher Fe^{3+} complexing capacity of G7 than for the G6 was not due to impurities. These results are consistent with those reported by López-Rayo et al.[44], where G7 presented a better complexing capacity than G6 for Mn in solution. Also, Clemens et al.[14] hypothesized that G7 has a higher complexing capacity than G6 due to previous studies that demonstrated its effectiveness to correct Fe chlorosis in calcareous soils.

The molecular weight distribution over 5000 Da for Fe^{3+}:G7 complexes at different molar ratios (Fig. 3f) is in agreement to the molecular weights of Fe^{3+}:sugars previously reported[18] analyzed by GFC on Sephadex® G-25 and G-100. Silva et al.[20], also observed the formation of oligomeric species for a 1:2 Fe^{3+}:citrate mixture at neutral pH. The high molecular weight obtained by the different molar ratios of the Fe^{3+}:G7 is attributed to the

formation of polynuclear structures, as was confirmed by the Mössbauer spectrum of the Fe^{3+}:G7 (1:2) (see Figure 1a). The formation of the polynuclear structures in the Fe^{3+}:G7 complexes involves carboxylate oxygen and deprotonated alcoholic hydroxy groups[45], as was detected in the FTIR spectra of the Fe^{3+}:G7 complexes (Fig. 2d). This FTIR spectra showed the binding of Fe^{3+} with carboxylic and hydroxyl groups, as well as the interaction Fe-O that can take place in the formation of the polynuclear complexes[18].

4.2 Effectiveness of the complexes at different Fe:ligand ratios to provide Fe under hydroponic conditions

The Fe^{3+}:G7 complexes at all the molar ratios were able to correct the Fe chlorosis when they were applied at the same Fe dose than the Fe^{3+}:HBED, based on the chlorophyll indices results (see Fig. 4). Moreover, the Fe concentration in the leaves did not show significant differences among the Fe treatments, being all within the Fe sufficient concentration range (50-150 μg g^{-1} DW) described by Marschner[1]. According to Ejraei[2], Fe plays an important role in the growth and development of tomato. Therefore, the increase in the length and the DW of roots for the Fe^{3+}:G7 (1:1) and the Fe^{3+}:G7 (1:2) can be an indicator of the ability of these complexes to provide Fe in hydroponic conditions. Carrasco-Gil et al.[46] also observed an increase in the DW of roots after the application of Fe to deficient tomato plants grown under hydroponic conditions. The effect of the treatments in the Fe nutrition was visible compared to the Fe-deficient plants, which presented a low chlorophyll index (see Fig. 4), and visible symptoms of chlorosis together with low Fe concentrations in the plant tissues. These plants also showed a marked decrease in the DW and root length, which values were similar to the Fe-deficient tomato plants grown under hydroponic conditions of other authors[4,47]. The high Fe concentration in the root of plants treated with Fe^{3+}:G7 (1:05) could suggest a possible Fe precipitation on the root surface, and, as a consequence of the low percentage of Fe TR to leaf (1.2-fold) as compared to the Fe^{3+}:HBED (Table 1), related to its low stability. This

457 accumulation was also observed by Kovács et al.[48] when using Fe^{3+}:citrate but no when
458 stable synthetic chelates were used. In contrast, the high percentage of Fe TR presented
459 by the Fe^{3+}:HBED (Table 1) was confirmed by a 2-fold increase in the Fe concentration in
460 stem as compared to the rest of the treatments. This result is in agreement with Martín-
461 Fernández et al.[49], who demonstrated a high percentage of Fe TR when the Fe^{3+}:HBED
462 was applied in early growth stages. A similar percentage of Fe TR to the Fe^{3+}:HBED was
463 presented by the Fe^{3+}:G7 (1:1) compared to the rest of molar ratios. These differences
464 may be due to the different stability of the complexes, as a main consequence of the
465 different molar ratios used for their preparation. According to Carrasco et al.[22], the
466 Fe:lignosulfonate molar ratio influenced in the coordination sites involved in the formation
467 of Fe complexes.

468 The Mn concentration in leaves of the plants treated with the complexes were above the
469 critical deficiency concentrations (10-20 μg g^{-1} DW) indicated by Marschner[1], presenting
470 the Fe^{3+}:G7 (1:1) the highest concentration in leaf and Fe^{3+}:G7 (1:2) in the stem (Table 2).
471 The larger elongation of the root for both treatments may be contributing to the better Mn
472 uptake (Table 2). Because the G7 has shown to be an efficient complexing agent to
473 maintain the Mn in solution under hydroponic conditions according to López-Rayo et al.[44],
474 it may explain the higher Mn uptake obtained in our experiment as compared to the Zn and
475 Cu. Besides, these results suggest that a higher Mn uptake by the plant may be promoted
476 by the application of Fe^{3+}:G7 (1:1 and 1:2) under the same conditions. In contrast, the
477 Fe^{3+}:G7 (1:3) showed a low Mn concentration, as well as the lowest Zn concentration in
478 leaf, even lower than the critical deficiency concentration (15-20 μg Zn g^{-1} DW) established
479 by Marschner[1]. The low concentrations of micronutrients obtained by Fe^{3+}:G7 (1:3) can be
480 due to the combined effect of an excess of complexing agent and a low Fe concentration.
481 These explanation was supported by the results obtained by Wallace et al.[25], where a Cu
482 deficiency was obtained in bush bean plants grown under hydroponic conditions growth

