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Determination of ^{67}Zn Distribution in Navy Bean (*Phaseolus vulgaris* L.) after Foliar Application of ^{67}Zn−Lignosulfonates Using Isotope Pattern Deconvolution

Ana Benedicto,[†,§] Lourdes Hernández-Apaolaza,[†] Inmaculada Rivas,[‡] and Juan J. Lucena*[,†]

[†]Departamento de Química Agrícola and [‡]Servicio Interdepartamental de Investigación, Universidad Autónoma de Madrid, C/Francisco Tomás y Valiente 7, 28049 Madrid, Spain

ABSTRACT: The improvement of Zn fertilizers requires new techniques to evaluate their efficacy. In this paper, the ^{67}Zn stable isotope was used as tracer of several Zn−lignosulfonate complexes to study the foliar-applied Zn uptake and distribution behavior in the plant, compared with ZnEDTA. Navy bean plants (*Phaseolus vulgaris* L.) were grown hydroponically in a Zn-free nutrient solution, and six modified lignosulfonates and EDTA complexed with ^{67}Zn were used in foliar application in the young leaves as Zn sources. Zinc isotopes in roots, stems, and sprayed and unsprayed leaves were determined by ICP-MS, and signal interferences caused by the compounds of the digested vegetal samples were corrected. The mathematical procedure of isotope pattern deconvolution allowed the minimization of the uncertainty in the measured molar fractions of Zn from fertilizer or from natural sources. Significant differences in Zn use and distribution were observed among the fertilizers when the calculated concentrations of Zn from the fertilizer were compared, whereas they were unnoticeable attending to the total Zn in plant tissues, usually determined at the conventional studies. By foliar spray, higher Zn uptake and mobilization to leaves and stems were achieved with ^{67}ZnEDTA than with ^{67}Zn−LS complexes. The ultrafiltered LS and phenolated LS showed slightly better ability to provide Zn to the bean plants than the other LS. The foliar-applied Zn use and distribution in the plant were related with the stability of the Zn−lignosulfonates complexes. Those presenting the lower stability versus pH, but the highest complexing capacity, were slightly more suitable to supply foliar-applied Zn to navy beans.

KEYWORDS: ^{67}Zn, lignosulfonates, fertilizers, foliar application, isotope pattern deconvolution

INTRODUCTION

Zinc deficiency is a widespread micronutrient disorder among different crops[1,2] affecting either the quantity or the nutritional quality of the harvest.[3] This fact becomes especially important because inadequate dietary Zn in a population has a high prevalence in the world (estimated at 31% in global population). Moreover, the risks of morbidity and mortality associated with Zn deficiency are relatively high.[4] Consequently, special efforts have been made in the study of Zn fertilization of crops.

Traditional fertilizer experiments are not able to distinguish between the Zn applied with the fertilizer and the background Zn level in plant tissues. However, the incorporation of an isotopic tracer allows a detailed knowledge of the uptake, transport, and accumulation of this element in plants. High precision and low detection thresholds reached with isotopic tracer techniques could allow finding differences normally not detected with conventional techniques. This fact is highly relevant in the comparison of new fertilizers, for which differences may not be assessed by using the traditional techniques, as the determination of total Zn in plant tissues. In the literature, there are several studies that use the radioactive isotope ^{65}Zn to evaluate the efficacy of Zn fertilizers[5−12] and the Zn availability in soils.[13] However, the development of sensitive analytical techniques, such as inductively coupled plasma mass spectrometry (ICP-MS), allows also the use of stable isotopes for this application. The use of stable instead of radioactive isotopes gives a high flexibility in the experimental designs used and can include field studies, because special safety measurements and trained staff are not required. Moreover, long-term assays can be carried out with no radioactivity decay over time. In addition, the generation of radioactive wastes is avoided. For this purpose, the stable isotope ^{68}Zn has been used in several studies to evaluate absorption and translocation of Zn in plants.[14−17] Also, ^{67}Zn, another stable isotope, should be considered. ^{67}Zn natural abundance (4.1%) is around 4 times lower than that of ^{68}Zn (18.75%), so a greater efficacy of the former as tracer could be expected. The stable isotope ^{67}Zn was previously used to track the amount of Zn provided by rainfall or acid fog in a forest;[18] however, as far as we know, this isotope has not been used as a tool to study the Zn nutrition in plants.

Accuracy and precision of stable isotope ratio measurements by ICP-MS in plant tissues require the correction of possible interferences and of the instrumental mass bias. For example, to determine ^{57}Fe in a plant matrix by ICP-MS, Rodríguez-Castrillón et al.[19] carried out the internal mass bias correction by using an isotope pattern deconvolution mathematical procedure as a technique for isolating distinct isotope signatures from mixtures of natural abundance and enriched tracers. It was observed that, for plants with low ^{57}Fe enrichment, isotope pattern deconvolution achieved lower tracer/tracee ratio uncertainties than the traditional methods, which permitted a more accurate assessment of the Fe from different sources.

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Table 1. Instrument Conditions ICP-MS Elan 6000

ICP RF power	1200 W
nebulizer gas flow	0.78 L min^{-1}
sampler/skimmer cone	Ni
lens voltage	9.50 V
analogue stage voltage	−2420 V
pulse stage voltage	1550 V
sweeps/reading	20
readings/replicate	1
replicates	3
scanning mode	peak hopping
dwell time	50 ms per point
rinse time	45 s 48 min^{-1}
sample uptake time	30 s 48 min^{-1}
read delay	30 s 20 min^{-1}

Several Zn−lignosulfonates (Zn−LS) with different physical−chemical characteristics are the fertilizers tested in this study, compared with the synthetic chelate ZnEDTA. Lignosulfonates are byproducts obtained from the pulp and paper industry used as complexing agents in >50% of the commercial Zn formulations available in the Spanish market in 2009.[20] Application of LS in the formulation of Zn fertilizers implies the reuse of the black liquor obtained in these industries, which is potentially a pollutant material. These Zn natural organic complexes are a more ecocompatible and less expensive alternative to the synthetic chelates. However, they are in many cases less effective than chelates because of the weak bonds between the metal and the complexing agent, which determines the low stability of these products.[21] The understanding of the relationship between their physical−chemical characteristics and their efficacy in plants will be decisive in choosing among LS from different types of wood available in the market and the most suitable modification processes required to improve their efficacy as fertilizers.

