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Humic-like substances from agro-industrial residues affect growth and nitrogen assimilation in maize (*Zea mays* L.) plantlets

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1. Introduction

For many years agricultural research has aimed at improving crop yields, while placing little importance on the quality of the products or environmental protection. More recently, the environmental impact of production methods, high production costs and the need to reduce chemical substances in the soil have become important agricultural objectives (Gastal and Lemaire, 2002). To promote efficient plant adsorption of nutrients and reduce environmental pollution, a number of chemical molecules have been studied (Ertani et al., 2009, 2011). These compounds are defined as biostimulants and include humic substances (HS), seaweeds and amino acids (Miller, 1990). Over recent years, it has been shown that these compounds are of a mainly organic nature and can increase plant growth and development, both in the laboratory and in the field, in a different way from normal fertilizers (Quilty and Cattle, 2011). The most frequent mechanisms of action are: stimulation of microbial activity, increased activity of a number of soil enzymes, increased production of hormones in the soil or growth regulators in plants and stimulation of numerous plant metabolism parameters (Nardi et al., 2009). Frankenberger and Arshad (1995) observed that biostimulants lead to a more efficient adsorption of nutrients due to better root development in the treated plants and a greater number of root hairs. Moreover, it was also demonstrated that these compounds increase photosynthetic efficiency by promoting an accumulation of sugars in fruit, fruit set, improved size and conservability (Presutto and Pezzutto, 2005). Some authors have shown that biostimulants can make a crop less sensitive to stressful conditions (drought, extreme temperatures, excessive moisture in the rhizosphere, over or under-exposure to light), due to a greater production of anti-oxidants (Ertani et al., 2011; Subler et al., 1998). Nevertheless, maximum efficiency is only achieved if they are administered at specific times, using the optimal dose which varies according to the crop and even from cultivar to cultivar (Zhang et al., 2003).

It is known that soil HS can affect plant growth by simulating the behaviour of plant hormones (Nardi et al., 2009; Trevisan et al., 2010; Jindo et al., 2012). The presence of indoleacetic acid (IAA) has been demonstrated using both immunological approaches (Muscolo et al., 1998) and gas-chromatography mass spectrometry (Canellas et al., 2002). Nevertheless, humic substances cannot be classified as real hormones. It is also known that HS influence the assimilation of nutrients depending on the pH and concentration of the humic substances themselves (Vaughan et al., 1985). Moreover, carboxylic and hydroxyl functional groups in HS have been correlated to their biochemical activity (Muscolo et al., 2007a,b).

Using similar procedures to those for extracting HS from the soil, it is possible to extract organic fractions, defined as humic-like (HL) substances, from the biomass. Different studies have shown that these substances have a number of properties. For example, Montoneri et al. (2008) took a humic-like fraction extracted from solid urban waste and studied its capacity to solubilize a textile colorant and soil contaminants like the aromatic, polycyclic hydrocarbons (PAH). This capacity was compared to that of sodium dodecyl sulfate (SDS). The results showed that there was a greater solubility of both the colorant and the soil pollutants when using the humic-like substance than when using SDS. In another study Montoneri et al. (2009) observed that some humic-like acids isolated from organic plant wastes had such excellent surfactant properties that they could be used as industrial surfactants.

This work aims at evaluating the possible biostimulant effect of humic-like substances extracted from agro-industrial wastes: rape, castor oil and flax plant and digestate residues. The extracts were characterized by means of elemental analysis, FT-InfraRed (FT-IR) and ^{13}C-NMR spectroscopy. The presence of bioactive molecules such as indoleacetic acid (IAA), total phenolic acids (TP) and flavonoids (FL) was determined and then the hormone-like activity was evaluated by a bioassay. HL extracts were then applied to maize plantlets and

their effect on the growth, nitrogen metabolism, and photosynthetic parameters of the plants was considered. The relationship between some chemical characteristics of HL and their overall biostimulant activity was then discussed.

2. Materials and Methods

2.1. Humic-like substances (HL)

The humic-like components (HL) were extracted from agro-industrial wastes including: oil extraction residues from rape (B-HL, *Brassica napus* L.), castor-oil plant (R-HL, *Ricinus communis* L.) and flax (L-HL, *Linum usitatissimum* L.) and from digestate (D-HL). The latter was obtained from an agro-livestock farm which uses an anaerobic digestion plant to produce biogas. The extraction procedure was inspired by a previously reported procedure (Montoneri et al., 2009) and is described below. 200 g of finely ground waste was treated with aqueous 0.1 mol L^{-1} KOH at a 1:5 w/v (waste/solution ratio). The suspension was shaken under N_2 for 20 hours, allowed to settle overnight then centrifuged (3,000 rpm for 20 min) to separate the supernatant. A triplicate series of alternate treatments with 0.1 mol L^{-1} KOH was carried out on solid residue in order to solubilise and totally remove the humic-like (HL) substances. The supernatant solutions obtained were collected and freeze dried.

2.2. Basic characterization of HL

The C, N, S content of B, R, L and D -HL was measured using an element analyzer (vario MACRO CNS , Hanau, Germany). The HL were characterised for total acidity group content (TA) in accordance with the barium hydroxide method proposed by Swift (1996). Soluble phenolic acids were extracted with 3 mL pure methanol (1:10 w/v). The extracts were maintained in an ice bath for 30 min and centrifuged at 5,000 *g* for 30 min at 4 °C. The

supernatants were stored at -20 °C until analysis. Total phenols were measured according to Arnaldos et al. (2001). Flavonoids were extracted from 1 g material using 50 mL of acidified methanol solution. The extracts were kept at 4°C for 16 h before measuring the absorbance at λ =300 nm. Flavonoids have been expressed as gallic acid equivalents.

2.3. FT-IR spectra

The FT-IR spectra were obtained with a Perkin-Elmer FT-IR 2000 spectrometer (Phoenix Equipment Inc., NY), equipped with an IR source, KBr beam splitter and DTGS KBr detector. For each sample, 64 scans were recorded with a resolution of 4 cm^{-1}, over a range of 4000-400 cm^{-1} , on pellets which were obtained by pressing (10,000 kg for 30 min) a mixture of 1 mg of HL sample and 400 mg dried KBr under reduced pressure.

