Forms and characteristics of insoluble Fe-humic substances used for Fe nutrition of cucumber (*Cucumis sativus* L.)

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Abstract

In the present research, different forms and reactions of insoluble Fe-humic substance complexes (Fe-HS) and a soluble Fe-EDDHA complex, were compared. Five Fe-HS complexes were synthesized by neutralizing Fe(II) ions and humic substances using different synthesis conditions. Analysis was centred on chemical extraction developed to study iron distribution associated with humic substance (HS). Insoluble Fe-HS showed characteristics that differ from those of poorly ordered Fe oxides, indicating the presence of Fe hydrolytic products that were co-precipitated with HS in large molecules. Fe-HS and Fe-EDDHA were tested for their ability to cure chlorosis in Fe-deficient cucumber plants; the sources were used applying 0.03, 0.3 or 3 µmol of Fe in a nutrient solution (300 mL) buffered to pH 7.5. Results indicate that both Fe sources allowed plants to recover from chlorosis, with the effectiveness depending on the Fe source applied. Data here presented suggest that insoluble Fe-HS complexes could represent an environment and friendly tool for preventing Fe deficiency in crops and for correcting Fe availability in soils.

Key Words

Insoluble Fe-Humic complexes, poorly ordered iron oxides, Fe deficiency, chlorosis.

Introduction

Iron deficiency is an important nutritional problem affecting many cultivated plant species, particularly in calcareous soils (Lucena 2003), causing relevant loss in quantity and quality of agricultural yields (Rombolà and Tagliavini 2006). This nutritional disorder, which triggers the yellowing of young leaves (chlorosis), is due to a low availability of iron, which depends on the low solubility of Fe crystalline oxides, particularly in alkaline conditions (Mengel 1994). On the other hand, it is well accepted that humified substance (HS) of soil organic matter plays an important role in the processes which control the levels of Fe in the soil solution via formation of Fe complexes (Stevenson 1994). For this reason, natural Fe-humates are of great interest in soil science and in surface chemistry. Humic substances are acidic, negatively charged polyelectrolytes of variable molecular weight. It has been largely demonstrated that HS of low and high molecular weight can strongly react with the surfaces of Fe oxides, reducing their crystallization process and the mineralogy of the iron phase (Schnitzer 1986). In addition, HS can enhance Fe availability for plants by formation of soluble complexes and by increasing Fe diffusion to roots (Cesco *et al.* 2000). It has been demonstrated that iron complexed to water soluble humic substances can be used as an Fe source by Fe-deficient plants grown in hydroponics; in dicots, this process occurs via reduction-based mechanism at the plasma-membrane level of Fe(III)-HS (Pinton *et al.* 1999), while *gramineae* use complexed Fe via ligand exchange between HS and phytosiderophores (Cesco *et al.* 2002). The objective of this research was to study the forms and the characteristics of insoluble Fe–HS complexes and to evaluate their efficacy as an Fe source for a cultivated plant species for preventing Fe deficiency in crops and for correcting Fe availability in soils.

Methods

Preparation and characterization of Fe complexes

In this research we used two different Fe sources: 1) five Fe-insoluble humic substance complexes (Fe-HS); 2) one soluble Fe-EDDHA complex (Fe–EDDHA). Fe-EDDHA was prepared following the setting of Cesco *et al*. 2000. Fe-HS samples were prepared mixing $FeSO_4$ with humic substances using a commercial liquid mixture of humic and fulvic acids (Leonardite extracted CIFOUMIC, CIFO). Five types of complexes were synthesized, modifying the order and the amount of Fe added to the humic substances (3-6 mmol Fe g /HS) and the OH/Fe molar ratios. A, B and E were prepared by adding 3 mmol of $FeSO_4$ acid solution, C and D with 3 mmol of $FeSO_4$, respectively, to the alkaline solutions of humic substances; for sample A and C. The pH was previously adjusted to 5.0, after 2 hours the suspensions were stabilized and 0.5 M NaOH solution was gradually added to reach pH 7.0; the pH of sample B and D was directly neutralized with NaOH up to

pH 7.0. Sample E was obtained as for sample A but inverting the sequential order of adding Fe and HS. Part of the Fe-HS complexes were freeze dried for chemical analysis, the rest was stored as a concentrated stock solution (1.6 g/L TOC), dialyzed and then purified for plant experiments. Dynamic light scattering (DLS) experiments were conducted with diluted sample suspensions to determine their relative size as a function of reflective properties. The light scattering setup consisted of a Zetasizer nano ZS laser delivering (www.malvern.com/zetasizernano, USA).