The aim of this study is to test the suitability of ^{67}Zn as a tool to compare the Zn uptake and distribution behavior in navy beans after the foliar application of six Zn−LS. The isotope was quantified by ICP-MS, the data were processed using a novel isotope pattern deconvolution mathematical procedure, and mass bias correction was performed internally. Interference studies and Zn isotope ratio measurements were also carried out in plant tissue digested samples.

MATERIALS AND METHODS

Zinc Determinations in Plant Tissues by ICP-MS and Isotope Pattern Deconvolution. *Instrumentation.* The instrument used was an ELAN 6000 quadrupole ICP-MS Perkin-Elmer Sciex. The sample introduction system consisted of a Ryton cross-flow nebulizer with a Ryton double-pass Scott spray chamber. Sample transport from the autosampler to the nebulizer was established by a peristaltic pump. Instrumental settings used for the analysis are summarized in Table 1. All standard and sample solutions were prepared by using water according to EN ISO 3696,[22] grade I, free of organic contaminants and HNO_3, Suprapur (Merck). Daily verification of the instrument was performed by using a 10 μg Li, Mg, Rh, Ba, Ce, and Pb L^{-1} mixture in 1% (w/w) HNO_3 solution to check the correct instrument response; the oxides (CeO) and double-charge ions (Ba^{2+}) formation levels were <3%, and the background levels at mass 5 was 220 and 260.

Table 2. Isotopic Abundance (Percent) of Natural Zn and the ^{67}Zn-Enriched Zn

	^{64}Zn	^{66}Zn	^{67}Zn	^{68}Zn	^{70}Zn
natural Zn (IUPAC)	48.63	27.90	4.10	18.75	0.62
^{67}Zn-enriched Zn	1.56	3.88	89.60	4.91	0.05

Quantitative analysis of isotopes 64, 66, 67, and 68 of Zn that will be used to determine the total Zn concentration in the samples was performed using external calibration and internal standardization. Standard solutions of 0.5, 1, 5, 10, 50, 100, 500, and 1000 μg Zn L^{-1} were prepared from Zn monoelemental standard solutions CertiPur (Merck), and linear calibration was established for each isotope measured. Solutions of 3, 30, and 300 μg Zn L^{-1} were used to check percentage recovery immediately after the calibration and during the sample analyses. ^{72}Ge CertiPur (Merck) was added as internal standard to the blanks, calibration standards, and samples in a concentration of 30 μg L^{-1} to control plasma fluctuations and to correct for ion signal instability.

The Zn isotope ratio measurements in the digested plant samples were obtained from 64, 66, 67, and 68 isotope signal intensities monitored in the quantitative analysis previously corrected as was determined in the interferences study below.

Interferences Study. Taking into account common content of elements in plants and abundances of their proper isotopes, more serious interferences in the quantitative determination of Zn isotopes could be expected in the case of Ti (up to 1 μg g^{-1} in plant material), Ca (up to 20 000 μg g^{-1}), Ba (up to 100 μg g^{-1}), S (up to 5000 μg g^{-1}), and Ni (up to 5 μg g^{-1}).[23] Therefore, concentration ranges of these elements and Zn in the samples were estimated by semiquantitative analysis. Calibration was performed using a mixed solution of ICP multielement standard solution VI CertiPur (Merck) and solution I (Accustandard) at a concentration of 30 μg L^{-1}. ^{103}Rh CertiPur (Merck) was added as internal standard to samples, calibration standards, and blanks to the semiquantitative analysis at a concentration of 30 μg L^{-1}. On the basis of these results, two different concentrated Zn solutions were prepared, to which different amounts of Ti, Ca, Ba, S, and Ni CertiPur (Merck) were added. The quantitative analysis of the mixed solutions as described below was carried out to evaluate possible interferences.

Mathematical Procedures. Molar Fraction from Fertilizer or Natural Source Calculation. As a consequence of the application of ^{67}Zn-enriched treatments, Zn in plants tissues (N_{plant}) after their development can be distributed in two different sources with different original isotopic distributions. Therefore, the total amount of Zn in a given plant sample (N_{plant}) can be expressed in terms of the Zn from the fertilizer (N_{fer}) and the Zn from seeds and from the nutrient solution (N_{nat}) (eq 1). Isotopic distribution of the former is assumed to be the same as that of the ^{67}Zn-enriched Zn treatment applied, and the isotopic distribution of the latter is assumed to be the natural isotopic distribution of Zn (Table 2).

$$N_{plant} = N_{fer} + N_{nat} \quad (1)$$

The mass balance for each Zn isotope can be expressed as shown by eq 2, illustrated for isotope 67 as an example:

$$N_{plant} \times {}^{67}A_{plant} = N_{fer} \times {}^{67}A_{fer} + N_{nat} \times {}^{67}A_{nat} \quad (2)$$

In eq 2, $^{67}A_{plant}$ is the isotope abundance of ^{67}Zn in the vegetal sample, $^{67}A_{fer}$ is the corresponding isotope abundance in the tracer, and $^{67}A_{nat}$ is the natural isotope abundance. When eq 2 is divided by eq 1, the expression

$$^{67}A_{plant} = x_{fer} \times {}^{67}A_{fer} + x_{nat} \times {}^{67}A_{nat} \quad (3)$$

is obtained, where x_{fer} and x_{nat} denote the molar fractions of Zn in the isotopically altered sample arising from the two different sources of the element (fertilizer or natural).

In the conventional procedure, the molar fractions x_{fer} and x_{nat} would be calculated using the single isotope ratio $^{67}A_{plant}/^{64}A_{plant}$. If more isotope measures are available, they can be calculated by isotope pattern deconvolution. Equations similar to eq 3 can be obtained for all of the isotopes and expressed in matrix notation as

$$\begin{bmatrix} {}^{64}A_{plant} \\ {}^{66}A_{plant} \\ {}^{67}A_{plant} \\ {}^{68}A_{plant} \end{bmatrix} = \begin{bmatrix} {}^{64}A_{fer} & {}^{64}A_{nat} \\ {}^{66}A_{fer} & {}^{66}A_{nat} \\ {}^{67}A_{fer} & {}^{67}A_{nat} \\ {}^{68}A_{fer} & {}^{68}A_{nat} \end{bmatrix} \times \begin{bmatrix} x_{fer} \\ x_{nat} \end{bmatrix} + \begin{bmatrix} {}^{64}e \\ {}^{66}e \\ {}^{67}e \\ {}^{68}e \end{bmatrix} \quad (4)$$

As there are more parameters (isotope abundances) than unknowns (molar fractions), an error vector is included in eq 4. The best values of x_{nat} and x_{enr} are found by least-squares fitting of the error vector e (minimizing the square sum of errors). If the vector of the isotope abundances in the sample is named y, the matrix of the isotope abundances of the pure components A, and the vector of the unknown molar fractions x, the least-squares solution of this overdetermined system of equations can be calculated as[24,25]

$$x = (A' \times A)^{-1} \times (A' \times y) \quad (5)$$

where A' indicates the transpose of A and superscript -1 the inverse.

