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Characterization of Fe−Leonardite Complexes as Novel Natural Iron Fertilizers

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Supporting Information

ABSTRACT: Water-soluble humic substances (denoted by LN) extracted at alkaline pH from leonardite are proposed to be used as complexing agents to overcome micronutrient deficiencies in plants such as iron chlorosis. LN presents oxidized functional groups that can bind Fe^{2+} and Fe^{3+}. The knowledge of the environment of Fe in the Fe−LN complexes is a key point in the studies on their efficacy as Fe fertilizers. The aim of this work was to study the Fe^{2+}/Fe^{3+} species formed in Fe−LN complexes with ^{57}Fe Mössbauer spectroscopy under different experimental conditions in relation to the Fe-complexing capacities, chemical characteristics, and efficiency to provide iron in hydroponics. A high oxidation rate of Fe^{2+} to Fe^{3+} was found when samples were prepared with Fe^{2+}, although no well-crystalline magnetically ordered ferric oxide formation could be observed in slightly acidic or neutral media. It seems to be the case that the formation of Fe^{3+}−LN compounds is favored over Fe^{2+}−LN compounds, although at acidic pH no complex formation between Fe^{3+} and LN occurred. The Fe^{2+}/Fe^{3+} speciation provided by the Mössbauer data showed that Fe^{2+}−LN could be efficient in hydroponics while Fe^{3+}−LN is suggested to be used more effectively under calcareous soil conditions. However, according to the biological assay, Fe^{3+}−LN proved to be effective as a chlorosis corrector applied to iron-deficient cucumber in nutrient solution.

KEYWORDS: *iron, leonardite, complexes, Mössbauer spectroscopy, fertilizers*

INTRODUCTION

Traditionally, the most common approach used to improve physical and chemical soil properties is to incorporate organic residues (compost or agricultural/farm wastes) into the soil to increase its organic matter content.[1,2] In that way, humic substances (HS) are the most important natural soil conditioners because they improve the physical and chemical properties that are essential for plant growth. Different studies have reported that the beneficial effect of HS on the growth of different plant species is directly related to the ability of these complexes to supply available macronutrients and micronutrients[3−8] due to their high specific surface area and cation exchange capacity. HS can act as a carrier of polyvalent cations by forming stable complexes,[9] mainly with carboxyl and phenolic groups.[3,10,11] Chen and co-workers[6,12] have suggested that growth enhancement of plants grown in nutrient solution containing HS is related to significant improvement of the Fe and possibly Zn nutrition. In addition, HS display hormonelike activity[13,14] and influence plant metabolism and morphology by interacting with a variety of biochemical mechanisms and physiological processes. They increase the chlorophyll content and stimulate growth and enzyme activities related to the photosynthetic pathway.[15,16]

For this reason, HS are potentially useful complexing agents of natural origin that can be utilized to overcome micronutrient deficiencies such as iron deficiency, which is a worldwide problem affecting plants grown especially on calcareous soils.[17] Natural complexing agents used as iron fertilizers have lower efficacies than the synthetic compounds, but they are cheaper and biodegradable. The inclusion of complexing agents in the EU Fertilizer Directive[18] is under discussion, while the Spanish Regulation[19] already allows the use of humic, fulvic, lignosulfonic, gluconic, and heptagluconic acids, free amino acids, and citric acid in fertigation and foliar application. HS extracted from lignites, such as leonardite, are currently used in the whole Mediterranean area as liquid concentrates in drip-irrigated cultures and fruit tree plantations. These HS are similar to soil HS since lignites come from carboniferous plant species. It is supposed that they improve the physical and chemical soil characteristics in the same way as the native soil HS. HS derived from leonardite contain oxidized functional groups that can bind different metal ions (e.g., Fe^{2+} and Fe^{3+}) or heavy metals.[20] According to the structural properties and literature data on the positive effects of purified HS on plant development and iron nutrition,[20−22] Leonardite-derived HS seem to be a potentially useful natural iron fertilizer. However, the nature of the binding between this material and the metals is not well-known.

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In the present work, the structure, Fe-complexing ability, and Fe speciation in the water-soluble humic acid fraction isolated from leonardite (denoted as LN) has been studied with the help of diffuse-reflectance FTIR (DRIFT), ^{13}C NMR, and ^{57}Fe transmission Mössbauer spectroscopy. DRIFT and ^{13}C NMR spectroscopy provided information on the structural characteristics of LN,[23−35] while ^{57}Fe Mössbauer spectroscopy gave information on the Fe speciation in the Fe−LN compounds. It is of high interest to have information on the speciation of Fe in Fe−LN, since it should play an important role in the Fe availability, uptake, and translocation in plants. Knowing the environment of Fe in the Fe^{3+}−LN complexes is a key point in the studies on their efficacy as Fe fertilizers, for the selection of the most appropriate conditions for their production, and on the possible chemical modifications of LN to obtain products of optimal efficacy. Mössbauer spectroscopy is a suitable tool for studying the chemical environment of iron in different compounds, including various synthetic and natural Fe complexes and plant-related iron compounds.[26] The aim of this work was to study the Fe^{2+}/Fe^{3+} species formed in Fe−LN complexes with ^{57}Fe Mössbauer spectroscopy, varying the conditions of preparation, including the iron source (Fe^{2+} or Fe^{3+}), pH, and Fe:LN ratio. This could increase the stability and the quality of this type of fertilizer and presumably the amount of Fe provided to the plants when these products are applied. Moreover, the efficiency of the Fe^{3+}−LN complex prepared at an Fe:LN ratio of 1:1.1 was tested with the help of iron-deficient cucumber plants supplied with Fe^{3+}−LN in nutrient solution.

EXPERIMENTAL SECTION

Reagents. All reagents used were of recognized analytical grade, and solutions were prepared with type-I grade water according to ISO 3696:1987,[27] free of organic contaminants (Millipore, Milford, USA). $^{57}FeSO_4$ solution was prepared from metallic ^{57}Fe (Isoflex, 95.70% ^{57}Fe isotopic enrichment) by dissolving it in 1 M H_2SO_4 (Aldrich). The $^{57}FeCl_3$ solution was prepared from the same metallic ^{57}Fe by dissolving it in 20% HCl (Aldrich) and then oxidizing it with few drops of 3% H_2O_2 solution.

Chemical Properties of the LN Extract. The LN used in this work was a solid commercial product named "dry Energy" that was originally from North Dakota and was provided by the Spanish company Agrichem S.A. The product was completely soluble since it was produced after KOH extraction, filtration, and drying of the bulk material. Moisture, total organic matter (OM), total humic extract (THE), and humic and fulvic acid (HA and FA) content were measured in the soluble fraction of LN. Moisture was measured after heating the LN overnight at 105 °C, and OM was determined by weight loss after calcination for 4 h at 550 °C. For THE, the sample was extracted in 0.1 M NaOH and 0.1 M $Na_4P_2O_7$. HA was obtained after precipitation with H_2SO_4 at pH 1. The carbon contents in THE and HA were determined after oxidation with $K_2Cr_2O_7$ and determination of the excess $Cr_2O_7^{2-}$ with $Fe(NH_4)SO_4$. Conversion of C to THE and HA was made using the 1.724 factor. The FA content was determined by the difference between THE and HA. Organic nitrogen was determined as the difference between the total nitrogen and inorganic nitrogen contents. The pH was measured in an LN/water mixture at a ratio of 1:2.5. The Fe, Mn, Cu, and Zn concentrations were determined by atomic absorption spectroscopy (AAS) after mineralization of the sample in a closed-vessel microwave system (MARSXpress, CEM, Matthews, NC, USA) following the procedure established by García-Delgado et al.[28] The elemental analysis of C, H, N, and S contents was carried out by total oxidation of the samples through an instant and complete combustion in a LECO CHNS-932 elemental analyzer.

