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REGULAR ARTICLE

Azotochelin and N-dihydroxy-N,N'-diisopropylhexanediamide as Fe sources to cucumber plants in hydroponic cultures

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ABSTRACT

Environmental concerns related to the use of synthetic iron chelates, usually non-biodegradable, for overcoming iron chlorosis motivates the search for alternative compounds. Thus, the main aim of this work was to evaluate siderophore, azotochelin, and a siderophore mimic, N-dihydroxy-N,N'-diisopropylhexanediamide (DPH) as potential sources of iron to cucumber plants grown in hydroponic cultures. The behavior of the iron chelates of azotochelin and DPH, as a substrate of ferric chelate reductase (FCR) and the ability as iron suppliers for chlorotic plants was studied and compared with *o,o*-EDDHA/Fe^{3+} and EDTA/Fe^{3+} chelates, traditionally used for this purpose. The rate of reduction of DPH/Fe^{3+}, by FCR, was comparable to *o,o*-EDDHA/Fe^{3+} but lesser than the obtained for EDTA/Fe^{+3}. The rate of reduction for azotochelin/Fe^{3+} was not possible to determine. Both azotochelin/Fe^{3+} and DPH/Fe^{3+} chelates were effective in supplying iron to cucumber plants. After 7 and 21 days, all the plants treated with the iron chelates (10 μM, Fe) of DPH and azotochelin showed significantly higher SPAD index, leaf dry weight and leaf Fe concentration than the control plants (2μM, Fe). In conclusion, azotochelin/Fe^{3+} and DPH/Fe^{3+} can be considered as iron sources for cucumber plants when growing in hydroponic culture.

Keywords: Iron chlorosis; Ferric chelate reductase; Siderophore; Hydroponic culture

INTRODUCTION

Iron chlorosis is a common and complex nutritional disorder that affects plant growth with insufficient quantities of available iron, especially in calcareous soils where calcium carbonate buffers soil solution at pH 7.5-8.5 and the concentration of bicarbonate is high. It is characterized by leaf yellowing that affects the development and decreases the yield of many crops (Villen et al., 2007; Rodriguez-Lucena et al., 2010b, 2011; Maqueda et al., 2011; Nadal et al., 2012).

Higher plants have developed efficient mechanisms for acquiring Fe in response to Fe deficiency, known as "Strategy I" and "Strategy II". Strategy I, found in dicotyledonous and non-graminaceous monocotyledonous, involves i) Fe(III) solubilization, usually by rhizosphere acidification, through proton extrusion, ii) the release of reductants and chelating compounds for Fe(III) complexation and mobilization and iii) the reduction of Fe(III) before iv) uptake into roots cell by a specific transporter for Fe(II) (Marschner and Romheld, 1994; Vansuyt et.al. 2007; Ma and Ling, 2009; Jin, et al., 2014). While Strategy I is characterized by soil acidification and Fe reduction for absorption of Fe(III), Strategy II plants secrete mugineic acid family phytosiderophores (MAs), synthesized by gramineous plants, to dissolve Fe in the rizosphere and acquire iron as Fe(III)-MAs complexes (Ding et al. 2009; Xiong et al. 2013).

Moreover, the direct uptake of Fe(III)-siderophore complexes without the requirement of a reduction step

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can also occur and the direct uptake of siderophores by strategy I plants was proposed by several authors. This ability was considered in the development of strategies to protect crops from Fe-limiting environments. Peanut/maize intercropping has shown to be a sustainable cropping system in farming practicy, which effectively improves Fe nutrition in peanuts, a strategy I plant species intercroped with maize, a strategy II plant species, in limed soils, when compared with peanut monoculture (Ding et al. 2009; Xiong et al. 2013). According to Ding et.al. (2009; 2010), this beneficial effect was associated with the increased siderophore secretion by maize and the increased Fe(III) reductase activity and transcript levels AhFRO1 by peanut. Xiong et al. reported that phytosiderophores released by Fe-deficient wheat promoted Fe acquisition and improved Fe nutrition in nearby peanuts, and the phytosiderophore deoxymugeinic acid (DMA) was detected in the roots of intercropped peanuts. Additionally the yellow stripe1-like (YSL) family of genes, which are homologous to maize yellow stripe 1 (ZmYS1), and that transport Fe(III)-MAs, where identified in peanut roots, suggesting that Fe(III)-DMA dissolved by maize might be absorbed directly by neibouring peanuts in peanut/maize intercropping system (Xiong et al. 2013).

Nowadays, the most efficient remedy to overcome and control iron chlorosis is the application of iron chelates to soils. However, most of these chelates are expensive and poorly biodegradable. After the chelate splitting (dechelation), these ligands lead to environmental concerns that arise from their persistence in the environment where they can solubilize heavy metals from soils and sediments and thus to increase the presence of metals in ground and drinking waters (Rodriguez-Lucena et.al. 2010a, b, 2011).

So, alternative compounds with Fe-complex forming comparable to the usual synthetic aminopolycarboxylates (APCAs) used and showing better biodegradability are needed to be introduced. Many siderophores [low molecular weight (<1500 Da) chelating agents produced by a wide range of organisms (plant, fungal and bacterial) under iron limiting conditions (Crumbliss and Harrington, 2009)], namely those containing catechol and hydroxamic acid groups, evidence important characteristics (are Fe-effective chelators, more selective to Fe(III) than to other divalent metals and better biodegradable than synthetic APCAs) that make them potentially substitutes of the synthetic APCAs, traditionally used for Fe fertilization.

The N,N'-dihydroxy-N,N'-diisopropylhexamide (DPH) (Fig. 1) is a biological and physicochemical model of the siderophore rhodotorulic acid, a dihydroxamic acid produced by *Rhodotorula pilimane* and related yeasts (Barclay et al., 1984). A recent study demonstrated the ability of DPH to maintain iron in a soluble form up to pH 9.5 under

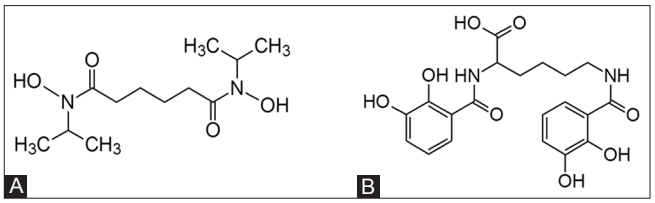


Fig 1. Structure of (A) N-Dihydroxy-N,N'-diisopropylhexanediamide (DPH) and (B) (N, N´)-2,6– Bis(2,3-dihydroxybenzoyl)-L-lysine] (azotochelin).

hydroponic conditions (Martins et.al. 2017). Moreover, the siderophore N,N'-2,6-bis(2,3-dihydroxybenzoyl)-L-lisine, usually named as azotochelin, (Fig. 1) is a bis(catecholamide) siderophore produced by the nitrogen-fixing soil bacterium *Azotobacter vinelandii* (Bellenger et al., 2007). The high efficiency of azotochelin, as iron chelator, has already been reported (Duhme et al., 1996, 1997; Cornish and Page, 1998). However, as far as we know, the ability of these two compounds for correcting iron deficiency of plants has never been evaluated. Based on these facts, the main purpose of this work was to evaluate the efficacy of the iron chelates of DPH and azotochelin to provide iron to cucumber (iron-efficient) plants in hydroponic cultures and to compare the results with those obtained with the iron chelates of o,o-ethylenediamine-N,N´bis(o-hydroxy-phenylacetic) acid (o,o-EDDHA) and ethylenediaminetetraacetic acid (EDTA), commonly used in iron fertilization. Because the reduction of iron(III) to iron(II) is a mechanism widely described for iron uptake by dicotyledonous plants, the ability of iron chelates of azotochelin or iron chelates of DPH, to act as substrates in enzymatic reduction, by the ferric chelate reductase (FCR), at pH 7.5, was also evaluated.

