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REGULAR ARTICLE

Biostimulant activity of humic substances extracted from leonardites

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Abstract
Background and aims Biostimulants are natural compounds that enhance plant growth and plant nutrient use efficiency. In this study, biostimulant effects of humic substances (HS) extracted from leonardites were analysed on the metabolism of maize plants grown in hydroponic conditions.
Methods HS extracted from four leonardites were tested for their auxin-like and gibberellin-like activities. Then, 11 day old maize seedlings were treated for 48 h with five concentrations (0, 0.1, 0.5, 1, and 10 mg C L^{-1}) of HS. After sampling, root growth and morphology, glutamine synthetase (GS) activity, glutamate synthase (GOGAT) activity, total protein content, soluble sugars content, phenylalanine ammonia-lyase (PAL) activity, soluble phenols, and free phenolic acids were analysed.
Results HS from leonardites had similar spectroscopic pattern, with small differences. The HS from the South Dakota lignite (HS_USA) had more carboxylic groups, whereas the three from Turkish mines had more aromatic and aliphatic structures. HS_USA best enhanced total root growth, root surface area, and proliferation of secondary roots. Plant nutrient use efficiency was enhanced by HS_4, HS_USA and HS_B, with increment of GS and GOGAT enzymes activity and total protein production. HS stimulated also PAL enzyme activity, followed by a higher production of total soluble phenols, *p*-hydroxybenzoic acid, *p*-coumarilic acid, and chlorogenic acid.
Conclusion This study found that, although the activity of the HS depended on the origin of the leonardite, these compounds can be attributed to the biostimulant products, eliciting plant growth, nitrogen metabolism, and accumulation of phenolic substances.

Keywords Humic substances · Leonardite · Biostimulant · Glutamine synthetase · Glutamate synthase · Phenylalanine ammonia-lyase · FT-IR

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Introduction

The development of sustainable and environmentally friendly agricultural systems represents a major policy challenge in many countries (Povero et al. 2016). In the last two decades, farming expectations have changed and farmers are expected to produce food, whilst

protecting biodiversity, soil, air and water quality (OECD 2013). Although fertilizers are powerful tools for increasing yield and plant health (Hirel et al. 2001), farmers must optimize product application to avoid nutrient pollution and to preserve the economic margin.

One of the most promising solutions to achieve these goals is the use of plant biostimulants. In 2012 Europe has become a leading market for biostimulants, while the biostimulants global market is expected to grow over the next years, reaching $2524.02 million in sales by 2019, with an annual growth rate of 12.5% (Calvo et al. 2014; Povero et al. 2016).

Biostimulants are compounds containing substances and microorganisms able to enhance plant nutrient uptake, nutrient efficiency, tolerance to abiotic/biotic stresses, and crop quality (European Biostimulant Industry Council 2013). Furthermore, when applied to the soil, biostimulants may stimulate rhizosphere microbes and soil enzymes, the photosynthetic process, and the production of hormones or growth regulators in plants (Calvo et al. 2014).

Biostimulants are considered as borderline substances between plant protection products and fertilizers, as they do not give direct protection against pest and do not have nutritional activity (La Torre et al. 2016). For this reason, there is not yet a legal definition of biostimulants (du Jardin 2015). Despite this, international organizations and scientists recognized six main categories: microorganisms, protein hydrolysates, seaweed extracts, chitosan, inorganic compounds and humic substances (HS) (Calvo et al. 2014; du Jardin 2015; European Biostimulant Industry Council 2013).

Among these categories, HS or humates have a positive effect on the uptake of macro and micro nutrients that considerably improve the metabolism, the growth and yields of relevant agricultural crops (Bronick and Lal 2005; Ferreras et al. 2006; Nardi et al. 2009; Puglisi et al. 2009). The positive effects of HS on plant metabolism are well recognized as hormone-like activity (auxin, gibberellin or cytokine-like activity) in terms of changes in root architecture through the lateral roots and root hair production (Canellas et al. 2011; Mora et al. 2012; Pizzeghello et al. 2013; Trevisan et al. 2010b). HS increase root plasma membrane H^+-ATPase activity, enhancing nitrate and other nutrient uptake, contributing to cell wall loosening, cell enlargement and organ growth (Jindo et al. 2012; Zandonadi et al. 2007). Moreover, TCA cycle, phenylpropanoid metabolism, and uptake and metabolism of nitrate have been found to be positively influenced by treatment with HS (Quaggiotti et al. 2004; Vaccaro et al. 2009).

However, HS effects on plant growth cannot be overgeneralized due to their different origin (e.g. from volcanic soil, compost, vermicompost or brown coal), dosage (differs from types culture media) as well as plant species (Nardi et al. 2009; Rose et al. 2014).

Leonardite is an oxidized form of lignite with a medium-brown coal-like appearance. It is found at shallow depth over more compact coal in various coal mines (Stevenson 1979) around the world, mainly in the USA (Fernandez et al. 1996). This brown coal, particularly enriched in humic C (30–80%), is used to manufacture a wide range of commercial HS products.

Akinremi et al. (2000) demonstrated that leonardite increased dry matter yield and nutrient uptake (N, P, K, and S) when applied to canola. In greenhouse conditions, HS from leonardites enhanced the resistance of tomato plants under salinity stress (Casierra-Posada et al. 2009). *Arnica montana* L. treated with HS from leonardite had higher floral stems' number, flower heads' number, and yield compared to control plants (Sugier et al. 2013). A low molecular weight fraction of HS from leonardite enhanced the seedling's root surface area, root length, and total root number of snap bean (Qian et al. 2015). David et al. (2014) demonstrated that potassium humate salts extracted from lignite, and potassium humate regenerated from lignite with two oxidizing agents (nitric acid and hydrogen peroxide), positively influenced root growth and division, starch and protein contents in treated *Zea mays* seedlings.

Leonardite is thus referred to as a benchmark humic material with respect to responses on plant growth. Although the effects of leonardite on crop production, resistance to stress, and soil microbial activity have already been reported, much less attention has been devoted to their impact on plant physiology and biochemistry (Bulgari et al. 2015). Moreover, comparison of leonardite from different sources and the growth effects of these differently sourced materials are scarse in the literature. This study seeks to gain a better understanding of the importance of leonardite origin on their biological activity.

HS extracted from four leonardites were characterized by FT-IR. The effects of these HS on *Zea mays* plants grown under controlled conditions were studied by evaluating: (1) roots growth parameters like total root length, area, diameter, thin roots length, and number of tips and forks; (2) the responses of enzymes involved in

nitrogen and phenylpropanoid metabolism, (3) proteins, sugars, and total phenols content in roots and leaves.

Materials and methods

Leonardites origin and humic substances extraction

Leonardites have been supplied by LandLab srl (Quinto Vicentino, Vicenza, Italy). The leonardite named LE_USA is from South Dakota mines, while the others, LETU_4, LE_A, and LE_B are from Turkish mines.

Humic substances were extracted from the four leonardites (LE_USA; LETU_4; LE_A; LE_B) with 0.1 M KOH (1:10 *w*/*v*) at 130 rpm for 16 h at 50 °C. The extracts were centrifuged at 7000 rpm for 30 min, and filtered on Whatman filter N. 2 paper (Whatman, Boston, USA). Humic extract was desalted by using 14 kDa cut-off dialysis Visking (Medicell, London, UK) tubing with distilled water. Distilled water was changed daily until neutral pH was reached. Subsequently, the extracts were desalted on ion exchange Amberlite IR-120 (H^+ form) (Stevenson 1994). 20 ml of humic extracts were freeze-dried for IR, CNS, and ash content determination and the remaining extracts were kept frozen for use on plant treatments.

Humic carbon content of the extracts in each step of the extraction was determined in triplicate by following a modified version of the method of Walkley and Black (1934). Humic substances (HS) were labelled as HS_USA, HS_4, HS_A, and HS_B.