2.4. Solid-state CP MAS ^{13}CNMR spectra

The solid-state CP MAS ^{13}C NMR spectra of the samples were acquired at 10 kHz on a Bruker AMX 600 spectrometer (Bruker BioSpin GmbH, Rheinstetten) using a 4 mm CP-MAS probe. The pulse repetition rate was set at 0.5 s, the contact time at 1 ms and the number of scans was 1800. A contact time of 1 ms was obtained after the VCT experiments. The error in signal acquisition caused by using the average contact time was determined by comparing the signal intensity in the absence of carbon relaxation – I_0 and the intensity of the signal I_{tcp} measured at the optimal contact time. In these conditions, it was shown that CP MAS ^{13}C NMR provides a quantitative representation of the C content in humic substances (Conte et al., 2002). The reference standard for the chemical shift scale of the CPMAS-^{13}C NMR spectra was tetramethylsilane (δ = 0 ppm). For a semi-quantitative approach, the ^{13}C -NMR spectra were subdivided into five regions. Five types of carbon can be distinguished in the NMR spectrum (Table 1): (i) short chain aliphatic carbon (e.g., volatile fatty acid and steroid-like

molecules) (Réveillé et al., 2003) (0–28 ppm); (ii) long chain aliphatic carbon (e.g., plant aliphatic biopolymers) (Pereira et al., 2005) and proteins (Dignac et al., 2000) (28–47 ppm); (iii) O-alkyl carbon (e.g., polysaccharides) (Kögel-Knabner, 2002) (47–113 ppm); (iv) aromatic carbon (e.g. lignin) (Ussiri and Johnson, 2003) (113–160 ppm); and (v) carbonyl carbon in aliphatic esters, carboxyl groups, and amide carboxyl (160–210 ppm). Spectra were elaborated using TOPSPIN 1.3 software (Bruker BioSpin GmbH, Rheinstetten, Germany).

2.5. Indoleacetic acid quantification

Indole-3-acetic acid (IAA) was quantified by using an enzyme linked immuno-sorbent assay (ELISA) (Phytodetek, Sigma, St. Louis, MO). The ELISA test utilized monoclonal antibodies sensitive in the 0.05–100 picomol IAA range. The tracer and standard solutions were prepared following the manufacturer's instructions, and the absorbance values were read at λ = 405 nm with a Biorad microplate reader (Hercules, CA). Details of this test as applied to HS have been previously reported (Schiavon et al., 2010).

2.6. Phytohormone-like activity

The hormone-like activity of B-HL, R-HL, L-HL and D-HL was assessed by measuring the reduction in root growth in watercress (*Lepidium sativum* L.) (Audus, 1972). Watercress seeds were surface-sterilized by immersion in 8% hydrogen peroxide for 15 min. After rinsing 5 times with sterile distilled water, 15 seeds were placed on sterile filter paper in a sterile Petri dish. The filter paper was wetted with 1.2 mL 1 mM $CaSO_4$ (control), or 1.2 mL of 10, 1, 0.1, or 0.01 mg L^{-1} indoleacetic acid (IAA) (Sigma, St. Louis, MO) to obtain the calibration curve, or 1.2 mL of a HL solution ranging from 10 mg mL^{-1} - 0.01 μg mL^{-1}. The seeds were allowed to germinate in the dark at 25°C. After 48 h the seedlings were removed and the root lengths were measured with a TESA-CAL IP67 electronic caliper (TESA, Renens, CH) and Data

Direct software, version 1 (ArtWare, Asti, IT). Data from the standard curve were transformed on natural logarithmic scale to obtain the best linear fitting. Regression analysis was used to calculate the HL dose–response curves. Both the standard curve and the progression of HL dilutions were repeated twice with three replicates per experiment. The standard deviations were always less than 5% with n = c.a. 90.

2.7. Plant material

Zea mays L. seeds (var. DKc 5783, DeKalb, Lodi, IT) were soaked in distilled water for one night and then surface-sterilized in 5% (v/v) sodium hypochlorite for 10 min while shaking. The seeds were left to germinate for 60 h in the dark at 25°C on a filter paper wetted with 1 mM $CaSO_4$ (Nardi et al., 2000). Germinated seedlings were transplanted into 3 L pots containing an aerated modified Hoagland solution (Hoagland and Arnon, 1950), with a density of 24 plants per pot. 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), $CuCl_2 * 2H_2O$ (0.036), $MnCl_2 * 4H_2O$ (0.9), $ZnCl_2$ (0.09), NaMoO * $2H_2O$ (0.01). Plants were cultivated in a climate chamber under a14 h light/10 h dark cycle, with an air temperature of 27/21°C, relative humidity of 70/85% and at a photon flux density of 280 mol $m^{-2}s^{-1}$. Twelve days after transplanting B, R, L or D -HL was added to the nutrient solution contained in the pots at different concentrations: 0 (Control, CTR), 0.1 and 1 mL L^{-1} from a initial solution of 100 mg 10 mL^{-1}. After 48 h, plants were randomly harvested from three pots per treatment and then fresh samples of roots and leaves were carefully washed and dried with blotting paper. A sub-sample of the plant material was immediately frozen with liquid nitrogen and kept at -80 °C for physiological analyses. For dry weight measurement, thirty plants were used (ten per treatment from each pot). Plants were divided into roots and leaves, and weighed separately. The samples were placed in a drying

oven for 2 d at 70° C and allowed to cool for 2 h inside a closed bell jar. The dry weight was measured per plant.

To evaluate the possible physiological effect of a high K^+ or NH_4^+ concentration in the extracts, the concentration of the two ions in the solutions used for the treatments was determined. Aqueous solutions containing HL (100mg $10mL^{-1}$) were diluted 1:1000 (v:v) and analysed for K^+ and NH_4^+ content by ICP and ion chromatography, respectively. The amount of K^+ was 0.49, 0.49, 0.50 and 1.13 mg L-1 for B-HL, R-HL, L-HL, and D-HL, respectively. The NH_4^+ content was 0.03, 0.05, 0.13 and 0.05 mg L-1 for B-HL, R-HL, L - HL and D-HL, respectively.

