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Potential alteration of iron–humate complexes by plant root exudates and microbial siderophores

Assunta Nuzzo^{1*}, Antonio De Martino², Vincenzo Di Meo² and Alessandro Piccolo^{1*}

Abstract

Background: Two humic acids extracted from a volcanic soil (HA₁) and a leonardite (HA₂) were used to form insoluble complexes with iron metals. To simulate the rhizospheric processes that displace iron from complexes of humic molecules and solubilize the soil humeome, the insoluble iron–humates were treated with a solution of siderophore deferroxamine mesylate (DFOM) and a mixture of citric, oxalic, tartaric and ketoglutaric acids, at different concentrations and contact times.

Results: Results showed that the removal of iron from humic complexes varied depending on the concentration of the extractants and extraction times. At large concentration, the mixture of organic acids was generally a better extractant than the siderophore, probably because of their lower solution pH. However, at smaller concentrations, the extracting capacity of the DFOM solution and the mixture of organic acids was generally similar. Differences in iron extractability between the two iron–humates were attributed to both the humic molecular composition and the steric hindrance of the extractants. Iron was more easily released from complexes formed with HA₁ rich in aliphatic C than from those of HA₂ rich in aromatic C, possibly because the more flexible conformational structure of HA₁ was more accessible to the bulky DFOM than the rigid conformation of iron complexes made by the largely aromatic HA₂.

Conclusions: This work provided evidence that iron–humate complexes may be potentially used to enhance iron nutrition of plants, whose exudates rich in organic acids, together with the siderophores produced by rhizospheric microbes, can displace iron from complexes and enhance its solubility. Concomitantly, the same process may solubilize humic molecules from the same iron–humates, thereby enhancing the concentration of bioactive humic matter in the soil solution.

Keywords: Siderophore, Iron, Humic complexes, Humic acids, Organic acids, Rhizosphere, Iron chlorosis, Organic ligands, Iron extraction, Plant root exudates

Background

Iron (Fe) is an essential microelement for the growth and life of plants. Iron chlorosis is a widespread disease that affects plant growth and reduces the yield of many crops [1]. Iron deficiency results in a decrease of chlorophyll in plant leaves, leading to a progressive yellowing of plants,

starting from the younger leaves and proceeding to the older parts of plants.

The causes of plant iron deficiency can be different, ranging from nutritional disorder to infections caused by microorganisms. Soil pH plays a key role in iron supply to plants [2], since iron solubility is enhanced in acidic soils and depressed in alkaline soils. In high pH calcareous soils, iron availability can be dramatically reduced [3], and iron chlorosis is widely diffused [4], because soluble Fe may be as low as 10^{-10} M, while a normal requirement for plant growth is $\geq 10^{-7}$ M [5].

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Fertilization with synthetic chelates is the most common practice to solve iron chlorosis, and ethylenediamine-*N,N'*-bis (*o*-hydroxyphenyl) acetic acid (EDDHA) is one of the most efficient iron chelating agents [6]. However, a natural plant survival process is the exudation in the rhizosphere of a variety of natural organic ligands, which can form soluble complexes with Fe³⁺, thus favoring the mobilization of Fe from soil oxides/hydroxides [7, 8] or Fe–humates [9–11]. Microbial siderophores [12], plant root exudates including phytosiderophores [13], organic acids [14, 15] and phenolic compounds [16, 17] belong to the natural organic ligands that facilitate iron uptake by plants. It is reported that more than 95% of the total plant-available Fe in the soil solution may be represented by this organic Fe pool [18].

An important Fe source in soil is represented by the insoluble Fe complexes with humic substances (HS). Iron complexation by humic substances is attributed to the oxygen-containing functional groups (carboxylic, phenolic and carbonyl) and, to a lesser extent, nitrogen- or sulfur-containing functions [19]. This iron can be made soluble by displacing humic molecules through a ligand exchange mechanism. In fact, the stability constants of Fe complexes with organic acids [20] and with siderophores [21] are generally much larger than those calculated for iron–humate complexes [22].

HS can be defined as ubiquitous natural compounds arising from the chemical and biological degradations of plant and animal residues [23]. A general consensus regards HS as supramolecular associations of heterogeneous and relatively small molecules, which are held together in only apparently large molecular sizes by weak forces, such as hydrogen and hydrophobic bonds [24], and whose conformations can be disrupted by the action of weak organic acids [25]. Based on their solubility in aqueous solutions as a function of pH, HS can be divided into three fractions: humic acids (HA) is the fraction that is soluble under alkaline pH conditions, fulvic acids (FA) is the fraction soluble in water at all pH values, and humin is the insoluble fraction.