Chemical and FTIR characterization

The ash content of leonardites and humic substances was determined gravimetrically after dry combustion in a muffle furnace at 550 °C for 6 h. The moisture content was determined at 105 °C. The pH was measured potentiometrically on pulverized samples by adding deionized water (1:2.5 *w*/*v*, dry weight basis). The electrical conductivity (EC) for leonardites only, was potentiometrically determined after water extraction (2:5 *w*/*v*) and filtration through Whatman filter N. 2 paper.

Total C, N, and S contents were measured in triplicate on each sample by using CNS Vario Macro elemental analyzer (Elementar, Hanau, Germany).

IR spectra were recorded with a Bruker ALFA FT-IR Spectrophotometer (Bruker, Ettlingen, Germany) equipped with a single reflection ATR sampling module. The spectra were collected from 4000 to 400 cm^{-1} and averaged over 64 scans (resolution 4 cm^{-1}). The spectral data were processed with Grams/386 spectroscopic software (Galactic Industries, Salem, NH, USA).

Curve-fitting analysis in the region between 1900 and 900 cm^{-1} was used to determine the area under each of the individual bands by using Grams/386 spectroscopic software (version 6.00, Galactic Industries Corporation, Salem, NH).

Bioassay to test the biological activity of HS from leonardites

The biological activity of HS_USA, HS_4, HS_A, and HS_B was assessed by checking the growth reduction of watercress (*Lepidum sativum* L.) roots and the increase of the length of lettuce (*Lactuca sativa* L.) shoots (Audus 1972).

Watercress and lettuce seeds were surface-sterilized by immersion in 8% hydrogen peroxide (Sigma, St. Louis, MO) for 15 min. After rinsing 5 times with sterile distilled water, 20 seeds were aseptically placed on filter paper in a Petri dish. For watercress, the filter paper was wetted with 1.2 mL of H_2O (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 (10 mg C L^{-1}, 1 mg C L^{-1}, 0.1 mg C L^{-1}, 0.001 mg C L^{-1}, and 0.00001 mg C L^{-1}) of the HS. 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 HS (dilutions 10 mg C L^{-1}, 1 mg C L^{-1}, 0.1 mg C L^{-1}, 0.001 mg C L^{-1}, and 0.00001 mg C L^{-1}) and the calibration curve was a serial dilution 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.

A linear regression model (Y = a + bX) was applied to describe the dose-response relationship. In the case of IAA, GA and HS doses a mathematical transformation to log(x) (where x is the original dose value) was needed before regression analysis (Pizzeghello et al. 2013).

Plant material and growth conditions

Plant material was grown as reported in Carletti et al. (2008). Seeds of *Zea mays L.* (var. DKC 5401, DeKalb, Italy) were soaked in distilled water for one night. 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 (Hoagland and Arnon 1950) with a density of 24 plants per beaker.

The nutrient solution was renewed every 48 h and had the following composition: 40 μM KH_2PO_4, 200 μM $Ca(NO_3)_2$, 200 μM KNO_3, 200 μM $MgSO_4$,10 μM FeNaEDTA, 4.68 μM H_3BO_3, 0.036 μM $CuCl_2 \cdot 2H_2O$, 0.9 μM $MnCl_2 \cdot 4H_2O$, 0.086 μM $ZnCl_2$, 0.011 μM $NaMoO \cdot 2H_2O$.

Plants were grown in a climate chamber with 11 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}. Nine days after transplanting, HS_USA, HS_4, HS_A, and HS_B were added to the nutrient solution contained in the beakers at different concentrations: 0 (control), 0.1, 0.5, 1 and 10 mg C L^{-1}. Each concentration was replicated 3 times. The addition of the products to the nutrient solution was performed only once. After 48 h, plants were randomly harvested, fresh samples of roots and leaves were carefully washed and dried with blotting paper, and weighted (data not shown). The treatment period was chosen according to previous experience on studies of HS from various origins and their related biostimulant activity (Ertani et al. 2011; Quaggiotti et al. 2004).

A subsample of the plant material was immediately frozen with liquid nitrogen and kept at −80 °C for physiological analyses. Dry weight measurement was performed in triplicate for each treatment using aliquots of approximately 1 g plant fresh tissue.

For each beaker roots and leaves were weighed. 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 (data not shown).

Root scanning

Root scanning was rapidly performed before the sampling process using an Epson Expression 10000XL 1.0 system (Regent Instruments Company, Canada) as reported in Ding et al. (2014). Three plants for each beaker were randomly picked for root scanning, for a total of 9 plants for each measurement. The following parameters were recorded with a root image analysis system using the image analysis software WinRHIZO Pro (Regent Instruments, QC, Canada): root total length (TRL) (cm), surface area (cm^2), average diameter (mm), number of tips, and length of fine roots (cm) ($0 < L < 0.5$).

Protein extraction and determination

Fresh leaf and root samples were ground to a homogenous powder in liquid nitrogen (N_2). Proteins were extracted with 38 mM KH_2PO_4 and 62 mM K_2HPO_4 buffer at a pH 7. The protein concentration in the extract was determined according to Bradford (1976), using a Jasco V-530 UV/vis spectrophotometer (Jasco Corporation, Tokyo, Japan) at 595 nm. The protein concentration was expressed as mg of protein per g of fresh root or leaf.

Enzyme extraction and essay conditions

To extract the enzymes involved in N reduction and assimilation, fresh leaves and roots were ground to a homogenous powder in liquid N_2. For the extraction of the enzymes two different buffers were used. Each activity essay was done in triplicate.

Glutamine synthetase (GS; EC 6.3.1.2) was extracted by homogenising 0.6 g of ground roots or leaves with 2.4 mL of a 1 mM Tris(hydroxymethyl)aminomethane HCl (Tris-HCl), 25 mM KH_2PO_4, 10 mM L-cysteine hydrochloride monohydrate, 3% (*w*/*v*) bovine serum albumin solution, at 4 °C at pH 7.8 (Baglieri et al. 2014). After 10 min, the extract was filtered through three layers of muslin and centrifuged at 15000 *g* for 25 min at 4 °C. 200 μL of supernatant was incubated with 200 μL of reaction buffer (50 mM Tris-HCl, 20 mM $MgSO_4$, 80 mM L-glutamate, 30 mM NH_2OH, 24 mM ATP; pH 7.8) at 37 °C for 25 min. The reaction was stopped with stopping solution (370 mM $FeCl_2 \cdot 6H_2O$ and 670 mM HCl). Samples were centrifuged at 15000 *g* for 15 min. The amount of γ-glutamyl hydroxamate in the supernatant was photometrically (540 nm) determined against an immediately stopped parallel sample (Jezek et al. 2015). A standard curve was made using γ-glutamyl hydroxamate (GHA) (Sigma). The enzyme activity was expressed as μmol of GHA produced per g of fresh root or leaf per minute.

Glutamate synthase (GOGAT; EC 1.4.7.1) was extracted by homogenizing 0.5 g of ground roots or leaves with 2 mL of a 100 mM Tris-HCl at pH 8.2, 10 mM $MgCl_2 \cdot 6H_2O$, 2 mM β-mercaptoethanol, 10% (*v*/v) glycerol and 1 mM Na_2EDTA solution. After 15 min, the extract was filtered through two layers of muslin and centrifuged at

15000 *g* for 30 min at 4 °C. The supernatant was centrifuged a second time at 15000 *g* for 15 min at 4 °C. For the enzymatic essay, 100 μL of extract were added to 900 μL of reaction buffer (41.6 mM HEPES at pH 7.5, 1 mM NADH, 10 mM EDTA, 20 mM glutamine) and 300 μL (for leaf extract) or 900 μL (for root extract) of 10 mM α-ketoglutaric acid. GOGAT was assayed spectrophotometrically by monitoring NADH oxidation at 340 nm according to Avila et al. 1987. GOGAT activity was expressed as nmol NADH reduced per g of fresh root or leaf per minute.