with a low Fe concentration (10^{-5} M) and an excess of EDTA. In the case of the Cu in leaf, the Fe^{3+}:G7 complexes presented concentrations above the critical deficiency concentrations (1-5 μg g^{-1} DW) in agreement with Marschner[1], but not the Fe^{3+}:HBED treatment (Table 2). High concentrations of Mn, Cu and Zn in some tissues of the Fe-deficient plants were also observed in tomato grown under hydroponic conditions in Fe-free conditions[46].

4.3 Effectiveness of Fe:G7 and Fe:G6 complexes to provide Fe in calcareous soil conditions

This second experiment in calcareous soil was performed with soybean plants instead of tomato plants. Tomato has shown low sensitivity to Fe deficiency[50] and, consequently, lower differences are expected among the Fe treatments under soil conditions. Despite the soil used was selected for its low Fe availability, this could be enough to supply sufficient Fe to the tomato plants. Moreover, the Fe^{3+}:EDTA presented the highest chlorophyll index at 7 DAT (Fig. 5a) and, both, the Fe^{3+}:EDTA and Fe^{3+}:G7 (1:2) presented the highest Fe concentration in leaf at 7 DAT (Table 3). Iron chelates have shown a faster effect for the Fe chlorosis recovery than the Fe complexes. A long-lasting effect prevails in these compounds because the Fe is mainly accumulated in the soil available fraction (Table 3); however, the remaining Fe in the soluble fraction was not analyzed in our experiment due to the instrumental limitations. This long-lasting effect of the studied Fe complexes such as Fe^{3+}:G6 (1:2) and Fe^{3+}:LS, and the fast effect of the Fe chelates such as Fe^{3+}:EDDHA have been also observed by Martín-Fernández et al.[10]. At 21 DAT, the Fe^{3+}:G7 (1:0.5 and 1:3) and Fe^{3+}:G6 (1:1) showed that the chlorophyll indices for the third and fifth leaf level were variable (Fig. 5a and b, respectively), except to the Fe^{3+}:G7 (1:1 and 1:2) that presented a similar increase to the Fe^{3+}:EDTA for both leaf levels. The fact that re-greening occurred in both leaf levels for the Fe^{3+}:G7 (1:1 and 1:2) suggests a higher Fe distribution in the leaves, similar to the Fe^{3+}:EDTA, as has been shown by Martín-

509 Fernández et al.[11] for Fe^{3+}:G6 (1:2) and Fe^{3+}:lignosulfonate. In addition, the chlorophyll
510 indices measured in the fifth leaf level presented a similar tendency to the total Fe content
511 in leaves. Most of them were above the Fe critical concentrations (45-50 mg kg^{-1} DW in
512 leaf) in soybean estimated by Adams et al.[51], with the exception of Fe^{3+}:G6 (1:1) and
513 Fe^{3+}:G7 (1:3), which values were lower or closer to the critical concentration. A Fe:Mn
514 molar ratio in leaf within the range 1.5-2.5 indicates an adequate nutrition according to
515 Adriano[43] but, in this study, values below 1.5 were obtained due to the low Fe dose used in
516 the treatments to obtained a better comparison of the treatments. However, at 7 DAT, the
517 values were higher than the values obtained by García-Marco et al.[52] under hydroponic
518 conditions, with soybean (0.67-0.54) grown for 14 days with low Fe concentrations of
519 Fe^{3+}:EDDHA. At 21 DAT, only the Fe^{3+}:EDTA and the Fe^{3+}:G7 (1:0.5) were higher than
520 those values since our study was conducted for 7 days more.

521 Fertilization with Fe^{3+}:G6 (1:1) resulted in a low chlorophyll index at 21 DAT in the fifth leaf
522 level which can be associated to its lower complexing capacity in comparison with G7 (Fig.
523 1), which has already been hypothesized by Clemens et al.[14]. This treatment also showed
524 a decrease in the concentrations of Fe, Mn, Zn and Cu in soybean tissues (Table 3 y 4).
525 This fact is in good agreement with the low stability of G6 to maintain Mn and Zn in
526 calcareous soil previously reported[53].

