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Effect of Commercial Lignosulfonate-Humate on *Zea mays* L. Metabolism

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ABSTRACT: Lignosulfonate-humate a and lignosulfonate-humate b, derived by an industrial process from lignin, were studied chemically and biologically, and their effects on maize metabolism compared with the responses induced by humic substances obtained from leonardite. Lignosulfonate-humate a and lignosulfonate-humate b elicited hormonelike activity and leonardite displayed giberellin properties. To improve our understanding of their biological action, lignosulfonate-humate a, lignosulfonate-humate b and leonardite were supplied to maize plants and their effect was studied on growth, nitrogen metabolism and photosynthesis. All products increased root and leaf growth. Glutamine-synthetase, glutamate-synthase enzyme activities and protein content were all increased. The treatments also increased chlorophyll content, glucose, fructose and rubisco enzyme activity, suggesting a positive role of lignosulfonate-humate a, lignosulfonate-humate b and leonardite in the photosynthetic process. In addition, an increase in phenol content was observed. In light of these results, being environmentally friendly products, lignosulfonate-humate a and lignosulfonate-humate b could be used to increase crop yield.

KEYWORDS: lignosulfonate-humate, leonardite, biological activity, *Zea mays*, nitrogen metabolism, rubisco enzyme

INTRODUCTION

The extensive use of mineral fertilizers and intensification of cropping systems have affected soil quality by limiting its biological activity and rapidly reducing the organic matter content.[1,2] This has caused an impoverishment of soil nutrient contents and worsened the physical properties. The physical−chemical characteristics of soil are an important prerequisite to endow long-term fertility in a system based on dynamic balance, with a constant exchange of signals between soil and plant.[3]

Traditionally, the most common approach used to improve physical and chemical properties is to incorporate organic residues (compost or agriculture wastes) into the soil to increase its organic matter content.[4,5] However, the application of these organic residues might be a source of harmful microorganisms[6] and weed seeds, and their use could create environmental problems, such as pollution from toxic organic compounds[7] and heavy metals.[8]

In this context, it is necessary to stimulate the search for alternative materials, environmentally friendly compounds, such as protein hydrolysates,[9] seaweed extract,[10] or humic-like substances produced by manufactures from various kinds of raw materials (bark, sawdust, straw, wheat/buckwheat/rice husk, corn ears, etc.) that can differ considerably in their composition and activity.

Lignosulfonates, isolated from spent sulfite pulping liquors of lignin,[11] are widely used in several industrial fields[12] and as fertilizers in agriculture.[13,14] Lignohumate is manufactured in a controlled process of organic substance synthesis. In principle, the process includes the following steps: a primary raw material (potassium lingosulfonan) is homogenized in a specified proportion with potassium hydroxide and exposed to hydrolysis in the process line where, subjected to high pressure and specific temperatures, humic substances are created from a material that does not primarily contain such substances. In fact, what happens is the simulation and acceleration of a process that would take many years in nature. The outcome of the process is a concentrate of humates that have properties of humic acids without being typical humates.

Although researchers have partly elucidated the primary structure of these polymers, few studies have investigated their effects on plant growth and production. Lignosulfonates seem to show similar properties to humic substances (HS) in terms of chelation, buffering and cation exchange capacity due to significant concentrations of carboxylic and phenol groups bonded to the aromatic ring.[15,16] These properties suggest a possible use of lignosulfonates as soil conditioners and plant stimulants.

HS are the most important natural soil conditioners because they improve the physical and chemical properties that are essential for plant growth. In addition, HS display hormone-like activity[17,18] and influence plant metabolism and morphology by interacting with a variety of biochemical mechanisms and physiological processes. They stimulate growth and increase the total amount of nutrients[19] uptaken by plants, influence glycolysis and respiration pathways, and exert a direct effect on the expression of genes encoding H^+-ATPase isoforms and nitrate transport.[20,21]

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Only fragmentary and not very recent investigations are available on photosynthesis. The most prominent effect of HS on plants was an increase in chlorophyll content and in the stimulation of the enzyme activities related to the photosynthetic pathway.[22] A positive effect of HS has also been observed on the main photosynthetic metabolism in maize leaves, where a decrease in starch content was accompanied by an increase in soluble sugars.[23] This change appeared to be mediated by variations in the activity of the main enzymes involved in carbohydrate metabolism.

The present study aimed to elucidate the effects of two lignosulfonate-humates (LHa and LHb) on *Zea mays* plants grown under controlled conditions. In order to verify their action on plant metabolism, glutamine-synthetase (GS) and glutamate-synthase (GOGAT), which are key enzymes involved in N-assimilation, were evaluated. The chlorophyll content and rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase, E.C.4.1.1.39) activity were considered. The results were compared with the physiological responses induced by leonardite humic acid (PH). Structural details were elucidated by using Fourier transform infrared (FT-IR) and nuclear magnetic resonance (NMR) spectroscopies.

MATERIALS AND METHODS

Chemical and Spectroscopic Characterization. Lignosulfonate-humates (LHa and LHb) and leonardite humic acid (PH) were supplied by ILSA (Arzignano, Vicenza, Italy). Their C, H and N contents were determined by using a dry combustion procedure in an element analyzer (vario MACRO CNS, Hanau, Germany). The percentage of ash content was quantified by calcinations of samples (ca. 558 mg) in a porcelain crucible kept for 6 h in a muffle furnace at 900 °C until reaching a constant weight.

IR spectra were recorded using a Nicolet 5700 FT-IR equipped with a diamond attenuated total reflectance (ATR) accessory and a DTGS detector. The total number of scans averaged for each spectrum was 64 with a resolution of 4 cm^{-1}. The region between 1800 and 400 cm^{-1} was observed as being the most sensitive to spectral differences between samples. Spectra analysis was performed with Grams/386 spectral software (Galactic Industries, Salem, NH).

^1H NMR spectra were recorded at 22 ± 1 °C with a Bruker Avance 400 spectrometer (Bruker, Karslruhe, Germany) equipped with a dual $^1H/^{13}C$ probe, operating at 400.13 and 100.61 MHz, respectively. NMR spectra were recorded on samples dissolved in deuterated water (D_2O). The standard implementations of monodimensional (1D) Bruker NMR experiments were used.