For the phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) essay, 1 g of ground leaves were homogenized with 0.1 g of poly(vinylpolypyrrolidone) (PVPP) and 5 mL of 100 mM potassium phosphate buffer (pH 8.0) containing 1.4 mM β-mercaptoethanol. After 10 min, the extract was filtered through two layers of muslin and centrifuged at 15000 *g* for 20 min at 4 °C. 60 μL of supernatant was incubated with 400 μL of 100 mM Tris-HCl buffer (pH 8.8), 140 μL of 100 mM phosphate buffer and 200 μL of 40 mM phenylalanine at 37 °C for 30 min. The reaction was stopped with 200 μL 6 M HCl (El-Shora 2002). After centrifuging at 10000 g for 15 min, the absorbance of the supernatant was measured at 280 nm against an immediately stopped parallel sample. A standard curve was made using cinnamic acid (Sigma). PAL activity was expressed as nmol cinnamic acid produced per mg protein per minute.

Extraction and measurement of soluble phenols

Soluble phenolic acids were extracted by homogenizing 200 mg of crushed leaves with 600 mL of pure methanol. The extract was maintained in ice for 30 min and centrifuged at 15000 *g* for 30 min at 4 °C. Total phenols were measured according to Arnaldos et al. (2001). 1 mL of 2% Na_2CO_3 and 75 μL of Folin-Ciocalteau reagent (Sigma-Aldrich) were added to 50 μL of phenolic extract. After 15 min of incubation at 25 °C in the dark, the absorbance was measured at 725 nm. A standard curve was made by using gallic acid (Sigma). The soluble phenols content was expressed as mg of gallic acid equivalent (GAE) per g of fresh leaf.

Quantitative determination of free phenolic acids by HPLC

Leaves (5 g) were homogenized in methanol (20 mL) with an Ultra Turrax T25 dispenser at 13500 rpm for 30 s until uniform consistency. Samples were filtered the first time through a filter paper (589 Schleicher) and a then through cellulose acetate syringe filters (0.45 μm). The extract was then ready for HPLC analysis.

The liquid chromatography system was a Jasco X-LC system consisting of a PU-2080 pump, a MD-2015 multi-wavelength detector, a AS-2055 autosampler, and a CO-2060 column oven. The separation of phenolic acids was carried out on a Tracer Extrasil ODS2 column (5 μm, 250 × 4.6 mm, Teknokroma) operating at 35 °C. The flow rate was set to 1 mL min^{-1}. The mobile phase consisted of water (0.1% formic acid)(A) and methanol (0.1% formic acid) (B). The gradient elution was as follows: 25–70% B over 15 min and 70–100% B over 5 min to clean the column. Chlorogenic acid, ferulic acid, *p*-coumaric acid, caffeic acid, and gallic acid were quantified using an HPLC diode array detector (DAD) at 325 nm. Identification of *p*-coumaric acid was performed at 310 nm. ChromNAV chromatography data system was used as software.. All standards were dissolved in methanol and the calibration curves were generated with concentrations ranging from 0.3 mg L^{-1} to 30 mg L^{-1} (Nicoletto et al. 2013).

Quantitative determination of sugars by HPLC

The liquid chromatography used in these analyses was the same reported above. The separation of sugars was achieved on a HyperRez XP Carbohydrate Pb^{++} analytical column (8 μm, 300 × 7.7 mm, ThermoScientific), operating at 80 °C. Isocratic elution was made using water at a flow rate of 0.6 mL min^{-1}. Standards of β-D-glucose, β-D-fructose, and sucrose were dissolved in water and the calibration curves were generated with concentrations ranging from 100 mg L^{-1} to 1000 mg L^{-1} (Nicoletto et al. 2013).

Statistical analysis

The data represent the means of measurements from three different beakers per treatment. For each measurement, the average ± standard error is reported. Analysis of variance (two-way ANOVA) was performed using the SPSS 23 (IBM Corp) software with leonardite type and concentration as factors, and was followed by pairwise post hoc analyses (Student-Newman Keuls test) to determine which means differed significantly at $P \leq 0.05$. Levene and Mauchly's tests were applied to check homoscedasticity and sphericity, respectively, to

ensure that assumptions of the model were met as recommended in Field 2013.

For each analytical parameter, linear and logarithmic regressions were also performed using SPSS 23 software with concentrations of HS as independent variable to evaluate the presence of dose-response curves.

Results

Chemical characterization

The main chemical characteristics for each leonardite are displayed in Table 1. The pH ranged from alkaline for LE_USA to acid for LETU_4, LE_A, and LE_B. It is interesting to note that the EC was high in LE_USA and low in LETU_4. LE_USA had high C, N, and S content while LETU_4 had a high mineral content.

Characteristics of HS are shown in Table 2. HS had similar pH values as a consequence of Amberlite IR-120 treatment. HS_4 had the highest C content (50.48%), whereas HS_USA had the highest S content (5.63%).

FTIR characterization

The main functional groups of HS_USA, HS_4, HS_A, and HS_B are displayed in Fig. 1. In all spectra there are vibrational bands which are assigned to the same functional groups such as the broad band from 4000 to 3000 cm^{-1} arises from (O–H) stretching vibration. The shape of this region suggests that the O-H groups formed several hydrogen bonds. The broad shoulder in the 2700–2400 cm^{-1} region is undoubtedly due to OH stretching of intermolecular hydrogen bonding in carboxylic acids or alcohols (Rao 1963). The presence of peaks at around 1700 and 1220 cm^{-1} might be due to C = O and C–O stretching motions of carboxylic acids. The peaks appearing at 2910 and 2852 cm^{-1} toghether with those at around 1420 and 1370 cm^{-1} are due to (C-H) stretching and bending motions in aliphatic substances respectively. The strong band at around 1580 cm^{-1} is highly characteristic of aromatic rings skeletal vibration (Bellamy 1975). Since the position and intensity of this band are dependent on the type of substitution, the conjugation with C = C or C = O should justify the great intensification of this band in all spectra. Other bands that can be useful in identifying the aromatic compounds are the C-H stretching motion at around 3100–3000 cm^{-1} and the C-H out of plane deformation between 900 cm^{-1} and 650 cm^{-1}. The variable intensity of the bands at around 1030 cm^{-1}, 520 cm^{-1} and 463 cm^{-1} may be due to mineral impurities.

Deconvolution fitting procedure on the pattern of HS from different leonardites gave eight Gaussian curve centered at 1700, 1580, 1420, 1210, 1130, 1030, 917 cm^{-1} (Fig. 2). The percentage area for each band considerably changed in relation to different leonardite origin. In particular, the content of COOH (1700 cm^{-1}) linked to aromatic rings (Bellamy 1975) was higher in HS_USA and progressively decreased in others. In contrast, the aromatic C = C skeletal stretching (1580 cm^{-1}) gradually increased from HS_USA to HS_B. The considerable intensification of this band in HS_A and HS_B might suggest the presence of different polar substituents in the aromatic ring (Bellamy 1975). As well as the coupled C-O stretching and OH in plane deformation modes (1210 cm^{-1}), which are typical in aryl acids and phenols (Bellamy 1975), was highest in HS_A and HS_B. At lower frequencies, the C–O and C-C (1130 cm^{-1}) were only present in HS_USA and HS_4, with highest amount in HS_4. The C-O-C

Table 1 Physical and chemical parameters of leonardites from different origin. Data are mean ± SE, $n = 3$