Spectroscopic Characterization of the LN. DRIFT and ^{13}C NMR analyses of solid LN samples were performed in order to characterize the structure of LN. The DRIFT spectrum of a mixture of LN with KBr (1 mg of sample + 99 mg of dry KBr) from 7000 to 560 cm^{-1} was recorded on a Bruker IFS66v FTIR spectrophotometer fitted with an apparatus for diffuse reflectance. The DRIFT spectrum was the average of 250 scans, and the resolution was set at 4 cm^{-1}. The ^{13}C NMR spectrum of LN was recorded at 100.32 MHz with a rotation speed of 10 kHz in a Bruker WB-400-V spectrometer in a 4 mm triple-channel probe with ZrO_2 rotors and a Kel-F cap at room temperature. The cumulative number of scans was 1024, and the pulse sequence employed to acquire the spectrum was cross-polarization magic-angle-spinning (CP-MAS) $^1H-^{13}C$ using a spectral width of 35 kHz, a contact time of 3.5 ms, and a relaxation delay of 4 s, with ^1H decoupling type TPPM15 at a rate of 80 kHz.

Determination of Maximum Complexing Capacities (MCCs) of LN with Fe^{2+} and Fe^{3+}. For the determination of the Fe MCCs, the official method CEN/TC 260 (EN 15962:2011)[29] based on the work of Villén et al.[30] was applied. In short, increasing volumes of a c_{Fe} = 200 g L^{-1} solution of $FeSO_4 \cdot 7H_2O$ for Fe^{2+} and $FeCl_3 \cdot 6H_2O$ for Fe^{3+} were added to 20 mL of the LN solution (c_{LN} = 100 g L^{-1}). After the addition of two drops of H_2O_2 (33% w/v), the pH was raised to 9.0 with NaOH. After 1 day standing in dark, the pH was increased again to 9.0; after 2 h, the solutions were transferred to a 100 mL volumetric flask, and the volume was made up to 100 mL. Afterward, the solutions were centrifuged at 7500 min^{-1} (at 10691*g*) at room temperature for 10 min, and the supernatants were filtered using 0.45 μm filters (Millipore). After the removal of the organic compound in accordance with method 9.3 (EU Directive 2003/2003)[18] using H_2O_2 (33% w/v) and 0.5 M HCl for the digestion, the complexed element was determined by AAS (AAnalyst 800; PerkinElmer, Shelton, CT, USA) using 0.5% La, 0.2% Cs, and 5% HCl as a matrix modifier. MCC determinations were made in triplicate.

Mössbauer Characterization. *Sample Preparation.* Two types of samples were made. One set of samples was prepared by freeze-drying the Fe−LN complex solutions containing nonlabeled iron salts (solid samples); the other one was prepared with iron salts enriched in ^{57}Fe isotope, applying a rapid-freezing (quenching) method (frozen solution samples).

The solid samples were prepared by mixing an aliquot of a 200 g L^{-1} solution of $FeSO_4$ for Fe^{2+}−LN or $FeCl_3$ for Fe^{3+}−LN with the suitable amount of the complexing agent calculated on the basis of their highest complexing ability. The LN:Fe ratio was varied among 1.1:1, 1.5:1, and 2:1 according to the maximum complexing capacity data, where the 1:1 LN:Fe ratio is equal to the maximum capacity of binding. Then the pH of the samples was set to 7.0 or 4.0 with 0.05 and 0.005 M NaOH solutions, and all of the solutions were allowed to stand overnight in darkness. Thereafter, the pH of the solutions was readjusted to 7.0 or 4.0, and they were filtered through a 0.45 μm Millipore membrane and diluted to the final volume with Milli-Q water. The samples were freeze-dried using a Thermo Scientific freeze-dryer (model Heto PowerDry LL3000).

The frozen solution samples obtained by the application of the rapid-freezing (quenching) method were prepared by the same procedure as described above, applying $^{57}FeSO_4$ or $^{57}FeCl_3$ salts prepared from ^{57}Fe-enriched metallic iron as 0.1 M stock solutions. The Fe^{2+} and Fe^{3+} contents of the stock $^{57}FeSO_4$ and $^{57}FeCl_3$ solutions were checked by Mössbauer spectroscopy measurements, which confirmed the absence of Fe^{3+} in the $^{57}FeSO_4$ solution and the absence of Fe^{2+} in the $^{57}FeCl_3$ solution.

The final concentration of ^{57}Fe in the solutions was set to 0.01 M with deionized water. The pH of the Fe−LN solutions was adjusted to 7.0 or 4.0 with 0.05 and 0.005 M NaOH solutions. Any precipitates formed were removed by centrifuging the solution at 10000*g* at room temperature for 5 min. Then 400 μL of the resulting supernatant was rapidly frozen within a few seconds in liquid nitrogen (freeze-quench method).[31]

Mössbauer Spectroscopy Measurements. All of the Mössbauer spectroscopy measurements were performed at $T \sim 80$ K (with each sample kept in a cryostat filled with liquid nitrogen) using a

conventional constant-acceleration Mössbauer spectrometer and a ^{57}Co(Rh) source with an activity of ∼10^9 Bq. The spectra were evaluated by least-squares fitting of spectral lines using the MOSSWINN 4.0 software[32] with the assumption of Lorenzian line shapes. In the case of some Fe^{3+}−LN samples, broad absorption lines could be observed. We made an attempt to take this broadening into account by the assumption of Gaussian broadening in the spectral lines, and consequently, we applied Voigt functions (approximated by pseudo-Voigt functions[32]) instead of Lorentzians to fit the spectra. The calculated parameters were the isomer shift (δ, mm s^{-1}), the quadrupole splitting (Δ, mm s^{-1}), the experimentally observed line width [full width at half-maximum, Lorentzian line width (LLW) and Gaussian line width (GLW), mm s^{-1}], and the partial resonant absorption area (S_r, %) for each spectral component. The parameter S_r was used to represent the relative content of the related iron species, assuming a common recoilless fraction for all iron components in a sample contributing to the spectrum at low temperature. The spectrometer (WissEl, Germany) was calibrated with α-iron at room temperature.

Bioassay. *Plant Growth.* Cucumber seeds (*Cucumis sativus* L. cv Ashley) were germinated in Petri dishes on wet filter paper for 2 days in darkness at 30 °C. The seeds with radicles of similar length were planted on a plastic net placed between polystyrene disks, which were stored for an additional day in PVC cups, each containing 200 mL of 0.5 mM $CaSO_4$ solution. This achieved a slightly faster radicle elongation rate under the isosmotic conditions ensured by the salt solution. They were covered with wet filter paper to keep the plants in a dark, moisture-filled atmosphere at 26 °C for an additional 1 day. Afterward, the plants were transferred into 400 mL of preculture solution with the following composition: 0.9 mM KNO_3, 1.0 mM $Ca(NO_3)_2$, 0.3 mM $MgSO_4$, 0.1 mM KH_2PO_4, 10 μM H_3BO_3, 2.5 μM $MnCl_2{\cdot}4H_2O$, 10 μM $ZnSO_4{\cdot}7H_2O$, 0.05 μM $Na_2MoO_4{\cdot}2H_2O$, 1 μM $CuSO_4{\cdot}5H_2O$, 1 μM $CoSO_4$, 1 μM $NiSO_4{\cdot}7H_2O$, 35 μM NaCl, 100 μM K_2EDTA (dipotassium salt of ethylenediaminetetraacetic acid), 0.1 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 0.1 g/L $CaCO_3$, and 5 μM Fe^{3+}−EDTA. The solution was continuously aerated. After the preculture, the plants were transferred to a nutrient solution of the same volume and composition except for iron, which was not included, and they were grown for an additional 7 days. These plants were supplied with 20 μM Fe^{3+}−LN or Fe^{3+}−*o*,*o*-EDDHA [ethylenediamine-*N*,*N*′-bis(*o*-hydroxyphenylacetic acid)] in nutrient solution with the same composition as used for preculture for 1 day before the harvest. As a reference (+Fe control), some plants were grown all along in the preculture solution using 20 μM Fe^{3+}−EDTA.