Three different types of 1D proton spectra were acquired by using (a) conventional composite pulse sequence[24] with 1.5 s water-presaturation during relaxation delay, 8 kHz spectroscopic width, 32K data points, and 32 scans; (b) water-suppressed spin−echo Carr−Purcell−Meiboom−Gill (CPMG) sequence[25] with 1.5 s water presaturation during a relaxation delay of 1 ms echo time and 360 ms total spin−spin relaxation delay, 8 kHz spectroscopic width, 32K data points, and 256 scans; and (c) sequence for diffusion measurements based on stimulated echo and bipolar-gradient pulses with Δ 200 ms, eddy current delay t_e 5 ms, δ 2 × 2 ms, fine shaped gradient with 32 G/cm followed by a 200 μs delay for gradient recovery, 8 kHz spectroscopic width, 8K data points, and 256 scans.[26] For each lignosulfonate humate the integration area was performed on specific spectral regions, as reported throughout the results. Each integrated area is the average value from three independent calculations; standard error is ≤5%.

Bioassays To Test the Biological Activity of LHa, LHb and PH. The biological activity of LHa, LHb and PH was assessed by checking the growth reduction of watercress (*Lepidium sativum* L.) roots and the increase in the length of lettuce (*Lactuca sativa* L.) shoots.[27]

Watercress and lettuce seeds were surface-sterilized by immersion in 8% hydrogen peroxide for 15 min. After rinsing 5 times with sterile distilled water, 10 seeds were aseptically placed on filter paper contained in a Petri dish. For watercress, the filter paper was wetted with 1.2 mL of 1 mM $CaSO_4$ (control); or 1.2 mL of 0.1, 1, 10, and 20 mg L^{-1} indoleacetic acid (IAA) (Sigma, St. Louis, MO) to obtain the calibration curve; or 1.2 mL of a serial dilution of the products into 1 mM $CaSO_4$. For lettuce, the experimental design was the same as for watercress except that the sterile filter paper was wetted with 1.4 mL of the above solutions and the calibration curve was a progression of 0, 0.01, 0.1, and 10 mg L^{-1} gibberellic acid (GA) (Sigma).

The seeds were germinated in the dark at 25 °C. After 48 h for watercress and 72 h for lettuce, the seedlings were removed and the root or shoot lengths were measured.

Plant Material. Seeds of *Zea mays* L. (var. DKc 5783, DeKalb, Italy) were soaked in distilled water for one night and then surface-sterilized in 5% (v/v) sodium hypochlorite for 10 min while shaking. Seeds were left to germinate on filter paper wetted with 1 mM $CaSO_4$ for 60 h in the dark at 25 °C. Germinated seedlings were transplanted into 3 L beakers containing an aerated Hoagland solution,[28] with a density of 24 plants per beaker $^{-1}$.

The nutrient solution was renewed every 48 h and had the following composition (μM): KH_2PO_4 (40), $Ca(NO_3)_2$ (200), KNO_3 (200), $MgSO_4$ (200), FeNaEDTA (10), H_3BO_3 (4.6), $CuCl_2 \cdot 2H_2O$ (0.036), $MnCl_2 \cdot 4H_2O$ (0.9), $ZnCl_2$ (0.09), $NaMoO \cdot 2H_2O$ (0.01).

Plants were grown in a climate chamber with 14 h of light per day, air temperature between 21 and 27 °C, relative humidity of 70/85%, photon flux density of 280 mol m^{-2} s^{-1}. Twelve days after transplanting, LHa, LHb and PH were added to the nutrient solution contained in the beakers at different concentrations: 0 (control), 0.5 and 1 mg of carbon liter (mg C L^{-1}). The addition of the products to the nutrient solution was performed only once. After 48 h, plants were randomly harvested and then fresh samples of roots and leaves were carefully washed and dried with blotting paper.

A subsample of the plant material was immediately frozen with liquid nitrogen and kept at −80 °C for physiological analyses. For dry weight measurement, 30 plants were used (ten per treatment from each beaker).

For each plant, roots and leaves were weighed separately. The samples were placed in a drying oven for 2 days at 70 °C and allowed to cool for 2 h inside a closed bell jar. The dry weight was measured per plant.

Determination of Chlorophyll Content. For the determination of chlorophyll content, fresh foliar tissue (300 mg) was ground in liquid nitrogen and extracted with 15 mL of ethanol (96% v/v). The samples were kept in the dark for 2 days at 4 °C, and the extracts were filtered and then analyzed spectrophotometrically (UV/vis Lambda 1; PerkinElmer, Norwalk, CT) at λ = 665 nm for chlorophyll a (Chl*a*) and 649 nm for chlorophyll b (Chl*b*). The concentration of Chl*a* and Chl*b* in each sample was calculated using the Welburn and Lichtenthaler[29] formula and expressed in mg of pigment g^{-1} of leaf fresh weight. Two measurements were performed for each plant, on six plants per treatment.

Analysis of Total Nitrogen, Soluble Proteins and Sugars. The nitrogen content was measured using a dry combustion procedure inside an element analyzer (vario MACRO CNS, Hanau, Germany). For the extraction of proteins, foliar and root tissues (100 mg) of five representative plants per beaker were ground in liquid nitrogen, vortexed with 5 mL of extraction buffer (100 mM Tris-HCl pH 7.5, 1 mM Na_2EDTA, 5 mM DTT), and centrifuged at 14000*g*. The supernatants were mixed with 10% (w/v) trichloroacetic acid and centrifuged. The pellets obtained were resuspended in 0.1 N NaOH. The protein concentration was analyzed according to Bradford[30] using a UV/vis spectrophotometer (Lambda 1, Perkin-Elmer, Monza, Italy) at

λ = 595 nm. The soluble protein concentrations are expressed as mg of protein g^{-1} fresh weight.