MATERIALS AND METHODS

All chemicals were of analytical grade. The chelating agents, o,o-EDDHA, 94,49% (LGC Standards), Na_2H_2EDTA 99% (86% as free acid) (Titriplex III, Merck), were purchased from the market. The titrimetric purity of o,o-EDDHA, expressed with respect to the acidic form, was determined as described in Yunta et al. (2003a).

Synthesis of chelating agents

All solvents used in the synthesis of (N, N´)-2,6–Bis(2,3-dihydroxybenzoyl)-L-lysine (azotochelin) and N-Dihydroxy-.N,N'-diisopropylhexanediamide **(**DPH) were purified by standard methods and distilled before use (Perrin et al., 1980). Elemental analyses were performed with a Thermo Finnigan-CE Flash EA 1112 CHNS series analyser. NMR spectra were recorded either on a Bruker AMX- 400MHz or a Bruker Avance-600MHz apparatus, using as an internal standard, the residual peak of H_2O.

Synthesis of azotochelin

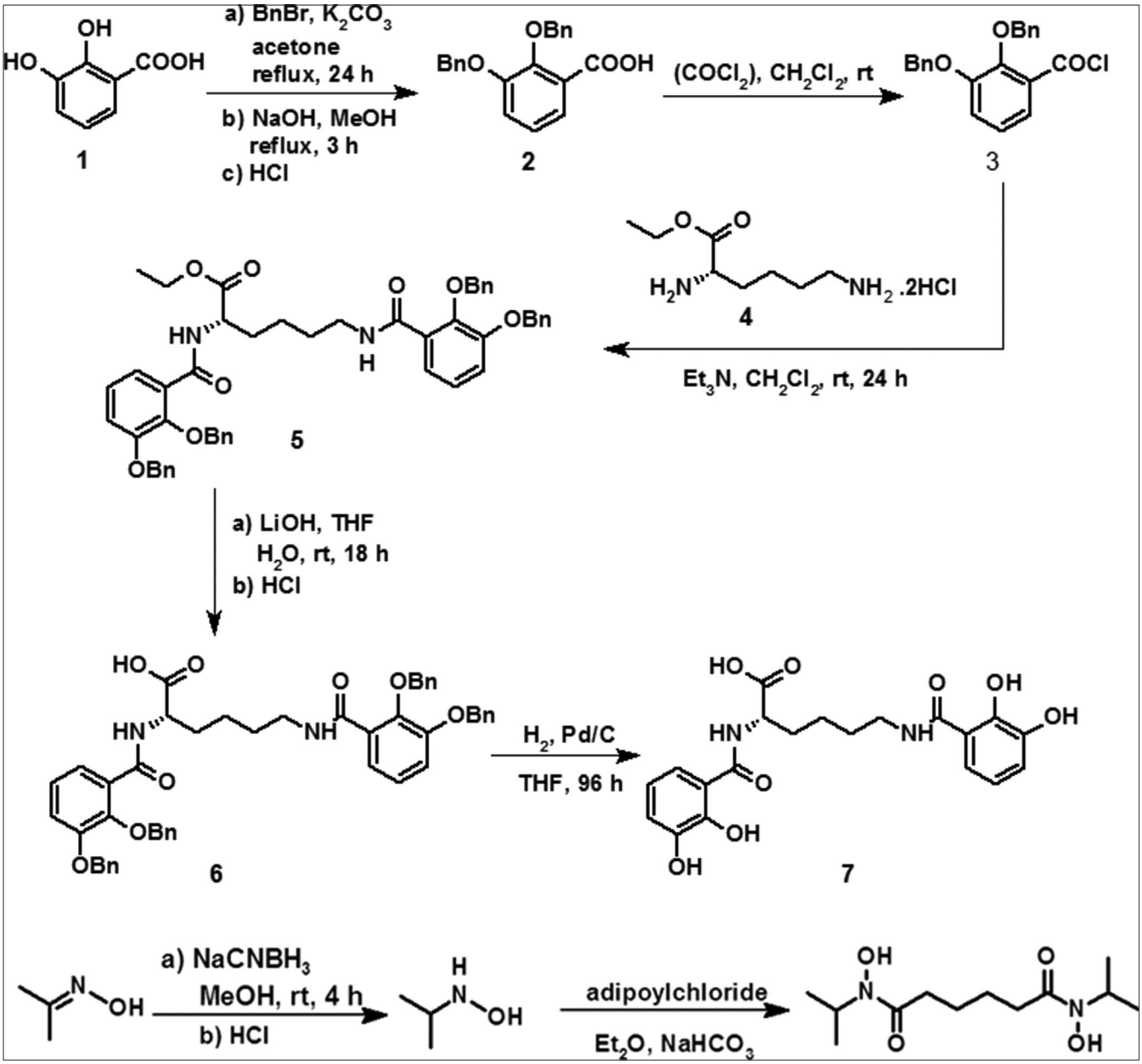
Azotochelin was synthesized based on a literature procedure (Leydier et al., 2008) with an overall yield of 30%, using 2,3-dihydroxybenzoic acid (1) and (S)-ethyl 2,6-diaminohexanoate (4) (scheme A). ^{1}H NMR (DMSO-d_6): δ 12.09 (br s, 1H), 9.13 (br s), 8.88 (d, J = 7.4 Hz, 1H), 8.77 (m, 1H), 7.45 (d, J = 7.8 Hz, 2H), 7.29 (d, J = 7.9 Hz, 2H), 6.92–6.99 (m, 2H), 6.67–6.72 (m, 2H), 4.44– 4.48 (m, 1H), 3.30–3.34 (m, 2H), 1.67–1.72 (m, 2H), 1.35–1.58 (m, 4H). ^{13}C NMR: δ 173.7, 170.1, 169.9, 150.0, 149.5, 146.5, 129.2, 128.5, 119.1, 118.5, 118.2, 117.4, 115.5, 115.3, 52.7, 39.1, 30.6, 28.7, 23.6 Anal. Calcd (%) for $C_{20}H_{22}N_2O_8$.THF: C, 58.89; H, 5.97; N, 5.72; O, 29.42. Found: C, 59.05; H, 6.22; N, 5.38.