Selective chemical extraction
Four selective dissolutions were carried out on the Fe-HS complexes depending on the different forms of Fe: 1) Fe total content (Fe_t) in the Fe–HS complexes was determined following acid digestion with HCl and HNO_3. 2) DTPA-extractable (Diethylene-Triamine-Penta-Acetate) Fe (Fe-DTPA) was determined extracting with DTPA + 0.01 mol/L $CaCl_2$ + 0.01 mol/L TEA, pH 7.3 for 2 h; 3) the Fe short-range ordered phase (Fe_o) was extracted with ammonium oxalate $((NH_4)_2C_2O_4)$ 0.2 M , pH 3 for 4 h in the dark; 4) Fe exchangeable and complexed to organic matter (Fe_p) was extracted with Na-pyrophosphate 0.1 M, pH 10.0 for 16 h. The extractions were carried out at room temperature in 100 ml polypropylene bottles, using 1 g of air-dried sample. After each chemical treatment, the extracts were separated by centrifugation and the Fe in the supernatant was determined by ICP-AES. Although the extractions used are not specific for a given Fe pool and for Fe availability, some of them are reasonably specific as nutrition tests for most soils. Following commonly used interpretations, Fe pools were grouped as follows, ordered by decreasing stability and affinity with humic substances: Fe_o/Fe_t = total Fe in poorly ordered Fe oxides; Fe_p/Fe_t = Fe exchangeable and complexed to organic matter; Fe-DTPA/Fe_t = Fe bonded to organic matter.

Plant material and growth conditions
To evaluate the contribution of these Fe sources in the micronutrient acquisition by plants, the Fe-HS sample A was used according the suitable chemical properties of the complexes (i.e. better solubility, high Fe coprecipitated). For this propose, cucumber (*Cucumis sativus* L.) plants had been grown for 15 days in hydroponics with pH buffered at 7.5 and without any supplies of exogenous Fe sources. The plants had then been grown for a further 11 days, divided into the following treatments: 1) not supply with any exogenous Fe sources (-Fe control); 2) supplied with sample A that was characterized by Fe complexed to poorly soluble, high molecular–weight-humic substances (Fe-HS); 3) supplied with a soluble synthetic Fe chelate (Fe-EDDHA), widely used in agriculture to cure and prevent Fe chlorosis. In a 300 mL nutrient solution, 0.03, 0.3 or 3 µmol of each Fe sources was supplied; the soluble Fe fraction had been measured via ICP-AES after 0.2-µm filtration of the solutions. SPAD-index values of leaves had been measured along the treatments. At the end of the experiment, pictures of root and leaf tissues were taken.

Results
The relationships between the Fe pools released with different chemical extractions can provide information about the sources from which Fe is extracted. The chemical extractions of the insoluble Fe-HS complexes are summarized in Table 1. Acid oxalate is commonly used to estimate short-range-order Fe hydroxides and oxihydroxides and Fe bonded to organic matter. The ratio Fe_o/Fe_t and Fe_p/Fe *et al.* so is a measure of the relative distribution of Fe between the mineral and the organic phases of the organic complexes. Chemical extractions show that Fe_o and Fe_p values were very high in all samples, the Fe pool is dominated by short-range order Fe phases strongly associated with organic complexes. All samples showed $Fe_o/Fe_t >0.9$ while the ratio was reduced (Fe_p/Fe_t = 0.57 and 0.77) in the samples obtained with 6 mmol Fe g /HS. In spite of the dominance of the mineral Fe reactive pool in most of the studied complexes, the pool of reactive Fe is large in all samples, as indicated by the fact that pyrophosphate extracts an amount of Fe_p very similar to Fe_o in particular in the samples A, B and E. The amounts extracted by DTPA were smaller than those obtained by using the oxalate and pyrophosphate methods and with very comparable values in the five samples. In particular the chemical data show that A, B and E samples contained similar Fe forms (poorly ordered, organic complexes, exchangeable and soluble), while sample C and D, obtained with 6 mmol Fe g /HS, have an excess of insoluble iron that could indicate a low degree of iron availability. The low amount of DTPA extractable Fe may be ascribed to Fe exchange between HS and DTPA. In soil available Fe was normally high correlate with Fe extracted by oxalate and citrate–ascorbate-extractable (De Santiago and Delgado 2006).

Table 1. Fe forms according to the chemical extractions of Fe-HS samples.