Foliar tissues (100 mg) of five representative plants per beaker were dried for 48 h at 80 °C, ground in liquid nitrogen and then extracted with 2.5 mL of 0.1 N H_2SO_4. Samples were incubated in a heating block for 40 min at 60 °C and then centrifuged at 6000*g* for 10 min at 4 °C. After filtration (0.2 μm, Membra-Fil Whatman Brand, Whatman, Milan, Italy) the supernatants were analyzed by HPLC (Perkin-Elmer 410). The soluble sugars were separated through a Biorad Aminex 87 C column (300 × 7.8 mm) using H_2O as eluent at a flow rate of 0.6 mL min^{-1}. Sugar concentration is expressed as mg g^{-1} dry weight.

Determination of GS, GOGAT and Rubisco Enzyme Activity. For the extraction of GS, GOGAT and rubisco enzymes, root and leaf tissues (1 g) were ground in a mortar with 10 mL of 100 mM Hepes−NaOH solution at pH 7.5, a 5 mM $MgCl_2$ solution and a 1 mM dithiothreitol solution. The ratio of plant material to mixture solution was 1:3. The extract was filtered through two layers of muslin and centrifuged at 20000*g* for 15 min. The supernatant was used for enzymatic analysis. All steps were performed at 4 °C.

For glutamine synthetase (GS EC 6.3.1.2) assay, the mixture contained 90 mM imidazole-HCl (pH 7.0), 60 mM hydroxylamine (neutralized), 20 mM $KAsO_4$, 3 mM $MnCl_2$, 0.4 mM ADP, 120 mM glutamine and the appropriate amount of enzyme extract. The assay was performed in a final volume of 750 μL. The enzymatic reaction was developed for 15 min at 37 °C. The α-glutamyl hydroxamate was colorimetrically determined by addition of 250 μL of a mixture (1:1:1) of 10% (w/v) $FeCl_3 \cdot 6H_2O$ in 0.2 M HCl, 24% (w/v) trichloroacetic acid and 50% (w/v) HCl. The optical density was recorded at λ = 540 nm. Enzyme activity was expressed in $\mu mol^{-1}\ g^{-1}$ fw, representing the amount of enzyme catalyzing the formation of 1 nmol of c-glutamylhydroxamate min^{-1}.[31]

Glutamate synthase (GOGAT EC 1.4.7.1) assay contained 25 mM Hepes−NaOH (pH 7.5), 2 mM L-glutamine, 1 mM α-ketoglutaric acid, 0.1 mM NADH, 1 mM Na_2EDTA and 100 μL of enzyme extract. GOGAT activity was measured spectrophotometrically by monitoring NADH oxidation at λ = 340 nm.[32] The enzyme activity was expressed in $\mu mol^{-1}\ g^{-1}$ fw, representing the amount of enzyme catalyzing the oxidation of 1 μmol of NADH min^{-1}.

The activity of rubisco (EC 4.1.1.39) was determined spectrophotometrically in a coupled assay by measuring the production of 3-phosphoglycerate after a 5 min period of incubation with 2 mL of 10 mM $MgCl_2$ and 20 mM $NaHCO_3$.[33] Rubisco enzyme activity was expressed in μmol CO_2 mg^{-1} prot.

All reported activities were measured in triplicate on each sample (five subsamples per sample). For the enzyme activities five replicates were performed per treatment and the absorbance in the samples was measured using a JASCO V-530 UV/vis spectrophotometer.

Analysis of Soluble Phenols. Phenolic acids were extracted according to the procedure previously described by Pizzighello et al.[34] The extracted phenols were filtered at 0.45 μm and directly analyzed by using a HPLC 2700 coupled with a 1806 UV/vis (Finningan) detector. The stationary phase was constituted by the column (Supelcosil TM-LC 18) and precolumn (Pelliguard TM-LC 18) of Supelco. The mobile phase (1 L) was constituted by *n*-butanol (Sigma-Aldrich) (18 mL) and acetic acid (Sigma-Aldrich) 50% (1.5 mL). Phenolic compounds were separated at room temperature (loop 20 μL) and with a flux of 1.2 mL min^{-1}. The analyzer was a UV detector at 275 nm. Each run lasted 30 min. Five standards, namely, protocatechuic, caffeic, *p*-coumaric, ferulic and *p*-hydroxybenzoic acid (Sigma-Aldrich), were used for the calibration curves.

Statistical Analysis. A linear regression model ($Y = a + bX$) was applied to describe the dose−response relationship. In the case of IAA and LHa doses a mathematical transformation to $\sqrt{(x)}$ (where x is the original dose value) was needed before regression analysis.

Table 1. Elemental Analysis and Ash Content (Average ± SD) of Lignosulfonate-Humates (LHa and LHb) and Leonardite Humic Acid (PH)

	content, %			
samples	C	H	N	ash
LHa	59 ± 0.7	5.1 ± 0.1	3.0 ± 0.04	5.8 ± 0.01
LHb	57 ± 0.5	5.6 ± 0.5	2.7 ± 0.01	5.0 ± 0.02
PH	50 ± 0.1	3.5 ± 0.2	1.0 ± 0.02	21 ± 0.01

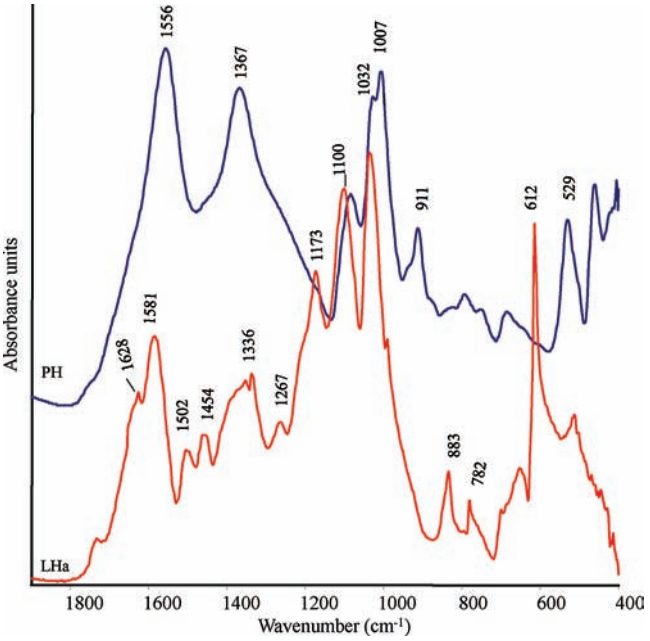

Figure 1. Attenuated total reflectance (ATR) spectra of lignosulfonate-humates LHa (line red) and leonardite humic acid (PH) (line black).