The plants were grown during the preculture and the growth period in a controlled environment at 22−26 °C and 65−70% humidity and illuminated with metal halogen lamps and fluorescent tubes with a photosynthetic photon flux density (PPFD) of 150 μmol m^{-2} s^{-1} and a 14 h/10 h light/dark photoperiod.

Pigment Determination. Chlorophyll contents were determined in 80% (v/v) acetone extracts using a UV−vis spectrophotometer (Shimadzu, Japan) and the absorption coefficients of Porra et al.[33]

Chloroplast Isolation. Cucumber leaves were homogenized in isolation buffer [50 mM HEPES−KOH, pH 7.0, 330 mM sorbitol, 2 mM EDTA, 2 mM $MgCl_2$, 0.1% (w/v) bovine serum albumin (BSA), 0.1% (w/v) sodium ascorbate] using a Waring blender for 5 s. The homogenate was filtered on four layers of gauze and two layers of Miracloth (Calbiochem-Novabiochem), and centrifuged at 1500*g* for 5 min. All of the centrifugation steps were carried out in a swing-out rotor. The pellet was resuspended in washing buffer (50 mM HEPES−KOH, pH 7.0, 330 mM sorbitol, 2 mM $MgCl_2$), and centrifuged at 2000*g* for 15 min. The pellet was resuspended in washing buffer. The number of chloroplasts was counted in a Bürker chamber using a Nikon Optiphot-2 microscope (Zeiss Apochromatic 40/0.95 160/0.17 objective) equipped with a Nikon D70 DSLR camera. Chloroplast numbers were counted with ImageJ software (http://rsbweb.nih.gov/ij) using the Cell Counter plugin.

Measurement of Chloroplast Iron Uptake by the BPDS Method. Chloroplasts were resuspended in 0.25 mL of washing buffer and solubilized in 1% sodium dodecyl sulfate (SDS), 1% dithiothreitol (DTT) solution at room temperature for 30 min. Nonsolubilized material (starch) was removed by centrifugation of samples at 10000*g* for 5 min. The iron content of solubilized chloroplast material (as the $[Fe(BPDS)_3]^{4-}$ complex) was measured photometrically at 535 nm using a UV−vis spectrophotometer (Shimadzu, Japan) after the addition of 100 μM ascorbic acid (to reduce the total iron content of the samples into Fe^{2+}) and 300 μM bathophenanthroline disulfonic acid disodium salt (BPDS) (Sigma). Steady-state absorbance was reached after incubation for 60 min in darkness at room temperature. An absorption coefficient of ε = 22 140 L mol^{-1} cm^{-1} given by Smith et al.[34] was used to calculate the iron contents of the chloroplasts before and after iron uptake.

Chlorophyll a Fluorescence Induction. Fluorescence induction measurements of leaf samples were performed using a PAM 101-102-103 chlorophyll fluorometer (Walz, Effeltrich, Germany). Leaves were adapted to dark for 30 min. F_0 values were measured after eliminating reduced electron carriers by a 3 s far-red light impulse. The maximum fluorescence yields F_m in the dark-adapted state were measured by applying a 0.7 s pulse of white light (PPFD of 3500 μmol m^{-2} s^{-1}; KL 1500 electronic light source, Schott, Mainz, Germany), which saturated the PSII electron transport, thus closing all of the PSII traps. The maximal efficiency of PSII centers was determined as $F_v/F_m = (F_m - F_0)/F_m$.

RESULTS AND DISCUSSION

Chemical and Spectroscopic Characterization of LN. The main chemical properties are summarized in Table 1. Most of the OM is present in the THE and it presents a high K content, both as consequences of its production system, which already includes base extraction. The high O content is remarkable.

Table 1. Chemical Properties of LN

parameter[a]	result
moisture (g kg^{-1} ww)	140.0 ± 1.3
total OM (g kg^{-1} ww)	583 ± 15
THE (g kg^{-1} ww)	566 ± 24
HA (g kg^{-1} ww)	436 ± 18
FA (g kg^{-1} ww)[b]	130
organic N (g kg^{-1} ww)	8.3 ± 0.2
K (g kg^{-1} ww)	134 ± 4
pH (1:2.5) H_2O	9.76 ± 0.03
micronutrient concentration (mg kg^{-1} dw)	
Fe	5695 ± 21
Mn	160 ± 10
Cu	17.3 ± 1.6
Zn	62.9 ± 6.8
elemental analysis (g kg^{-1} dw)	
C	353.9 ± 0.0
H	32.4 ± 0.2
N	6.7 ± 0.1
S	2.9 ± 1.2
O[c]	443.3
C/N	52.8

[a]ww = wet weight; dw = dry weight. [b]FA was determined as the difference between THE and HA. [c]O =1000 − (C + H + N + S + K + Fe + Mn + Cu + Zn).

The DRIFT spectrum of LN, shown in Figure 1, was interpreted using a reference of infrared spectral bands.[35] Bands from 2800 to 2980 cm^{-1} can be attributed to strong C−H (alkane) stretching vibrations. They are superimposed on a broad absorption envelope of stretching O−H moieties of several functional groups (3700−2600 cm^{-1}) The band at 1698

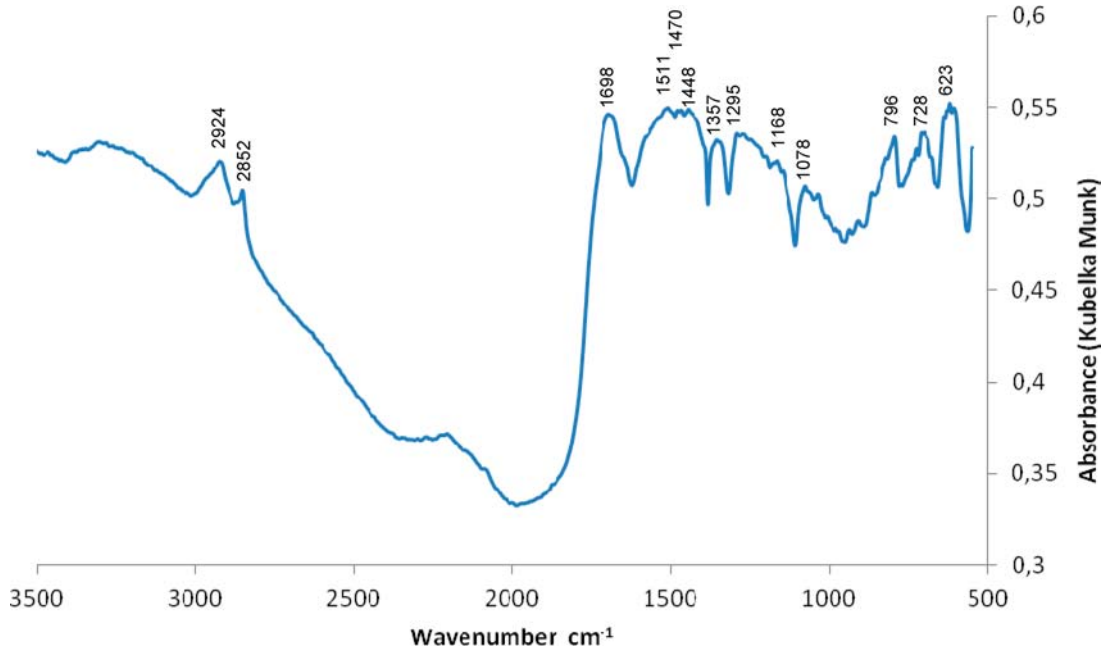

Figure 1. DRIFT spectrum of LN powder in a KBr matrix.