Treatment	$[H^+]$ pH	EC (μS cm^{-1})	C* (g kg^{-1})	N (g kg^{-1})	S (g kg^{-1})	DW (%)	Moisture (%)	Ash** (%)
LE_USA	8.57 ± 0.05	1400 ± 58	29.81 ± 0.51	3.39 ± 0.15	6.69 ± 0.12	90.00 ± 0.14	10.00 ± 0.14	62.29 ± 0.64
LETU_4	5.29 ± 0.03	128 ± 5	17.84 ± 0.45	0.38 ± 0.02	1.37 ± 0.23	92.55 ± 3.84	7.45 ± 0.86	73.76 ± 1.13
LE_A	3.81 ± 0.05	1002 ± 21	16.04 ± 1.10	0.55 ± 0.04	1.07 ± 0.31	81.00 ± 0.40	19.00 ± 0.40	66.69 ± 0.85
LE_B	3.85 ± 0.04	906 ± 11	17.35 ± 0.64	0.56 ± 0.05	1.02 ± 0.15	81.14 ± 0.37	18.86 ± 0.37	66.94 ± 1.23

EC = Electrical Conductivity

DW = Dry Weight

* = g kg^{-1} of DW** = % of DW

Table 2 Elemental analysis and pH of humic substances extracted from leonardites. Data are mean ± SE, $n = 3$

Treatment	$[H^+]$ pH*	C** (g kg^{-1})	N (g kg^{-1})	S (g kg^{-1})
HS_USA	2.35 ± 0.01	45.16 ± 0.31	1.06 ± 0.09	5.63 ± 0.52
HS_4	2.64 ± 0.04	50.48 ± 0.15	1.38 ± 0.13	2.86 ± 0.38
HS_A	2.63 ± 0.03	46.94 ± 0.23	1.72 ± 0.27	1.35 ± 0.29
HS_B	2.58 ± 0.01	47.95 ± 0.17	1.76 ± 0.02	1.51 ± 0.41

*= pH measured after amberlite purification of the extract

** = g kg^{-1} of DW

stretching in ethers (1030 cm^{-1}) appeared in all samples but the lowest percentage was detected in HS_4. Finally, the coupled C-OH bending out of plane and CH bending (917 cm^{-1}) was considerably higher in USA.

Bioassay to test the biological activity

IAA concentration in growth media inhibited the elongation of watercress roots in a dose-dependent response ($P \leq 0.001$) (Table 3). In a similar way, the increasing concentrations of HS_USA, USA_4, and HS_A caused significant decreases of watercress roots elongation. In all cases, a logarithmic model explained the best fit of our data (R^2 = 98–99%, $P \leq 0.05$). Only in HS_B there was not a significant dose-dependent response. The b value coefficient was used to compare the auxin-like effect between HS (Table 3). The lower b value was related to the higher auxin-like activity: HS_A had the highest activity, HS_4 the lowest one, while in HS_USA b value was intermediate. For the gibberellin-like activity (Table 3), the dose-dependent response induced by GA was significant ($P \leq 0.05$) in the elongation of lettuce shoots. Also in this experiment, the best fit was obtained with logarithmic dose-response curve (R^2 = 99%) for HS_USA ($P \leq 0.05$) and HS_A ($P \leq 0.05$). The high b value corresponded to a large GA-like activity. Consequently, HS_USA had the highest GA-like activity, and HS_A the lowest one. No GA-like activity was observed for HS_4 and HS_B.

Total root length and other morphological parameters of maize plants

Total radicular length for each seedling was calculated as the sum of the lengths of all radicular nodal segments, using automatic linearization with WinRHIZO software. In maize seedlings treated for two days with HS, root diameter, number of forks, and number of thin roots

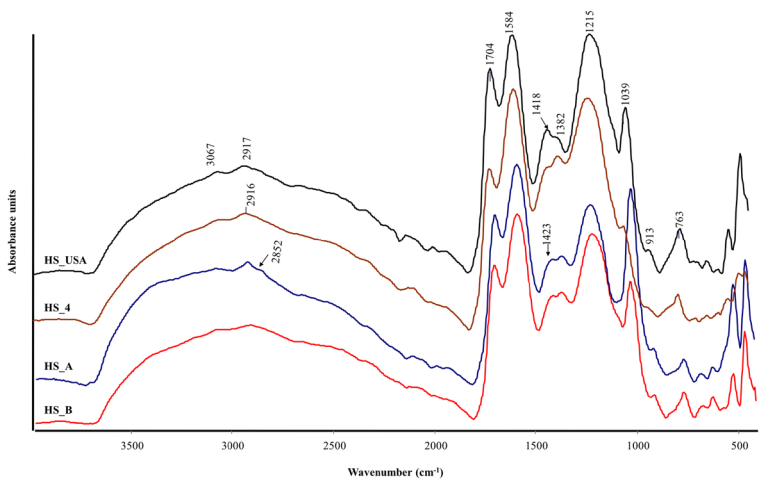

Fig. 1 FT-IR spectra of HS extracted from leonardites (HS_USA, HS_4, HS_A, and HS_B)

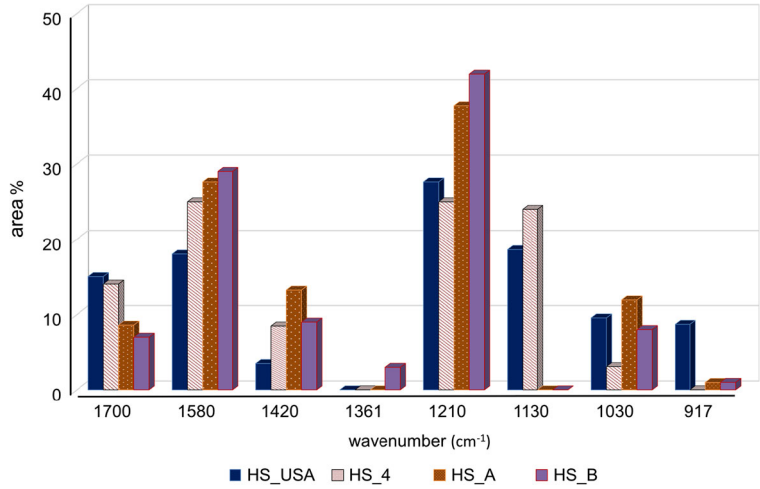

Fig. 2 Histograms of HS of the ATR/FTIR peak areas processed by Gaussian curve fitting

were significantly affected by treatment ($P \leq 0.05$) and concentration ($P \leq 0.05$) (Table 4). As a result, a stimulation of total root length (TRL) ($P \leq 0.05$), and enhanced root surface area ($P \leq 0.05$) was obtained. Among HS and with respect to untreated plants, HS_USA affected the greatest number of forks (2305 vs 974), number of thin roots (624 vs 349), TRL (743 mm vs 429 mm), and root surface area (89 cm^2 vs 51 cm^2), whereas HS_A had the highest effect on root diameter (0.39 mm vs 0.38 mm) and number of tips (477 vs 396). The concentrations stimulated in a wider way the aforesaid parameters (Table 4). Indeed, TRL was up to 1.92 fold higher compared to untreated for HS_4 at 0.1 mg C L^{-1} (treatment × concentration interaction significant at $P \leq 0.05$), the number of forks raised up to 2.96 fold compared to untreated for HS_USA at 0.5 mg C L^{-1} (treatment × concentration interaction significant at $P \leq 0.05$), and the number of thin roots were up to 1.99 fold compared to untreated for HS_B at 1 mg C L^{-1} (treatment × concentration interaction significant at $P \leq 0.05$). Finally, although not statistically significant, HS_A at 0.1 mg C L^{-1} gave the highest number in root tips (1.30 fold compared to untreated).