Internal Mass Bias Correction Using Isotope Pattern Deconvolution. As shown in eq 4 there are more parameters than unknowns in the calculations, so the standard uncertainties for those parameters can also be determined using the variance−covariance matrix, $V(x)$. The diagonal elements of this matrix are the variances of the variables x_{nat} and x_{enr}. From the multivariate linear regression this matrix can be calculated as

$$V(x) = (A' \times A)^{-1} \times S_e^2 \quad (6)$$

where S^2_e is the variance of the regression model (the square sum of errors divided by the degrees of freedom). It can be demonstrated that the variance of the regression model is a function of the mass bias factor applied for correction and is minimal when the "right" mass bias factor is used.

The same procedure used by Rodríguez-Castrillón et al.[19] to correct the internal mass bias was followed by applying the exponential model

$$R_{cor} = \frac{R_{exp}}{e^{K \times \Delta M}} \quad (7)$$

where R_{cor} is the corrected isotope ratio, R_{exp} is the measured isotope ratio, and ΔM is the mass difference between the isotopes considered. An arbitrary initial value of the mass bias factor K is given. Then, corrected isotope abundances in the samples are calculated using the following equation:

$$A^i_{plant} = \frac{R^i_{cor}}{\sum_{i=1}^{n} R^i_{cor}} \quad (8)$$

When these corrected abundances are introduced in the isotope pattern deconvolution procedure, the internal mass bias correction factor, K, is calculated by minimizing the variance of the regression, s^2_e. For iteration, the SOLVER application in Excel was used (change K until s^2_e is minimum).

Total Zn Concentration Determination. Total Zn concentration was calculated by the sum of the Zn isotope concentrations. The ^{70}Zn concentration was estimated on the basis of the molar fraction of Zn from fertilizer (x_{fer}) and natural source (x_{nat}) in the sample and on the basis of the fertilizer and natural isotopic distribution (Table 2).

Lignosulfonates (LS). Six LS, kindly provided by Lignotech Ibérica S.A., were tested in this study. They were obtained through sulfite treatment of hardwood (eucalyptus; Euc.LS1) and softwood (SpruceLS) sources. Euc.LS2, Euc.LS3, and Euc.LS4 were obtained from Euc.LS1 by oxidation, sulfonation, and phenolation industrial modifications, respectively, to increase the amount of functional groups of the polymer capable of complexing Zn. Euc.LS5 was obtained through ultrafiltration to reduce the molecular weight of the polymers to facilitate leaf uptake.

Table 3. Chemical Characteristics of the Products[a]

	SpruceLS	Euc.LS1	Euc.LS2	Euc.LS3	Euc.LS4	Euc.LS5
pH	3.6	4.3	6.8	4.7	3.5	2.3
LS content	835	587	559	653	504	390
organic-S	55	51	45	57	45	55
phenolic −OH	19	19	18	18	31	18
−COOH	26	35	77	67	63	58
av M_w	25732	6275	7550	7903	6259	4975

[a] LS content, organic S, phenolic −OH and −COOH are expressed in g kg^{-1} dry weight. The average molecular weight (M_w) is expressed in g mol^{-1}.

SpruceLS, Euc.LS1, Euc.LS2, and Euc.LS3 were previously described elsewhere.[26] Euc.LS4 and Euc.LS5 were characterized in the same way, by the determination of pH, LS content,[27] average molecular weight,[28] organic S,[29] and carboxylic and phenolic group[30] content. Chemical characterization of the products (Table 3) indicates that each modification has changed not only one physical−chemical characteristic of LS but also others, which must be considered in the interpretation of results.

^{67}Zn−Lignosulfonates and Chelates. Labeled treatments (the six ^{67}Zn−LS and ^{67}ZnEDTA) to be applied by foliar spray to navy bean plants were prepared in a Zn concentration of 4 mM by using ^{67}Zn provided by Isoflex with the isotopic distribution presented in Table 2. ^{67}Zn-enriched Zn was dissolved in H_2SO_4 Suprapur (Merck). The ^{67}Zn−LS were prepared by mixing ^{67}Zn-enriched Zn with the suitable amount of LS to complex it, calculated on the basis of their maximum complexing capacity determined below. To ensure that all Zn added is complexed, a 10% extra amount of LS was added. For the preparation of the ^{67}ZnEDTA solution, an amount of ^{67}Zn-enriched Zn, calculated to be 5% in excess of the molar amount of ligand, was slowly added to a solution of Na_2EDTA (as Tritiplex III, Merck). During the chelation process the pH was maintained between 6.0 and 8.0. Finally, the pH of all solutions was adjusted to 5.0 with 0.1 M NaOH to avoid altering the ion exchange properties of the cuticle of the leaves.[31] Then, they were left to stand overnight, filtered through a 0.45 μm Millipore membrane, and made up to volume.

Foliar Application of ^{67}Zn−LS Complexes to Bean Plants. The Zn uptake and distribution in navy bean plants (*P. vulgaris* L. cv. Negra polo), which are highly sensitive to Zn deficiency, were studied after foliar application of the six ^{67}Zn−LS complexes in comparison with ^{67}ZnEDTA. Experiments were carried out in hydroponic culture conditions. Seeds were germinated in the dark at 30 °C on filter paper moistened with distilled water. After germination, seedlings were transferred to a Dycometal-type CCK growth chamber, where they grew until the end of the experiment under controlled climatic conditions: day/night photoperiod, 16/8 h; temperature (day/night) 30/25 °C; relative humidity (day/night) 50/70%. The composition of the Zn-free nutrient solution was the following: (macronutrients in mM) 1.0 $Ca(NO_3)_2$, 0.9 KNO_3, 0.3 $MgSO_4$, 0.1 KH_2PO_4; (cationic micronutrients in μM as buffered micronutrient solution) 2.5 $MnSO_4$, 1.0 $CuSO_4$, 1.0 $NiCl_2$, 1.0 $CoCl_2$, 105.5 Na_2EDTA, 231.0 KOH, 20.0 FeEDDHA; (anionic micronutrients in μM) 35.0 NaCl, 10.0 H_3BO_3, 0.05 Na_2MoO_4. The nutrient solution during all of the experiments was continuously aerated, and the pH was buffered with 1.0×10^{-4} M HEPES and adjusted at 7.5−8.0 with 1.0 M KOH. First, seedlings were placed on containers filled with 1/5 diluted nutrient solution with a