cm^{-1} can be due to C═C stretching of alkene groups and/or C═O stretching of amide groups and quinonic and/or H-bonded conjugated ketones.[36] Stretching of C═C in aromatic rings can be found in the spectral region between 1400 to 1600 cm^{-1} with medium-weak intensity. Peaks situated in the range from 1150 to 1360 cm^{-1} with medium-weak intensity can be attributed to stretching vibrations of C−N in amines and/or C−O in alcoholic and phenolic groups. The bands around 1050 cm^{-1} might be due to C−O vibrations in acids, ethers, and alcoholic groups.[37] The band at 802 cm^{-1} can be associated with strong bending of ═C−H in alkene groups.

In the ^{13}C NMR spectrum obtained under our conditions (Figure 2), only three main bands can be observed. According

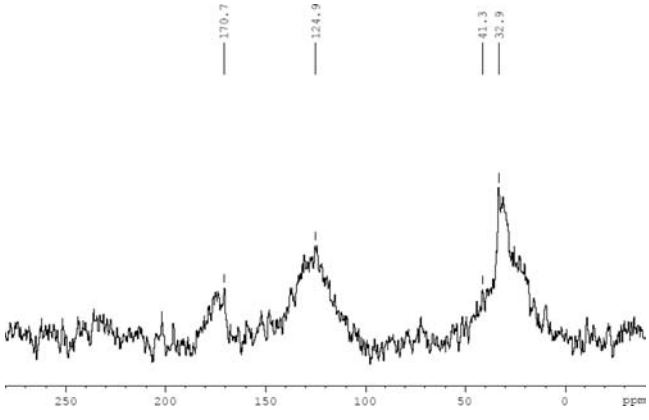

Figure 2. ^{13}C NMR spectrum of solid LN.

to Fernández et al.,[38] the peaks presented in the region between 220 and 160 ppm correspond to carboxylic acids, ketones, esters, and amides. Those in the region from 160 to 110 ppm are associated with phenolic-type aromatic carbons, while aliphatic carbons are present in the range from 110 to 0 ppm.

Comparing the FTIR and ^{13}C NMR spectra for LN with those obtained by Fernández et al.,[36,38] we can conclude that the LN used in this work is in a highly oxidized state. This has been attributed to the presence of unsaturations (aromatic and carboxylic groups) that dominate in both spectra. These data are also in good agreement with the high oxygen content found in LN (see Table 1). With respect to the bulk material proposed by the International Humic Substance Society (IHSS) as standard leonardite from North Dakota (www.humicsubstances.org) the data are also in good agreement, but in our case the aliphatic content of LN is higher than from the source material of the IHSS as result of the KOH extraction and high oxidation in the commercial sample.

Maximum Complexing Capacity of LN. Figure 3 shows the titration curves obtained for the calculation of the Fe complexing capacity of LN either with Fe^{2+} or Fe^{3+}. The iron that remains in solution (complexed) is represented versus the Fe added. The rising segment corresponds to the Fe-complexing process by LN, while the decreasing one is probably due to the coagulation of material by an excess of metal.[30] Lines were obtained from the linear regression for the rising and decreasing segments, and the intersection of the two segments represents the maximum complexing capacity (MCC). The MCC with Fe^{3+} (131 ± 12 mg_{Fe} $g_{product}^{-1}$) was higher than with Fe^{2+} (94 ± 6 mg_{Fe} $g_{product}^{-1}$). This could be related to a higher stability of the Fe^{3+}−LN complex at alkaline pH compared with Fe^{2+}−LN. However, a certain amount of the Fe^{2+} added to the complexing agent may be oxidized in air, which could lead to the formation of iron oxides and hydroxides that decrease the soluble amount of the metal.

Mössbauer Characterization of Solid Ferrous and Ferric LN Complexes: Effect of the pH and the LN:Fe ratio on the Formation. The Mössbauer spectra of solid Fe^{2+}−LN complexes prepared at pH 4 are shown in Figure 4. The spectra can be evaluated assuming three components with corresponding Mössbauer parameters listed in Table 2. The Mössbauer parameters of the Fe^{2+} components ($\delta \approx 1.4$ mm s^{-1} and $\Delta \approx 2.9$ and 2.2 mm s^{-1}) are in good agreement with those of high-spin Fe^{2+} in a distorted octahedral O_6 environment. The doublet with $\Delta \approx 2.9$ mm s^{-1} (D1) has already been reported for Fe^{2+}−humates and is associated with

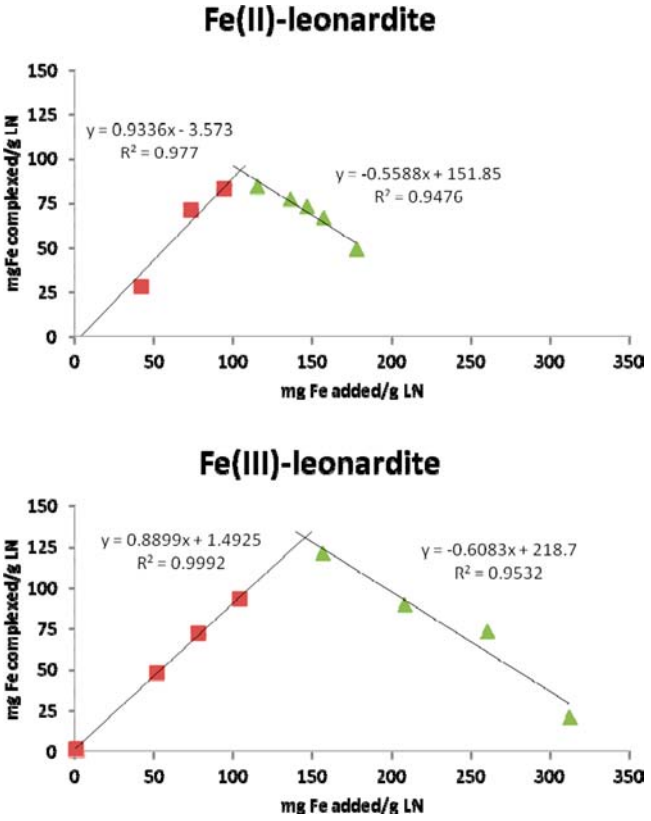

Figure 3. Titration curves for the determination of the maximum complexing capacities (MCCs) of LN with (top) Fe^{2+} and (bottom) Fe^{3+}.