2.8. Analysis of total nitrogen, nitrate, and soluble proteins

The nitrogen content was measured using a dry combustion procedure inside an element analyzer (vario MACRO CNS,Hanau, Germany). Root and leaf tissues (1 g) of five representative plants per pot were frozen in liquid nitrogen and homogenized (1:5 w/v) in 10 mM HCl to determine nitrate content. The extract was filtered through two layers of muslin and clarified by centrifugation at 35 000 g for 15min. All the steps were performed at 4°C. The supernatant was filtered (0.22 μm; Membra-Fil® Whatman Brand, Whatman, Milano, Italy). The quantification of NO_3^- was performed by HPLC using an AS 4S-SC anionic exchange column (Dionex, Sunnyvale, CA, USA), equipped with a Dionex suppressor and a 431 conductivity detector (Waters-Millipore, Milford, MA, USA). The eluent was a solution of sodium bicarbonate and sodium carbonate (1.7 mM $NaHCO_3$/1.8 mM Na_2CO_3) at a 2mL min–1 flow rate. Sodium nitrate (Fluka, Buchs, Switzerland).was used as a reference standard. Nitrate concentration is expressed as NO_3^- μmol g^{-1} fresh weight.

To extract the proteins, the 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 14,000 *g*. The supernatants were mixed with 10% (w/v) trichloroacetic acid and centrifuged. The pellets obtained were re-suspended in 0.1 N NaOH. The protein concentration was analyzed according to Bradford (1976) using a UV/vis spectrophotometer (Lambda 1, Perkin-Elmer, Monza, Italy) at $\lambda = 595$ nm. The soluble protein concentrations are expressed as mg of protein g-1 fresh weight.

2.9. Determination of chlorophyll content

To determine the chlorophyll content, 300 mg of fresh foliar tissue from five representative plants per pot were ground in liquid nitrogen and extracted with 15 mL ethanol (96% v/v). The samples were kept in the dark for 2 d at 4 °C, and the extracts were filtered and then analyzed spectrophotometrically (UV/VIS Lambda 1; PerkinElmer, Norwalk, CT) at $\lambda = 665$ nm for chlorophyll *a*, 649 nm for chlorophyll *b*, and 470 nm for total carotene (TC). Chlorophyll *a*, *b* and TC concentrations were calculated using the Welburn and Lichtenthaler (1984) formula and expressed in mg of pigment per g^{-1} of leaf fresh weight. Determination of the relative chlorophyll concentration was also performed using a non-destructive method: a SPAD (Soil Plant Analysis Development) chlorophyll meter (Minolta, SPAD-502, Osaka, Japan) on the last expanded leaf of the maize plants. Three measurements were performed for each plant, on six plants per treatment.

2.10. Enzyme extraction and assay conditions

N reduction and assimilation enzymes were extracted by grinding root and leaf tissues (1 g) in a mortar with a 100 mM Hepes-NaOH solution at pH 7.5, 5 mM $MgCl_2$ solution, and 1 mM dithiothreitol (DTT) solution. The ratio of plant material to solution mixture was 1:3. The extract was filtered through two layers of muslin and clarified by centrifugation at 20,000 *g* for 15 min. The supernatant was used for enzymatic analysis. All the steps were carefully

performed at 4 °C. Nitrate reductase (NR) (EC 1.7.1.1) activity was determined in an assay containing 100 mM KH_2PO_4, 100 mM KNO_3 and 400 µL of enzyme extract. The activity was measured spectrophotometrically at λ= 540 nm and the calibration curve was carried out with known concentrations of $NaNO_2$ (Lewis et al., 1982). For the 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 adding 250 µL of a mixture (1:1:1) of 10% (w/v) $FeCl_3*6H_2O$ in 0.2 M HCl, 24% (w/v) trichloroacetic acid and 50% (w/v) HCl. The optical density was recorded at λ = 540 nm (Canovas et al., 1991). The glutamate synthase (GOGAT, EC 1.4.7.1) assay contained 25 mM Hepes-NaOH (pH 7.5), 2 mM 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 (Avila et al., 1987). Enzyme activities were repeated twice with three replicates per experiment.

2.11. Statistical analysis

Barlett's test was used on the data to test the homogeneity of variance. A two-way ANOVA was used to compare treatment effects. The factors considered were the type of HL and its concentration. The LSD test was applied to compare the difference between group means. Correlations between variables were determined using Spearman's coefficient. All statistical analyses were performed using SPSS for Windows software, version 18.0 (SPSS, Chicago, IL).

3. Results and Discussion

3.1. Basic characterization of HL

The characterization data for HL are shown in Table 1. The ash content is highest for D-HL and lowest for R-HL. The ash content of more than 500 g Kg^{-1} found in the humic-like substances extracted from digestate attest to the presence of more inorganic than organic material. However, the high ash content in all the HL emphasize the importance of a purification step to obtain ash-free humic or humic-like fractions. In the present study, the purification step was intentionally omitted in order to make the extraction process faster and easier.

On a moisture and ash free basis, therefore, the following considerations can be made regarding the organic component of each fraction. The percentage of C is highest for R-HL and lowest for D-HL, while for B-HL and L-HL the contents are similar. The value of carbon for R-HL agrees with those determined for humic-like acids extracted from cattle manure, municipal solid waste and composted herbaceous material (Piccolo et al., 1992; Garcia et al., 1989). The values obtained for B-HL and L-HL are also similar to those determined for humic-like substances from farmyard and poultry litter (about 30% of Carbon - Pandeya, 1992; Prasad and Sinha, 1984). The content of D-HL is very close to that found for fulvic acid-like extracts from sewage sludges (approximately 40% of C; Sposito et al., 1982).

The nitrogen level is high for humic-like acids extracted from the agro-industrial residues while it is low for the extract from the digestate. The latter result is probably due to a loss of this element after anaerobic digestion by microorganisms. The values found for B-HL, R-HL and L-HL are similar to those found by other authors (Senesi et al., 1996) for humic-like acid extracted from various agricultural wastes. As a consequence of the above, the C / N ratio is lowest for B-HL and highest for D-HL.