concentration of 2 μM Zn as ZnEDTA. After 5 days, the diluted nutrient solution was replaced by the full-strength Zn-free nutrient solution. Seedlings were kept in this solution for 6 days to induce Zn deficiency. Then the plants were transferred to polyethylene pots (three pairs of plants per pot) containing 2 L of the full-strength Zn-free nutrient solution. $CaCO_3$ (0.1 g L^{-1}) was added to simulate calcareous conditions. At this time, the treatments (^{67}ZnEDTA and the six ^{67}Zn−LS tested) were applied in the two bottom levels of leaves of the plants, on both the adaxial and abaxial leaf surfaces, with a nebulizer system. A dose of 8 μmol of ^{67}Zn per pair of plants was applied as 4 mM ^{67}Zn foliar solutions at pH 5. Surface-active agents were not added because of the surfactant property of the LS. The nutrient solution and the treatments were renewed weekly. Three replicates (three pots) per treatment were used. Two samplings were carried out: 1 and 3 weeks after the first foliar application of treatments, corresponding to 18 and 25 days after germination of seeds, respectively. The sampled roots, stems, and leaves (sprayed and unsprayed) were separated and washed,[32] weighed, and dried at 65 °C for 3 days. Zinc isotope ratios and total Zn concentration in the plant organs after dry mineralization were determined as described above.

Study of the Stability of Zn−LS Complexes. The efficacy of a chelate or a complex to correct Zn deficiency is related to its capacity to maintain Zn in solution, which is function of the chelate/complex stability. The chelate/complex stability depends on the chemical characteristics of the ligand and the environmental conditions such as pH, ionic strength, redox potential, or presence of competing cations.[33] Two experiments were carried out to study the stability of Zn−LS complexes: (I) Zn complexing capacity determination of LS applying AOAC modified method; and (II) stability study of Zn−LS complexes as a function of pH in presence of Ca as competing cation.

(I) Zinc Complexing Capacity. The Zn complexing capacity determination of the six LS products was carried out according to the titration method described by Villén et al.[34] In brief, increasing volumes (from 0.2 to 7 mL) of 100 g $ZnSO_4 \cdot H_2O$ L^{-1} (Merck) were added to 20 mL of sample solution (100 g L^{-1}). The pH was increased to 9.0 with 0.5 M NaOH and again after 30 min. The solution was allowed to stand for 1 day in the dark. Afterward, the pH was readjusted to 9.0, and the samples were diluted to 100 mL. These solutions were centrifuged at 7500 rpm and then filtered through a 0.45 μm Millipore filter. Zinc concentration in the filtrate, considered as complexed Zn, was determined by atomic absorption spectrometry (AAS) (Perkin-Elmer 4000) after the removal of the organic compounds in accordance with method 9.3 in regulation EC 2003/2003.[35] A solution of 0.5% La, 0.2% Cs, and 5% HCl was used as matrix modifier.

(II) Stability of Zn−LS Complexes as a Function of pH in the Presence of Ca as Competing Cation. Nonlabeled Zn−LS solutions (0.76 mM Zn) were prepared using a solution of 100 g $ZnSO_4 \cdot H_2O$ L^{-1} (Merck) according to the same procedure used for ^{67}Zn−LS. An aliquot of 5 mL of the Zn−LS solutions was added to 15 mL of 0.01 M buffer solution (HEPES, MES, CAPS, AMPSO) at different pH values (4.0, 5.0, 6.0, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, and 11.0) and as well as 5 mL of 0.05 M $CaCl_2$ solution. The $CaCl_2$ solution was added to study the effect of Ca as competing cation. Three replicates per each pH value were done. Samples were shaken for 3 days at 25 °C and 56 min^{-1}. Solutions were filtered through 0.45 μm Millipore membranes, and the pH was measured with an Orion Research (Ion Analyzer EA920). Zinc concentration in the filtrate was determined by AAS. As comparison, the stability of $ZnCl_2$ as a function of pH was calculated using the equilibrium speciation model VMINTEQ program[36] in the presence of ε-ZnOH as solid phase and in the same conditions of the experiment.

Statistical Analyses. Data were evaluated by analysis of variance (ANOVA). When the p value was <0.05 in the ANOVA test, means were also compared using Duncan's test at $p < 0.05$ to find significant differences among treatments. Pearson correlation analysis was applied to study the correlation between the Zn complexing capacity and plant parameters. In all cases, the program used for the analysis was SPSS 15.0.

Table 4. ICP-MS Recovery Percentage (% R) of ^{64}Zn, ^{66}Zn, ^{67}Zn, and ^{68}Zn Isotopes in Mixed Standard Solutions of Zn with S, Ca, Ti, and Ba

	% R ^{64}Zn	% R ^{66}Zn	% R ^{67}Zn	% R ^{68}Zn
5 μg Zn L^{-1} + 5 mg S L^{-1}	120.3[a]	110.4	108.6	108.2
30 μg Zn L^{-1} + 25 mg S L^{-1}	121.9[a]	114.8	115.0	113.1
5 μg Zn L^{-1} + 30 mg Ca L^{-1}	112.8	102.2	125.8[a]	104.5
30 μg Zn L^{-1} + 100 mg Ca L^{-1}	104.1	105.4	105.0	103.9
5 μg Zn L^{-1} + 30 μg Ti L^{-1}	109.4	104.5	123.8[a]	103.5
30 μg Zn L^{-1} + 100 μg Ti L^{-1}	107.5	105.1	106.3	105.0
5 μg Zn L^{-1} + 10 μg Ni L^{-1}	110.4	108.5	127.7[a]	110.3
30 μg Zn L^{-1} + 30 μg Ni L^{-1}	110.2	102.4	104.5	102.5
5 μg Zn L^{-1}+ 5 μg Ba L^{-1}	101.5	100.1	119.4	106.2
30 μg Zn L^{-1}+ 30 μg Ba L^{-1}	105.7	106.7	113.2	111.1

[a] % R > 120 considered to interference corrections.