The data represent the means of measurements from three different beakers per treatment. For each measurement the average ± standard deviation (SD) of three plants was used. Analysis of variance (ANOVA) was performed using the SPSS software, and was followed by pairwise post hoc analyses (Student−Newman−Keuls test) to determine which means differed significantly at $P \leq 5\%$.[35]

RESULTS

Characterization of Humates. LHa and LHb showed similar C and N contents (Table 1) as also supported by spectroscopic investigation (see below). The main changes of functional groups for PH, LHa and LHb samples were observed in the region between 1800−400 cm^{-1} (Figure 1). The PH spectrum is characterized by two broad bands at 1556 cm^{-1} and 1367 cm^{-1} assigned to asymmetric and symmetric stretching of carboxylate bound to aromatic rings, respectively. This is in conformity with the NMR spectrum. The bands in the region between 1100 and 600 cm^{-1} are prevalently assigned to mineral compounds.[36] In particular, silicates have intense adsorption in the region 1100−1000 cm^{-1}, although we cannot exclude the presence of a component due to the C−O−C stretching vibration at around 1010 cm^{-1}.[37]

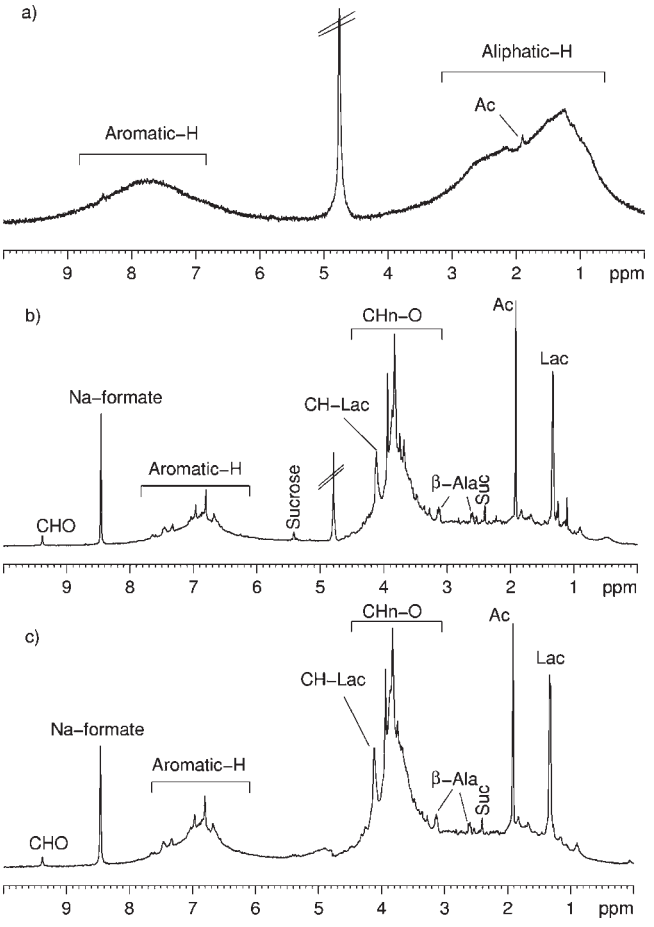

Figure 2. ^{1}H NMR spectra of leonardite humic acid [PH] (a), lignosulfonate-humate [LHa] (b) and lignosulfonate-humate [LHb] (c) are reported. Abbreviations: β-alanine (β-Ala), succinate (Suc), acetate (Ac), lactate (Lac).

The LHa and LHb spectra are totally superimposable, therefore we discuss only one (Figure 1). A weak band at 1742 cm^{-1} is due to C−O stretching motion in ester; the shoulder at 1628 cm^{-1} is assigned to $-COO^-$ group stretch, C5C stretch, aromatic and nonaromatic; the band at 1581 cm^{-1} is due to C5C aromatic stretch; and the bands at 1502 cm^{-1} (aromatic skeletal vibration), 1267 cm^{-1} (guaiacyl ring breathing with carbonyl stretching) and 1032 cm^{-1} (C−H deformation in guaiacyl with C−O deformation in the primary alcohol) suggest the presence of hardwood lignin residues.[38]

However the relative intensity of the band at 1032 cm^{-1}, being greater than that of the 1100 cm^{-1} band (C−H in-plane deformation of the syringyl unit), gives an idea of the presence of softwood lignin traces. The additional band at 1173 (C−O−C stretching skeletal vibration) might suggest the existence of cellulose residues.[39] The band at 1454 cm^{-1} is assigned to C−H deformation in the methoxyl ($-O-CH_3$) group. Since ester carbonyl groups (1742 cm^{-1}) are predominately found in hemicellulose, we have considered this band (1742 cm^{-1}) as indicative of hemicellulose residues and the peak at about 1502 cm^{-1} as a measure of the lignin component.

1D ^{1}H NMR spectra of samples are shown in Figure 2. The PH spectrum (Figure 2a) was characterized by a typical profile of humic materials with high coalification rank.[40]

Table 2. Integration Area of the ^{1}H NMR Spectra in D_2O of Lignosulfonate-Humates (LHa and LHb)

	area, %	
	LHa	LHb
aromatic H	11	10
sugar (CH_n-O)	32	36
sucrose	0.31	nd[a]
acetate	8	9
lactate	3	5

[a] Not detected.