Synthesis of DPH

DPH was synthesized according to scheme B, using the method described by Smith and Raymond (1980) as a white powder, with an overall yield of 60% as its hydrochloride salt. ^{1}H NMR (DMSO-d_6): δ 4.55 (m, 2H), 2.32 (t, J = 6.4 Hz, 4H), 1.48 (t, J = 6.4 Hz, 4H), 1.04 (d, J = 6.6 Hz, 12 H). ^{13}C NMR: δ 177.2, 50.9, 36.9, 29.1, 24.2 Anal. Calcd (%) for $C_{12}H_{26}Cl_2N_2O_4$: C, 43.25; H, 7.86; N, 8.41. Found: C, 46.51: H, 7.94: N, 7.86.

Preparation of the iron chelates

For the preparation of the Fe chelate solutions, used in the hydroponic experiments, the amount of iron added was 5% in excess of the calculated requirements in order to



scheme

ensure a complete metalation of the ligand. For reductase experiments, 5% excess of ligand was used to ensure total metal complexation. The $Fe(NO)_3$ solution was added slowly to a solution of the ligand, previously dissolved in sufficient NaOH. The formation of Fe:L complexes with stoichiometry of 3:2 was considered in the case of DPH (Barclay et al., 1984) and azotochelin (Cornish and Page, 1998) while the formation of 1:1 complexes was considered for EDTA and o,o-EDDHA.

During the chelation, the pH was maintained between 6.0 and 8.0. The pH was adjusted to 7.5 for azotochelin/Fe^{3+} and DPH/Fe^{3+}, 7.0 for o,o-EDDHA/Fe^{3+} and 6.5 for EDTA/Fe^{3+}. Solutions were left to stand overnight to allow the precipitation of Fe in excess as (hydr)oxides. In the case of azotochelin/Fe^{3+} chelate, the solutions were stirred for 62h and then left to stand overnight. Final solutions were filtered through a 0.45-μm cellulose membrane (Milipore) and made up to volume to obtain the desired concentration with type I water (electrical conductivity max: 0.056 μS cm^{-1} at 25ºC; total organic C max: 100μg L^{-1}; Na^+ max: 1μg L^{-1}; Cl^- max: 1μg L^{-1}; total Si max: 3 μg L^{-1}). In order to prevent chelate photodecomposition, light exposure was avoided during preparation and storage of chelate solutions.

Stability of the iron chelates of DPH and azotochelin

In order to compare the ligands effectiveness for iron chelation, theoretical computer calculations were performed to calculate the pFe values, at pH 7.5, for the iron chelates of DPH, EDTA and o,o-EDDHA. The pFe values were determined based on chemical speciation calculations using the MINEQL+ Version 4.5 software (Schecher and McAvoy, 2001). Chemical equilibrium concentrations of all species considered in the model by the program reactions were generated based on the component stability constants and molar concentrations. Total ligand and iron concentrations of 10^{-5} and 10^{-6} M, respectively, which are usually described in the literature for this purpose (Zhang et al., 2009; Hider and Kong, 2010), were used together with the protonation and the stability constants between Fe^{3+} and DPH (Martell and Smith, 2004), o,o-EDDHA (Yunta et.al., 2003b) and EDTA (Martell and Smith, 2004). Precipitation of iron hydroxides was considered by introducing the solubility equilibrium for $Fe(OH)_3(s)$ (log β=-2.94) (Martell and Smith, 2004).

The stability of the iron chelates of azotochelin or DPH in $CaCl_2$ solutions (Ca^{2+}, 1.6 x 10^{-3} M) was determined at different pH values. An aliquot from the stock solution of the chelate prepared at pH 7.5, as described in section 2.1, using 5% excess of ligand was added to a solution of $CaCl_2$. After adding a proper buffer (MES, HEPES or CAPSO, which do not complex iron (Ferreira et.al. 2015), the pH of each solution was adjusted to pH values between 4 and 11, with HCl or NaOH solutions, as needed; blanks were prepared at pH below 1. Two replicates per pH were performed. The volume was then raised to 50 mL and the samples were shaken at 25 ºC and 56 rpm for 3 days. At the end of each period, the pH of the solutions was measured using a Crison 52 09 pH combined electrode and a Crison MicropH 2002 meter. Additionally, the total soluble iron was determined by atomic absorption spectrometry with flame atomization (AAS-FA) using a Perkin-Elmer Analyst AA400 spectrophotometer after previous filtration of the samples with a 0.45 μm Milipore membrane.

Azotochelin/Fe^{3+} and DPH/Fe^{3+} as substrate for FCR activity in stressed cucumber plants

Cucumber seeds (*Cucumis sativus* L. cv. Ashley) were germinated using a standard seed-growing procedure in sterilized trays (Garcia-Marco et al., 2006). The seeds were washed with water for 30 min and then placed in trays between two sheets of cellulose paper moistened with distilled water. The trays were kept in darkness at 28 ºC for 4 days in a thermostated incubator.

After germination, seedlings were placed on a holed plate, floating in containers with a continuously aerated EDTA buffered (100 μM excess) nutrient solution with the following composition: macronutrients (mM) – 1.0 $Ca(NO_3)_2$, 0.9 KNO_3, 0.3 $MgSO_4$, 0.1 KH_2PO_4; cationic micronutrients (μM) – 5.0 HBED/Fe^{3+}, 2.5 $MnSO_4$, 1.0 $CuSO_4$, 10 $ZnSO_4$, 1.0 $CoSO_4$, 1.0 $NiCl_2$, 115.5 $EDTANa_2$; anionic micronutrients (μM) – 35 NaCl, 10 H_3BO_3, 0.05 Na_2MoO_4; and 0.1 mM HEPES, which is a non-complexing compound (Ferreira et al. 2015), as pH buffer.

The pH of the solution was adjusted to 7.5 with KOH 1M. Plants were grown in this nutrient solution for 6 days in a Dycometal-type CCKF 0/16985 growth chamber, where they were grown under controlled climatic conditions: day/night photoperiod, 16/8 h; temperature (day/night) 30/25 ºC; relative humidity (RH) (day/night) 50/70%. The amount of Fe added (5μM) was found by Lucena and Chaney (2006) to be the most adequate to produce green but stressed cucumber plants.

Then, uniform seedlings, regarding the shoot growth, were selected and the stems of two individual plants were wrapped together with polyurethane foam and placed in a 12-L polypropylene bucket (12 pairs of plants per bucket) in a continuously aerated EDTA buffered nutrient solution, with the same composition as described above. The pH was buffered at 7.5 with HEPES 0,1 mM and 2.4g $CaCO_3$ per pot were added to simulate the conditions of calcareous soils. After 3 days, the plants were used for the reductase assay (RA).