RESULTS AND DISCUSSION

Correction of Interferences in Isotope Ratio Measurement by ICP-MS. The concentration ranges of several elements in the digested samples of the spiked bean plants were determined by ICP-MS semiquantitative analyses. These elements were selected because they could cause more serious interferences[23] as indicated under Materials and Methods. Concentration ranges of 5−30 μg Zn L^{-1}, 20−300 mg Ca L^{-1}, 0−300 μg Ti L^{-1}, 2−100 μg Ni L^{-1}, 0−60 Ba μg L^{-1}, and 5−25 mg S L^{-1} were found. Taking into account these values, different concentrated mixed standard solutions of Zn and these elements were prepared, and Zn isotopes recoveries in the ICP-MS quantitative analyses were determined (Table 4). The ^{67}Zn recoveries were >120% in the mixed solutions of 5 μg Zn L^{-1} with Ni, Ti, and Ba. However, corrections are not considered necessary in these cases because the samples are enriched in ^{67}Zn isotope. The ^{64}Zn recovery is mainly affected for the SO_2 polyatomic interference. The SO_2 intensity signal at mass 64 was monitored in standard solutions of 50, 100, and 200 mg S L^{-1}, and an average correction factor was calculated from the net interference signal/net S signal ratio. Isobaric interference of ^{64}Ni in the ^{64}Zn isotope signal is corrected straightforwardly by the software using the ^{60}Ni isotope. Both correction factors are included in eq 13:

$$^{64}\mathrm{Zn} = \frac{100}{48.63} \times (\mathrm{mass64} - 0.00028 \times \mathrm{mass32} - 0.035297 \times \mathrm{mass60}) \quad (13)$$

Once isotope signal intensities have been corrected, their processing by pattern deconvolution allows the mass bias correction to be performed internally. Therefore, no natural abundance standard needs to be measured, and so the number of necessary ICP measurements is reduced. The uncertainty of the molar fractions x_{fer} and x_{nat} can be minimized because several

Table 5. Dry Weight (DW) and Total Zn Concentration in Roots, Stems, and Leaves of Bean Plants after 1 and 3 Weeks of Treatment[a]

	DW (g), 1 week			DW (g), 3 weeks		
treatment	roots	stems	leaves	roots	stems	leaves
SpruceLS	0.71 ns	0.85 ns	1.12 ns	1.88 a	1.48 b	2.21 b
Euc.LS1	0.76	0.78	0.92	1.82 a	1.43 b	2.13 b
Euc.LS2	0.56	0.97	1.19	2.48 a	1.97 a	2.95 a
Euc.LS3	0.70	0.66	1.11	1.86 a	1.42 b	2.15 b
Euc.LS4	0.87	0.81	1.15	2.00 a	1.50 b	2.23 b
Euc.LS5	0.87	0.73	1.05	2.11 a	1.62 b	2.33 ab
^{67}ZnEDTA	0.57	0.78	1.19	1.02 b	1.32 b	1.44 c
	total Zn (mmol kg^{-1}), 1 week			total Zn (mmol kg^{-1}), 3 weeks		
treatment	roots	stems	leaves[b]	roots	stems	leaves[b]
SpruceLS	0.191 ns	0.137 b	0.093 ns	0.162 ns	0.086 c	0.074 b
Euc.LS1	0.222	0.134 b	0.132	0.148	0.095 bc	0.078 b
Euc.LS2	0.318	0.138 b	0.111	0.151	0.118 b	0.108 b
Euc.LS3	0.226	0.119 b	0.126	0.167	0.101 bc	0.079 b
Euc.LS4	0.269	0.120 b	0.116	0.179	0.097 bc	0.111 b
Euc.LS5	0.272	0.132 b	0.170	0.165	0.108 bc	0.101 b
^{67}ZnEDTA	0.281	0.242 a	0.133	0.179	0.173 a	0.318 a

[a] In each data range, different letters denote significant differences among the treatments according to Duncan's test ($p < 0.05$). ns, not significant. [b] Total Zn concentration in leaves is referred to unsprayed leaves.

isotopic lines are used by the pattern deconvolution procedure instead of the two lines used in the classical single isotope ratio with external mass bias correction procedure.

Foliar-Applied Zn Uptake and Distribution in Navy Beans with Six ^{67}Zn−LS and ^{67}ZnEDTA. The nutrition status of the navy beans in the experiment was evaluated prior to the study of the Zn uptake and distribution in the plants with the isotopic techniques. For this purpose, the dry weight and total Zn concentration in the different parts of the bean plants were determined, and the data are presented in Table 5. In the first sampling after 1 week of treatment (and also after 2 weeks, data not shown) the growth of the bean plants has not been affected by the Zn sources. After 3 weeks of treatment, the dry weight of stems and leaves was higher in the plants treated with Euc.LS2, but differences among the rest of the ^{67}Zn−LS were not found. ^{67}ZnEDTA showed the lowest root and leaf growth. Three weeks after the first foliar spray, the Zn concentrations in the stems and unsprayed leaves of plants treated with ^{67}ZnEDTA reached significantly higher values (around 11 and 21 mg kg^{-1}, respectively) than those with ^{67}Zn-LS (in the range of 5−8 mg kg^{-1}), the latter considered to be in the deficiency range. On the other hand, Zn concentrations between 0.38 and 1.22 mmol kg^{-1} (25−80 mg kg^{-1}) were found in sprayed leaves treated with ^{67}Zn−LS against 3.44 mmol Zn kg^{-1} (around 225 mg kg^{-1}) found in sprayed leaves treated with ^{67}ZnEDTA. This value is too high and probably toxic for the plants and could explain the lower weight of those treated three times with ^{67}ZnEDTA.

Some studies have proved that foliar-applied ZnEDTA is suitable for providing adequate Zn nutrition to plants.[9,11,37,38] On the contrary, other authors reported that ZnEDTA did not improve significantly the Zn concentration in the leaves after its foliar application[39] and that the foliar-applied chelated Zn is not as efficient or economical as Zn sulfate.[40] In the case of Zn−LS complexes, their capacity to supply Zn to the plants in hydroponic culture conditions[41] and by foliar spray[9,38,42] has been reported. In this experiment, the dose of applied Zn probably was enough for the treated parts of the plants but not high enough for the total recovery of the untreated parts with the ^{67}Zn−LS. This dose was selected to find significant differences in the Zn uptake and distribution among the tested products.

The ^{67}Zn tracer technique was used to monitor Zn coming directly from the fertilizer. The Zn isotope also allows distinguishing if the differences observed are due to the different efficacies of the fertilizer or to the variability related to Zn contained in the seeds.