The first region between 0.8 and 2.0 ppm is commonly assigned to aliphatic protons, whereas those in the range 2.0−3.0 ppm can be attributed to aliphatic groups linked to high electronegative atoms (O or N). In particular, the components between 0.9 and 1.3 ppm can also be assigned to protons of methyl groups of highly branched aliphatic structures and terminal methyl groups of methylene chains.[41]

The signals at 1.3 ppm have also been attributed by Wilson et al.[42] to hydrogens that are β or γ attached to benzene rings. The peaks appearing in the 1.8−2.5 ppm interval are due to protons on C atoms adjacent to carbonyl, carboxyl or aromatic rings.[41,42] Finally, the third region between 6.5 and 8.5 ppm can be attributed to the presence of highly substituted aromatic ring hydrogens.[42]

The ^{1}H NMR spectra of LHa and LHb are similar, but they slightly differ by the percentage of protons (Table 2). The region of aliphatic H (0.8 and 2.0 ppm) is characterized by two strong signals, arising from lactate (signal at 1.33 and 4.11 ppm) and acetate (signal at 1.91 ppm).[41,43,44] A slight increase in lactate appeared in LHb while acetate did not show much variation in either spectrum. The region between 2.0 and 3.0 ppm shows weak resonances due to succinate and β alanine.[44] A strong signal with some narrow components appearing in the 3.0−4.0 ppm region is attributed to sugar-like components (CH_3-O, $-CH_2-O$, $-CH-O$). This region differed in both spectra and was significantly higher in LHb. A weak resonance at 5.42 ppm, assigned to protons in sucrose, was only observed in LHa. The aromatic region (6.6−8.0 ppm), due to aromatic and phenolic H, did not seem to change quantitatively in either sample. Finally the strong resonance at 8.49 ppm arises from formate, and the weak one at 9.38 ppm is due to aldehydic group H.

Audus Test. Audus[27] test results are summarized in Figures 3 and 4. A linear regression model was performed to estimate the dose−response between lignosulfonate-humates, PH and watercress root growth. It was observed that root growth was negatively correlated with both LHa (r = −0.96) and LHb (r = −0.97) doses, revealing an IAA acid-like dose-dependent response. By contrast, no statistically significant dose-dependent response was induced by PH treatment.

The linear regression analysis between treatments (LHa, LHb and PH) and increase in shoot length is shown in Figure 4. Shoot length was positively correlated with different doses of LHa (r = 0.97), LHb (r = 0.94) and PH (r = 0.88), confirming a GA-like dose-dependent response.

Growth of Maize Plants. The effect of LHa, LHb and PH on maize plant growth is reported in Table 3. LHa, LHb and PH stimulated root growth. More specifically, LHa and LHb at the higher concentration increased root weight (+24% and +18%)

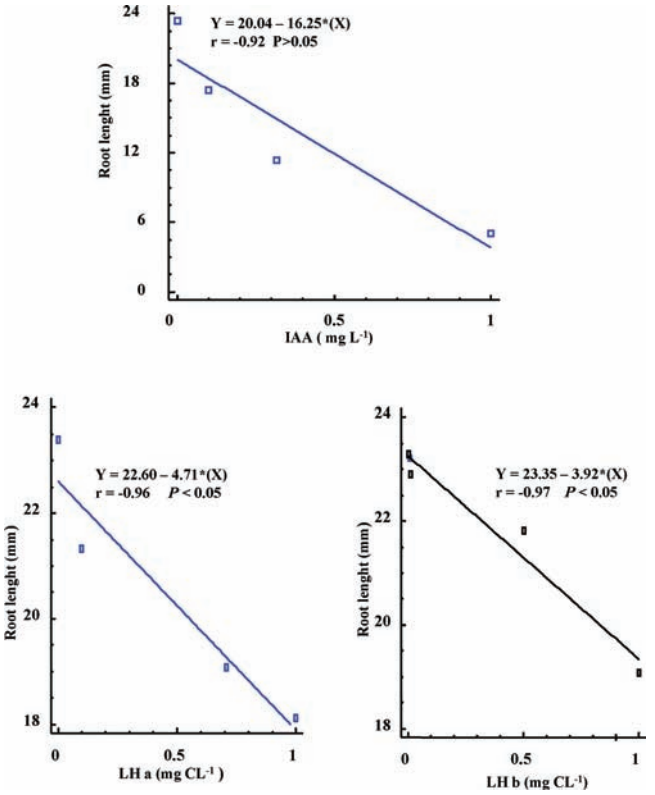

Figure 3. Auxin-like activities of LHa and LHb evaluated via Audus test, i.e. by measuring the increase in root length (mm) of watercress. The linear regression analysis was performed on 20 samples and an average of five replications.

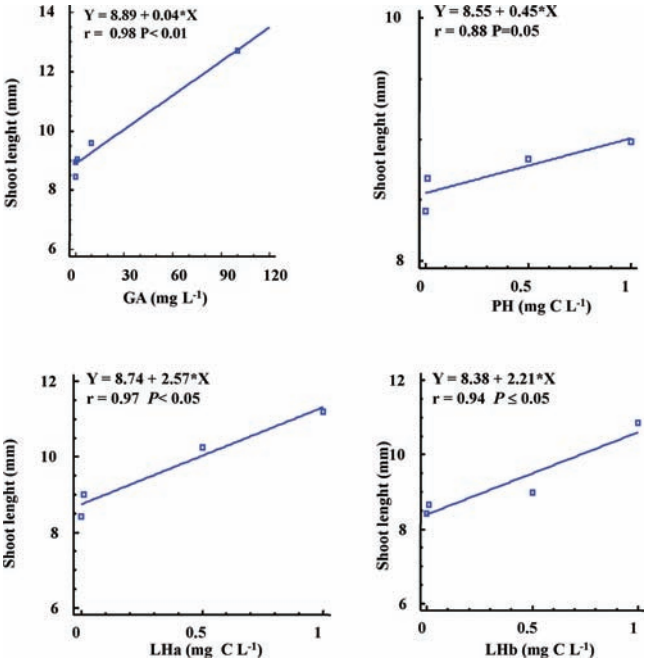

Figure 4. Gibberellin-like activities of LHa and LHb evaluated via Audus test, i.e. by measuring the reduction in shoot length (mm) of lettuce. The linear regression analysis was performed on 20 samples and an average of five replications.

with respect to untreated plants. PH at the lower concentration upgraded root dry weight (+11%), while 1 mg of C L^{-1} did not influence root biomass. LHa and LHb slightly decreased leaf biomass of plants, while PH significantly enhanced leaf dry weight at both test doses (0.5 mg of C L^{-1} and 1 mg of C L^{-1}).