Table 3 Parameters of the regression curves [Y = a + b*log(X)] between concentration and root length of watercress plantlets or steam length of lettuce plantlets treated with indoleacetic acid (IAA) or gibberellic acid (GA) or with the humic substances extracted from leonardites

Treatment	R^2	*b*	*P*	df
IAA	0.99	−1.76	0.001	1215
HS_USA	0.99	−0.21	0.040	277
HS_4	0.99	−0.11	0.050	291
HS_A	0.98	−0.46	0.043	316
HS_B	ns	ns	ns	ns
GA	0.93	0.72	0.037	262
HS_USA	0.99	0.29	0.048	249
HS_4	ns	ns	ns	ns
HS_A	0.99	0.07	0.050	247
HS_B	ns	ns	ns	ns

df = degree of freedom;
ns = not significant

For all the HS linear and logarithmic regression models were tested and they did not explain the data distribution of tested root growth parameters. Parameters (R^2 and *P* value) are reported in Supplementary material (Table S1).

GS and GOGAT activity

Treatment significantly influenced both roots ($P \leq 0.001$) and leaves GS enzyme activity ($P \leq 0.001$) (Table 5). Among treatments, HS_4 and HS_B were the most effective in roots ($P \leq 0.05$), whereas HS_4, HS_A, and HS_USA were the most active in leaves ($P \leq 0.05$). In particular, HS_4 always showed high values in GS enzyme activity, up to 1.63 fold higher than untreated roots (1 mg C L^{-1}) ($P \leq 0.05$), and 1.49 fold higher than untreated leaves (10 mg C L^{-1}) ($P \leq 0.05$). The activity of GOGAT enzyme was also affected by treatment in both roots ($P \leq 0.001$) and leaves ($P \leq 0.001$) (Table 5). In roots,

Table 4 Effect of different concentrations of HS extracted from leonardites on root growth morphological parameters of maize seedlings analysed by WhinRhizo software. Total root length (TRL) is calculated as the sum of the length of primary and lateral roots

Treatment	Dose (mg C L^{-1})	TRL (cm)	Area (cm^2)	Diameter (mm)	Tips (n)	Forks (n)	Thin roots (cm)
Control	0	$429 \pm 26^{cC c*}$	$51 \pm 3^{cC b}$	$0.38 \pm 0.006^{abcdAB ab}$	396 ± 21	$974 \pm 78^{deB b}$	$349 \pm 25^{bC d}$
HS_USA	0.1	734 ± 12^{abc}	90 ± 16^{abc}	0.38 ± 0.013^{abc}	492 ± 59	2436 ± 555^{ab}	615 ± 101^{ab}
	0.5	757 ± 97^{ab}	97 ± 12^{ab}	0.41 ± 0.009^{ab}	372 ± 41	2880 ± 394^{a}	618 ± 81^{ab}
	1	787 ± 28^{a}	87 ± 3^{abc}	0.35 ± 0.009^{cd}	428 ± 63	2130 ± 98^{abc}	669 ± 36^{a}
	10	695 ± 96^{abc}	79 ± 10^{abc}	0.36 ± 0.007^{abcd}	390 ± 27	1774 ± 347^{bcde}	594 ± 94^{ab}
		743 ± 43^{A}	89 ± 5^{A}	0.379 ± 0.006^{AB}	420 ± 25	2305 ± 199^{A}	624 ± 38^{A}
HS_4	0.1	825 ± 117^{a}	98 ± 12^{a}	0.38 ± 0.008^{abcd}	483 ± 72	2005 ± 372^{bcd}	716 ± 107^{a}
	0.5	505 ± 72^{abc}	57 ± 8^{abc}	0.35 ± 0.007^{abcd}	415 ± 35	1054 ± 148^{de}	429 ± 68^{ab}
	1	606 ± 46^{abc}	65 ± 6^{abc}	0.34 ± 0.007^{cd}	431 ± 23	1321 ± 127^{cde}	530 ± 44^{ab}
	10	594 ± 73^{abc}	67 ± 10^{abc}	0.35 ± 0.015^{bcd}	488 ± 43	1341 ± 286^{cde}	507 ± 64^{ab}
		615 ± 41^{B}	69 ± 5^{B}	0.359 ± 0.005^{C}	451 ± 20	1378 ± 127^{B}	530 ± 37^{B}
HS_A	0.1	613 ± 61^{abc}	76 ± 8^{abc}	0.39 ± 0.007^{abc}	516 ± 45	1401 ± 148^{cde}	494 ± 47^{ab}
	0.5	562 ± 59^{abc}	74 ± 10^{abc}	0.41 ± 0.020^{a}	490 ± 49	1194 ± 188^{cde}	438 ± 41^{ab}
	1	665 ± 35^{abc}	83 ± 6^{abc}	0.39 ± 0.01^{abc}	433 ± 43	1813 ± 120^{bcde}	568 ± 27^{ab}
	10	442 ± 18^{bc}	52 ± 2^{c}	0.37 ± 0.005^{abcd}	458 ± 10	940 ± 43^{de}	354 ± 19^{b}
		562 ± 29^{B}	70 ± 4^{B}	0.394 ± 0.006^{A}	477 ± 19	1299 ± 92^{B}	455 ± 24^{B}
HS_B	0.1	551 ± 44^{abc}	58 ± 5^{abc}	0.33 ± 0.007^{d}	327 ± 27	1139 ± 121^{cde}	491 ± 42^{ab}
	0.5	444 ± 35^{bc}	55 ± 5^{bc}	0.39 ± 0.010^{abc}	340 ± 14	871 ± 95^{e}	363 ± 29^{b}
	1	798 ± 140^{a}	95 ± 17^{ab}	0.38 ± 0.007^{abcd}	512 ± 108	1814 ± 401^{bcde}	694 ± 118^{a}
	10	630 ± 53^{abc}	73 ± 6^{abc}	0.37 ± 0.010^{abcd}	455 ± 16	1447 ± 173^{cde}	537 ± 51^{ab}
		588 ± 40^{B}	68 ± 5^{B}	0.368 ± 0.006^{BC}	399 ± 25	1273 ± 113^{B}	506 ± 36^{B}
	0.1	665 ± 45^{ab}	78 ± 6^{a}	0.373 ± 0.007^{b}	421 ± 27	1688 ± 185^{a}	564 ± 38^{ab}
	0.5	559 ± 40^{b}	69 ± 5^{a}	0.392 ± 0.007^{a}	414 ± 22	1451 ± 198^{a}	456 ± 33^{c}
	1	706 ± 37^{a}	81 ± 4^{a}	0.365 ± 0.006^{b}	464 ± 28	1741 ± 116^{a}	609 ± 32^{a}
	10	586 ± 35^{b}	67 ± 4^{a}	0.367 ± 0.005^{b}	450 ± 14	1358 ± 124^{a}	494 ± 33^{bc}

*In the same column differences among treatment mean (capital letters), concentration (italicized letters) and treatment × concentration (lowercase) were at $P \leq 0.05$ by Student Newman Keuls test

strong effects were induced by HS_USA and HS_4, and in leaves by HS_B. In fact, GOGAT activity was 1.97 and 1.87 fold higher than untreated roots (HS_USA 0.1 C mg L^{-1}) ($P \leq 0.05$) and leaves (HS_B 1 mg C L^{-1}) ($P \leq 0.05$), respectively (treatment × concentration interaction significant at $P \leq 0.05$).

Proteins and soluble sugars content

The treatment with HS affected the proteins content and concentration ($P \leq 0.05$) of maize plants ($P \leq 0.005$) (Table 6). In roots, HS_4 and HS_USA had the highest effects at 1 and 10 mg L^{-1} ($P \leq 0.05$) highlighting values 1.81 and 1.73 fold untreated, respectively. In leaves only HS_A at 0.5 mg C L^{-1} increased the protein content (1.19 fold untreated) ($P \leq 0.05$).