Table 2. Mössbauer Parameters (T = 80 K) of the Solid Fe^{2+}–LN Samples Prepared at LN:Fe Ratios of 1.1:1, 1.5:1, and 2:1 at pH 4[a]

	LN:Fe = 1.1:1	LN:Fe = 1.5:1	LN:Fe = 2:1
		Fe^{2+} (D1)	
S_r	59%	49%	44%
δ	1.34(1)	1.35(1)	1.34(1)
Δ	2.87(1)	2.92(3)	2.90(2)
LLW	0.47(1)[b]	0.47(2)[b]	0.46(2)[b]
		Fe^{2+} (D2)	
S_r	24%	25%	22%
δ	1.35(1)	1.40(2)	1.38(2)
Δ	2.17(2)	2.24(5)	2.17(4)
LLW	0.47(1)[b]	0.47(2)[b]	0.46(2)[b]
		Fe^{3+} (D3)	
S_r	17%	26%	33%
δ	0.46(1)	0.47(3)	0.45(4)
Δ	0.73(2)	0.80(5)	0.80(7)
LLW	0.50(3)	0.55(7)	0.75(9)

[a]Errors in the last digits are given in parentheses; for S_r (%), the relative error is 5%. [b]Line widths were constrained to be the same.

Fe^{2+} coordinated in its first coordination sphere with carboxylate, phenolic, and/or alcoholic hydroxyl groups.[39] The doublet with $\Delta \approx 2.2$ mm s^{-1} (D2), representing another Fe^{2+} species, has not been identified earlier. The two different coordination sites may arise from the different dehydration during freeze-drying since Fe^{2+} is prone to retain water molecules in its hydration shell,[39] which can be (partly) replaced by the functional groups of LN upon dehydration. We should note that the line width of each component is rather large (~0.45 mm s^{-1}), suggesting the slightly different

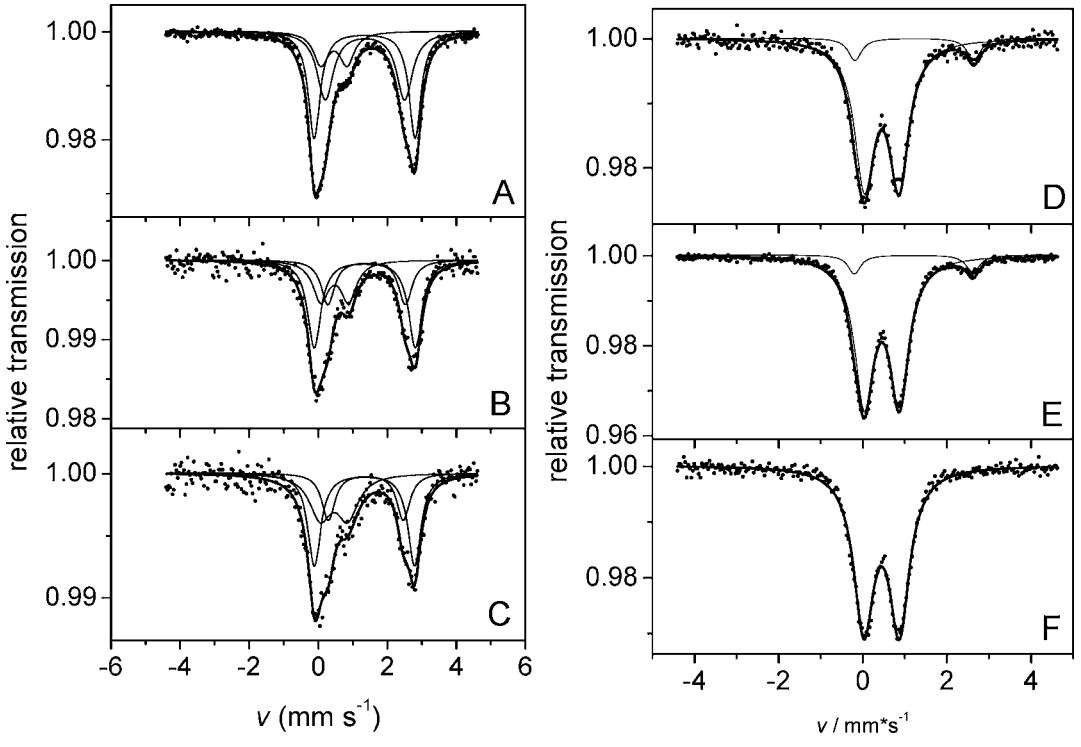

Figure 4. Mössbauer spectra at T = 80 K for solid Fe^{2+}–LN samples prepared at LN:Fe^{2+} ratios of (A) 1.1:1, (B) 1.5:1, (C) 2:1 at pH 4 and at LN:Fe ratios (D) 1.1:1, (E) 1.5:1, (F) 2:1 at pH 7.

geometrical arrangement of the ligands present in the first coordination sphere of the Fe^{2+} ions.

Besides the D1 and D2 components, a third doublet with parameters of $\delta \approx 0.5$ mm s^{-1} and $\Delta \approx 0.8$ mm s^{-1} (D3) can also be seen in the spectra of the solid Fe^{2+}−LN complexes. According to its Mössbauer parameters, D3 can be identified as high-spin Fe^{3+} in distorted octahedral O_6 coordination. The presence of the +3 oxidation state shows that Fe^{2+} is partly oxidized to Fe^{3+}, possibly as a result of the oxygen content of air during preparation. This finding confirms that the divalent iron attached to LN does not form a stable complex (e.g., remains as partly hydrated Fe^{2+}) and thus it is sensitive to oxidation even at slightly acidic pH. The resulting Fe^{3+} can be complexed by the carboxylic or hydroxo groups of LN and can also partly form ferric oxides and/or hydroxides. Since the solutions of Fe^{2+}−LN complexes were filtered before freeze-drying, we can assume that the spectral contribution of D3 may arise mainly from Fe^{3+} complexed by LN; however, the large line widths (LLW > 0.50 mm s^{-1}) of D3 suggest the presence of different similar Fe^{3+} environments (e.g., in partly hydrolyzed Fe^{3+} species). The presence of finely dispersed iron(III) oxide or hydroxide (as γ-FeOOH or γ-Fe_2O_3) has been shown in a recent work,[40] although in that case the pH of the purified humic compounds was significantly higher (final pH was maintained at 10.0), which can modify the hydrolysis of Fe^{3+}.

The relative occurrence of D3 increases as the LN/Fe^{3+} ratio is increased. This indicates a preference of LN to complex Fe^{3+} compared with Fe^{2+}, which can be understood according to the MCC data of Fe^{2+}−LN complexes, as LN was shown to have higher binding capacity for Fe^{3+} than for Fe^{2+} and the Fe^{2+} was suggested to form only weak adducts with LN.