Effects of LHa, LHb and PH on Chlorophyll, Protein, Total Nitrogen and Sugar Content. To establish the stimulatory effect of LHa, LHb and PH on maize plants the chlorophyll and soluble proteins contents (Table 4) were evaluated.

LHa, LHb and PH upgraded the level of Chl*a* and Chl*b*, mainly at 1 mg of C L^{-1} (+17%, +45% and +56%, respectively) compared to untreated plants. At the same concentration Chl*b* was also increased by 70% for LHa, 61% for LHb and 84% for PH. At this dose, protein accumulation was enhanced in the leaves, +21% for LHb and +18% for PH treatments, while LHa did not influence this parameter.

The amount of total nitrogen in roots and leaves of plants treated at both concentrations showed no changes with respect to untreated plants (data not shown).

Among soluble sugars, glucose and fructose increased in roots and leaves of plants treated with LHa, LHb and PH at both tested concentrations (Table 5). In particular, the maximum values of glucose and fructose in roots were recorded in plants grown with LHa at 1 mg of C L^{-1} (+110% and +75%) and in leaves treated with LHb at the same concentration (+231% and +233%) with respect to untreated plants. PH slightly increased the content of detected sugars in roots, while in leaves the increase ranged from 79% to 126% at the lower concentration, and from 104% to 109% at 1 mg of C L^{-1}.

Table 3. Effect of LHa, LHb and PH Treatment on Root and Leaf Dry Weight of *Z. mays* Plants Grown for 12 Days in a Hoagland Modified Nutrient Solution and Treated for 2 Days with LHa, LHb and PH at 0.5 or 1 mg of C L^{-1}[a]

	roots		leaves	
treatment	g	%	g	%
control	2.02 ± 0.06 c	100	4.77 ± 0.10 b	100
LHa 0.5	2.49 ± 0.07 a	123	5.03 ± 0.18 ab	105
LHa 1	2.50 ± 0.12 a	124	4.29 ± 0.14 c	90
LHb 0.5	2.37 ± 0.09 b	117	4.31 ± 0.04 c	90
LHb 1	2.38 ± 0.07 b	118	4.74 ± 0.14 b	99
PH 0.5	2.25 ± 0.06 b	111	5.39 ± 0.12 a	113
PH 1	2.04 ± 0.05 c	101	5.28 ± 0.15 a	111

[a] Percentage values refer to the root and leaf growth of plants treated with LHa or LHb or PH compared to the control (=100%). Data represent the means of three measurements with ten plants in each (± SD). Values in the same column following the same letter are not statistically different at $P < 0.05$ according to Student–Newman–Keuls test.

Effects of LHa, LHb and PH on GS, GOGAT and Rubisco Enzyme Activity. The effects of LHa, LHb and PH on maize plants were investigated by measuring the activities of enzymes that catalyze key steps in nitrogen organication (GS and GOGAT) and in the Calvin–Benson cycle (rubisco). At the concentration of 1 mg of C L^{-1}, the activity of GS was enhanced compared to the controls by 84% and 65% in roots of plants treated with LHb and PH respectively, and by 7% and 93% in leaves (Table 6), while LHa increased GS activity by 30% at 0.5 mg of C L^{-1} and about 20% at 1 mg of C L^{-1}.

The tested products strongly enhanced GOGAT activity in roots with the sole exception of LHa at 1 mg of C L^{-1}. In particular, PH at 0.5 mg of C L^{-1} upgraded the GOGAT enzyme activity in the roots (+176%) and leaves (+204%) of maize plants in comparison with the controls.

Rubisco enzyme activity was stimulated in plants treated with LHa and PH at 0.5 mg of C L^{-1} (+24% and +40%, respectively); on the contrary, this concentration did not influence rubisco activity.

Table 4. Effect of LHa, LHb and PH Treatment on Chlorophyll and Protein Content of *Z. mays* Plants Grown for 12 Days in a Nutrient Solution and Treated for 2 Days with LHa, LHb and PH at 0.5 or 1.0 mg of C L^{-1}[a]

	mg g^{-1} fw			
			proteins	
treatment	Chl*a*	Chl*b*	roots	leaves
control	5.13 ± 0.009 c	1.11 ± 0.007	1.05 ± 0.01 c	2.90 ± 0.01 c
LHa 0.5	6.51 ± 0.05 b	2.00 ± 0.08 a	1.08 ± 0.02 c	2.90 ± 0.01 c
LHa 1	6.05 ± 0.19 b	1.89 ± 0.08 a	1.12 ± 0.002 b	2.90 ± 0.03 c
LHb 0.5	5.65 ± 0.07 bc	1.64 ± 0.03 b	1.06 ± 0.01 c	3.61 ± 0.03 a
LHb 1	7.45 ± 0.06 ab	1.79 ± 0.02 b	1.09 ± 0.01 c	3.53 ± 0.04 b
PH 0.5	6.37 ± 0.02 b	1.73 ± 0.01 b	1.04 ± 0.01 c	3.24 ± 0.09 bc
PH 1	8.01 ± 0.11 a	2.05 ± 0.09 a	1.28 ± 0.02 a	3.44 ± 0.06 b

[a] For chlorophylls, two measurements were performed for each plant on six plants per treatment. For protein determination, data represent the means of 3 measurements with three plants in each (± SD). Values in the same column following the same letter are not statistically different at $P < 0.05$ according to Student−Newman−Keuls test.

Phenol Content. Differential accumulation of phenol compounds was found in plants in response to LHa, LHb and PH treatments (Table 7). Two derivatives of benzoic acid were detected (protocatechuic, *p*-hydroxybenzoic acid) and three of cinnamic acid (caffeic, *p*-coumaric and ferulic). The content of protocatechuic and *p*-hydroxybenzoic acid was higher in roots of plants treated with LHa, LHb and PH than the controls. Ferulic acid was not detected, while caffeic acid was present in controls and in plants treated with LHa at 1 mg of C L^{-1} and LHb at 0.5 mg of C L^{-1}.