Sucrose content was considerably influenced by treatments ($P \leq 0.005$) (Table 7). This led a general decrease in sugars with respect to the control, however for HS_4 the effect was the opposite at low doses (0.1 and 0.5 mg C L^{-1}), with sucrose content increasing up to 3.39 and 2.07 fold compared to untreated plants, respectively. Fructose content was positively influenced by HS treatment ($P \leq 0.05$). Whereas for glucose content HS treatment had little effect. Although not statistically significant, the trend showed that HS_B induced a widespread increase in the amount of both glucose and fructose.

Table 5 Glutamine synthetase (GS) and glutamate synthase (GOGAT) activities of leaves and roots per plant of maize seedlings after treatment with HS extracted from leonardites. Data are mean ± SE, $n = 9$

Treatment	Dose (mg C L^{-1})	GS (μmol GHA g^{-1} fw min^{-1})		GOGAT (nmol NADH g^{-1} fw min^{-1})	
		Roots	Leaves	Roots	Leaves
Control	0	$0.101 \pm 0.008^{cdefCc*}$	0.090 ± 0.004^{cdCc}	0.36 ± 0.05^{bcB}	0.08 ± 0.08^{bcBC}
HS_USA	0.1	0.098 ± 0.009^{cdef}	0.102 ± 0.008^{abcd}	0.71 ± 0.01^{a}	0.09 ± 0.09^{bc}
	0.5	0.084 ± 0.005^{def}	0.110 ± 0.007^{abcd}	0.59 ± 0.02^{ab}	0.05 ± 0.05^{c}
	1	0.091 ± 0.001^{cdef}	0.102 ± 0.006^{abcd}	0.56 ± 0.03^{ab}	0.07 ± 0.07^{bc}
	10	0.135 ± 0.003^{abc}	0.119 ± 0.007^{abc}	0.62 ± 0.05^{ab}	0.07 ± 0.07^{bc}
		0.102 ± 0.004^{C}	0.108 ± 0.003^{B}	0.62 ± 0.02^{A}	0.07 ± 0.005^{C}
HS_4	0.1	0.148 ± 0.008^{ab}	0.126 ± 0.004^{ab}	0.63 ± 0.08^{ab}	0.10 ± 0.10^{b}
	0.5	0.157 ± 0.005^{ab}	0.128 ± 0.010^{ab}	0.61 ± 0.09^{ab}	0.10 ± 0.10^{b}
	1	0.165 ± 0.012^{a}	0.119 ± 0.008^{abc}	0.65 ± 0.06^{a}	0.07 ± 0.07^{bc}
	10	0.155 ± 0.007^{ab}	0.134 ± 0.008^{a}	0.62 ± 0.06^{ab}	0.10 ± 0.10^{b}
		0.156 ± 0.004^{A}	0.127 ± 0.003^{A}	0.63 ± 0.03^{A}	0.09 ± 0.005^{B}
HS_A	0.1	0.085 ± 0.008^{def}	0.097 ± 0.004^{bcd}	0.13 ± 0.00^{c}	0.08 ± 0.08^{bc}
	0.5	0.073 ± 0.001^{ef}	0.108 ± 0.008^{abcd}	0.13 ± 0.03^{c}	0.10 ± 0.10^{b}
	1	0.057 ± 0.008^{f}	0.108 ± 0.003^{abcd}	0.12 ± 0.00^{c}	0.08 ± 0.08^{bc}
	10	0.098 ± 0.018^{cdef}	0.119 ± 0.003^{abc}	0.08 ± 0.03^{c}	0.08 ± 0.08^{bc}
		0.078 ± 0.006^{D}	0.108 ± 0.003^{B}	0.12 ± 0.01^{C}	0.08 ± 0.004^{BC}
HS_B	0.1	0.125 ± 0.001^{abcd}	0.088 ± 0.005^{cd}	0.15 ± 0.01^{c}	0.12 ± 0.12^{ab}
	0.5	0.115 ± 0.002^{bcde}	0.102 ± 0.001^{abcd}	0.17 ± 0.02^{c}	0.14 ± 0.14^{a}
	1	0.119 ± 0.003^{bcde}	0.083 ± 0.007^{d}	0.20 ± 0.01^{c}	0.15 ± 0.15^{a}
	10	$0.131 \pm 0.011_{abcd}$	0.092 ± 0.002^{cd}	0.18 ± 0.01^{c}	0.14 ± 0.14^{a}
		0.122 ± 0.003^{B}	0.091 ± 0.002^{C}	0.18 ± 0.00^{C}	0.14 ± 0.004^{A}
	0.1	0.114 ± 0.006^{b}	0.103 ± 0.004^{b}	0.40 ± 0.07	0.09 ± 0.006
	0.5	0.107 ± 0.007^{c}	0.112 ± 0.004^{a}	0.38 ± 0.06	0.09 ± 0.009
	1	0.108 ± 0.008^{c}	0.103 ± 0.004^{b}	0.38 ± 0.06	0.09 ± 0.010
	10	0.130 ± 0.006^{a}	0.116 ± 0.004^{a}	0.37 ± 0.06	0.10 ± 0.007

*In the same column differences among treatment mean (capital letters), concentration (italicized letters) and treatment × concentration (lowercase) were at $P \leq 0.05$ by Student Newman Keuls test

PAL activity, soluble phenols content, and phenolic acids

PAL activity in maize leaves was significantly influenced by treatment ($P \leq 0.001$) and concentration ($P \leq 0.001$) (Table 8). HS_4, in the range at 0.5 and 1 mg C L^{-1} gave the highest PAL activity. In particular, at 0.5 mg C L^{-1} the PAL activity increased up to 3.70 fold relative to the control (treatment × concentration interaction significant at $P \leq 0.01$). PAL activity was also increased by HS_A at 1 mg C L^{-1} up to 1.54 fold compared to untreated. A general increase of soluble phenolic content was induced by HS treatment ($P \leq 0.001$) (Table 8)with HS_4 and HS_A showing the highest effects. In particular, HS_A at 10 mg C L^{-1} and 0.5 mg C L^{-1} showed a strong effect in the content of *p*-hydroxybenzoic acid (3.25 fold untreated) and *p*-coumaric acid (2.3 fold untreated), respectively. Finally, HS_USA at 1 mg C L^{-1} and HS_B at 10 mg C L^{-1} increased the chlorogenic acid (1.21 fold compared to untreated) (Table 8).

Linear and logarithmic regression curves were tested and they did not explain the data distribution of the enzymes activities, phenols, phenolic acids, proteins, and sugars contents. Parameters (R^2 and *P* value) are reported in Table S1.

Table 6 Roots and leaves protein content per plant of maize seedlings after treatment with HS extracted from leonardites. Data are mean ± SE, $n = 9$

Treatment	Dose (mg C L^{-1})	Protein (mg g fw^{-1})	
		Roots	Leaves
Control	0	$0.99 \pm 0.07^{bCb*}$	8.56 ± 0.34^{bcABbc}
HS_USA	0.1	1.16 ± 0.06^{b}	8.23 ± 0.50^{cd}
	0.5	1.09 ± 0.04^{b}	7.56 ± 0.26^{cd}
	1	1.52 ± 0.36^{ab}	9.37 ± 0.35^{a}
	10	1.72 ± 0.11^{a}	7.16 ± 0.36^{d}
		1.37 ± 0.10^{B}	8.08 ± 0.24^{B}
HS_4	0.1	1.54 ± 0.12^{ab}	8.82 ± 0.27^{b}
	0.5	1.58 ± 0.02^{ab}	9.08 ± 0.34^{ab}
	1	1.79 ± 0.02^{a}	9.06 ± 0.15^{ab}
	10	1.61 ± 0.09^{ab}	8.72 ± 0.63^{bc}
		1.63 ± 0.04^{A}	8.92 ± 0.18^{A}
HS_A	0.1	1.19 ± 0.12^{b}	8.33 ± 0.26^{cd}
	0.5	1.11 ± 0.07^{b}	10.2 ± 0.13^{a}
	1	1.17 ± 0.15^{b}	9.95 ± 0.08^{a}
	10	1.28 ± 0.06^{ab}	7.68 ± 0.12^{cd}
		1.19 ± 0.05^{C}	9.06 ± 0.23^{A}
HS_B	0.1	1.13 ± 0.03^{b}	8.43 ± 0.17^{abcd}
	0.5	1.01 ± 0.05^{b}	8.57 ± 0.25^{bc}
	1	1.02 ± 0.03^{b}	8.72 ± 0.25^{bc}
	10	1.09 ± 0.04^{b}	7.80 ± 0.43^{cd}
		1.06 ± 0.02^{C}	8.38 ± 0.15^{AB}
	0.1	1.25 ± 0.05^{ab}	8.45 ± 0.16^{abc}
	0.5	1.20 ± 0.05^{ab}	8.87 ± 0.23^{ab}
	1	1.37 ± 0.11^{a}	9.28 ± 0.14^{a}
	10	1.42 ± 0.06^{a}	7.84 ± 0.23^{c}