The values of total acidity content are higher for D-HL and L-HL than those determined for B-HL and R-HL. The D-HL and L-HL values obtained are higher than those reported by Pandeya (1992) and Prasad and Sinha (1984) for humic-like substances from farmyard and poultry litter, respectively (TA about 11.5 meq g^{-1}). The value obtained for R-HL is comparable to those determined for humic-like acids extracted from cattle manure, citrus-pulp, municipal solid waste and composted herbaceous material (Piccolo et al., 1992; Meli et al., 2007; Garcia et al., 1989; Hammouda and Adams, 1987). Finally, a value similar to that found in humic-like acids extracted from sewage sludges (TA about 8.0 meq g^{-1}; Garcia et al., 1989) was obtained for B-HL.

3.2. FT-IR spectra

The assignment of the bands in the FT-IR spectra for the four humic-like substances extracted was performed in accordance with Stevenson (1982), Senesi et al. (1996) and Baglieri et al. (2012).

The 2922 and 2848 cm^{-1} bands are attributed to the C-H stretching of the $–CH_2-$ aliphatic groups, the 1715 cm^{-1} band is assigned to the C=O stretching of the –COOH groups, the 1633 cm^{-1} band is attributed to the C=O stretching of the linked quinones and/or ketones or to the C=O stretching of the amidic groups (amide I band), the band at about 1515 cm^{-1} is attributed to the vibration of the C=C of the II amides or $-COO^-$ symmetric stretching, the 1460 cm^{-1} band is attributed to the C-H stretching of the aliphatic groups and the bands at 1125 cm^{-1} and 1030 cm^{-1} to the C-O stretching of the polysaccharides or to the Si-O stretching of silicates. The spectra (Fig. 1) obtained for B-HL and R-HL are very similar in shape. In both the peaks attributable to the C-H stretching of aliphatic groups -CH2- (2922, 2848 and 1460 cm^{-1}), the C=O stretching of COOH functional groups (1712 cm^{-1}), the C=O stretching of quinones and/or ketones or conjugated to the C=O stretching of the amide groups (amide I band) (1633

cm^{-1}) are very evident. The last assignment is the most probable for organic fractions rich in nitrogen such as those studied (Table 1).

The L-HL and D-HL spectra are also similar but different from those described previously. In the flax and digestate extracts the peaks attributed to the deformation of the aliphatic groups were less evident than in extracts from castor-oil plant and rape (2923, 2852 and 1460 cm^{-1}), the peak at 1715 cm^{-1} also disappeared in L-HL and D-HL. Finally, peaks attributable to the polysaccharides and inorganic impurities (1125 and 1028 cm^{-1} respectively) are evident in these HL. The inorganic impurity content would seem to agree with the high ash content of L-HL and D-HL (Table 1). However, the presence of compounds containing Si-O groups, such as silicate minerals, is unlikely in alkaline extracts from biomass.

3.4. ^{13}C CPMAS NMR analysis.

^{13}CPMAS-NMR provides qualitative and quantitative information on the composition of the humic-like components by identifying the main C-type constituent of the organic matter (Conte et al., 1997). The relative changes in the levels of C containing groups are presented in Table 2. The B-HL and L-HL spectra are characterized by signals in the 0-47 ppm area attributable to the presence of alkyl-C (fatty acids and lipids) but also to the presence of proteins (Dignac et al., 2000). Peaks in this region (15-25 ppm) were representative of the presence of short alkyl-C chains such as fatty acids (Tambone et al., 2009). On the other hand, peaks observed in all the samples at 30-40 ppm are indicative of the presence of aliphatic-C with long polymethylenic chains, typical of vegetables (e.g. cutine, waxes and suberine) and/or of long chain fatty acids present in the oil extraction residues (Dignac et al., 2000; Pichler et al., 2001). The area at 165-210, was due, above all, to the presence of carboxylic-C, probably associated with the presence of both short and long fatty acids.

The ^{13}C NMR spectra of D-HL is dominated by signals in the 47-113 ppm area (O-alkyl carbon, e.g., polysaccharides), in agreement with the FT-IR spectra results. Anaerobic digestion led to a change in the chemical composition of the ingestates. In particular, the process induces a great reduction in the aliphatic-C content and increases the O-alkyl-C content (Pereira et al., 2005). These results indicated that there was a widespread degradation of fatty acid during anaerobic digestion and that a relative concentration of the O-alkyl fraction occurred. This interpretation was confirmed by the fact that the -carboxyl-C area decreased proportionally to the alkyl-C area. The particular peak at 56 ppm corresponds to carbon atoms substituted by amino groups, i.e., in peptides and amino acids but it could also indicate the –OCH3 of both lignin and hemicelluloses. The peak close to 72 ppm, is due to the O-alkyl C of the C-2, C-3 and C-5 atoms of polysaccharides (cellulose and hemicelluloses). The 105 ppm peak represents the anomeric carbon atoms (C-1) of cellulose and the peak at 65 ppm is due to C-6 in hexose and/or C-5 in pentose (Veeken et al., 2001). In addition in the D-HL spectrum there is a peak at 158 ppm which is higher than that present in the B-HL and L-HL spectra, where it appears at 153 and 157 ppm, respectively (Fig. 2). On the contrary, this signal is not present in R-HL. In ^{13}C-NMR spectra of humic substances, the adsorption near 158 ppm is usually attributed to the methoxylic group bound to an aromatic ring carbon (Senesi et al., 1989).

Finally, the R-HL spectra is dominated by signals in the 47-113 ppm area (O-alkyl carbon). Interestingly, there are the peaks in this spectra in the 115-160 ppm area, indicating the presence of lignin (Ussiri and Johnson, 2003) but also of phenols and poly-phenols, which are typically contained in oil residues.

In conclusion, on the basis of the distribution of the various types of carbon, B-HL and L-HL are similar; R-HL is richer in substituted aliphatic compounds (eg, polysaccharides and

proteins) and more aromatic than the previous fractions. While D-HL has a similar aromatic C content to R-HL.