The FCR activity measurement was made in accordance with Lucena and Chaney (2006) at pH 7.5. The experiment was initiated within the following 2 h after the day-light period. A bunch of two plants was transplanted into 200 mL assay solution containing bathophenanthroline dissulfonic acid, disodium salt, (Na_2BDPS, 300 μM) and 5 mL of the corresponding treatment solution (o,oEDDHA/Fe^{3+}, EDTA/Fe^{3+}, azotochelin/ Fe^{3+}, DPH/Fe^{3+}) was added (time 0) so that the final concentration of iron was 100 μM. For each treatment, six replicates were arranged. In addition, 2 blank replicates, per chelate, consisting of solutions without plants were included in order to correct reduction rates for slow photoreduction. Aliquots of 3 mL were sampled at 0, 10, 20, 60 and 120 min and the fresh weight of the roots was quantified at the end of the experiment.

The $(BDPS)_3/Fe^{2+}$ concentration was calculated as in Lucena and Chaney (2006) by determining the absorbance, using a JASCO V-650 UV-Vis spectrophotometer (JASCO Corporation, Tokyo, Japan), at 535 nm (maximum absorbance of the $(BDPS)_3/Fe^{2+}$ complex) and at 480 nm, 375 nm and 630nm for the treatments with o,oEDDHA/Fe^{3+}, DPH/Fe^{3+} and azotochelin/Fe^{3+}, respectively, to consider the contribution of the applied treatments on the total absorbance. With the exception of EDTA/Fe^{3+} that did not present significant absorption at 535 nm, the concentration of each chelate, was calculated by solving the two-equation system, exemplified below for the case of o,oEDDHA/Fe^{3+}.

$$A_{535} = a_{FeBDPS535} \times \left[Fe(BDPS)_3\right] + a_{o,oEDDHA/Fe535} \times \left[o,oEDDHA/Fe\right]$$

$$A_{480} = a_{FeBDPS480} \times \left[Fe(BDPS)_3\right] + a_{o,oEDDHA/Fe480} \times \left[o,oEDDHA/Fe\right]$$

where A_{535} and A_{480} are the absorbance measured for each sample at 535 and 480 nm, respectively; $\alpha_{FeBDPS535}$, $\alpha_{FeBDPS480}$, $\alpha_{o,oEDDHA/Fe535}$ and $\alpha_{o,oEDDHA/Fe480}$ are the molar absorption coefficients in the experimental conditions, and [$Fe(BDPS)_3$] and [o,oEDDHA/Fe] are the concentration of the chelates.

Efficacy of azotochelin/Fe^{3+} and DPH/Fe^{3+} to provide Fe to cucumber plants in hydroponic culture

Cucumber seeds (*Cucumis sativus* L. cv. Ashley) were germinated using the same procedure as for reductase assays. After germination, seedlings of similar development (shoot growth) were placed on a holed plate, floating in containers with a continuously aerated nutrient solution with the same composition as in the FCR assays, but 1/5 diluted, for 6 days, in the growth chamber, where they were grown under controlled climatic conditions: day/night photoperiod, 16/8 h; temperature (day/night), 28/20 °C; relative humidity (RH) (day/night) 40/60%.

After this time, the stems of two plants were wrapped together with foam, and placed in 2 L polyethylene vessels [three holes in the lid, six plants (3 pairs) per pot] containing 2 L of a continuously aerated full strength nutrient solution with the same composition as in the reductase experiment. Iron was not added to this nutrient solution. The pH was adjusted to 7.5 with KOH 1.0 mol L^{-1} and buffered with HEPES 0.1 mM, and 0.4 g of solid $CaCO_3$ per pot. The 2L pots were covered with black plastic to avoid light exposure.

Plants were grown under these conditions until visual symptoms of iron deficiency were observed (6 days), when treatments were applied. The treatments with the iron (10 μM) chelates of o,o-EDDHA, $EDTA^{3+}$, DPH and azotochelin, respectively, as sources of iron were replicated five times in a completely randomized design, as well as the control containing 2μM HBED/Fe^{3+} (3 replicates). The growth chamber conditions were the same as those described above. Water was added every 2 days and the solution renewed weekly. During the experiment, Soil-Plant Analysis Development (SPAD) index readings, with a chlorophyll-meter (Minolta SPAD-502) were taken for all the leaf stages (average of three readings per leaf) at several times.

Whole plants were sampled 7 (two pairs of plants) and 21 (one pair of plants) days after application of the treatments. After sampling, plant nutritional status and Fe redistribution were studied. The sampled roots, stems and leaves were separated, weighed and washed with a 0.01% non-ionic detergent (Tween 80) in 0.1% HCl solution for 30 seconds and rinsed twice with ultrapure water following the procedure of Garcia-Marco et al. (2006). Then, samples were dried in a forced air oven at 65° C for 3 days. Fresh and dry (DW) weights were determined. After dry digestion in a muffle furnace (480 °C), the ashes were dissolved in 6 M HCl. Micronutrients were determined in stems and leaves. Fe, Mn, Cu were analysed by AAS-FA.

Statistical analysis

Statistical analyses were performed with SPSS statistical software (v.21; SPSS, Chicago, IL, USA). Differences among treatments were determined using a one-way analysis of variance (ANOVA). Significant differences were established at $p<0.05$ using the Duncan test.

RESULTS

Stability of the iron chelates of DPH and azotochelin

The pFe^{3+} value express the amount of "free iron" present in equilibrium under particular set of conditions. Usually, calculated pFe^{3+} values, assuming total ligand and iron concentrations of 10^{-5} and 10^{-6} M, respectively, at pH 7.4, are used for a direct comparison of the iron stability of the different chelates in solution (Zhang et al., 2009; Hider and Kong, 2010). Thus, assuming these conditions, computer chemical simulations were performed to calculate pFe^{3+} values at pH 7.5, for DPH, o,o-EDDHA, and EDTA in aqueous solution. The pFe value for azotochelin at pH 7.4 (23.1) was reported by Cornish and Page, (1998). These results predict that the iron chelates of o,o – EDDHA are the most stable at pH 7.5 (pFe^{3+}= 27.2). The pFe value for azotochelin is slightly higher than for EDTA (pFe^{3+}= 22.8) while DPH/Fe^{3+} chelates are the less stable (pFe^{3+}= 20.8) at pH 7.5.

Figs. 2A and 2B show the percentage of Fe remaining in DPH/Fe^{3+} and azotochelin/Fe^{3+} solutions, respectively, at different pH after 3 days of interaction with 1.6mM of $CaCl_2$. These results confirm the high stability of the iron chelates of azotochelin or DPH at pH 7.5. In the case of azotochelin, all Fe remained in solution between pH 6 and 10 while in the case of DPH a slight decrease of soluble Fe was observed above pH 6.5; nevertheless, 95% of the Fe remained in solution at pH 7.5.