In view of the sensitivity of Fe^{2+}−LN complexes to oxidation during preparation, possible redox transformations of Fe^{2+} in the solid material (after freeze-drying) may also occur. To get information about the effect of aging, one of the solid Fe^{2+}−LN complexes (prepared at pH 4 and a 2:1 LN:Fe ratio) was investigated also after storage for 12 months at room temperature in darkness (Figure 5; also see also Table S1 in the Supporting Information). Comparing the Mössbauer spectrum with the one taken immediately after preparation (Figure 4C) shows that the contribution of the central doublet D3 associated with Fe^{3+} species increased significantly (from 33% to 77%). This result clearly shows that Fe^{2+}−LN adducts

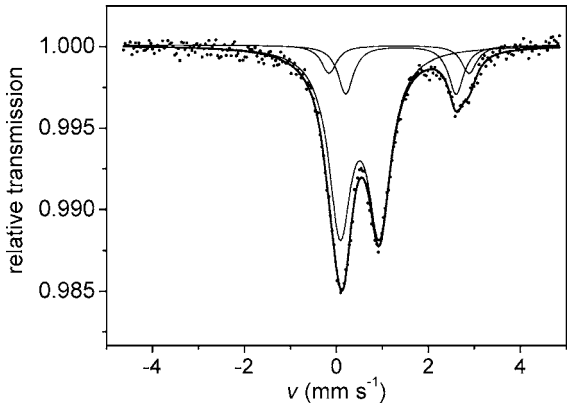

Figure 5. Mössbauer spectrum at T = 80 K for solid Fe^{2+}−LN samples prepared at pH 4 and 2:1 LN:Fe ratio recorded 1 year after preparation. The solid complex was allowed to stand in air for 12 months.

are not stable in air and that slow oxidation goes on even after dehydration. Although HS are reported to be capable of reducing metals, including Fe^{3+},[41] no reduction could be observed in the case of Fe−LN compounds. This can be explained by the highly oxidized state of LN, as the FTIR and ^{13}C NMR results have already shown. It also has to be mentioned that in spite of the oxidation of Fe^{2+} to Fe^{3+}, no well-crystalline, magnetically ordered iron oxide or oxide hydroxide formation could be observed in any of the $Fe^{2+/3+}$−LN samples. This is in good agreement with previous studies of several HS showing that high-molecular-weight humic fractions can stabilize hydrolyzed Fe^{3+} species and thus can prevent their further crystallization to more crystalline iron oxides.[42] This may play an important role in keeping iron available for plants in soils,[43] as was also demonstrated recently in a bioassay on iron-deficient cucumber plants grown in hydroponics containing iron-rich humic compounds.[40]

Mössbauer spectra of the Fe^{3+}−LN complexes prepared at pH 4 could not be measured since the iron content of these complexes was under the detection limit. This finding indicates that Fe^{3+}−LN complexes cannot be prepared under slightly acidic conditions because the main part of Fe^{3+} forms a precipitate upon mixing of the Fe^{3+} and LN solutions and thus is removed from the solution by the filtration process. This may be caused by the protonation of LN at acidic pH which can hinder complex formation between Fe^{3+} and LN. The pH dependence of the formation of Fe^{3+}−LN compounds indicates that Fe^{3+} is coordinated mainly to the COO^- and phenolic groups present in LN (e.g., humic and fulvic acids), and thus, the protonation/deprotonation processes due to the pH change strongly influence the complexing ability of LN.

When the pH was raised from 4 to 7, a significant decrease in the Fe^{2+}/Fe^{3+} ratio was observed in the case of Fe^{2+}−LN complexes, as shown in Figure 4D−F and Table 3. This

Table 3. Mössbauer Parameters (T = 80 K) of the Solid Fe^{2+}−LN Samples Prepared at LN:Fe Ratios of 1.1:1, 1.5:1, and 2:1 at pH 7[a]

	LN:Fe = 1.1:1	LN:Fe = 1.5:1	LN:Fe = 2:1
		Fe^{2+} (D1)	
S_r	10%	8%	
δ	1.23(2)	1.12(1)	
Δ	2.82(3)	2.82(1)	
LLW	0.38(4)	0.36(2)	
		Fe^{3+} (D3)	
S_r	90%	92%	100%
δ	0.45(1)	0.45(1)	0.45(2)
Δ	0.82(1)	0.84(1)	0.85(1)
LLW	0.56(1)	0.55(1)	0.58(1)

[a]Errors in the last digits are given in parentheses; for S_r (%), the relative error is 5%.

indicates a higher oxidation rate at more alkaline pH, which is well-known for Fe^{2+} in aqueous solutions[44] and shows again that LN is not capable of maintaining Fe^{2+} in the +2 oxidation state. The Mössbauer parameters of Fe^{2+} components are different at pH 7 compared with pH 4: at neutral pH, only one Fe^{2+} compound could be found, with slightly lower quadrupole splitting and isomer shift compared with D1 (Tables 2 and 3). Moreover, the line width of this Fe^{2+} component (0.36−0.38 mm s^{-1}) is significantly smaller than that found at pH 4, which

suggests that at pH 7 one well-defined coordination site is occupied by the Fe^{2+} ions.

In the case of Fe^{3+}−LN complexes prepared at pH 7, a wide quadrupole doublet can be observed in their Mössbauer spectra (Figure 6), which could be evaluated by supposing Gaussian line broadening (Table 4). Although the origin of this broadening (which is characteristic of all Fe^{3+}−LN samples in both solid and solution form prepared at pH 7) cannot be unambiguously determined on the basis of our measurements, we suggest that it is connected to the formation of different $Fe_x(OH)_y(LN)_z$-type oligomeric species formed by the partial hydrolysis of Fe^{3+} at neutral pH. The formation of such hydrolyzed polynuclear Fe^{3+} complex species (where the average molecular weight and short-range order depends mainly on the OH/Fe ratio, the ionic strength of the solution, and the corresponding anions of Fe^{3+}) has already been demonstrated in Fe^{3+} solutions by several authors.[44] The rather low stability of different metal−HS complexes in the pH range 6−9 compared with complexes of synthetic chelating agents (e.g., EDTA) or organic compounds (e.g., organic acids, siderophores)[43] also suggests that the formation of polynuclear −Fe−OH/O−Fe− species may be favored in the presence of LN. Similar compounds could be identified in the case of $Fe^{2+/3+}$−LN frozen solutions, too, as discussed in the next section.

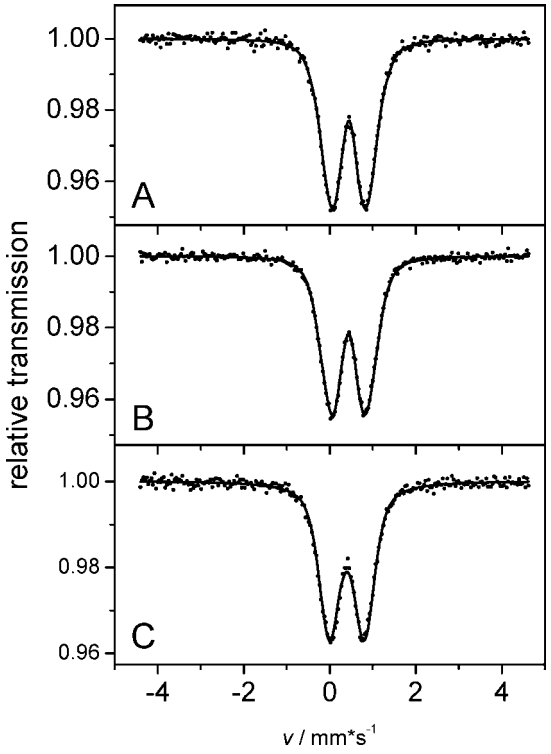

Figure 6. Mössbauer spectra at T = 80 K for solid Fe^{3+}−LN samples prepared at LN:Fe^{3+} ratios of (A) 1.1:1, (B) 1.5:1, and (C) 2:1 at pH 7.

Table 4. Mössbauer Parameters (T = 80 K) of the Solid Fe^{3+}−LN Samples Prepared at LN:Fe Ratios of 1.1:1, 1.5:1, and 2:1 at pH 7[a]

	LN:Fe = 1.1:1	LN:Fe = 1.5:1	LN:Fe = 2:1
Fe^{3+} S_r	100%	100%	100%
δ	0.44(1)	0.44(2)	0.40(1)
Δ	0.82(1)	0.82(1)	0.81(1)
LLW	0.37(2)	0.38(1)	0.34(2)
GLW	0.33(2)	0.32(2)	0.37(2)

[a]Errors in the last digits are given in parentheses.