Soil iron oxides were recently shown to preferentially sequester oxidized and highly unsaturated organic molecules, rich in aromatic rings, polyphenols and carboxyl groups [26]. The formation of Fe–O–C bonds in stable [27] and large-sized organometallic amorphous complexes [28] had likely the effect to enhance the recalcitrance of the soil humeome that is intended as the totality of humic molecules present in soil. In fact, organic compounds bound to iron were extracted by Humeomics technique, a sequential chemical fractionation developed to characterize the molecular

composition of a humified matter, only after stable molecular components had been progressively uncovered by first hydrolyzing ester and ether fractions [29].

The binding to iron enables the persistent fixation of humic molecules in soil, including the nitrogen-containing compounds [30], and, thus the formation of organo-Fe complexes can be reckoned as a major mechanism of molecular stabilization of soil organic carbon. Finally, it is plausible to believe that any plant- or microbe-mediated process that displaces iron from complexes with SOM exposes humic molecules to solubilization and enhances their bioactivity in soil.

A more detailed understanding of the ability of natural organic ligands to displace Fe from complexes with HS may be thus important not only to promote a more efficient iron uptake by crops, but also to solubilize in the rhizosphere humic compounds that may act as plant biostimulants. The aim of this work was hence to assess the solubilization of iron from Fe complexes with two different humic acids by various concentrations of a siderophore and a mixture of organic acids at different times of extraction.

Methods

Humic matter

HS were extracted from: (1) a volcanic soil (Typic Dystrandept) sampled in the caldera of Lake Vico near Rome (Italy), and (2) a North Dakota Leonardite (Mammoth Chem. Co.). Original materials were shaken overnight and in a 0.5 M NaOH and 0.1 M Na₄P₂O₇ solution under N₂ atmosphere [31]. The resulting humic acids were precipitated by lowering the pH to 1 with 6 M HCl, purified by three cycles of dissolution in 0.1 M NaOH and precipitation in 6 M HCl, treated with a 0.5% (v/v) HCl–HF solution for 36 h, dialyzed against deionized water until chloride-free (Spectrapore 3 dialysis tubes, 3500 Mw cutoff) and freeze-dried. They were then redissolved in 0.5 M NaOH solution and passed through a strong cation exchange resin (Dowex 50) to further eliminate divalent and trivalent metals and freeze-dried again. An aliquot of samples was subsequently suspended in deionized water and titrated (VIT 90 Videotitrator, Radiometer, Copenhagen, Denmark) to pH 7 with a CO₂-free solution of 0.5 M NaOH. After having reached a constant pH 7, the resulting sodium–humates were left under titration for 2 h, filtered through a Millipore 0.45 μm and freeze-dried. Total acidity, carboxylic and phenolic groups of humic samples were determined according to the methods described by Schnitzer [32]. The elemental composition (C, N, H) of humates was carried out by an EA 1108 Elemental Analyzer (Fisons Instruments).

Solid-state nuclear magnetic resonance (NMR) spectroscopy

A 300 MHz Bruker Avance spectrometer, equipped with a 4 mm wide-bore MAS probe, was used to run solid-state spectra of humic samples. Each fine-powdered sample (5 mg) was packed into a 4 mm zirconium rotor, stoppered by a Kel-F cap and spun at a rate of $13,000 \pm 1$ Hz. In particular, ^{13}C NMR spectra were acquired through the cross-polarization magic-angle spinning (CPMAS) technique, by using 2 s of recycle delay, 1 ms of contact time, 30 ms of acquisition time and 4000 scans.

Synthesis of iron–humate complexes

Humic samples were added with a 0.8 N FeCl_2 solution to completely neutralize, with the Fe^{2+} cations the total acidity of humic solutions (Table 1), thereby precipitating the formed iron–humate complexes. The insoluble complexes were separated from the supernatant by centrifugation, dialyzed and freeze-dried. The total Fe content in complexes was measured by atomic absorption spectrometer (AAS) (Perkin Elmer, Analyst 700) following mineralization of the iron–humates by a $\text{HNO}_3\text{--HClO}_4$ treatment at 300 °C.

The solid iron–humate complexes were then treated with the following extracting solutions: (1) a mixture of citric, oxalic, tartaric and ketoglutaric acids (Merck KGaA, Germany) and (2) an aqueous solution of the commercially available deferoxamine mesylate (DFOM) (Merck KGaA, Germany), mimicking the naturally occurring siderophore deferoxamine B. An extraction experiment was based on the concentration of 10^{-3} M for both extracting solutions, to simulate the concentration of organic acids in maize root exudates reported in literature [33]. Other extraction experiments were conducted with solution concentrations of an order of magnitude either above or below 10^{-3} M.