Zinc content (μmol/plant) was used instead of Zn concentration to study Zn distribution in the plant. The total Zn content (μmol/plant) was determined in roots, stems, and unsprayed leaves of bean plants treated with the six ^{67}Zn−LS and ^{67}ZnEDTA considering the dry weight of each plant organ. Zinc content from the fertilizer (Zn_{fer} content, μmol/plant) was obtained from the total Zn and the molar fraction of Zn from the fertilizer (x_{fer}) calculated with the mathematical procedure of internal mass bias correction using isotope pattern deconvolution. Data are presented in Figure 1. Statistical differences were not found among the treatments in the total Zn content 1 week after the first foliar application of products, whereas Zn_{fer} content showed statistical differences in stems. In this case the use of ^{67}Zn stable isotope and the isotope pattern deconvolution procedure allowed better distinction among the efficacies of the fertilizers. After 3 weeks of treatment, both total Zn and Zn_{fer} content in stems and leaves showed statistical differences among products. However, it is necessary to consider that the greater total Zn content in plant organs does not necessarily indicate better ability of the fertilizers to supply Zn to the plant. The presence of Zn from natural sources (N_{nat} in eq 1) such as seeds or Zn added to the nutrient solution in the initial development stages of the bean plants should be taken into account. This is especially important

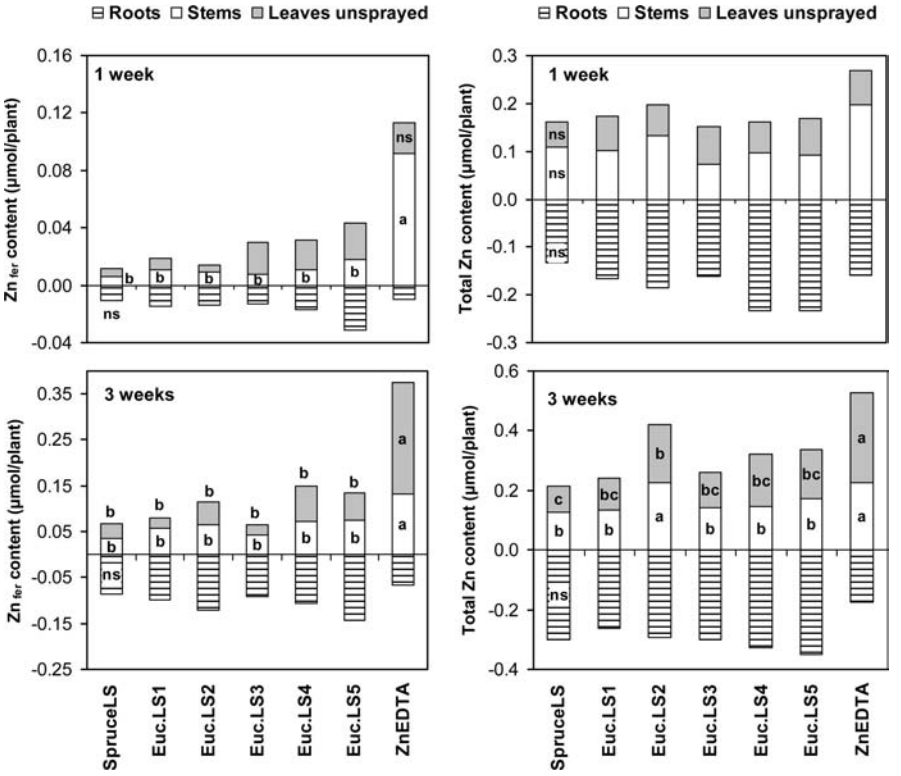

Figure 1. Total Zn (μmol/plant) (right) or Zn from the fertilizer content (Zn_{fer} content, μmol/plant) (left) in roots, stems, and unsprayed leaves after 1 week (top) or 3 weeks (bottom) of treatment. For each series different letters denote significant differences among the treatments according to Duncan's test ($p < 0.05$). ns, not significant. Negative values refer to Zn content in roots.

just after the application of products (first sampling), when the influence of Zn from natural sources is still high.

With respect to Zn_{fer} content (μmol/plant) (Figure 1) after 1 week of experiment, ^{67}ZnEDTA supplied higher Zn amount to the stems than ^{67}Zn−LS. After 3 weeks of experiment, Zn_{fer} content in stems and unsprayed leaves was higher in plants treated with ^{67}ZnEDTA than in plants treated with ^{67}Zn−LS. Statistical differences were not found in the foliar-applied Zn amount that reached the roots. However, a tendency of higher Zn translocation to roots was observed with the ^{67}Zn−LS than with the synthetic chelate.

With regard to the total Zn content (Figure 1), the amount found after 3 weeks of treatment in stems of plants treated with Euc.LS2 is higher than that found in stems of plants treated with the other ^{67}Zn−LS. Total Zn content in unsprayed leaves also was significantly higher with Euc.LS2 than with SpruceLS. However, a better behavior of Euc.LS2 was not observed with regard to Zn_{fer} content. This could be due to a higher Zn content in the seeds or other natural sources of the plants sprayed with Euc.LS2. With respect to only the total Zn content, the ability of this LS to supply foliar-applied Zn to the plant with respect to the other ones could be overestimated. Actually, the Zn amount supplied by the fertilizer (Zn_{fer}) to the plant is similar with Euc. LS2 to the Zn amount provided with the rest of LS or even lower compared with Euc.LS3 and Euc.LS4. In the case of contradictory data of total Zn content and Zn_{fer} content, the latter would reflect better the efficacy of the fertilizer.

Molar fractions of Zn from fertilizer (x_{fer}) in roots, stems, and unsprayed leaves are obtained directly from the isotope pattern deconvolution procedure. Data plotted in Figure 2 show that in general x_{fer} increased from 1 to 3 weeks of experiment. Statistical

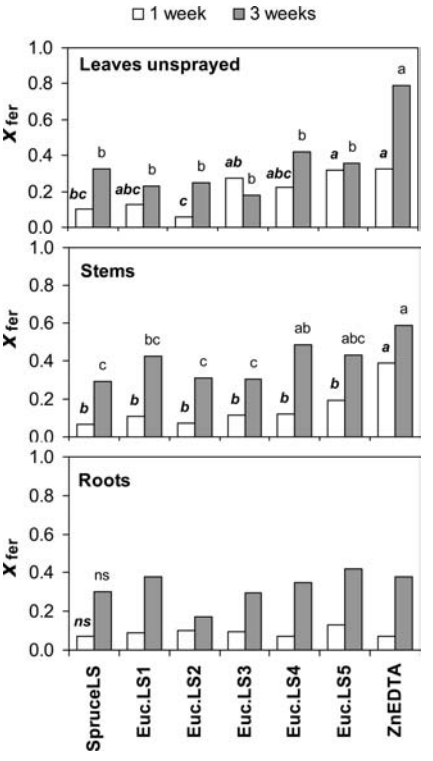

Figure 2. Molar fractions of Zn from fertilizer (x_{fer}) in leaves unsprayed, stems, and roots after 1 week and 3 weeks of treatment. For each series different letters denote significant differences among the treatments according to Duncan's test ($p < 0.05$). ns, not significant.