The amount of protocatechuic acid in leaves was increased by LHa, LHb and PH treatments, although it was not present in plants treated with LHb at 1 mg of C L^{-1}. A significant accumulation of caffeic acid was observed in leaves of plants grown with all tested products at all test doses. The amount of ferulic acid showed a remarkable decrease with respect to the controls, except for LHb treatment at 1 mg of C L^{-1}, while *p*-hydroxybenzoic acid was not present.

Table 5. Effect of LHa, LHb and PH Treatment on Leaf and Root Sugar Contents of *Z. mays* Plants Grown for 12 Days in a Nutrient Solution and Treated for 2 Days with LHa, LHb and PH at 0.5 or 1.0 mg of C L^{-1}[a]

	roots				leaves			
	glucose		fructose		glucose		fructose	
treatment	mg g^{-1} dw	%	mg g^{-1} dw	%	mg g^{-1} dw	%	mg g^{-1} dw	%
control	34.56 ± 1.11 d	100	19.18 ± 1.12 d	100	23.12 ± 1.03 e	100	6.12 ± 0.02 e	100
LHa 0.5	70.76 ± 1.22 a	205	25.01 ± 1.04 b	130	46.24 ± 0.12 d	200	12.52 ± 0.02 d	205
LHa 1	72.71 ± 0.98 a	210	33.58 ± 0.13 a	175	54.65 ± 0.11 c	236	17.94 ± 0.04 b	293
LHb 0.5	39.55 ± 0.77 c	114	21.92 ± 0.13 c	114	69.10 ± 0.14 b	229	18.71 ± 0.07 b	306
LHb 1	47.79 ± 0.16 b	138	22.71 ± 1.13 c	118	76.56 ± 0.22 a	331	20.41 ± 0.03 a	333
PH 0.5	39.05 ± 0.21 c	112	19.89 ± 1.15 d	104	41.45 ± 1.12 d	179	16.30 ± 0.12 c	226
PH 1	39.55 ± 1.10 c	114	21.52 ± 1.22 c	112	47.15 ± 2.20 d	204	12.82 ± 0.22 d	209

[a] Data are the means of three measurements with three plants in each (± SD). Values in the same column following the same letter are not statistically different at $P < 0.05$ according to Student−Newman−Keuls test.

Table 6. Effect of LHa, LHb and PH Treatment on GS, GOGAT and Rubisco Activity in Root and Leaf of *Z. mays* Grown for 12 Days in a Nutrient Solution and Treated for 2 Days with Either LHa or LHb or PH at 0.1 or 1.0 mg of C L^{-1}[a]

treatment	GS[b]		GOGAT[c]		rubisco[d]
	roots	leaves	roots	leaves	leaves
control	2.07 ± 0.06 c	3.68 ± 0.17 c	8.28 ± 0.15 d	11.62 ± 0.56 d	0.99 ± 0.006 c
LHa 0.5	4.23 ± 0.13 a	4.79 ± 0.58 b	18.6 ± 0.08 b	9.56 ± 0.15 e	1.23 ± 0.04 bc
LHa 1	0.46 ± 0.03 d	4.44 ± 0.32 bc	6.95 ± 0.07 d	20.73 ± 1.37 b	0.82 ± 0.001 c
LHb 0.5	1.57 ± 0.07 c	3.70 ± 0.18 c	12.48 ± 0.10 c	28.10 ± 0.11 a	0.93 ± 0.005 c
LHb 1	3.81 ± 0.29 b	3.95 ± 0.47 c	18.45 ± 0.21 b	8.32 ± 0.17 b	1.29 ± 0.01 b
PH 0.5	3.27 ± 0.54 b	5.68 ± 0.43 b	22.91 ± 1.01 a	23.72 ± 1.59 b	1.39 ± 0.01 b
PH 1	3.43 ± 0.30 b	7.13 ± 0.15 a	17.28 ± 0.15 b	17.74 ± 0.01 c	1.70 ± 0.01 a

[a] GS enzyme activity was expressed in μmol^{-1} glutammic acid g^{-1} fresh weight; GOGAT enzyme activity was expressed in μmol^{-1} Nicotinammide adenin dinucleotide (NADH) g^{-1} fw; Rubisco enzyme activity was expressed in μmol CO_2 mg^{-1} protein. Data are the means of three measurements with three plants in each (±SD). Values in the same column following the same letter are not statistically different at $P < 0.05$ according to Student−Newman−Keuls test. [b] μmol^{-1} ac glut g^{-1} fw. [c] μmol^{-1} NADH g^{-1} fw. [d] μmol^{-1} prot g^{-1} fw.

Table 7. Content of Different Phenolic Compounds in *Z. mays* Leaf and Root of Plants Grown for 12 Days in a Nutrient Solution and Treated for 2 Days with LHa, LHb and PH at 0.5 or 1 mg of C L^{-1}[a]

	content ($\mu g\ g^{-1}$ dw)						
	control	LHa 0.5	LHa 1.0	LHb 0.5	LHb 1.0	PH 0.5	PH 1.0
				Roots			
protocatechuic acid	0.10 ± 0.02 c	141.6 ± 1.11 a	46.1 ± 1.65 b	62.7 ± 1.30 b	94.0 ± 1.42 a	112.9 ± 3.28 b	86.0 ± 2.98 b
caffeic acid	3.21 ± 0.23 b	nd[b]	20.5 ± 2.13 c	15.9 ± 1.22 d	nd	nd	nd
p-coumaric acid	3.23 ± 0.13 b	16.3 ± 1.23 c	144.5 ± 2.75 a	32.7 ± 1.10 c	20.2 ± 3.10 c	333.2 ± 2.75 a	214.5 ± 3.60 a
ferulic acid	nd	nd	nd	nd	nd	nd	nd
p-hydroxybenzoic acid	15.22 ± 1.02 a	47.4 ± 2.15 b	39.9 ± 1.12 b	104.9 ± 2.17 a	55.1 ± 1.01 b	44.2 ± 2.12 c	41.8 ± 2.10 c
				Leaves			
protocatechuic acid	13.8 ± 1.73 c	116.4 ± 2.55 a	70.5 ± 1.11 a	124.2 ± 2.03 a	nd	112.9 ± 2.32 a	40.1 ± 1.18 a
caffeic acid	0.11 ± 1.23 d	47.4 ± 0.12 b	44.4 ± 0.73 b	42.2 ± 1.12 b	43.1 ± 0.78 b	46.0 ± 0.13 b	37.8 ± 0.95 a
p-coumaric acid	56.01 ± 4.03 b	16.3 ± 1.13 c	nd	32.7 ± 0.13 c	nd	12.87 ± 0.13 c	nd
ferulic acid	76.33 ± 5.62 a	17.1 ± 0.11 c	22.6 ± 1.08 c	20.0 ± 0.55 d	94.9 ± 1.12 a	18.6 ± 0.42 c	20.8 ± 1.30 b
p-hydroxybenzoic acid	nd	nd	nd	nd	nd	nd	nd