527 Based on the results obtained in these experiments, the influence of the variation of the
528 Fe:ligand ratio in the complexes may affect the following chemical mechanisms for the Fe
529 stability and availability to the plants: (I) When the Fe^{3+}:G7 (1:3) ratio is considered, the
530 equilibrium of the complexation reaction is shifted to the complexes formation due to the
531 excess of ligand. As a result, the bioavailability of the other micronutrients is reduced, and
532 even the Fe uptake by the plants can be retarded. (II) When the Fe^{3+}:G7 (1:0.5) ratio is
533 considered, the equilibrium of the complexation reaction is shifted to the release of Fe^{3+}
534 due to a decrease in the complexing agent. Because of the high pH in the media, the Fe is

535 more susceptible to precipitation as Fe-oxyhydroxides on root surfaces, thus, reducing the
536 Fe available to plants and affecting the Fe translocation. (III) When the Fe^{3+}:G7 (1:1) and
537 Fe^{3+}:G7 (1:2) ratios are considered, the equilibrium among micronutrients, as well as the
538 stability of the Fe and the complexing agent seems to be promoted. This effect could be
539 related to the affinity of the metal:ligand that can avoid competition among micronutrients
540 and the Fe-oxyhydroxides precipitation.

541 The proposed chemical mechanistic behavior of the studied complexes are consistent with
542 the low concentrations of Fe, Mn and Zn presented by soybean plants treated with
543 Fe^{3+}:G7 (1:3) (Table 3 and 4), as well as with the results obtained for the Fe^{3+}:G7 (1:3)
544 under hydroponic conditions (Table 2). This fact was also noticed by the low chlorophyll
545 index presented in the soybean at 21 DAT (Fig. 5b). This decrease in the plant uptake of
546 Fe, Mn, and Zn associated with the excess of ligands have already been described for
547 other ligands such as DTPA, BPDS and EDTA[24,25]. In our work the Fe^{3+}:G7 (1:1) showed
548 also the highest Mn and Cu concentrations (Table 4), indicating that this molar ratio can be
549 optimal for keeping and adequate levels of these micronutrients in the plants, which was
550 also supported by the highest Mn concentration founded by the experiment under
551 hydroponic conditions (Table 2). So, although Fe^{3+}:G7 (1:2) presented a low Fe
552 complexed fraction, it was able to sufficiently provide Fe to the plants due to the formation
553 of polynuclear Fe complexes, that can present variable stability depending on the
554 Fe:ligand ratio, confirming the hypothesis of this study. As it have been observed by
555 previous studies[21,54], where the complexes stability is high at low Fe:humic substances
556 ratios at alkaline pH (e.g 1:2 compared to 1:0.5 in this work).

557 **5 Conclusions**

558 The present work provides sufficient evidences to demonstrate that Fe can be used more
559 efficiently from Fe complexes with G7 than with G6 prepared at room temperature,

providing a more stable complex and high availability for the plant, and in both cases, a long-lasting effect due to the formation of polynuclear Fe complexes. Considering the Fe source, both complexing agents G6 and G7 have a higher affinity with Fe^{3+} than with Fe^{2+}, showing a better Fe complexing capacity and stability of the Fe^{3+} complexes at high pH. The Fe^{3+}:G7 molar ratios have shown to be critical for the Fe plant uptake under hydroponic conditions and calcareous soil. An excess of ligand with respect to the Fe^{3+} reduced the micronutrients uptake in the plant by the displacement of the equilibrium reaction towards the complex formation. The Fe^{3+}:G7 1:1 and 1:2 molar ratios were those highly improving the Fe uptake while high Fe^{3+}:G7 ratios are not adequate due to the Fe-oxyhydroxides precipitation. The results obtained also showed that a Fe^{3+}:G7 equimolar ratio can improve the uptake of other micronutrients such as Mn and Cu. Thus, the Fe^{3+}:G7 at equimolar ratio and 1:2 molar ratio can be an environmentally friendly alternative to less degradable synthetic chelates to correct Fe chlorosis in strategy I plants. Further studies with Fe^{3+}:G7 ratios of 1:1 and 1:2, but of low molecular weight synthetized according to other methodologies, as described by Nikolić et al.[29] would contribute to improving the knowledge of their effectiveness and their long-lasting effect.

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736 **Table 1:** Data of growth, Fe concentration and translocation (TR, leaf concentration/root
737 concentration) in tomato plants at 15 DAT, grown under hydroponic conditions.

Treatment	Length (cm)		DW (g plant^{-1})		Fe (mg Kg^{-1} DW)			Fe TR (%)
	Shoot	Root	Shoot	Root	Leaf	Stem	Root	Leaf/root
-Fe	22.8^{ns}	24.8^{c}	0.97^{c}	0.19^{c}	19.0^{b}	9.48^{e}	32.1^{d}	55.4^{b}
Fe^{3+}:G7 (1:0.5)	25.5	40.0^{b}	1.75^{b}	0.25^{c}	54.4^{a}	20.7^{d}	152^{a}	34.4^{c}
Fe^{3+}:G7 (1:1)	24.3	53.3^{a}	1.81^{b}	0.46^{ab}	59.5^{a}	31.4^{bc}	104^{b}	65.5^{ab}
Fe^{3+}:G7 (1:2)	24.1	48.3^{ab}	2.41^{a}	0.49^{a}	50.3^{a}	26.7^{c}	102^{b}	58.4^{b}
Fe^{3+}:G7 (1:3)	27.5	40.1^{b}	1.62^{b}	0.33^{bc}	55.2^{a}	32.9^{b}	101^{b}	58.6^{b}
Fe^{3+}:HBED	24.8	42.6^{b}	2.01^{ab}	0.33^{bc}	49.1^{a}	38.5^{a}	61.1^{c}	81.8^{a}

738 Different letters indicate significant differences among treatments according to Duncan´s
739 test ($p \leq 0.05$). ns, not significant.