Ferrous and Ferric LN Complexes in Frozen Solution. According to the results obtained with the solid Fe−LN complexes, the solution of the Fe^{2+}−LN complex was prepared at pH 4 and that of Fe^{3+}−LN at pH 7. The Mössbauer spectra of the frozen solutions (after removing the precipitate formed during preparation) of Fe^{2+}−LN (Figure 7A) and the calculated Mössbauer parameters (Table 5) are different from those of the solid Fe^{2+}−LN sample prepared at the same pH. In particular, one of the quadrupole doublets (D4, $\delta \approx 1.4$ mm s^{-1}, $\Delta \approx 3.4$ mm s^{-1}) representing the Fe^{2+} components is different from those found in the solid Fe^{2+}−LN complex. These parameters are in good agreement with those calculated for the $[Fe(H_2O)_6]^{2+}$ complex present in $^{57}FeSO_4$ solutions[45] and with the parameters measured for the original $^{57}FeSO_4$ solution used in the preparations (spectrum not shown). This indicates that approximately half of the total Fe^{2+} is not directly coordinated to the LN ligand in aqueous solution and also explains the weak coordination of Fe^{2+} in the solid Fe^{2+}−LN complexes discussed previously. The second Fe^{2+} species can be associated with D1 found also in the case of the solid samples, indicating the partial coordination of LN to Fe^{2+} ions as discussed previously.

The Mössbauer spectrum of the Fe^{3+}−LN complex in aqueous solution at pH 7 shows a well-defined quadrupole doublet (Figure 7B), which indicates that Fe^{3+}−LN complexes have an oligomeric structure and no monomeric Fe^{3+} species can be found in the solution.[33,46] This indicates a significant difference compared to Fe^{3+} chelates formed with EDTA or analogous ligands in the same concentration and pH range,[47,48] where mainly monomeric Fe^{3+} species were found. On the basis of literature data on the dimerization of Fe^{3+} species in aqueous solutions,[33,47] the presence of OH/O-bridged μ-$(OH/O)_xFe_yLN$-type complexes can be assumed, in good agreement with the data on the solid Fe^{3+}−LN samples discussed in the previous section.

Further information on the Fe−LN system can be obtained with the help of the Fe−LN solutions frozen without filtration. In the latter case, both the precipitate and the Fe species in the solution can be monitored. Comparing the Mössbauer spectra of the frozen suspensions (Figure 8) with those of the solutions (Figure 7), one can see that in the case of the Fe^{2+}−LN samples prepared at pH 4 without filtration (Figure 8A), the relative occurrence of Fe^{2+} is significantly lower than in those measured after filtration (Figure 7A). This indicates that the precipitate formed during preparation consists of Fe^{3+}, which according to its Mössbauer parameters (Table 6) may be assigned to polymeric −Fe−OH−Fe− compounds forming iron hydroxides or oxide hydroxides. However, 40% of the total iron remains in the +2 oxidation state, showing the presence of the same Fe^{2+}−LN species (D4, D1) that were already found in the Fe^{2+}−LN filtrates.

In the case of Fe^{3+}−LN samples prepared at pH 7, a broad quadrupole doublet similar to that for the solid Fe^{3+}−LN sample was obtained. This can represent both hydrolyzed polynuclear Fe^{3+} species and $Fe_x(OH)_y(LN)_z$-type oligomeric species present in the suspension. From these measurements

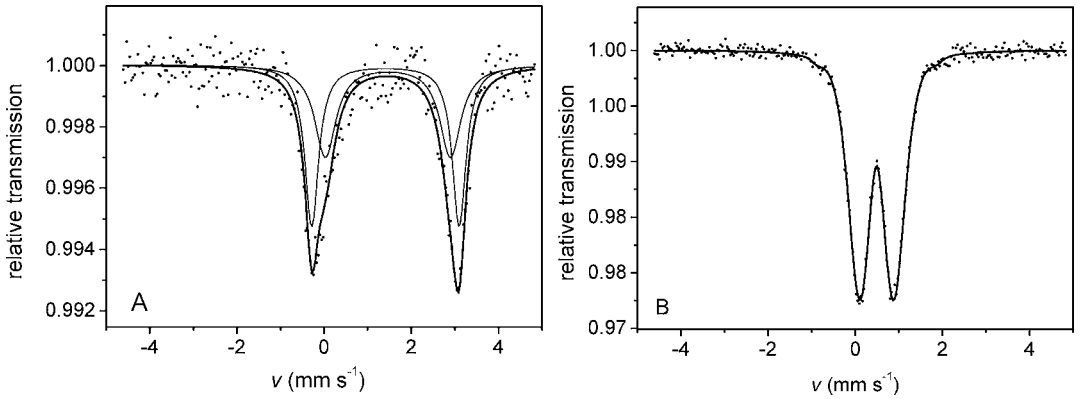

Figure 7. Mössbauer spectra of the filtered frozen solutions of (A) $^{57}Fe^{2+}$–LN at pH 4 and (B) $^{57}Fe^{3+}$–LN at pH 7. The LN:Fe^{3+} ratio was 1.1:1, and the total ^{57}Fe concentration was 0.01 M; measurements were taken at T = 80 K.

Table 5. Mössbauer Parameters (T = 80 K) of the Filtered Frozen Solutions of $^{57}Fe^{2+}$–LN at pH 4 and $^{57}Fe^{3+}$–LN at pH 7, Both at an LN:Fe Ratio of 1.1:1[a]

	Fe^{2+}–LN (pH 4)	Fe^{3+}–LN (pH 7)
Fe^{2+} (D1) S_r	47%	–
δ	1.45(3)	–
Δ	2.9(2)	–
LLW	0.59(5)	–
Fe^{3+} (D3) S_r	–	100%
δ	–	0.49(1)
Δ	–	0.82(1)
LLW	–	0.52(2)
Fe^{2+} (D4) S_r	53%	–
δ	1.41(1)	–
Δ	3.38(5)	–
LLW	0.38(6)	–

[a]Errors in the last digits are given in parentheses; for S_r (%), the relative error is 5%.

Table 6. Mössbauer Parameters (T = 80 K) of the Nonfiltered Frozen Solutions of $^{57}Fe^{2+}$–LN at pH 4 and $^{57}Fe^{3+}$–LN at pH 7, Both at an LN:Fe Ratio of 1.1:1[a]

	Fe^{2+}–LN (pH 4)	Fe^{3+}–LN (pH 7)
Fe^{2+} (D1) S_r	23% (4)	–
δ	1.34(1)	–
Δ	2.97(5)	–
LLW	0.44(3)	–
Fe^{3+} (D3) S_r	57% (4)	100%
δ	0.52(1)	0.48(1)
Δ	0.77(1)	0.80(1)
LLW	0.61(1)	0.36(1)
GLW	–	0.31(1)
Fe^{2+} (D4) S_r	20%	–
δ	1.37(1)	–
Δ	3.35(2)	–
LLW	0.30(3)	–

[a]Errors in the last digits are given in parentheses; for S_r (%), the relative error is 5%.

only, the relative contribution of the two components cannot be separated.