Azotochelin/Fe^{3+} and DPH/Fe^{3+} chelates as substrate for FCR activity in stressed cucumber plants

The ability of the iron chelates of azotochelin, DPH, EDDHA and EDTA to act as substrates in enzymatic reduction was evaluated; Fe-stressed, but still green, cucumber plants were used in these experiments.

The Fe reduction rate (μmol Fe(II) g^{-1} fresh root h^{-1}) using EDTA/Fe^{3+}, o,o-EDDHA/Fe^{3+} and DPH/Fe^{3+} chelates is shown in Fig. 3. For azotochelin/Fe^{3+} chelate, the reduction rate was not possible to be determined due to the non-negligible changes observed in the blanks that lead to misleading results (data not shown).

The reduction assays were conducted for 2 h. The reduction rate for EDTA/Fe^{3+} chelate was significantly higher than for the other iron chelates; DPH/Fe^{3+} chelate showed a similar activity to the observed for o,o-EDDHA/Fe^{3+} chelate. A slight decrease in the levels of reductase activity for DPH/Fe^{3+} was observed in the second hour.

Efficacy of azochelin/Fe^{3+} and DPH/Fe^{3+} chelates to provide iron to cucumber plants in hydroponic culture

In this experiment, four different Fe (10μM) chelate treatments were applied to Fe- efficient cucumber plants with visible chlorotic symptoms. The effect of the iron chelates of azotochelin/Fe^{3+}, DPH/Fe^{3+}, EDDHA/Fe^{3+} and EDTA/Fe^{3+} was evaluated and the results were compared with the control (Fe limited: 2μM Fe, HBED/Fe^{3+}).

The SPAD index was measured in the following days after the application of the treatments (DAT) to estimate the chlorophyll concentration and recovery during the experiment. Fig. 4 shows the time course of the SPAD index, measured in the first leaf level (the first developed after the cotyledons).

After the application of the treatments, all four iron chelates showed significantly higher SPAD values than the control in all leaf levels and in all stages of the experiment. These results are in agreement with the visual symptoms of iron deficiency exhibited by the control plants during the experiment. In the leaves of the first level, a marked increase of the SPAD index was observed after the third day of treatment with all chelates (Fig. 4) while, in the control, only a slight increase of the SPAD index was observed after the day 7.

Table 1 shows the SPAD index for the most recently developed leaves formed in different leaf levels after the

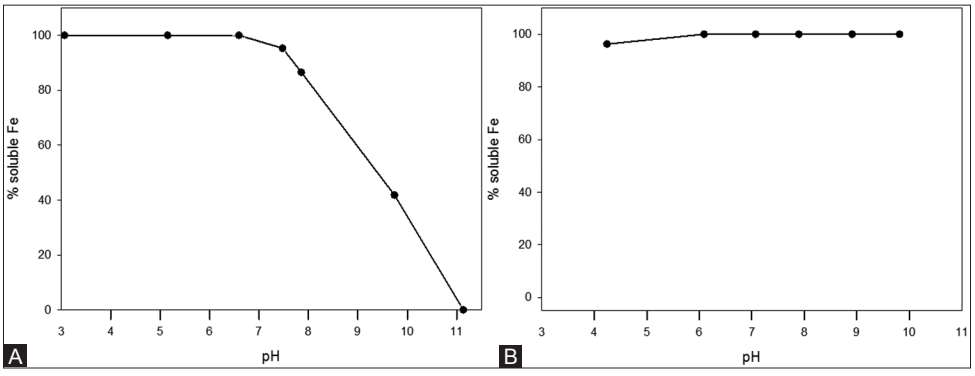


Fig 2. Effect of the pH on the percentage of soluble Fe, after 3 days of interaction: (A) DPH/Fe^{3+} (1.0 x 10^{-4} M Fe) and (B) azotochelin/Fe^{3+} (1.5 x 10^{-4} M Fe) in the presence of Ca^{2+} 1.6 x 10^{-3} M.

application of the treatments (3 DAT, second level; 7 DAT, third level; 14 DAT, fourth level and 21 DAT on second, third, fourth and fifth levels). At the end of the experiment (21 DAT), the SPAD measured in the upper leaf levels were similar in all the treated plants, with the exception of the fourth leaf level, where SPAD index in the leaves of the plants treated with EDTA/Fe^{3+} was slightly higher than the plants treated with azotochelin/Fe^{3+} and DPH/Fe^{3+}. It must be noted that the leaves in the fifth level were small and were present only in few plants in each treatment.

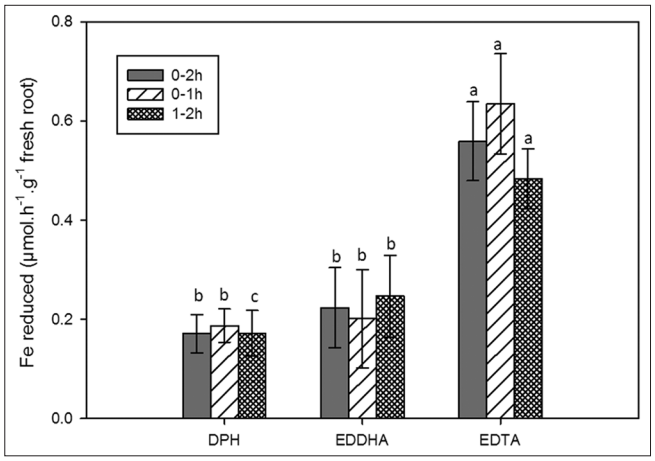


Fig 3. Rate of Fe^{3+} reduction of the iron chelates of DPH, EDDHA and EDTA, for cucumber plants. Error bars represent standard deviations (SD, n=6). Different letters in the same period denotes significant differences among treatments for Duncan test (p<0.05).

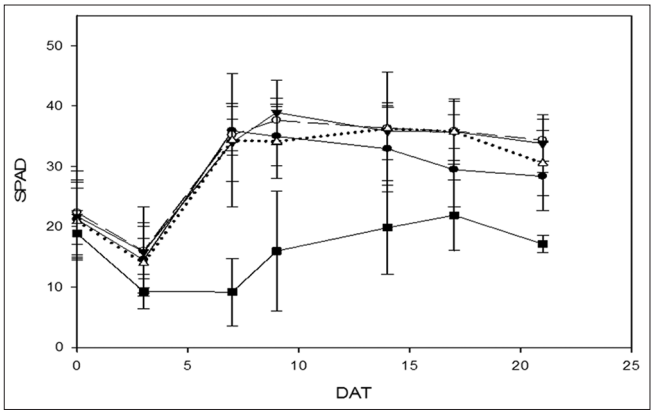


Fig 4. Effect of the different Fe chelate treatments on the SPAD index (± standard deviation; n = 5), measured in the first leaf level, of cucumber plants in hydroponic experiments: (•) Azotochelin ; (○) DPH; (▼) *o,o* – EDDHA; (Δ) EDTA; (■) Control.