740 **Table 2:** Cu, Mn and Zn concentrations in tomato plants tissues and Fe/Mn ratio in leaf at 15 DAT under hydroponic conditions.

Treatment	Mn (mg Kg^{-1} DW)			Fe:Mn	Zn (mg Kg^{-1} DW)			Cu (mg Kg^{-1} DW)		
	Leaf	Stem	Root	Leaf	Leaf	Stem	Root	Leaf	Stem	Root
-Fe	49.6^{a}	10.1^{b}	29.1^{a}	0.39^{d}	40.6^{a}	21.7^{a}	29.7^{a}	6.03^{a}	3.16^{ns}	14.6^{a}
Fe^{3+}:G7 (1:0.5)	39.3^{b}	10.1^{b}	34.0^{a}	1.40^{c}	17.9^{b}	15.7^{c}	26.7^{ab}	5.99^{a}	3.19	9.22^{bc}
Fe^{3+}:G7 (1:1)	43.3^{ab}	12.2^{ab}	18.4^{b}	1.53^{bc}	17.0^{b}	19.2^{b}	15.2^{d}	7.61^{a}	3.20	8.04^{c}
Fe^{3+}:G7 (1:2)	29.6^{c}	12.9^{a}	18.9^{b}	1.84^{ab}	14.9^{bc}	15.0^{c}	19.9^{bcd}	5.81^{a}	2.51	9.63^{bc}
Fe^{3+}:G7 (1:3)	25.4^{c}	10.1^{b}	22.4^{b}	2.18^{a}	11.1^{c}	17.0^{bc}	18.2^{cd}	5.89^{a}	3.36	7.93^{c}
Fe^{3+}:HBED	29.2^{c}	9.5^{b}	20.6^{b}	1.68^{bc}	18.1^{b}	14.9^{c}	23.5^{abc}	4.13^{b}	2.53	12.3^{ab}

741 Different letters indicate significant differences among treatments according to Duncan´s test ($p \leq 0.05$). ns, not significant.

742 **Table 3:** Fe concentrations in soybean tissues at 7 and 21 DAT in calcareous soil, and the
743 available fraction of metals extracted from soils at 21 DAT.

Treatment	7 DAT (mg Kg^{-1} DW)		21 DAT (mg Kg^{-1} DW)			Available metal in soil (mg Kg^{-1})			
	Leaf	Stem	Leaf	Stem	Root	Fe	Mn	Zn	Cu
-Fe	56.1^{cd}	29.7^{d}	42.6^{c}	35.7^{a}	618^{b}	6.31^{c}	5.51^{c}	0.91^{c}	1.03^{d}
Fe^{3+}:G7 (1:0.5)	64.4^{bc}	45.8^{bc}	58.5^{a}	20.1^{b}	662^{b}	6.46^{c}	4.66^{c}	0.88^{c}	0.95^{cd}
Fe^{3+}:G7 (1:1)	66.9^{bc}	33.9^{cd}	54.1^{a}	19.5^{b}	928^{a}	14.3^{b}	15.5^{b}	3.71^{a}	2.87^{a}
Fe^{3+}:G7 (1:2)	83.4^{a}	31.8^{d}	51.5^{ab}	24.0^{b}	791^{ab}	9.20^{c}	6.92^{c}	2.10^{b}	1.71^{bc}
Fe^{3+}:G7 (1:3)	47.3^{d}	51.3^{b}	45.7^{bc}	18.9^{b}	632^{b}	5.76^{c}	6.13^{c}	0.85^{c}	0.87^{cd}
Fe^{3+}:G6 (1:1)	49.3^{d}	30.5^{d}	43.7^{c}	18.4^{b}	589^{b}	6.08^{c}	8.00^{b}	0.66^{bc}	0.97^{cd}
Fe^{3+}:EDTA	72.5^{ab}	70.6^{a}	56.4^{a}	26.9^{b}	642^{b}	22.6^{a}	22.0^{a}	2.63^{bc}	2.58^{b}

744 Different letters indicate significant differences among treatments according to Duncan´s
745 test ($p \leq 0.05$). ns, not significant.

746 **Table 4:** Cu, Mn and Zn concentrations in plant soybean plants tissues at 7 and 21 DAT in calcareous soil.