Table 6. Zinc Found in the Unsprayed Organs with Respect to the Applied Zn ($Zn_{fer \rightarrow plant}$, % mol/mol) at the End of the Experiment[a]

	SpruceLS	Euc.LS1	Euc.LS2	Euc.LS3	Euc.LS4	Euc.LS5	^{67}ZnEDTA
$Zn_{fer \rightarrow plant}$	1.27 c	1.50 bc	1.97 bc	1.30 c	2.14 bc	2.31 b	3.69 a

[a] Different letters denote significant differences among the treatments according to Duncan's test ($p < 0.05$).

differences were found only 1 week after treatment in x_{fer} in leaves unsprayed, whereas they were not found in either total Zn or Zn_{fer} content (Figure 1). Plants treated with ^{67}ZnEDTA and Euc.LS5 presented higher x_{fer} values in leaves than those treated with SpruceLS and Euc.LS2. At this time, higher x_{fer} was also found in stems of plants treated with ^{67}ZnEDTA than in stems treated with the six ^{67}Zn−LS. Statistical differences were not found in roots 1 week after foliar treatment.

After 3 weeks, the highest x_{fer} values in unsprayed leaves were achieved with ^{67}ZnEDTA. x_{fer} values in stems after 3 weeks of treatment were higher with ^{67}ZnEDTA, Euc.LS4, and Euc.LS5. A similar tendency was found with regard to Zn_{fer} content (Figure 1) in stems and leaves at this time. Significant differences were not found in x_{fer} in roots among the treatments studied at 3 weeks after the first foliar application.

As the total amount of Zn applied with the fertilizer to the plants in the experiment is known (12 μmol/plant), it is possible to calculate the percentage of Zn found in the unsprayed organs with respect to the applied Zn ($Zn_{fer \rightarrow plant}$) as an index of the Zn fertilizer uptake and redistribution into the plant. Data presented in Table 6 indicate that around 1.2−2.3% of the Zn applied with ^{67}Zn−LS was taken up and distributed by bean plants at 3 weeks after treatment. $Zn_{fer \rightarrow plant}$ obtained with ^{67}ZnEDTA was higher (3.69%). With regard to the six ^{67}Zn−LS, the $Zn_{fer \rightarrow plant}$ obtained with each of them followed the tendency Euc.LS5 > Euc.LS4 > Euc.LS2 > Euc.LS1 > Euc.LS3 > SpruceLS.

In general, in the plant experiment, higher Zn uptake and mobilization in the bean plants were achieved with foliar application of ^{67}ZnEDTA than with ^{67}Zn−LS. On the contrary, differences were not found in the effect of the foliar application of ZnEDTA and Zn−LS in other studies.[9,38]

Several authors have concluded that the chelation of metals such Zn reduces the rate of foliar absorption, but increases the translocation of the absorbed nutrient in comparison with Zn inorganic sources.[43−46] Data in this experiment indicate that the entry and mobilization of Zn in the leaves and the stems are higher with the application of ^{67}ZnEDTA than with the ^{67}Zn−LS. However, the ^{67}Zn form which enters into the leaves (^{67}Zn as free ion or as ^{67}ZnEDTA) is not elucidated. The entry of the ^{67}ZnEDTA chelate into the leaves could explain their better response with respect to ^{67}Zn−LS complexes, the entry of which would be impeded by their high molecular weight. In the case that Zn^{2+} entered as a free ion into the leaves, a higher absorption of the Zn applied with ^{67}Zn−LS would be expected because ^{67}ZnEDTA is more stable than the ^{67}Zn−LS complexes. On the other hand, the tendency of the EDTA to form chelates with Ca, which is present in the cell walls, would favor the ^{67}Zn release from the ^{67}ZnEDTA. Both mechanisms probably occur at the same time. However, further research is necessary to understand Zn foliar absorption behavior.

Parameters studied in this experiment indicate that Zn sprayed with Euc.LS4 and Euc.LS5, modified by phenolation and ultrafiltration, respectively, was better absorbed and mobilized by the bean plants. On the contrary, Zn uptake and distribution were lower with SpruceLS. SpruceLS presents a larger molecular weight than the others (Table 3). This fact suggests again that the steric impediment could be implied in the foliar absorption of LS.

Table 7. Zinc Complexing Capacity (ZnCC) of Tested Products[a]

	SpruceLS	Euc.LS1	Euc.LS2	Euc.LS3	Euc.LS4	Euc.LS5
ZnCC	16.2	20.6	29.1	25.2	37.1	37.5

[a] Data are referred to amount of LS in the product (g Zn 100 g $^{-1}$ of LS).

Stability of Zn−Lignosulfonates Complexes and Its Relationship with Their Response in Plant. *Zinc Complexing Capacity of the LS.* Zinc complexing capacity, as the highest Zn amount that LS is able to maintain in solution at pH 9, is presented in Table 7. Data are referred to LS content (Table 3) in each product. LS from eucalyptus wood presented a higher Zn complexing capacity than LS from spruce wood. That is probably due to the low content of COOH groups presented by SpruceLS polymers (Table 3). On the other hand, the LS content in the product from spruce is larger than in the product from eucalyptus (Table 3), explaining the higher Zn complexing capacity of the complete product obtained from spruce than obtained from eucalyptus as observed by Martín-Ortiz et al.[41]

All of the modifications have improved the Zn complexing capacity of the initial product Euc.LS1. The increase of the Zn complexing capacity of Euc.LS2 (modified by oxidation) with regard to Euc.LS1 is probably due to its higher content of COOH groups. Euc.LS3 presents higher organic-S content (Table 3) and lower Zn complexing capacity than Euc.LS2 (Table 7), which indicates that organic-S content does not improve the Zn complexing capacity of the LS. The improvement of the Zn complexing capacity of Euc.LS4 (modified by phenolation) in comparison with Euc.LS1 can be attributed to the combined effect of both COOH and phenolic −OH groups. Pang et al.[47] studied Ca complexing capacity of LS from pine. After hydroxylmethylation, they found that hydroxyl groups are the main reason for the complexing capacity of the LS. Moreover, Ca complexation properties did not increase after sulfomethylation, which is in good agreement with our findings for Zn. Furthermore, they found that oxidation of the LS decreased its capacity to complex Ca because the hydroxyl groups in the LS molecule turn into carboxyls mostly after oxidation. However, Gonçalves and Benar[48] found that oxidation of the acetosolv lignin from eucalyptus increased the Cu chelating capacity, similar to our observations with Zn complexing capacity of the LS from eucalyptus. Transition metals such as Cu[48] or Zn (in our case) should be better complexed by carboxyls than hydroxyl groups. In addition, phenolic groups are found to be the best ligands for Zn in our work.