	Fe_t	Fe_o	Fe_p	FeDTPA	Fe_o/Fe_t	Fe_p/Fe_t	$FeDTPA/Fe_t$	$FeDTPA/Fe_p$
	(----------------g/kg-----------------)							
A	84.3	84.0	85.5	6.3	1.00	1.01	0.07	0.07
B	90.3	83.6	84.0	7.2	0.93	0.93	0.08	0.09
C	135.4	131.6	77.2	7.6	0.97	0.57	0.06	0.10
D	155.8	143.8	119.3	6.3	0.92	0.77	0.04	0.05
E	97.5	89.1	92.6	8.6	0.91	0.95	0.09	0.09

The Fe-HS complexes observed with the Dynamic light scattering (DLS) contain particles with a higher molecular mass than the ones used by Pinton *et al.* (1999), but in the range of that used by de Santiago and Delgado (2007). Figure 1a shows the size distribution obtained in sample A . The size distribution of Fe-HS complex A showed the dominant presence of large particles (81%) in the micron size range of 779 nm, with an additional 19% of 135 nm particles. Ferrihydrite showed instead a monomodal size distribution with small particles of 116 nm (Figure 1b).
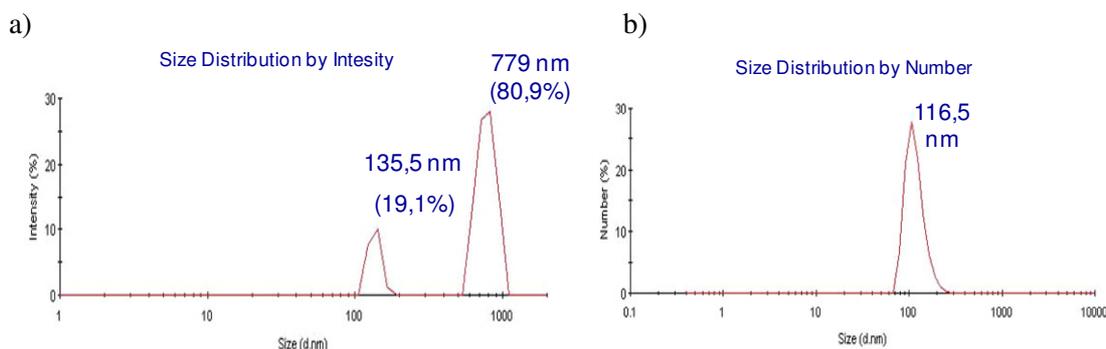
Figure 1. Intensity size distribution obtained with DLS of Fe-HS sample a) and Ferrihydrite b).