Treatment	Sampling (DAT)	Mn (mg Kg^{-1} DW)			Fe:Mn	Zn (mg Kg^{-1} DW)			Cu (mg Kg^{-1} DW)		
		Leaf	Stem	Root	Leaf	Leaf	Stem	Root	Leaf	Stem	Root
-Fe	7	73.5^{ns}	13.8^{ns}	---	0.76^{c}	39.0^{c}	14.0^{ns}	---	5.83^{b}	7.50^{bc}	---
Fe^{3+}:G7 (1:0.5)		72.1	14.9	---	0.95^{abc}	41.0^{c}	16.3	---	14.7^{a}	12.4^{a}	---
Fe^{3+}:G7 (1:1)		84.5	18.9	---	0.83^{bc}	55.5^{ab}	17.4	---	15.6^{a}	12.9^{a}	---
Fe^{3+}:G7 (1:2)		80.7	19.0	---	1.10^{ab}	47.0^{bc}	15.1	---	15.5^{a}	4.30^{c}	---
Fe^{3+}:G7 (1:3)		64.1	15.9	---	0.75^{c}	42.4^{c}	14.7	---	14.8^{a}	7.40^{bc}	---
Fe^{3+}:G6 (1:1)		63.6	16.2	---	0.82^{bc}	49.3^{bc}	15.4	---	16.0^{a}	10.1^{ab}	---
Fe^{3+}:EDTA		71.4	15.8	---	1.15^{a}	62.2^{a}	17.2	---	5.88^{b}	11.8^{a}	---
-Fe	21	102^{c}	14.3^{c}	19.8^{c}	0.43^{b}	42.0^{bc}	8.97^{b}	20.8^{cd}	10.9^{cd}	3.09^{c}	16.4^{b}
Fe^{3+}:G7 (1:0.5)		107^{c}	14.6^{c}	19.2^{c}	0.55^{a}	46.4^{b}	11.8^{b}	35.1^{a}	14.0^{ab}	2.96^{c}	22.6^{a}
Fe^{3+}:G7 (1:1)		161^{a}	23.0^{a}	46.6^{a}	0.35^{b}	47.1^{b}	16.4^{a}	25.3^{b}	15.0^{a}	5.48^{a}	23.0^{a}
Fe^{3+}:G7 (1:2)		128^{b}	18.8^{b}	33.9^{b}	0.43^{b}	40.9^{bc}	11.8^{b}	24.2^{bc}	13.2^{ab}	3.97^{b}	13.6^{c}
Fe^{3+}:G7 (1:3)		106^{c}	15.1^{bc}	18.5^{c}	0.43^{b}	27.2^{d}	9.10^{b}	22.9^{bcd}	10.6^{d}	2.83^{c}	17.8^{b}
Fe^{3+}:G6 (1:1)		101^{c}	15.2^{bc}	19.2^{c}	0.41^{b}	31.5^{cd}	9.81^{b}	19.5^{d}	10.4^{d}	3.23^{c}	13.6^{c}
Fe^{3+}:EDTA		89^{c}	16.3^{bc}	33.7^{b}	0.65^{a}	83.9^{a}	17.6^{a}	35.4^{a}	12.7^{bc}	4.98^{a}	16.3^{b}

747 Different letters indicate significant differences among treatments according to Duncan´s test ($p \leq 0.05$). ns, no significant.

Figure legends

Figure 1: Titration curves for the determination of the maximum complexing capacity of heptagluconate (G7) with a) Fe^{3+} and c) Fe^{2+}, and gluconate (G6) with b) Fe^{3+} and d) Fe^{2+}.

Figure 2: ^{13}C-NMR spectra of a) G6 and b) G7. FTIR spectra of c) G6 and Fe^{3+}:G6 (1:1) and d) G7 and the Fe^{3+}:G7 complexes.

Figure 3: ^{57}Fe Mössbauer spectra of a) Fe^{3+}:G7 (1:2) and b) Fe^{2+}:G7 (1:2) recorded at 298 K. The blue line indicates Fe^{3+} and the red line indicates Fe^{2+}. c) Absorption spectra of the molar ratios of Fe^{3+}:G7 and G7. d) Gel filtration chromatography of the Fe^{3+}:G7 complexes eluted on Sephadex® G-10. e) Elution of Fe^{3+}:G7 (1:3) on Sephadex® G-15 and f) elution of the molar ratios of Fe^{3+}:G7 on Sephadex® G-25 with 0.1 M NaCl at pH 6. V_o indicate the exclusion volume marker in each column.

Figure 4: Effect of the treatments applied to tomato plants under hydroponic conditions on a) chlorophyll index measured by Dualex in the fifth leaf level. Error bars indicate the standard error (N=5). Different letters represent significant differences among treatments following Duncan test ($p \leq 0.05$ level). ns, not significant.

Figure 5: Effect of the treatments applied on the chlorophyll index in the soybean plants grown in calcareous soil measured by Dualex in a) the third leaf level and b) fifth leaf level. Error bars indicate the standard error (N=6). Different letters represent significant differences among treatments at each day following Duncan test ($p \leq 0.05$ level). ns, not significant. Arrows indicate the days of application of the treatments.