3.5. Biochemical characterisation of HL

HL substances contained differing amounts of indoleacetic acid (IAA), total phenolic acids (TP), and flavonoids (FL) (Table 3). Of the bio-products, digestate had the highest contents of all three types of biomolecule, R-HL had the lowest amounts of IAA and TP, whereas L and B –HL had intermediate values of IAA and phenolic acids. In order to perform some activities HS and biostimulants must not only possess biomolecules but they should also be present in sufficient quantity to be physiologically active (Nardi et al., 2009; Quilty and Cattle, 2011). In HL IAA was present in a wide concentration range from 9.47 to 32.63 nmol L^{-1}. These amounts are consistent with those hydrolysed from alfalfa (18 nM) (Ertani et al., 2012) and in different humic fractions extracted from earthworm coprolites (27-34 nM) (Schiavon et al., 2010; Trevisan et al., 2010). Total phenolic acids were low when compared with dry apple and blueberry derived products (530 – 710 μM) (Ertani et al., 2011). To our knowledge no comparisons are possible for flavonoids as this is the first time that these compounds have been determined in bio-products to be used for plant nutrition.

3.6. Auxin-like activity

The bioactivity of the IAA present in HL was checked by evaluating the effect of HL on the growth of watercress (*Lepidium sativum* L.) which is a typical bioassay for auxins (Audus, 1972). Results showed that the type of treatment and concentration ($P \leq 0.05$) significantly affected watercress growth . The standard dose-response curve (IAA) fitted at a *P* value ≤0.001 and it was found that as the concentration of auxin increased root lengths shortened considerably (Fig. 3) (Table 4). HL dose-response curves behaved similarly with respect to

auxin and a significant shortening in the length of roots was found as the carbon (C) concentration increased (Fig. 3) (Table 4). The type of treatment affected watercress growth in the order IAA>D-HL>B-HL=L-HL>R-HL ($P \leq 0.05$). However, if the curve parameters (coefficient b) and C concentration intervals at which the curves significantly fitted are taken into consideration, the results show that both D and L –HL were more effective than B-HL. In fact, D and L -HL produced significant curves with a larger carbon concentration range than that of B-HL (Fig. 3). B-HL had a high modal value of b coefficient but was effective only in a narrow C concentration interval whereas R-HL had both a higher b coefficient and a narrower dosage interval, thus indicating a very low level of IAA activity (Table 4) (Fig. 3). The auxin-like effect of HS has long been recognized (for a review see Nardi et al., 2009) and the physiological role of the auxin entrapped in the HS structure has recently been confirmed by a molecular approach (Trevisan et al., 2010). Nevertheless, HS with the same amount of IAA but with a different molecular mass did not induce the same effect (Muscolo et al. 2007a,b). An auxin-like activity is exerted by other humic substance components such as the carboxylic groups, key receptors in triggering the bioavailability of IAA, the functional groups in general, and phenolic compounds (Jindo et al., 2012; Muscolo et al., 2007a,b; Rubery, 1981; Napier, 2001, 2004; Canellas et al., 2010). The role of phenolic acids in plant growth and metabolism has been the object of intensive studies (Inderjit, 1996; Mandal et al., 2010). At concentrations of 0.1 – 1 mM, many phenolic compounds are toxic to plants, especially seedlings. Nevertheless, at lower concentrations a number of phenolic acids have been shown to have effects similar to those of indoleacetic acid or gibberellic acid (Hrubcova et al., 2000; Pizzeghello et al., 2006). Flavonoids in plants protect against UV radiation, function as antioxidants and auxin transport regulators, have a role in plant micro-organism signalling, and as a defence against pathogens (Hassan and Mathesius, 2012). In the rhizosphere, as well, flavonoids can have multifunctional roles regulating root growth and

functions and influencing nutrient cycles such as N cycle (Cesco et al., 2012). They are present in the rhyzosphere in a concentration ranging from 1 μM to 10^{-10} M (Hassan and Mathesius, 2012), thus the concentration used in our experiments can be physiologically active. As a result of the above, IAA, total phenolic acids, and flavonoids were among the bioactive components in the four HL.

3.7. Biological activity in maize

Maize plantlets were significantly affected by the type of treatment and concentration used. Treatment affected the fresh and dry weight with slight increments in the roots and high increments in the (Tables 5). Root fresh weight was positively influenced when plants were treated with 0.1 mL L^{-1} of both D-HL (+14%) and L-HL (+12%) or 1 mL L^{-1} B-HL (+15%), whereas root dry weight was increased when plants were treated with L-HL at a concentration of 1 mL L^{-1} (+13%). Decrements or no differences with respect to the control were found for the other bio-products. The fresh to dry weight ratios (Table 5) also confirmed the effectiveness of D-HL on root fresh weight and of L-HL on the dry weight. As far as leaves are concerned, almost all the products induced significant increments both in fresh and dry weight (Table 5). The most bioactive products were B-HL at 0.1 ml L^{-1} (+31%) and D-HL at 1 mL^{-1} L (+28%) for the fresh weight, and L-HL (+26%), B-HL (+24%) and R-HL (+21%) at 0.1 mL L^{-1} for the dry weight. These results were confirmed by considering the fresh to dry weight ratio (Table 5). However, from the root to shoot ratio (Table 6) it can be seen that treatment induced decrements as compared to the controls, both for the fresh weight and dry weight and the lowest values were recorded for R-HL at 1 mL L^{-1} (0.265 and 0.420, respectively). Thus it appears that the bio-products mostly affect the plant weight at shoot level. It is known that HS induce a general increment in the fresh and dry weight of both roots and leaves according to the type and concentration of the HS and the plant species, age and

organ (Nardi et al., 2009). These results are also in accordance with the growth increments observed in maize roots and shoots treated with two protein-hydrolyzate-based fertilizers, one from alfalfa and one from meat flour (Ertani et al., 2009). Moreover, the addition of the different extracts to the Hoagland solution determined only a low increase in the K^+ and NH_4^+ ion content, therefore it is unlikely that the effects of HL on the plants is due to the presence of these ions.

With regard to the nitrate metabolism, it was evident that all the treatments induced a decrement in the nitrate content and an increment in the protein content, both in roots and leaves, against a steady level of total nitrogen content (Table 7). Both the type of treatment and concentration slightly influenced nitrate reductase (NR), whereas high increments were found in glutamine synthethase (GS) and glutamate synthase (GOGOAT) activity (Table 8). In roots, NR had a slightly positive effect induced by D, L and R –HL (+7%, +10%, and +5%, respectively). For GS and GOGAT, high increments were reported in the order D-HL>R-HL>B-HL. In particular, D-HL showed the highest increments of GS and GOGAT enzyme activity with values up to threefold those of the control. In leaves, slightly positive effects were recorded by L-HL and B-H in the activity of NR, whereas for GS and GOGAT high increments were found in the order D-HL>R-HL=B-HL>L-HL with values up to +190% more than the control.