For each extractant, 20 mL of solution was added to 50 mg of iron–humate sample placed in screwed-cap plastic vials and shaken on a rotating shaker for 1, 2, 4 and 16 h. The suspensions were then centrifuged and the supernatants separated. A second extraction was conducted by adding to the residue of the first extraction 20 mL more of the extractant and the vials shaken again for the same periods of time. All Fe extractions from complexes were carried out in triplicate. The

concentration of iron in the supernatants following the consecutive extractions was determined by AAS.

Results and discussion

Elemental composition, total, carboxyl and phenolic acidities of HA used in this study are reported in Table 1. The humic acid extracted from volcanic soil (HA_1) showed a smaller C content and a larger amount of acidic functional groups than the humic material extracted from leonardite (HA_2). HA contain multiple functional groups that can interact with metal ions through complex formation, as carboxyl and phenolic groups. The larger content of the total acidic functional groups found in HA_1 in comparison to HA_2 may explain the greater amount of Fe complexed by the former material (Table 1) and the establishment of a network of intra- and inter-molecular iron complexes.

Further chemical differences between HA_1 and HA_2 were shown by ^{13}C -CPMAS-NMR spectroscopy (Fig. 1). The NMR spectra were divided into the following intervals: 0–105 ppm (aliphatic C); 105–165 ppm (aromatic C); 165–190 (carboxyl C), and the relative percent distribution of signal areas in the different resonance intervals is reported in Table 2. HA_1 showed a larger content of aliphatic than aromatic carbons, contrary to HA_2 , in which the aromatic components were instead greatly prevalent. Moreover, the content of carboxyl carbons was larger in HA_2 than in HA_1 .

The percentages of iron extracted from the humic complexes after two consecutive extractions by either DFOM solution or the mixture of organic acids at different concentrations are reported in Tables 3 and 4. The amount of iron extracted varied with the molecular composition of humic materials, the concentration of the extracting solution and the time of extraction. In particular, the amount of iron recovered from HA_1 by extraction with the mixture of organic acids at the concentration of 0.01 M was significantly larger than that recovered with the same concentration of DFOM solution, regardless of the number of extractions and shaking time. These results indicate that the mixture of organic acids was significantly more efficient than the DFOM siderophore in displacing iron from complexes with HA (Table 3).

Table 1 Chemical properties of humic acids

Sample	C (%)	H (%)	N (%)	Ash (%)	Acidity (meq g^{-1})			Complexed Fe (%)
					Total	Carboxyl	Phenolic	
HA_1	50.8	4.2	2.3	1.0	10.7	4.7	6.0	7.57
HA_2	64.2	3.5	1.5	1.5	9.3	4.4	4.9	6.83

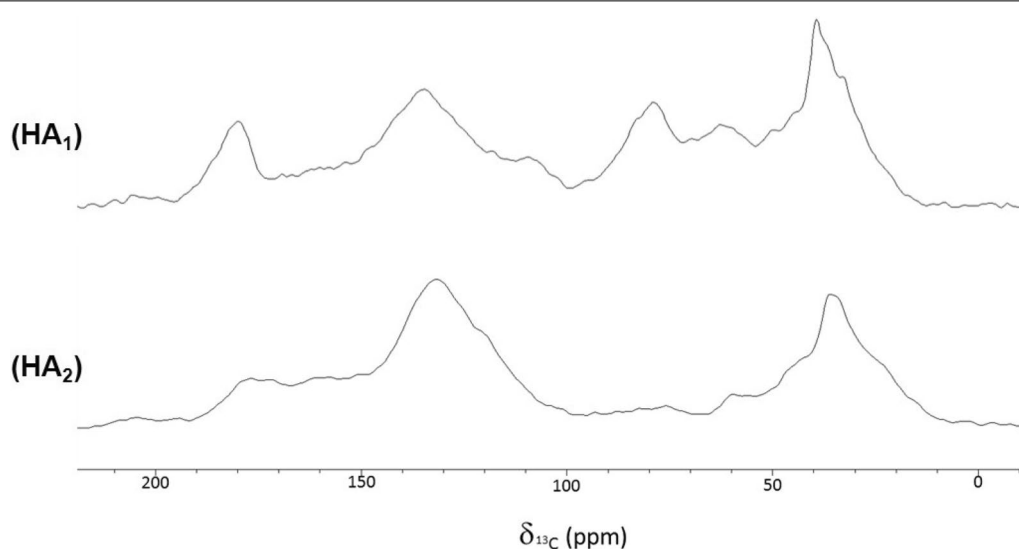


Fig. 1 ^{13}C -CPMAS-NMR spectra of humic acids extracted from a volcanic soil (HA_1) and a leonardite (HA_2)