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Effect of Fe:ligand ratios on hydroponic conditions and calcareous soil in Solanum lycopersicum L. and Glycine max L. fertilized with heptagluconate and gluconate

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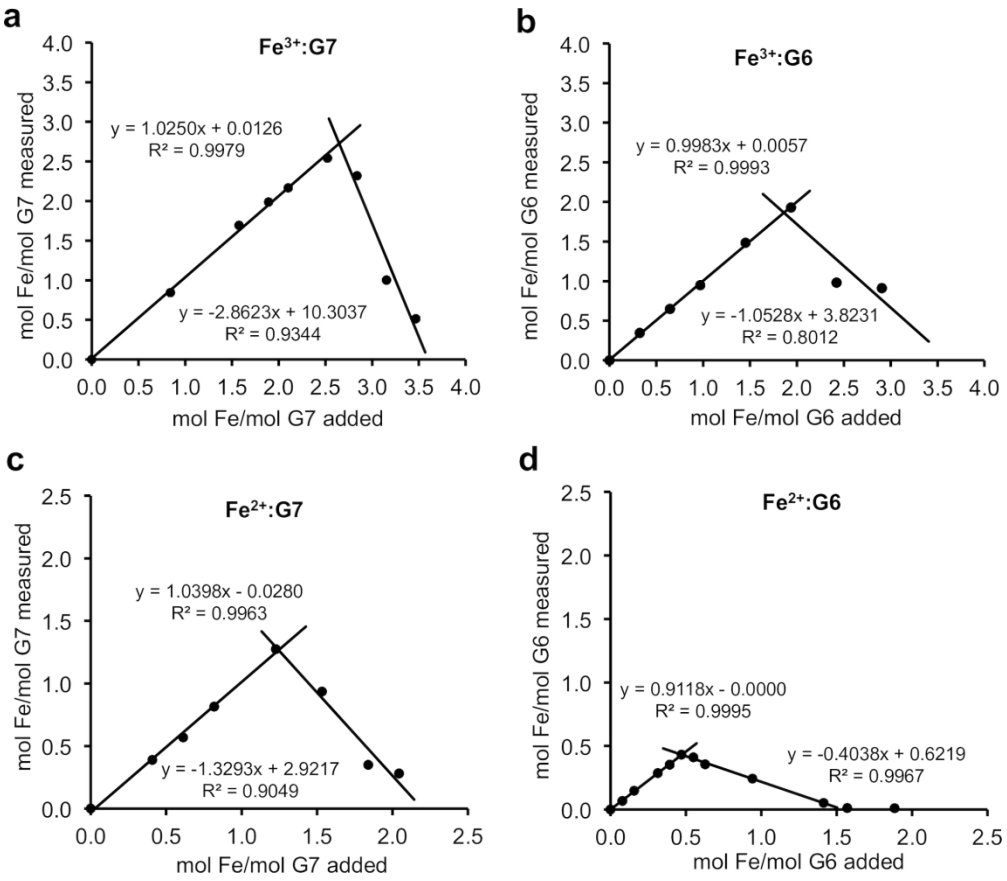

Figure 1: Titration curves for the determination of the maximum complexing capacity of heptagluconate (G7) with a) Fe3+ and c) Fe2+, and gluconate (G6) with b) Fe3+ and d) Fe2+.

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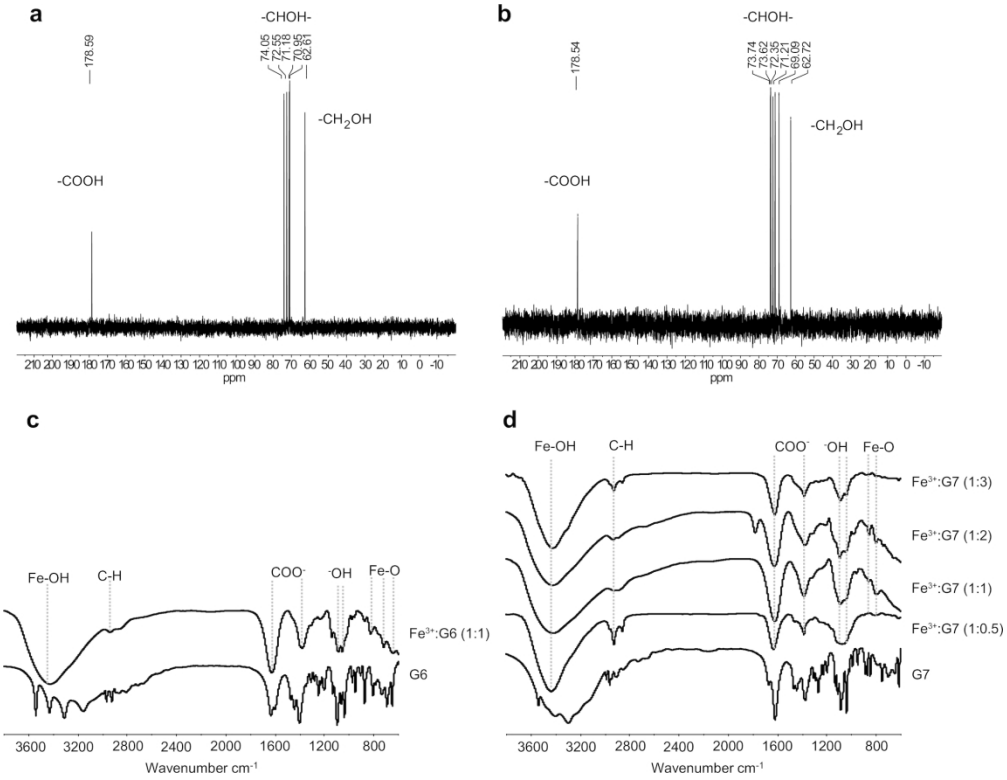

Figure 2: 13C-NMR spectra of a) G6 and b) G7. FTIR spectra of c) G6 and Fe3+:G6 (1:1) and d) G7 and the Fe3+:G7 complexes.