Nitrogen is the major limiting factor in plant growth and productivity, therefore it is of great importance to study the enzyme activity related to its organication. The enzymes nitrate reductase (NR) and nitrite reductase catalyze the two-step reduction of nitrate (NO_3^-) to ammonium (NH_4^+), which is rapidly incorporated into organic compounds through the activity of the enzyme GS (Lea and Ireland, 1999). GS works in association with the enzyme glutamate synthase (GOGAT) to produce glutamate (Glu) from glutamine and α-ketoglutarate in the GS/GOGAT cycle. Thus, from our results, the reduction in nitrate content and the

stimulation of the enzyme activity was transformed into an increment of the protein content both at root and leaf level. HL induced an increment in nitrogen organication, as reported for other biostimulants and humic substances (Chen et al., 2003; Quaggiotti et al., 2004; Ertani et al., 2009).

The interaction between N and photosynthesis is fundamental for crop production, and crosstalk was shown here by the high increase in nitrogen assimilation via GS and GOGAT enzyme activity following treatment. As compared to control plants, the chlorophyll contents and SPAD measurements indicated that treatment had induced significant effects on photosynthesis (Table 9). In particular, L-HL and B-HL treated plants had higher chlorophyll *a*, *b* and total carotene contents than the controls, whereas B-HL and D-HL induced the highest SPAD values. In support of this a correlation was also found between GS and GOGAT in roots (R^2= 0.98, p≤0.000), and between root and leaf GS (R^2= 0.67, p≤0.000), and GS and GOGAT in leaves (R^2= 0.97, p≤0.000) . A significant relationship between the SPAD index and photosynthesis-related N-metabolites such as chlorophyll *a* and *b* has been also reported (Debaeke et al., 2006). We found significant correlations between SPAD and GS in leaves (R^2= 0.30 p≤0.000), and between SPAD and GOGAT in leaves (R^2= 0.31 p≤0.000), while a strong correlation was seen between chlorophyll *a* and leaf NR (R^2= 0.81 p≤0.000). From our results it was evident that the SPAD index alone was not sufficient to evaluate photosynthetic efficiency and therefore chlorophylls and/or the activity of related enzymes must also be taken into consideration.

In conclusion, the four HL differed in their auxin, phenolic acid and flavonoid content and in their hormone-like activity. All the HL were efficient in increasing the protein content of maize and the activity of enzymes related to N assimilation and photosynthesis. Other bio-products, as humic-like substances from feces of earthworms and commercial lignosulfonate-humate, have recently been shown to affect plant metabolism by influencing nitrogen

assimilation, the Krebs cycle, and the phenylpropanoid metabolism mostly as a result of their hormone-like activity (Schiavon et al., 2008; Ertani et al., 2011). These results, added to the results of our own research, suggest the existence of divergent but overlapping regulation mechanisms between bio-products and humic substances. In summary, this study has found that the humic-like substance from digestate has improved the nutrition and metabolism of maize plants due to the following parameters: high IAA and TP content, the high total acidity and the carbon distributio; similar parameters were found in a bioactive low-molecular weight humic fraction (Nardi et al., 2007). However, further investigations are required for understanding the mode of action of humic-like substances regarding the biostimulation of plants.

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Figure captions

Fig. 1. FT-IR spectra of the humic-like (HL) substances isolated from: B, brassica, R, ricinus, L, linen and D, digestate.

Fig. 2. ^{13}C CPMAS-NMR spectra of the humic-like (HL) substances isolated from: B, brassica, R, ricinus, L, linen and D, digestate.

Fig. 3. Indoleacetic (IAA) standard curve and dose-response curves for the four humic-like (HL) substances obtained by checking the changes in length of watercress plantlet roots. In HL fitted points were: B, brassica, R, ricinus, L, linen and D, digestate.