Table 2 Relative carbon distribution (%) in different regions of chemical shift (ppm) in ^{13}C -CPMAS-NMR spectra of humic samples

Sample	Aliphatic C (0–105 ppm)	Aromatic C (105–165 ppm)	Carboxyl C (165–190 ppm)
HA_1	78.1	16.0	10.2
HA_2	51.7	39.4	16.6

Conversely, the Fe-removing capacity was similar for both extractants at the 0.001 M concentration in both the first and second extractions, except for a slightly larger Fe

amount extracted by the organic acids after 16 h of shaking. A similar behavior was observed for the 0.0001 M concentration at all shaking times in the first extraction, whereas in the second extraction the mixture of organic acids was again capable of removing a significantly larger Fe amount than the DFOM solution.

In the case of HA_2 , the capacity of both extractants to displace iron from the iron–humate complexes was significantly smaller than for HA_1 (Table 4). The largest iron-removing capacity was also shown in HA_2 by the mixture of organic acids at the 0.01 M concentration in both the first and second extractions and at increasing extraction times. The difference in iron-removing

Table 3 Fe recovered (%) from iron–humate complexes formed with HA_1 , after a first (I) and a second (II)^a extraction after different shaking times (*h*), with a solution of a DFOM siderophore and a mixture of four organic acids: citric, oxalic, tartaric and ketoglutaric acids (Mix) at different concentrations

Extractant	<i>h</i>	Concentration					
		0.01 M		0.001 M		0.0001 M	
		I	II	I	II	I	II
DFOM	1	24.3 ab	13.9 a	18.5 bcd	11.4 a	6.1 cde	2.1 a
	2	32.8 b	20.4 ab	21.6 defg	12.3 ab	6.4 cde	1.9 a
	4	53.6 c	22.6 ab	24.1 defgh	15.3 abc	6.9 de	1.9 a
	16	57.6 c	26.4 ab	24.9 efgh	19.6 cdef	6.7 de	1.9 a
Mix	1	68.4 cde	53.9 c	14.4 abc	12.6 ab	6.3 cde	5.0 bc
	2	77.2 def	65.3 cd	25.5 fgh	15.1 abc	7.0 de	5.5 bcd
	4	86.1 ef	95.2 f	28.5 hi	19.1 cde	7.3 e	6.5 cde
	16	88.2 f	78.9 def	33.1 i	26.6 gh	6.8 de	4.4 b

All values are average of three replications. Values followed by the same letter within columns are not significantly different by Tukey's test at the level of $P \leq 0.05$

^a Fe recovered after the second extraction was calculated on the basis of iron left in the solid iron–humates after the first extraction

Table 4 Fe recovered (%) from iron–humate complexes formed with HA₂, after a first (I) and a second (II)^a extraction at different hours of shaking (*h*), with a solution of the siderophore DFOM and a mixture of four organic acids: citric, oxalic, tartaric and ketoglutaric acids (Mix) at different concentrations

Extractant	<i>h</i>	Concentration					
		0.01 M		0.001 M		0.0001 M	
		I	II	I	II	I	II
DFOM	1	1.3 a	1.8 a	1.0 a	0.8 a	0.7 a	0.9 ab
	2	3.1 a	2.4 a	2.2 ab	1.1 a	1.1 abc	0.7 a
	4	4.0 a	2.7 a	2.6 ab	1.1 a	1.3 bcde	1.2 bcd
	16	9.2 a	7.4 a	3.8 bc	3.3 bc	1.8 f	1.7 ef
Mix	1	26.6 b	25.4 b	5.4 cd	6.5 de	1.1 abc	1.5 cdef
	2	42.0 c	38.1 c	6.9 de	7.7 e	1.5 cdef	2.3 g
	4	53.5 d	37.9 c	7.0 de	10.3 f	1.6 def	2.5 gh
	16	66.7 e	23.3 b	15.8 g	15.5 g	2.8 ih	3.1 i

All values are average of three replications. Values followed by the same letter within columns are not significantly different by Tukey's test at the level of $P \leq 0.05$

^a Fe recovered after the second extraction was calculated on the amount of iron left in the complexes after the first extraction

Table 5 Values of pH of a siderophore solution (DFOM) and a mixture of four organic acids: citric, oxalic, tartaric and ketoglutaric acids (Mix) at different concentrations

Extractant	Concentration		
	0.01 M	0.001 M	0.0001 M
DFOM	4.44	4.87	5.39
Mix	1.98	2.68	3.44

capacity between the mixture of organic acids and the DFOM solution remained significant at the 0.001 M concentration, whereas it disappeared at the 0.0001 M concentration except for the longest shaking time (16 h) of the first extraction and all shaking times of the second extraction.