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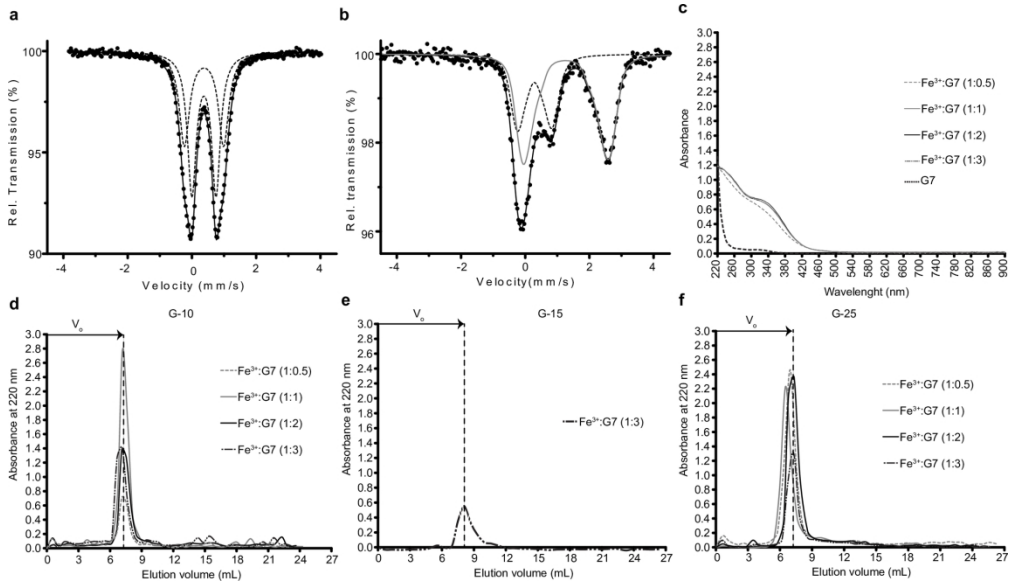

Figure 3: 57Fe Mössbauer spectra of a) Fe3+:G7 (1:2) and b) Fe2+:G7 (1:2) recorded at 298 K. The blue line indicates Fe3+ and the red line indicates Fe2+. c) Absorption spectra of the molar ratios of Fe3+:G7 and G7. d) Gel filtration chromatography of the Fe3+:G7 complexes eluted on Sephadex® G-10. e) Elution of Fe3+:G7 (1:3) on Sephadex® G-15 and f) elution of the molar ratios of Fe3+:G7 on Sephadex® G-25 with 0.1 M NaCl at pH 6. Vo indicate the exclusion volume marker in each column.

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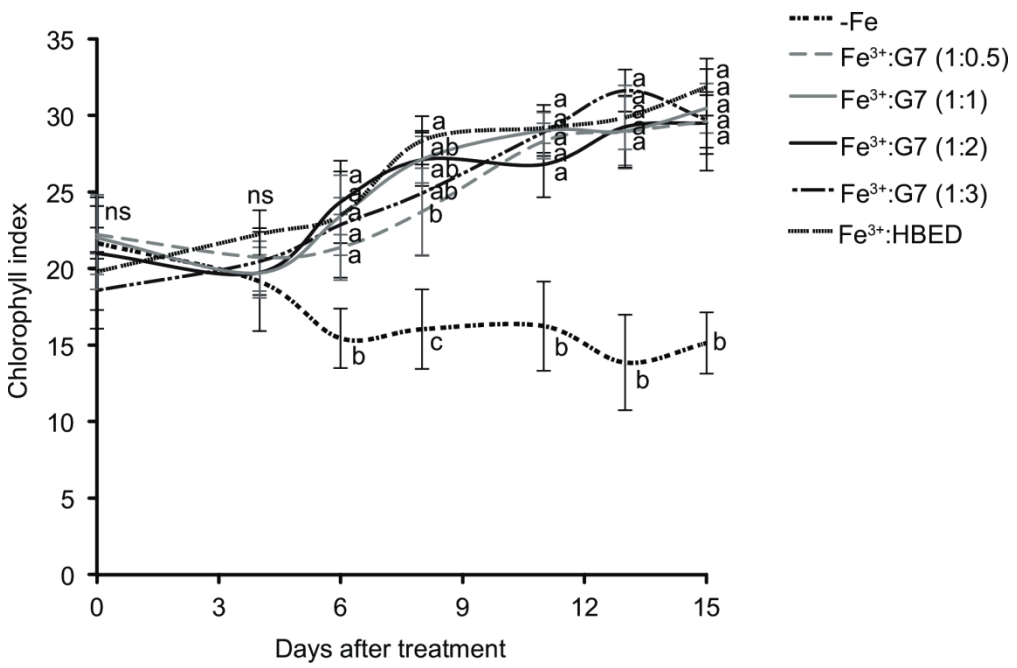