In both samplings (7 and 21 DAT), the leaf dry weight (Table 2) of all treated plants was always greater than the control (HBED/Fe^{3+}, 2µM) and followed the same trend as the SPAD index. In the case of the roots and stem dry weight (Table 2), the differences between the control and the treated plants were significant only at the end of the experiment (21 days). Only the stem dry weight of the plants treated with azotochelin/Fe^{3+} showed a slightly higher value at DAT 7. Among the treated plants at DAT 21, the dry weight of the leaves and stems of the plants treated with azotochelin/Fe^{3+} and DPH/Fe^{3+} was lower than EDTA/Fe^{3+} treatments but statistically comparable with the EDDHA/Fe^{3+} treatment.

The iron concentration in leaves (µmol g^{-1} DW), the total content of iron in leaves (µmol $plant^{-1}$) (Table 3), and the concentrations of Mn and Cu in leaves (Table 4) were determined to evaluate the mineral status of the plants. In the first sampling (7 DAT), the Fe concentration (µmol g^{-1} DW) and the total content of iron (µmol $plant^{-1}$) in leaves was similar in all plants treated with the iron chelates and higher than in the control (HBED, 2µM). At DAT 21, the total iron content (µmol $plant^{-1}$) in the leaves of the treated plants was also higher than in the control plants. Plants treated with EDDHA/Fe^{3+} showed the highest value. On the other hand, at DAT 21, the concentration of iron (µmol g^{-1} DW) measured in the leaves of the plants treated with EDTA/Fe^{3+} was similar to the control plants. However, the SPAD value (Fig. 4) and the visual observation of the plants (Fig. 5) clearly evidenced the recovery from the chlorosis symptoms of the plants treated EDTA/Fe^{3+} chelates, contrarily to the control plants.

The application of the iron chelates affected the uptake of other nutrients, which is evidenced by the high concentration of Mn and Cu (Table 4) measured in the

Table 1: Effect of the different Fe -chelate treatments on the SPAD index (± standard deviation) in the most recently developed leaves of cucumber plants formed in different leaf levels after the application of the Fe-chelates treatments in hydroponic experiments.

Treatment	DAT						
	3	7	14	21			
	Level 2	Level 3	Level 4	Level 2	Level 3	Level 4	Level 5
Control	-*	16±10^{b}	-*	15±5^{b}	15±5^{b}	15±3^{c}	19±8^{b}
Azotochelin	24±3^{a}	37±2^{a}	39±4ns	31±2^{a}	31±5^{a}	30±2^{b}	30±6^{a}
DPH	21±4^{a}	37±4^{a}	-*	33±3^{a}	29±3^{a}	32±3^{b}	28±3^{a}
EDDHA	19±4^{ab}	37±3^{a}	40±5	35±4^{a}	34±5a	33±5^{ab}	37±4^{a}
EDTA	12±7^{b}	39±1^{a}	39±2	32±2^{a}	31±5^{a}	36±2^{a}	34±2^{a}

Different letters in the same column denotes significant differences among treatments for Duncan test (p<0.05). ns: no significant differences, *Not complete leaf development at this level and time

Table 2: Effect of the different Fe chelate treatments on the dry weight (g; ± standard deviation) of leaf, root and stem in cucumber plants in hydroponic experiments

Treatment	Dry weight					
	leaves		roots		stems	
	7 DAT	21 DAT	7 DAT	21 DAT	7 DAT	21 DAT
Control	0.18±0.05^{b}	0.53±0.03^{c}	0.06±0.02^{ns}	0.08±0.01^{b}	0.04±0.01^{b}	0.08±0.01^{c}
Azotochelin	0.33±0.07^{a}	1.41±0.22^{b}	0.06±0.02	0.31±0.05^{a}	0.06±0.01^{a}	0.36±0.08^{b}
DPH	0.29±0.10^{a}	1.41±0.28^{b}	0.06±0.02	0.29±0.09^{a}	0.05±0.01^{ab}	0.33±0.04^{b}
EDDHA	0.29±0.09^{a}	1.69±0.44^{ab}	0.06±0.02	0.31±0.09^{a}	0.05±0.01^{b}	0.41±0.08^{ab}
EDTA	0.28±0.06^{a}	1.95±0.33^{a}	0.06±0.02	0.31±0.10^{a}	0.05±0.01^{ab}	0.45±0.09^{a}

Different letters in the same column denotes significant differences among treatments for Duncan test (p<0.05). NS: No significant differences

Table 3: Effect of the different Fe chelate treatments on the leaf Fe concentration (µmol g^{-1} DW; ± standard deviation) and Fe content (µmol plant^{-1}; ± standard deviation) in cucumber plants grown in hydroponic experiments

Treatment	Fe concentration in leaves (µmol g^{-1} DW)		Fe content in leaves (µmol plant^{-1} DW)	
	7 DAT	21 DAT	7 DAT	21 DAT
Control	0.47±0.12^{b}	0.97±0.17^{b}	0.17±0.02^{b}	0.51±0.06^{c}
Azotochelin	0.80±0.04^{a}	1.15±0.11^{a}	0.52±0.10^{a}	1.62±0.24^{b}
DPH	0.86±0.09^{a}	1.17±0.08^{a}	0.45±0.10^{a}	1.66±0.39^{b}
EDDHA	0.83±0.13^{a}	1.27±0.16^{a}	0.50±0.13^{a}	2.11±0.44^{a}
EDTA	0.80±0.12^{a}	0.94±0.11^{b}	0.45±0.05^{a}	1.79±0.10^{ab}

Different letters in the same column denotes significant differences among treatments for Duncan test (p<0.05). Ns: No significant differences

Table 4: Effect of the different Fe chelate treatments on the leaf Mn and Cu concentrations (µmol g^{-1} DW; ± standard deviation) and Fe/Mn ratio in cucumber plants grown in hydroponic experiments

Treatment	Concentration in leaves (µmol g^{-1} DW)				Fe/Mn	
	Mn		Cu			
	7 DAT	21 DAT	7 DAT	21 DAT	7 DAT	21 DAT
Control	3.3±0.4^{a}	4.4±0.5^{a}	0.25±0.02^{a}	0.30±0.02^{a}	0.15±0.04^{c}	0.22±0.04^{d}
Azotochelin	2.5±0.3^{b}	3.1±0.2^{b}	0.19±0.01^{c}	0.19±0.01^{c}	0.33±0.03^{ab}	0.37±0.03^{bc}
DPH	2.6±0.6^{b}	2.8±0.5^{b}	0.25±0.03^{a}	0.22±0.03^{b}	0.33±0.03^{ab}	0.43±0.03^{b}
EDDHA	2.3±0.4^{b}	1.9±0.1^{c}	0.18±0.01^{c}	0.18±0.03^{c}	0.35±0.03^{a}	0.64±0.03^{a}
EDTA	3.2±0.2^{a}	2.9±0.3^{b}	0.21±0.02^{b}	0.21±0.01^{b}	0.25±0.03^{b}	0.32±0.03^{c}