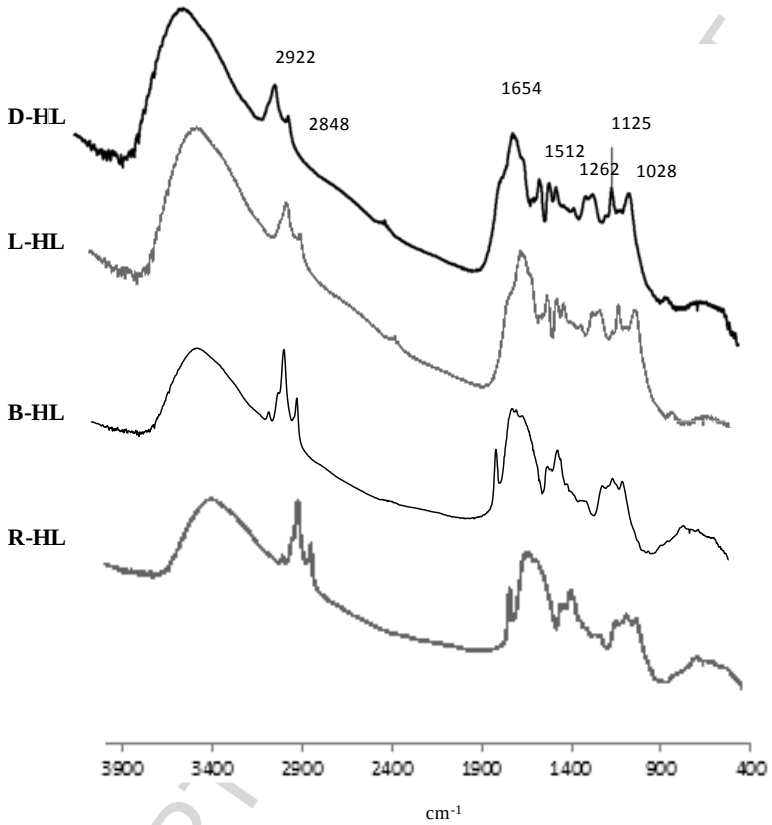

Fig. 1

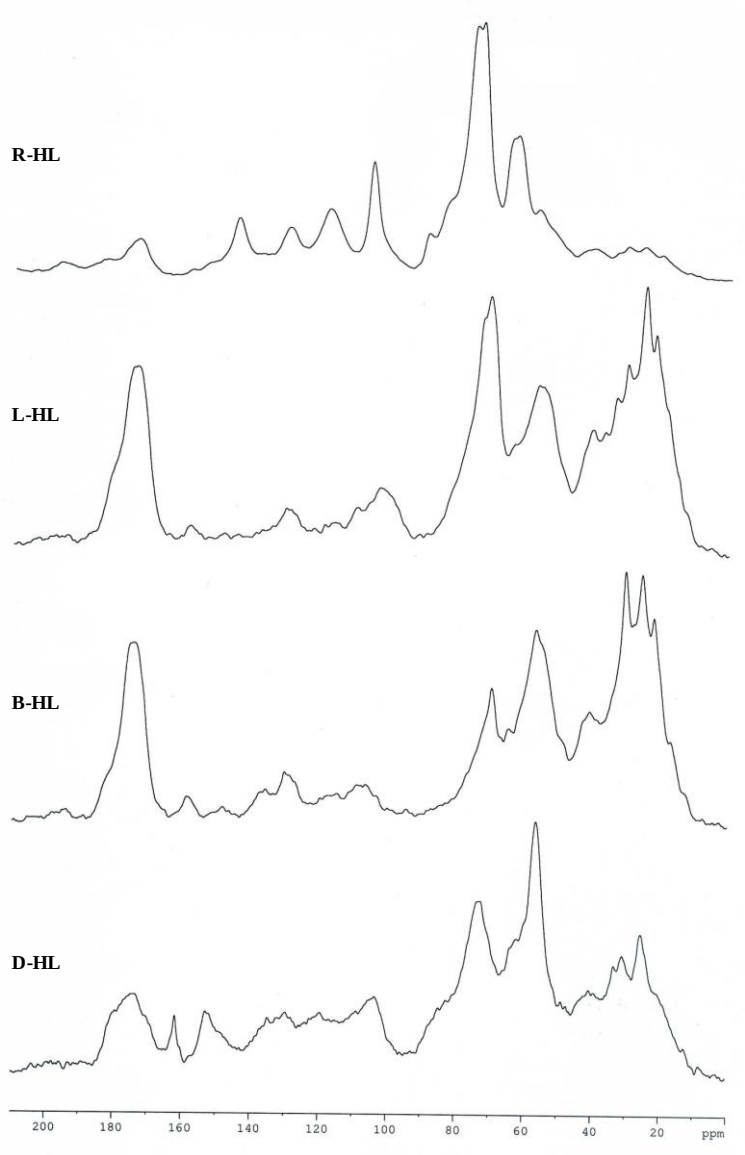

Fig. 2

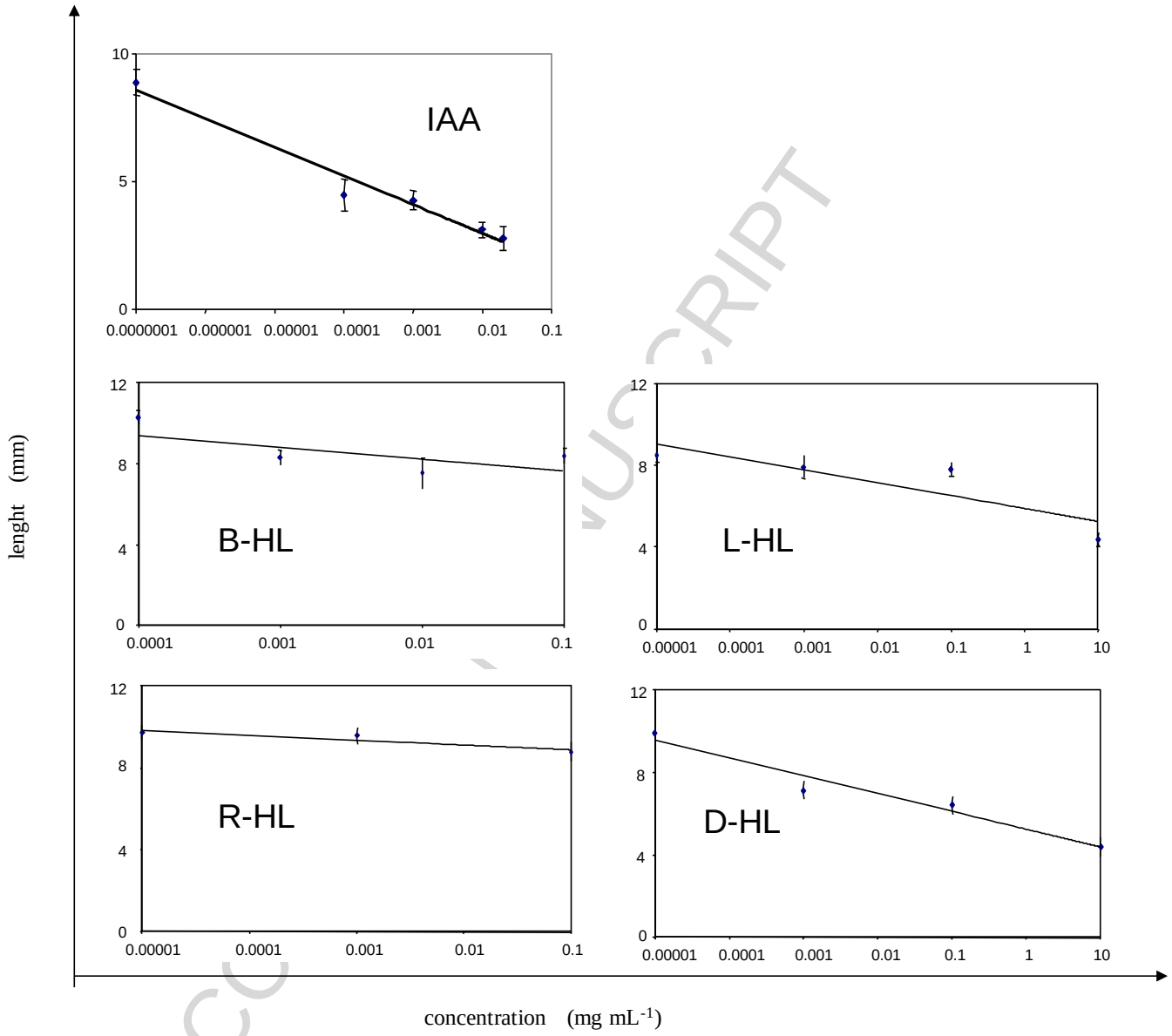

Fig. 3

Table 1. Ash, elemental composition, atomic ratio and total acidity content of the four bio-products.