Figure 4: Effect of the treatments applied to tomato plants under hydroponic conditions on a) chlorophyll index measured by Dualex in the fifth leaf level. Error bars indicate the standard error (N=5). Different letters represent significant differences among treatments following Duncan test ($p < 0.05$ level). ns, not significant.

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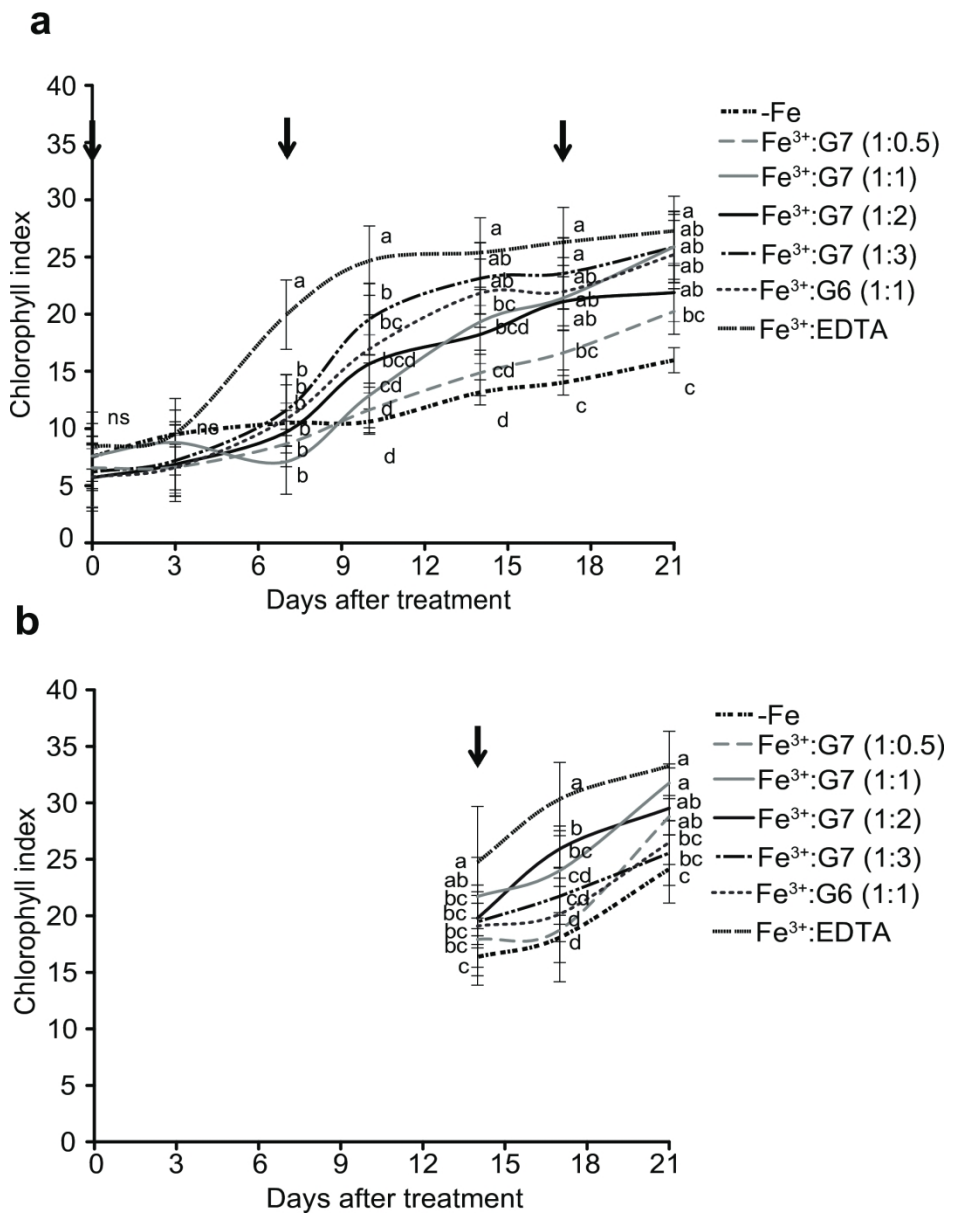

Figure 5: Effect of the treatments applied on the chlorophyll index in the soybean plants grown in calcareous soil measured by Dualex in a) the third leaf level and b) fifth leaf level. Error bars indicate the standard error (N=6). Different letters represent significant differences among treatments at each day following Duncan test ($p < 0.05$ level). ns, not significant. Arrows indicate the days of application of the treatments.

170x218mm (300 x 300 DPI)