Different letters in the same column denotes significant differences among treatments for Duncan test (p<0.05). ns: no significant differences

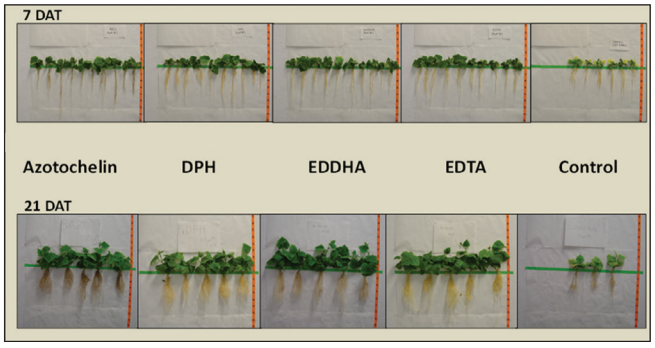


Fig 5. Cucumber plants after 7 and 21 days of treatment, in hydroponic culture, with the iron chelates (10µM Fe) of Azotochelin, DPH, o,o-EDDHA, EDTA and control plants (2µM Fe).

leaves of the untreated plants, especially at DAT 21. At DAT 7, the concentration of Mn in plants treated with EDTA/Fe^{3+} and the concentration of Cu in plants treated with DPH/Fe^{3+} were similar to the control plants.

The Fe/Mn ratio (Table 4) has been considered as an index of adequate iron nutrition for several crops mainly those grown in hydroponic conditions (Garcia-Marco et al., 2006). An increase of the Fe/Mn ratio implies a recovery from iron chlorosis. At DAT 7, the Fe/Mn ratio in all the treated plants was higher than in the control. At DAT 21, the plants treated with EDTA/Fe^{3+} showed the lowest Fe/Mn ratio but higher than the control; on the other hand, the highest value was observed in plants treated with o,o-EDDHA/Fe^{3+}. The Fe/Mn ratio obtained for DPH/Fe^{3+} and azotochelin/Fe^{3+} treatments were similar to EDDHA/Fe^{3+}, at day 7, but lower at day 21.

DISCUSSION

Plants treated with DPH/Fe^{3+} or azotochelin/Fe^{3+} chelates showed similar SPAD values (Table 1), leaf dry weight (Table 2) and iron concentration in leaves (µmol g^{-1} DW) (Table 3) as those treated with o,o-EDDHA/Fe^{3+} chelate; these results suggest the same level of recovery from the iron chlorosis symptoms.

At the end of the experiment (DAT 21), the plants treated with EDTA/Fe^{3+} chelates showed lower values of iron

concentration (µmol g^{-1} DW) and iron content (µmol $plant^{-1}$ DW) in leaves than those treated with EDDHA/Fe^{3+} chelate; these differences are in good agreement with published results (Nadal et al., 2012). However, at DAT 21, no significant differences were observed in leaf iron concentration (µmol g^{-1} DW) measured in control plants and in plants treated with EDTA/Fe^{3+} (Table 3). These results are not in agreement with the leaf SPAD (Table 1; 21 DAT, levels 4 and 5) and dry biomass (Table 2; 21 DAT) results nor with the visible recovery from the chlorosis symptoms (Fig. 5) showed by plants treated with EDTA/Fe^{3+}.

No differences were observed in the leaf dry weight of plants treated with all iron chelates at DAT 7. However, at DAT 21, the plants treated with EDTA showed a similar SPAD value (or higher at level 4) (Table 1) and a lower Fe concentration (Table 3), but a higher leaf dry biomass (Table 2) values when compared with the siderophores treatments, which suggests a possible dilution effect.

A good correlation between the SPAD values (Table 1) and the amount of iron in leaves was obtained when the total iron content in leaves (µmol $plant^{-1}$ DW) was determined. Therefore, this parameter seems to be more adequate than the leaf iron concentration (µmol g^{-1} DW) for comparing the iron uptake from different treatments: DPH/Fe^{3+}, azotochelin/Fe^{3+}, EDTA/Fe^{3+} and o,o-EDDHA/Fe^{3+}.

At DAT 7, all plants treated with the iron chelates showed similar leaf iron content; these results suggest that DPH/Fe^{3+} and azotochelin/Fe^{3+} are as efficient as EDTA/Fe^{3+} and o,o-EDDHA/Fe^{3+} for the treatment of plants showing symptoms of iron chlorosis. However, after the initial recovery from the symptoms, o,o-EDDHA/Fe^{3+} was more efficient in supplying iron to plants; at DAT 21, plants treated with o,o-EDDHA/Fe^{3+} showed the highest iron content in leaves.

The effectiveness of the Fe chelates, as Fe sources to plants, depends, not only of the formation constant of the Fe chelate, and thus on the ability to maintain Fe in solution, but also of the equilibrium constants of the reactions between the ligand with the competing cations (Alcaniz et.al. 2017; Alvarez-Fernandez et.al. 1997). An important characteristic of siderophores is the high selectivity for iron (Hider and Kong, 2010) that ensures the stability of the iron chelates in the presence of other metal ions. In the case of EDTA, the chelated iron can be displaced by other cations in solution that form stable complexes with EDTA and thus reduces the soluble Fe in solution available for the plants (Villen et al., 2007). However, the same behaviour is not expected to occur in the case of DPH and o,o-EDDHA under hydroponic conditions up to pH 9 (Martins et.al. 2017).

Besides the stability of the iron chelate, its behavior when it is used as a substrate of the enzyme FCR is also considered an important step in the evaluation of the efficiency of a chelating agent to provide iron to plants. The relationship between the efficiency of a chelate to deliver iron to plants and the reduction of iron(III) chelated by the reductase is not yet clear and is still in discussion (Lucena et al., 2008; Escudero et al. 2012). Several authors have demonstrated that the amount of iron(III) reduced by the plants gives only partial information about the uptake process (Lucena and Chaney, 2006; Nadal et al., 2012). In fact, higher reduction rate observed with EDTA/Fe^{3+} chelate did not lead to higher amount of iron in the leaves (Table 3). This fact may be due to the high stability of the EDTA/Fe^{2+} chelate that serves as an Fe^{2+} trapping agent and prevents most of the iron uptake by the plants and/or due to the possible dilution effect, already discussed above (Lucena and Chaney, 2006; Nadal et al., 2012). On the contrary, siderophores possess a higher affinity for iron(III) than iron(II) (Villen et al., 2007; Hider and Kong, 2010). Therefore, it was expected that DPH/Fe^{3+} and especially azotochelin/Fe^{3+} were more efficient than EDTA/Fe^{3+} in supplying iron to plants, which was not observed (Table 3). The low redox potential, common in microbial siderophores, probably limited the Fe acquisition from DPH/Fe^{3+} and azotochelin/Fe^{3+} chelates.