Bioassay. The chloroplast Fe content, total chlorophyll (Chl a + b) content, and maximal photosystem II (PSII) quantum efficiency (F_v/F_m) in leaves of iron-deficient cucumber supplied with Fe^{3+}–LN and Fe^{3+}–o,o-EDDHA for 1 day are shown in Figure 9. Data are relative to those obtained by the +Fe control treatment (100%). In iron-deficient cucumber leaves, the iron content of chloroplasts decreased. Both Chl biosynthesis and the development and function of photosynthetic apparatus depend on Fe availability.[49−52] As a result of iron deficiency, both the total Chl content (Chl a + b) and the maximal quantum efficiency of PSII reaction centers F_v/F_m (the main indicator of the function of the photosynthetic apparatus)[53] showed significant decreases. Both Fe^{3+}–o,o-

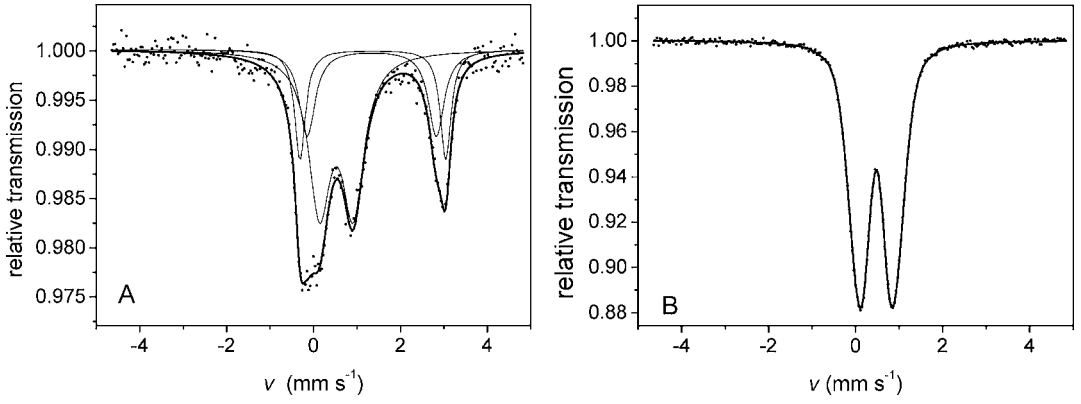

Figure 8. Mössbauer spectra of the nonfiltered frozen solutions of (A) $^{57}Fe^{2+}$–LN at pH 4 and (B) $^{57}Fe^{3+}$–LN at pH 7, both at an LN:Fe ratio of 1.1:1. The total ^{57}Fe concentration was 0.01 M, measurements were taken at T = 80 K.

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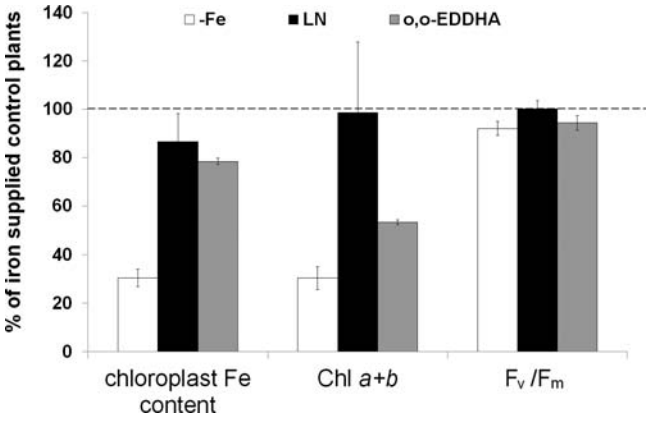

Figure 9. Chloroplast Fe content, total Chl (Chl $a + b$) content, and maximal PSII quantum efficiency (F_v/F_m) in leaves of iron-deficient plants after resupply with 20 μM Fe^{3+}−LN and 20 μM Fe^{3+}-*o*,*o*-EDDHA. Parameters are shown as relative percentage with respect to the iron-supplied plants (+Fe control: 20 μM Fe^{3+}−EDTA), where the chloroplast iron content was 269 ± 15 amol of Fe chloroplast^{-1}, Chl $a + b$ was 13.8 ± 1.1 μg of Chl $a + b$ cm^{-2}, and F_v/F_m was 0.803 ± 0.010.

EDDHA and Fe−LN supply recovered the parameters. However, Fe^{3+}−LN proved to be more effective in restoring the chloroplast iron content and the photosynthetic apparatus. In 1 day of recovery treatment, both the total Chl content and F_v/F_m were restored completely (there was no difference between Fe-supplied and Fe−LN-treated plants), whereas *o*,*o*-EDDHA was a less effective chelator to improve the physiological parameters.

In conclusion, ferrous and ferric LN complexes were investigated with the help of ^{57}Fe Mössbauer spectroscopy. In the case of Fe^{2+}−LN samples, a high oxidation rate of Fe^{2+} to Fe^{3+} was found, indicating the sensitivity of Fe^{2+} to oxidation in air both in the solution and in the solid form. In addition, the relatively oxidized composition of the LN may enhance this process. The aging of the solid Fe^{2+}−LN is also noteworthy, as after 12 months the relative occurrence of the Fe^{3+} component was twice as much as immediately after preparation. This redox transformation has to be taken into account in the case of applying Fe^{2+}−LN as an iron fertilizer, since the oxidation state of iron may strongly influence the availability of iron for plants. However, in spite of the oxidation, no well-crystalline magnetically ordered ferric oxide formation could be observed, which may indicate that LN can prevent further crystallization of Fe^{3+} oxides or hydroxides in slightly acidic or neutral media. Thus, it can help to keep Fe^{3+} soluble in the soil solution available to the plants. According to the Mössbauer parameters, two different coordination sites for Fe^{2+} could be identified, and the presence of H_2O ligands in the first coordination sphere could also be suggested.

In agreement with the chemical characteristics and the MCC and Mössbauer data, it was shown that the formation of Fe^{3+}−LN compounds is favored compared with Fe^{2+}. However, we should note that no complex formation occurred between Fe^{3+} and LN at acidic pH, so these compounds are suggested to be prepared only at neutral or at slightly alkaline pH. LN was shown to form oligomeric $Fe_x(OH)_y(LN)_z$-type species with Fe^{3+}, and besides the complex formation, a significant amount of amorphous ferric hydroxides was also formed during preparation.

The Fe^{2+}/Fe^{3+} speciation provided by the Mössbauer data has significant agronomical relevance to the applicability of Fe−LN compounds as iron fertilizers. In previous works it was shown that weak Fe complexes such as Fe^{2+}−lignosulfonate complexes could be efficient in hydroponics while more stable Fe^{3+}−lignosulfonate complexes were suggested to be used in foliar treatment.[54,55] Comparing these results with the data obtained on $Fe^{2+/3+}$−LN compounds indicates that similar applications of $Fe^{2+/3+}$−LN can be recommended. However, in the case of Fe^{3+}−LN complexes, the slightly acidic pH (∼5) applied in foliar treatments to avoid altering the ion exchange properties of the cuticle[55] could lead to Fe^{3+} precipitation, which has to be avoided in foliar sprays. Therefore, Fe^{3+}−LN complexes are suggested to be used more effectively directly to the soil under calcareous soil conditions. In fact, the Fe^{3+}−LN treatment of iron-deficient cucumber plants grown in hydroponics demonstrated the efficiency of the Fe^{3+}−LN compound as a chlorosis corrector. Cucumber plants were able to take up and metabolize the iron from Fe^{3+}−LN faster than the iron from the highly stable synthetic chelate Fe^{3+}−*o*,*o*EDDHA after the resupply treatments. Further work is necessary to elucidate the agronomic applications of Fe−LN complexes.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Mössbauer parameters of the aged 2:1 LN:Fe sample. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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