Finally, LS polymers of Euc.LS5 (modified by ultrafiltration) are able to complex the largest amount of Zn, indicating that also the reduction of average molecular weight of LS polymers could have a positive influence in their Zn complexing capacity.

Stability of Zn−LS Complexes as a Function of pH in the Presence of Ca. The percentage of soluble Zn remaining in

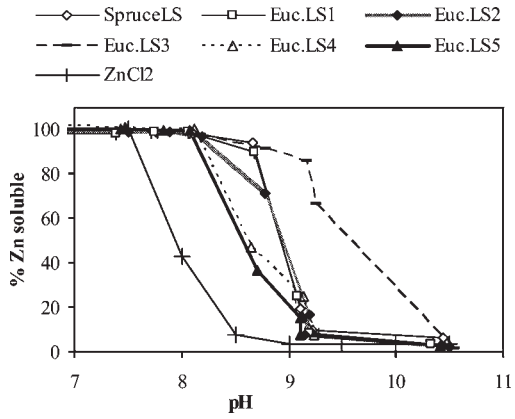

Figure 3. Percentage of soluble Zn that remained in a 0.01 M $CaCl_2$ solution as a function of pH for the tested products. Data represent the means. SEs for different tested products for each pH run in triplicate were lower than 1.80.

Table 8. Correlation of Zn from the Fertilizer Content (Zn_{fer} Content), Molar Fractions of Zn from Fertilizer (x_{fer}), or Zn Found in the Unsprayed Organs with Respect to the Applied Zn ($Zn_{fer \rightarrow plant}$) at the End of the Experiment with the Zn Complexing Capacity of LS (g Zn 100 g $^{-1}$ of LS)

	unsprayed leaves	stems	roots	unsprayed organs
Zn_{fer} content (μmol/plant)	0.61[a]	0.63[a]	0.34	
x_{fer} (mol/mol)	0.36	0.52[b]	0.20	
$Zn_{fer \rightarrow plant}$ (% mol/mol)				0.70[b]

[a] Significant Pearson correlation at level <0.05 (bilateral). [b] Significant Pearson correlation at level <0.01 (bilateral).

solution as a function of pH for each tested Zn−LS is plotted in Figure 3 (data for pH below 7 are not shown, being near 100% in all cases). At pH 9.2 all of the LS except Euc.LS3 allowed Zn precipitation almost completely. Complexes formed with Euc. LS4 and Euc.LS5 presented the lowest stability between pH 8 and 9. Similarly stable complexes formed with SpruceLS, Euc. LS1, and Euc.LS2 were observed.

Stability−Response Relationship in Plant of Zn−LS Complexes. The study of the foliar-applied Zn uptake and distribution in the bean plants using the ^{67}Zn stable isotope in combination with an isotope pattern deconvolution calculation procedure allowed the observation of differences among the six ^{67}Zn−LS. These differences would be undetectable without the use of the tracer, especially after little time of application of the fertilizers. Also, misleading conclusions that could be obtained from the total amount of Zn in plant tissues were avoided by the elimination of the masking effect due to the natural Zn in the bean plants. For this reason, the evaluation of the relationship between the stability of Zn−LS complexes and their ability to supply foliar-applied Zn to the plants was carried out using the plant parameters obtained from isotope pattern deconvolution procedure: Zn from fertilizer content (Zn_{fer} content), molar fractions of Zn from fertilizer (x_{fer}), or Zn found in the unsprayed organs with respect to the applied Zn ($Zn_{fer \rightarrow plant}$) at the end of the experiment.

To relate Zn analyzed in plant tissues with the Zn complexing capacity of the applied ^{67}Zn−LS, correlation analyses were performed. Pearson correlation values are presented in Table 8 for each parameter studied. Results show a significant ($\alpha < 0.05$) and positive correlation of Zn complexing capacity of the corresponding LS with the Zn_{fer} content in unsprayed leaves and stems and with the x_{fer} in stems at the end of the experiment. This was not observed in roots. Also, significant and positive correlation was found between Zn complexing capacity and the percentage of Zn of the fertilizer extracted by the plants after 3 weeks of treatment.

On the other hand, results indicated that a higher ^{67}Zn−LS stability versus pH in the presence of Ca as competing cation does not necessarily imply better ability to provide foliar-applied Zn to the plants. Complexes formed with the LS of the highest Zn complexing capacity, Euc.LS4 and Euc.LS5, showed the lowest stability as a function of pH in the presence of Ca, indicating that part of the Zn can be complexed by less selective complexing sites of Euc.LS4 and Euc.LS5 macromolecules. A higher Zn complexed amount linked more weakly to the LS ligands could favor the foliar uptake and translocation of Zn in the plant.

Several modifications of the LS from eucalyptus have improved the chemical properties and the ability to provide Zn to the bean plants of the corresponding Zn−LS by foliar application. However, the modifications increase the price of the products. The low price of LS because of its byproduct nature is one of its main advantages in relation to synthetic chelates. On the other hand, the use of complexing agents with higher Zn complexing capacity implies the use of less Zn inorganic salts to form the corresponding complex; as a consequence, fertilizer production costs could be reduced. A compromise between the cost and the efficacy of the product should be reached. Phenolation and ultrafiltration in Euc.LS4 and Euc.LS5, respectively, improved Zn complexing capacity and the ability to supply foliar-applied Zn to the bean plants in our experimental conditions, so feasibility studies of both modifications to foliar fertilizer are proposed as the most worthwhile options to be considered.

AUTHOR INFORMATION

Corresponding Author

*E-mail: juanjose.lucena@uam.es. Fax: +34 91 497 38 25.

Present Addresses

[§]Environmental Department, Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT), Av. Complutense 22, 28040 Madrid, Spain.

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