In fact, in the reductase assays, the reduction rate obtained for Fe(III)-DPH was significantly lower than for Fe(III)-EDTA. Furthermore, the redox potentials determined for rhodotorulic acid (-0.419 V vs NHE) (Crumbliss and Harrington, 2009) and for several dihydroxamic acids compounds at high-pH conditions (-0.261 to -0.446 V *vs* NHE) (Crumbliss and Harrington, 2009) suggest that the redox potential of Fe(III)-DPH chelate should be much lower than the redox potential of Fe(III)-EDTA chelate (+0.120 V, vs NHE) (Gomez-Gallego et al., 2005).

Catechol-based siderophores, such as azotochelin, are effective iron chelators, but the low redox potential of these compounds can prevent the reduction by most biological reductases (Hider and Kong, 2010). However, even though, the results of FCR experiments for Fe(III)-azotochelin were inconclusive, Fe(III)-azotochelin was able to deliver iron to plants (Table 3).

The reduction and iron release from cathecholamides siderophores, observed in several microorganisms, involves the shift of the coordination binding mode from the cathecolate to the salicylate mode after the protonation of the distal hydroxyl donor at each cathecolamide donor group (Abergel et al., 2006; Harrington et al., 2012).

In plants, it is possible that this binding mode shift occurs near the roots because it is known that a deficiency of

iron increases the production and concentration of organic acids that acidifies the rhizosphere (Marschner and Romheld, 1994). Additionally, dicots mainly use Strategy I for iron uptake; this strategy involves the reduction of iron (III) chelate by reductase before the transport across the plasma but it also generates a proton gradient to facilitate iron(III) solubilization outside the cell (Bellenger et al., 2007). Despite the use of 0,1 mM HEPES, as a pH buffer, the increased activity of H^+-ATPase in roots during iron deprivation acidifies the rhizosphere by excreting H^+ and may decrease the pH of the rizhosphere by 0.5-1 pH units. This is consistent with the fact that the FCR activity decreases with increasing pH (Lucena and Chaney, 2007).

The shift from the cathecolate to the salicylate mode not only lowers the affinity for binding trivalent metal cations but also shifts the redox potentials of iron(III) complexes into the range where iron(III) can be reduced by biological reductants (Bellenger et al., 2007; Harrington et al., 2012). Thus, the decrease of the absorbance at both 535 and 630 nm in the reductase assay with azotochelin (data not shown) may be due to the reduction of iron(III) to iron(II), after the shift to the salicylate coordination mode, with the formation of colorless Fe(II)-catechol complexes (Hider et al., 1981). The increase of the absorbance at 535nm (data not shown), after the first 20 minutes, suggests the release of iron(II) from azotochelin by competition with BDPS. Therefore, as in the case of EDTA/Fe^{3+} chelate, the affinity for iron(II), showed by azotochelin, lowers the release efficiency of $Fe^{2+}$and may also affect the supply of iron to plants (Miethke 2013).

The redox potential of o,o-EDDHA/Fe^{3+} chelate (- 0.560 V, vs NHE) (Gomez-Gallego et al., 2005) is also very low, probably lower than the redox potential of DPH/Fe^{3+}, assuming the values described in the literature for several dihyroxamate compounds. From this and considering the lower stability of the DPH/Fe^{3+} chelate compared with o,o-EDDHA/Fe^{3+} chelate, it was expected that the DPH/Fe^{3+} chelate would be a better substrate for FCR than o,o-EDDHA/Fe^{3+} chelate. However, as in the case of azotochelin, a mechanism, involving changes in the coordination of the o,o-EDDHA/Fe^{3+} chelate at a more acidic pH in the vicinity of the roots, has been already proposed (Gomez-Gallego et al., 2005). This fact can probably explain the effective reduction of o,o-EDDHA/Fe^{3+} chelate (Fig. 3) and the iron uptake from o,o-EDDHA/Fe^{3+} chelate by plants despite the low redox potential of the very stable iron(III) chelate formed with o,o-EDDHA.

Significant differences between the treatments were observed only at DAT 21 when higher content of iron was measured in leaves of plants treated with o,o-EDDHA (Table 3). These results may suggest that, not only the reduction mechanism, but also the iron transport within the plant may depend on the chelate used. In fact, we cannot exclude the direct uptake of Fe(III)-DPH or Fe(III)-azotochelin complexes by the plants. This strategy should involve the incorporation of the bacterial siderophore, by analogy to the iron uptake Strategy II, through their incorporation in roots when chelated with iron, as it was already proposed by other authors (Chen et.al. 2000; Vansuyt et.al. 2007).

In the various treatments, the uptake of Cu and Mn by plants was affected by the uptake of iron. The leaf concentrations of Cu and Mn (Table 4) evidenced the favored uptake of those cations under iron deficiency conditions. The higher concentration of Cu and Mn in the leaves of the untreated plants (Control, 2μM Fe), that was evident at the end of the experiment, is related with the involvement of the Iron Regulated Trasporter 1 (IRT1), not only in the uptake of iron, but also in the absorption of those cations, by the roots (Rodriguez-Lucena et al., 2010b; Maqueda et al., 2011). Moreover, the high affinity of o,o-EDDHA for Cu normally reduces the Cu concentration in leaves when o,o-EDDHA/Fe^{3+} is used in hydroponics conditions (Yunta et al., 2003c).

The Fe/Mn ratio obtained with the treatments of DPH/Fe^{3+} and azotochelin/Fe^{3+}chelates were similar in both samplings (7 and 21 DAT) and much higher than those obtained in the untreated plants. These results indicate a good recovery from iron chlorosis. However, these results were lower than the ones obtained in the second sampling (21 DAT) of the treatment with o,o-EDDHA/Fe^{3+}.

The results obtained in this work evidence that the iron chelates of the siderophore azotochelin and the siderophore mimic DPH are able to supply iron to plants, in hydroponics solution at pH 7.5, to correct iron chlorosis and to maintain a good nutritional status of the plants. Both iron chelates were as efficient as Fe(III)-EDTA and Fe(III)-EDDHA chelates in treating plants with visible symptoms of iron chlorosis, in hydroponic culture, at pH 7.5.

EDTA and *o,o*-EDDHA are the most widely used synthetic chelates for correcting iron chlorosis but they are not biodegradable. The catecholate and the hydroxamate groups, present in the azotochelin and in DPH compounds, respectively, are the most common in microbial siderophores and although DPH is not produced naturally, it is well known the existence of hydroxamate degrading microorganisms (Hördt et al., 2000). Thus, taking into account the nature of these compounds, the results obtained in this work open the possibility of application of the iron(III) chelates of azotochelin and DPH for more environmental friendly iron fertilization of plants grown in calcareous soils, which is presently under study.

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