*In the same column differences among treatment mean (capital letters), concentration (italicized letters) and treatment × concentration (lowercase) were at $P \leq 0.05$ by Student Newman Keuls test

Table 7 Sucrose, glucose, and fructose amount in leaf per plant of maize seedlings after treatment with HS extracted from leonardites. Data are mean ± SE, $n = 9$

Treatment	Dose (mg C L^{-1})	Sucrose (mg kg^{-1} fw)	Glucose	Fructose
Control	0	$611 \pm 288^{AB*}$	5820 ± 485	1978 ± 185
HS_USA	0.1	159 ± 14	6273 ± 580	2334 ± 52
	0.5	130 ± 67	6242 ± 637	2403 ± 290
	1	143 ± 4	5999 ± 604	2211 ± 254
	10	143 ± 14	5520 ± 221	2427 ± 124
		144 ± 13^{B}	6009 ± 233	2343 ± 83
HS_4	0.1	2073 ± 472	5833 ± 210	1666 ± 125
	0.5	1266 ± 404	6265 ± 165	2034 ± 47
	1	529 ± 509	4973 ± 803	1609 ± 200
	10	88 ± 40	6297 ± 636	2895 ± 184
		989 ± 323^{A}	5842 ± 284	2051 ± 202
HS_A	0.1	162 ± 16	6099 ± 137	2347 ± 858
	0.5	92 ± 10	5334 ± 434	2325 ± 428
	1	145 ± 13	5287 ± 568	2048 ± 334
	10	174 ± 36	5453 ± 678	2254 ± 320
		143 ± 14^{B}	5543 ± 343	2243 ± 206
HS_B	0.1	160 ± 10	6123 ± 800	2408 ± 257
	0.5	193 ± 26	7792 ± 761	3153 ± 169
	1	219 ± 61	7128 ± 520	2933 ± 354
	10	210 ± 59	5945 ± 452	2192 ± 171
		195 ± 19^{B}	6747 ± 376	2672 ± 174
	0.1	639 ± 325	6082 ± 328	2189 ± 206
	0.5	420 ± 200	6408 ± 392	2479 ± 187
	1	259 ± 114	5847 ± 394	2200 ± 211
	10	154 ± 22	5804 ± 237	2442 ± 131

*In the same column differences among treatment mean (capital letters) were at $P \leq 0.05$ by Student Newman Keuls test

Discussion

Several studies have reported possible relationships between the effects of leonardite on plant growth and their capacity to improve nutrient uptake and assimilation (Aguirre et al. 2009; Tahiri et al. 2015). Ertani et al. (2011) demonstrated that, in maize, HS from leonardite enhanced the production of N assimilates and promoted photosynthesis through the increase in chlorophyll content and stimulation of RuBisCo enzyme activity. These effects were mainly attributed to a complex macromolecular system mainly composed of polyaromatic rings, and may depend on the origin and characteristics of HS (Nardi et al. 2009).

This study showed that four different HS from leonardites had the same main functional components, but deconvolution fitting analysis gave more information on semi-quantitative differences between leonardites. In terms of functional groups distribution, HS_USA had the highest content in carboxyl groups bound to aromatic rings with few polar substituents. In contrast, HS_4 and in particular in HS_A and HS_B, the aromatic component was dominant and was composed by polar substituents and aliphatic structures. This feature has been related to

Table 8 Phenylalanine ammonia-lyase activity (PAL), soluble phenols content, and phenolic acid profile per plant of maize seedlings after treatment with HS extracted from leonardites. Data are mean ± SE, $n = 9$

Treatment	Dose (mg C L^{-1})	PAL	Soluble phenols	Phenolic acid (mg kg^{-1} fw)			
		(nmol cinn. Acid mg^{-1} prot min^{-1})	(mg gallic acid g^{-1} fw)	Chlorogenic	*p*-Cumaric	Ferulic	*p*-Hydroxybenzoic
Control	0	$2.52 \pm 0.25^{bC}b^{*}$	15.8 ± 0.2^{bC}	80 ± 5	4.3 ± 0.3	5.0 ± 0.9	12 ± 3^{cB}
HS_USA	0.1	1.96 ± 0.53^{b}	18.5 ± 0.3^{a}	88 ± 1	5.0 ± 0.4	6.0 ± 1.0	10 ± 2
	0.5	2.15 ± 0.47^{b}	15.7 ± 0.2^{b}	96 ± 2	5.4 ± 0.1	7.0 ± 1.6	13 ± 0
	1	2.80 ± 0.55^{b}	16.0 ± 0.2^{b}	97 ± 1	4.8 ± 0.1	5.0 ± 1.3	18 ± 0
	10	2.43 ± 0.80^{b}	15.4 ± 0.1^{b}	11 ± 2	5.7 ± 1.2	5.0 ± 0.6	24 ± 6
		2.34 ± 0.28^{C}	16.4 ± 0.3^{C}	98 ± 8	5.2 ± 0.2	6.2 ± 0.5	16 ± 2^{AB}
HS_4	0.1	3.19 ± 0.57^{b}	19.7 ± 0.4^{a}	82 ± 1	4.3 ± 1.0	8.0 ± 2	13 ± 0
	0.5	9.32 ± 0.60^{a}	18.0 ± 0.2^{a}	73 ± 6	4.0 ± 0.8	6.0 ± 2.4	nd
	1	8.17 ± 0.55^{a}	19.6 ± 0.4^{a}	90 ± 7	3.8 ± 1.0	6.0 ± 1.8	nd
	10	7.60 ± 0.39^{a}	19.6 ± 0.3^{a}	70 ± 0	4.2 ± 0.3	5.0 ± 1.6	nd
		7.07 ± 0.73^{A}	19.2 ± 0.23^{A}	79 ± 5	4.1 ± 0.3	6.6 ± 0.8	13 ± 0^{B}
HS_A	0.1	3.60 ± 1.19^{b}	19.1 ± 0.5^{a}	79 ± 2	3.6 ± 0.1	2.0 ± 0.2	25 ± 2
	0.5	3.70 ± 0.52^{b}	18.8 ± 0.6^{a}	47 ± 1	10 ± 6.7	6.0 ± 4.1	9 ± 3
	1	3.88 ± 0.82^{b}	18.5 ± 0.2^{a}	84 ± 1	4.2 ± 0.0	2.0 ± 0.2	29 ± 1
	10	2.80 ± 0.69^{b}	19.9 ± 0.4^{a}	83 ± 1	4.0 ± 0.0	2.0 ± 0.4	39 ± 6
		3.50 ± 0.40^{B}	19.1 ± 0.24^{A}	73 ± 7	5.5 ± 1.6	3.4 ± 0.9	26 ± 4^{A}
HS_B	0.1	2.18 ± 0.18^{b}	16.0 ± 0.3^{b}	71 ± 9	4.3 ± 0.4	4.0 ± 0.6	16 ± 3
	0.5	1.46 ± 0.48^{b}	18.7 ± 0.3^{a}	93 ± 2	5.2 ± 0.8	6.0 ± 1.9	23 ± 0
	1	2.57 ± 0.61^{b}	18.6 ± 0.8^{a}	94 ± 5	4.6 ± 0.0	5.0 ± 0.1	29 ± 2
	10	1.69 ± 0.38^{b}	16.7 ± 0.4^{b}	97 ± 0	4.9 ± 0.0	6.0 ± 0.5	28 ± 5
		1.97 ± 0.22^{C}	17.5 ± 0.34^{B}	89 ± 6	4.8 ± 0.2	5.5 ± 0.5	24 ± 2^{A}
	0.1	2.67 ± 0.39^{b}	18.3 ± 0.3	80 ± 6	4.3 ± 0.2	5.3 ± 0.9	$14. \pm 3.0^{ab}$
	0.5	3.42 ± 0.62^{a}	17.8 ± 0.3	77 ± 1	6.2 ± 1.5	6.8 ± 1.0	$11. \pm 3.2^{b}$
	1	3.81 ± 0.52^{a}	18.2 ± 0.3	91 ± 3	4.4 ± 0.2	4.7 ± 0.6	$19. \pm 5.0^{ab}$
	10	3.06 ± 0.52^{ab}	17.9 ± 0.4	90 ± 7	4.7 ± 0.3	5.0 ± 0.5	$23. \pm 5.8^{a}$