[a] Data are the means of three measurements with three plants in each (± SD). Values on the same row following the same letter are not statistically different at $P < 0.05$ according to Student−Newman−Keuls test. [b] Not detectable.

DISCUSSION

Both lignosulfonate humates (LHa and LHb) were characterized by similar chemical composition and structural features that can be considered typical for lignin derivatives.[45] As shown by the spectroscopic study, the aromatic rings and the carboxylic and ether groups appear to be the main structure of these materials. In addition, LHa and LHb differed from leonardite humic acid (PH) that instead has a complex macromolecular system mainly composed of polyaromatc rings.

Bioassays are commonly used to test and quantify the biostimulant activity of organic molecules on plants. These assays allowed the physiological responses they induce to be compared with the responses elicited by hormones.[46] Among bioassays, the Audus test is the most reliable in terms of reproducibility and repeatability, and allows determination of whether the studied molecules possess auxin- and/or giberellin-like activity.[47,48]

LHa and LHb were shown to possess biostimulant properties because the Audus test confirmed that they displayed both auxin- and giberellin-like activities. These activities may be in part due to the biological action of phenol compounds, as highlighted by Muscolo et al.,[49] who reported that phenol-C groups present in humic substances were responsible for the diverse biological responses during the early stages of seed germination. Moreover, Pizzeghello et al.[34] described the hormone-like activity of several phenolic acids present in the dissolved organic matter of forest soils; in particular, they showed that protocatechuic acid displayed IAA-like activity, while hydroxybenzoic acid exhibited GA-like activity. In addition, protocatechuic acid, hydroxybenzoic acid and *p*-coumaric acid have been identified as potential allelophatic agents.[50] They influence membrane perturbation, which is followed by a cascade of physiological effects that include improvement of plant−water relationships, stomatal function and rates of photosynthesis and respiration. These phenolic substances also interact with several phytohormones and enzymes, determining a different biosynthesis and flow of carbon into metabolites.[51] In our study the LHa, LHb and PH treatments improved nitrogen assimilation in maize plants through the stimulation of GS and GOGAT enzyme activities. In support of this we observed a decrease in nitrate content (data not shown) and increase in N organic compounds (chlorophyll and proteins) against a steady level of total N percentage.

Induced production of N assimilates by humic substances in maize plants was also shown in a previous work.[52] The stimulation of GS and GOGAT determined by the treatment with LHa, LHb and PH also promoted the growth of root and leaf biomass. All tested products had only a slight effect on the dry weight of leaves. These effects are consistent with those induced by humic substances in which short incubation times increase plant root growth and only long periods determine significant effects on shoots and leaves.[16] These increments in root dry weight may lead to a higher successful transplanting and to an overall plant biomass productivity, and consequently to better yields.[53]

Despite the remarkable amount of biochemical and physiological data on the effects of humic substances on plant growth,[16] the influence of HS on photosynthesis has not previously been investigated. Our results have shown that LHa, LHb and PH were effective in promoting photosynthesis through the increase of chlorophyll content and stimulation of rubisco enzyme activity. In fact, the amount of chlorophylls and rubisco enzyme activity are strictly correlated as they are considered indices of light harvesting capacity of leaves. While increased chlorophyll directly enhances the light-harvesting reactions in photosystems, rubisco activity is regulated by rubisco activase, which in turn is activated by thioredoxin and thus by light reactions. Moreover, sucrose, which is synthesized from carbohydrates produced by the fixation of atmospheric CO_2 via the reductive pentose phosphate (Calvin−Benson) pathway, represents the starting compound for the respiratory pathway, and its increase may justify the improved activity of rubisco and nitrogen assimilation.[54] Since HS stimulate C and N metabolism[55] and these are in turn involved in cross-talk with phenylpropanoid metabolism, we hypothesized that humates might also directly or indirectly influence phenolic level. To support this hypothesis we investigated the presence of phenolic acid in roots and leaves of plants treated with LHa, LHb and PH. Our data have shown that phenol compounds (protocatechuic, caffeic, *p*-coumaric,

ferulic and *p*-hydroxybenzoic acids) found in plants treated with LHa, LHb and PH are in line with those described in a previous study.[56] The patterns of phenolic monomers in the roots were the same as those in the leaves, except that the former did not contain ferulic acids, while the leaves had no *p*-hydroxybenzoic acid, as reported by Senè et al.[57] According to Muscolo and Sidari[58] the greater concentration of phenols recorded in plants after LHa, LHb and PH treatment could be responsible for a weak uncoupling of oxidative phosphorylation, which in turn would increase the metabolic processes requiring glucose.

In conclusion, the present work showed that the application of lignosulfonate-humates, derived by an industrial process, to hydroponically grown maize seedlings led to effects comparable to those produced by the treatment with a proven biological activity compound (humic acid from leonardite). Continued use of N−P−K fertilizers in soils poor in humic substances has caused many serious ecological problems. Emphasizing the importance of humic substances and their value as fertilizer ingredients has never been more urgent than it is today. Indeed, the use of commercial humates can contribute to contrasting the organic matter deficiency in soils and reducing inorganic fertilizer pollution in agriculture.

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