As expected, the Fe-HS complex sample A exhibited a poor solubility in the conditions used in this work (pH 7.5); in the 0.03-µmol Fe treatments, the amount of soluble-Fe fraction in the nutrient solution from Fe-HS complex was about 3 times lower than that measured when equal amount of Fe-EDDHA was applied, while in the case of the 3-µmol-Fe treatments, the difference increased to 20 times (data not shown). On the other hand, when used for curing Fe chlorosis on cucumber plants, both Fe sources were able to alleviate Fe-deficiency symptoms (SPAD-index values; Figure 2) and to allow a shoot root and higher biomass accumulation (Figure 3) in cucumber plants; however the effectiveness, particularly at a low concentration, was dependent on the Fe source used (FeEDDHA>Fe-HS). Results of the present work show that Fe coprecipitated with insoluble humic substances can be absorbed by roots and translocated to the shoot of cucumber plants.
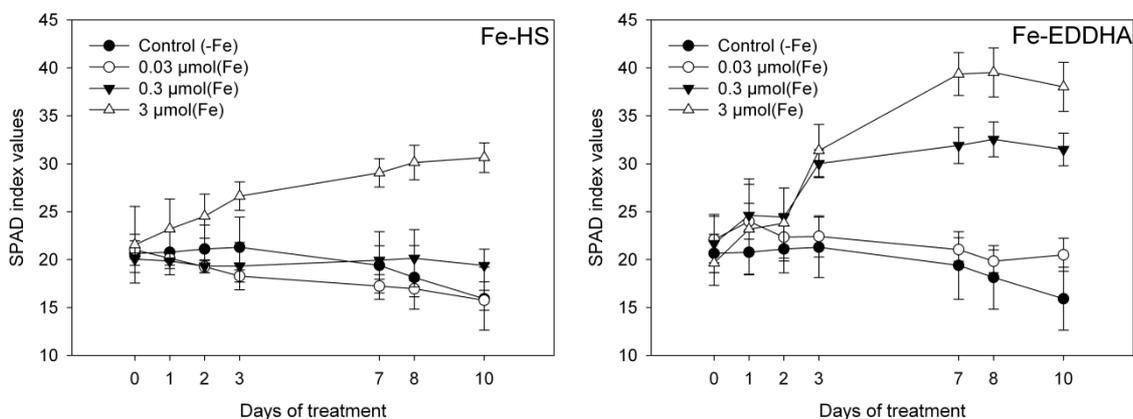
Figure 2. SPAD-index values of Fe-deficient cucumber plants treated for 11 days with 0.03, 0.3 or 3 µmol Fe of Fe-HS sample A or Fe-EDDHA.
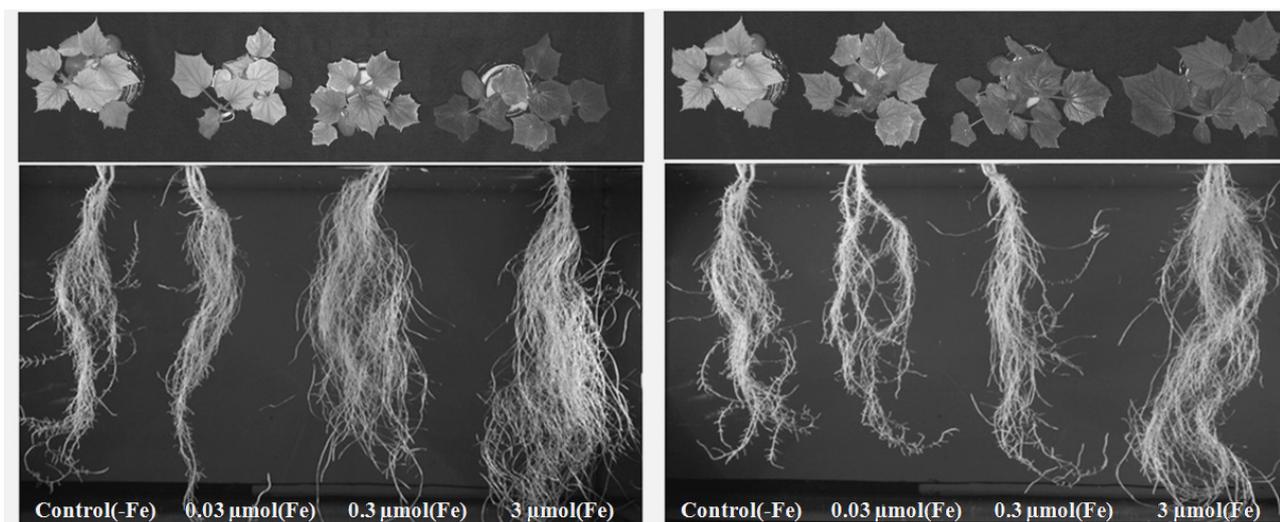
Figure 3. Fe-deficient-cucumber plants treated for 11 days with 0 (Control, -Fe), 0.03, 0.3 or 3 µmol Fe of Fe-HS sample A (left) or Fe-EDDHA (right).

Conclusions

In this preliminary study, analysis centred on chemical extraction procedures was applied to study iron distribution associated with humic substance (HS). The content in poorly crystalline Fe oxides (ferrihydrite), estimated by extraction with oxalate (Fe_o) showed similar values compared with Fe exchangeable and complexed to organic matter (Fe_p) in the sample with 3 mmol Fe g /HS. Dynamic light scattering (DLS) observation indicated that the Fe-HS showed characteristics that differed from those of poorly ordered Fe oxides (ferrihydrite), indicating the presence of large molecules formed by hydrolytic products of Fe co-precipitated with humic substances. Fe-HS seemed to contain multiple forms of Fe, with different behavior when treated with various chemical extractants. Selected Fe-HS were also used by cucumber plants as a Fe source, although the effectiveness of Fe-HS in curing Fe chlorosis at low concentration was lower than that of a synthetic Fe source, which is widely used in agriculture and were particularly expensive and not eco-compatible. Insoluble Fe-HS seems to play a significant role in the Fe nutrition and is consistent with the observation that it can be a good source of Fe for plants. In conclusion, all the data here presented indicate that these organo-mineral complexes could represent an environmentally friendly tool for preventing Fe deficiency in crops and for correcting Fe availability in soils.

Acknowledgments

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