nd, not detected

*In the same column differences among treatment mean (capital letters), concentration (italicized letters) and treatment × concentration (lowercase) were at $P \leq 0.05$ by Student Newman Keuls test

low rank coals. Overall, HS differently but significantly affected the growth of watercress root and lettuce seedlings in a logarithmic curve model. These effects are reported to reflect strong biostimulant properties (Scaglia et al. 2016). However, the magnitude and nature of these effects were different depending on the type of leonardite. IAA-like activity decreased in the order HS_A > HS_USA > HS_4, whereas HS_USA and HS_A had the highest and lowest GA-like activity, respectively. These results partially agree with those of Ertani et al. (2011) who found GA-like activity in a humic acid from leonardite, while other authors reported auxin-like properties (O'Donnell 1973). However, the intensities of IAA and GA-like activities were comparable with those found from humic and fulvic acids extracted from soil of natural ecosystems (Pizzeghello et al. 2015) and HS extracted from vermicompost (Scaglia et al. 2016).

The relationship between the biological activity and the structure of HS is very important to understand their effects in treated plants (Muscolo et al. 2007). However, the molecular structure is still debated. Recently, HS are described as supramolecular structures formed by relatively small molecules held together by non-covalent intermolecular interactions (e.g., hydrogen-bonding, charge-transfer, van der Waals and π-π) (Piccolo 2001). The exterior domain consists of polar groups (e.g., carboxylic acids) where their distribution is particularly relevant to determine HS solubility and biological reactivity (Muscolo et al. 2007). High content of carboxylic acids, proteins, and amino acids has been related to IAA-like activity. The carboxyl groups presence is a proxy for the bioavailability of auxin entrapped into the HS molecular structure (Napier 2004). Whereas the hydrophobic domain, composed by aromatics and amides functional groups, is related to GA-like activity (Pizzeghello et al. 2015). Such distinction might not always be applicable and it is possible that leonardite properties might be modified according to the functional groups exposed to the surrounding aqueous environment (Carletti et al. 2010). Nevertheless, our FT-IR spectra of HS confirmed a different content in carboxyl and aromatics rings that may justify their biological activity.

The most general trait in plant responses to HS pertains to growth and architecture of the rooting system, mainly affecting lateral root formation (Canellas and Olivares 2014; Nardi et al. 2009). Rooting is vital for plant survival in relation to nutrition and growth requirements, synthesis and accumulation of secondary metabolites and interaction with nitrogen-fixing organisms (Saini et al. 2013). Root is also the first plant organ targeted by HS in soil. In this study, among the leonardites, HS_USA had the strongest effect in maize root architecture, leading to an overall stimulation of elongation and proliferation of secondary roots as well increasing root diameter. HS_A and HS_USA, which showed high auxin-like activity in the bioassay, showed the highest morphological changes on maize root apparatus, resulting in a higher root surface area, increase of total radicular length and root diameter. This confirms the effect of the carboxylic components in HS. Primary root elongation, and increasing lateral roots are known to be an auxin-triggered mechanism (De Smet et al. 2006), which has been recently proven to be driven by the auxin entrapped in the HS themselves (Trevisan et al. 2010a). Effects on root architecture are indeed accompanied by changes in the biochemistry of energy generation and transport system across plasma membranes (Canellas et al. 2002; Zandonadi et al. 2007). Regarding root diameter, our results agree with previous findings which demonstrated that HS induced a higher rate of differentiation of cells of the root central cylinder relative to untreated plants. The augmented thickness of cells wall has been shown to be due to a higher production of lignin in HS-treated plants (Concheri et al. 1996; Nardi et al. 2000).

HS increased the enzymes involved in N assimilation (Baglieri et al. 2014). In particular, GS and GOGAT enzyme activities were widely affected by the presence of HS. These enzymes work in close association as the incorporation of ammonium (NH_4^+) into organic compounds by GS leads to the production of glutamate from glutamine and α-ketoglutarate by GOGAT. The GS/GOGAT system is the main metabolic route for N assimilation in higher plants (Mokhele et al. 2012), and its stimulation confirms the capability of HS from leonardites, to interact with the plant nitrate metabolism inducing an increase in N organic compounds, as supported by the augmented protein content recorded. Such results are consistent with previous ones, obtained with HS from other sources such as earthworms coprolites and lignosulfonate-humates (Carletti et al. 2008; Ertani et al. 2011).

In addition to nitrogen metabolism, HS may modulate C metabolism by increasing the activity of enzymes involved in glycolysis and the Krebs cycle (Nardi et al. 2007). In our study, after the application of HS from leonardites, the content of carbohydrates, such as glucose and fructose, sharply increased in the leaves. Carbohydrates, which represent the basis of plant metabolism (Winter and Huber 2000), not only provide the energy required for various metabolic pathways, but also provide carbon skeletons for nitrogen metabolism, thus their increase may justify the improved activity of nitrogen assimilation.

The activity of PAL, synthesized in response to HS treatment, results in the accumulation of phenolic compounds. PAL is an enzyme which, catalyzing the first metabolic step from primary to secondary metabolism (Douglas 1996), deaminates phenylalanine to produce cinnamic acid. As a consequence, HS_4 and HS_A enhanced the soluble phenols and strongly increased phenolics such *p*-hydroxybenzoic acid, *p*-coumaric acid and chlorogenic acid. The stimulation of secondary metabolism is also justified by the enhanced activity of primary metabolism. In addition, a greater concentration of phenols recorded in plants after treatment with leonardites is likely to be the result of a weak uncoupling of oxidative phosphorylation, which in turn increases the metabolic processes requiring glucose (Muscolo and Sidari 2006).

In conclusion, with this work, we aimed to test and compare the biostimulant activity of HS extracted from different leonardites. Overall, HS from leonardites positively affected root architecture, with a stimulation of the elongation and proliferation of secondary roots. They enhanced plant nutrient uptake and nutrient use efficiency, and influenced N metabolism, increasing GOGAT and GS enzymes activity, and hence protein production. The carboxyl groups resulted as proxy for the bioavailability of the auxin entrapped into the HS molecular structure, whereas the hydrophobic domain is related to GA-like activity. From this study it was found that: (1) HS from leonardites have strong biostimulant properties, (2) the leonardite origin has an influence on composition of the main functional groups, and, as a consequence, on their biological activity.

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