These findings suggest that the Fe displacement from humic complexes is governed by different factors. The pH of the extracting solutions is an important parameter that varies with the concentration of solutions (Table 5). In fact, the pH for the mixture of organic acids was significantly lower than for the DFOM solution at all the applied concentrations. This difference in pH values may partially account for the larger Fe recovery observed for the mixture of organic acids with respect to the siderophore solution. Moreover, the presence of different carboxyl functional groups in the mixture of organic acids, which are most effectively metal complexing agents, may further explain the greater Fe extracting capacity of the organic acids mixture as compared to the siderophore.

Other important factors that control the process of alteration of iron–humate complexes are the molecular composition of the humic acids and the possible steric hindrance of the two extractants that may prevent a direct contact with the iron-complexing sites. In fact, the large aliphatic characteristics of HA₁ extracted from soil may confer a conformational flexibility that may favor an easier iron displacement from the humic-complexing groups than for HA₂, whose prevalent aromatic composition and consequent abundance of π – π stacking interactions is instead conducive to a more rigid conformational structure that prevents the accessibility of complexed iron by the extracting solutions. In fact, a physical inhibition to reach the iron-complexing sites may explain the significant reduction of Fe extracted by DFOM when passing from HA₁ to HA₂. Moreover, it may be conceivable that the bulky siderophore molecule, with respect to the simpler structures of organic acids, was sterically hindered from reaching the iron complexed by the HA₂ functional groups.

Conversely, the bulky character of the DFOM molecule may have been less determinant in displacing iron from the more sterically flexible Fe–humate complex in HA₁, thereby accounting the Fe extraction mainly to the large stability of the iron–siderophore complex [21]. In fact, the stability constants of complexes formed between Fe and DFOM are greater than for the iron–humates complexes and for the complexes with the organic acids [20]. Therefore, the large stability of the DFOM–iron complex was the thermodynamic drive that determined the quantitative iron extraction from the iron–humates complexes.

Conclusions

This study showed that iron complexed by humic acids can be displaced and solubilized by the action of microbially produced siderophores or root-exuded organic acids. The major drive for the iron removal from the iron complexes is attributed to the stability constants with the new ligands, which are larger than those between iron and the humic acids. However, the extent of Fe displacement was also dependent on both the molecular composition and consequent conformational flexibility of humic materials and the steric hindrance of ligands. Complexes made by humic acids rich in aromatic carbon were less potentially susceptible to release iron to organic ligands present in the soil solution than complexes formed with alkyl-rich humic acids. In fact, the highly aromatic humic acids are liable to be more conformationally rigid and inhibit the accessibility of the bulky siderophore molecules to the iron-complexing sites.

These findings suggest not only that iron–humate products may be usefully employed to increase plant availability of iron metals in alkaline soils, but also that the humic molecules may be displaced from the iron complexes that stabilize organic matter in soils [30] and play a bioactive role in the rhizosphere. These results hence indicate that both plant-exuded organic acids and microbial siderophores play a fundamental role in regulating the dynamics and turnovers of humic molecules in soil, thereby ultimately controlling both the nutrition of crops and the stability of soil organic matter.

Abbreviations

HA₁: humic acid extracted from a volcanic soil; HA₂: humic acid extracted from a leonardite; DFOM: deferoxamine mesylate; Fe: iron; EDDHA: ethylenediamine-*N,N'*-bis (*o*-hydroxyphenyl) acetic acid; HS: humic substances; HA: humic acids; FA: fulvic acids; SOM: soil organic matter; NMR: nuclear magnetic resonance; CPAS: cross-polarization magic-angle spinning; AAS: atomic absorption spectrometer; Mix: a mixture of four organic acids: citric, oxalic, tartaric and ketoglutaric acids.

Authors' contributions

All authors have contributed equally to the work. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Consent for publication

All authors give their personal consent for publication.

Ethics approval and consent to participate

Not applicable.

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