Sample	Ash	C	N	S	C/N	Total acidity
	(%)				Atomic ratio	meq g^{-1}
B-HL	19.2	39.3	9.5	0.8	4.8	8.4
R-HL	13.2	48.0	7.5	1.8	7.5	4.3
L-HL	27.6	36.3	6.0	0.6	7.1	13.1
D-HL	54.7	29.2	2.4	0.7	14.2	18.7

Table 2. ^{13}CPMAS-NMR integrated area of different carbon types for the four bio-products.

Samples	C-type			
	0-47 ppm total aliphatic C	47-113 ppm N-alkyl O-alkyl C di-O-alkyl C	113-160 ppm aromatic C	160-210 ppm carboxyl C keto C
B-HL	39.8	35.8	8.6	15.8
R-HL	10.9	63.8	17.8	7.5
L-HL	34.7	42.2	7.0	16.1
D-HL	23.8	48.6	16.0	11.6

Table 3. Indoleacetic acid (IAA), total phenolic acid (TP), and flavonoid (FL) content in the 4 humic-like (HL) substances (brassica, B, ricinus, R, linen, L and digestate, D).

Treatment	IAA nmol L^{-1}	TP nmo lL^{-1}	FL mmol L^{-1}
B-HL	14.42±0.51b*	5.48±0.18c	1.86±31c
R-HL	9.47±0.38d	4.49±0.11d	2.52±15b
L-HL	11.54±0.42c	7.22±0.25b	0.96±33d
D-HL	32.63±1.17a	13.11±0.47a	3.66±45a

*Values in the same column followed by the same letter are not statistically different at $P<0.05$ as per the LSD test.

Table 4. Parameters of the regression curve (Y = a + b*log(X)) of the standard (IAA) and humic-like (HL) substances (brassica, B, ricinus, R, linen, L, and digestate, D) on the root reduction of 48h-old watercress plantlets.

Treatment	R2	*P*≤	a	b
IAA	0.970	0.001	4.10	-0.490
B-HL	0.509	0.020	6.99	-0.281
R-HL	0.877	0.050	8.65	-0.101
L-HL	0.734	0.001	5.90	-0.270
D-HL	0.953	0.001	5.21	-0.376

R^2 = R-Squared; P ≤ 95%; a = intercept; b= slope.

Table 5. Mean values of fresh weight (FW), dry weight (DW), and FW:DW ratio of 14 d-old maize plantlets treated with brassica (B), ricinus (R), linen (L) and digestate (D) humic-like (HL) substances.

Factor		Root			Leaf		
		FW g	DW g	FW/DW	FW	DW	FW/DW
T	CTR*	0.806B**	0.108A	7.48	2.026C*	0.173D	11.70
	B-HL	0.837A	0.105B	7.96	2.550A	0.207AB	12.33
	R-HL	0.702C	0.092C	7.66	2.405AB	0.200B	10.03
	L-HL	0.792B	0.113A	7.02	2.275B	0.217A	10.47
	D-HL	0.897A	0.088C	10.21	2.413AB	0.184C	13.08
C	0	0.806*a*	0.108*a*	7.48	2.026*b*	0.173*b*	11.70
	0.1	0.832*a*	0.100*b*	8.32	2.442*a*	0.204*a*	11.98
	1	0.777*b*	0.099*b*	7.89	2.370*a*	0.200*a*	11.85
TxC	CTR	0.806c	0.108b	7.48	2.026d	0.173c	11.70
	B-HL 0.1	0.732d	0.102b	7.19	2.651a	0.214a	12.36
	B-HL 1	0.931a	0.108b	8.60	2.460ab	0.200b	12.31
	R-HL 0.1	0.749d	0.101b	7.39	2.390b	0.209a	11.45
	R-HL 1	0.643e	0.079c	8.09	2.424ab	0.189bc	12.83
	L-HL 0.1	0.907a	0.107b	8.46	2.516ab	0.218a	11.54
	L-HL 1	0.612e	0.122a	5.02	1.896d	0.216a	8.77
	D-HL 0.1	0.921a	0.088c	10.47	2.224c	0.171c	12.98
	D-HL 1	0.872b	0.088c	9.94	2.602a	0.198b	13.17

*CTR= control.

[**]Values in the same column followed by the same letter are not statistically different at P<0.05 as per the LSD test. T = treatment; C = concentration; TxC = interaction.

Table 6. Root to shoot ratio of fresh weight (FW) and dry weight (DW) of 14 d-old maize plantlets treated with brassica (B), ricinus (R), linen (L) and digestate (D) humic-like (HL) substances.

Factor		Root/Shoot	
		FW	DW
T	CTR	0.398A**	0.622A
	B-HL	0.328B	0.509B
	R-HL	0.292C	0.458C
	L-HL	0.348AB	0.520B
	D-HL	0.372A	0.476C
C	0	0.398*a*	0.622a
	0.1	0.341*b*	0.491b
	1	0.328*c*	0.493b
TxC	CTR	0.398ab	0.622a
	B-HL 0.1	0.276c	0.475
	B-HL 1	0.376b	0.542b
	R-HL 0.1	0.313b	0.485c
	R-HL 1	0.265c	0.420d
	L-HL 0.1	0.361b	0.491c
	L-HL 1	0.323b	0.564b
	D-HL 0.1	0.414a	0.513bc
	D-HL 1	0.335b	0.444d

*CTR= control.

**Values in the same column followed by the same letter are not statistically different at $P<0.05$ as per the LSD test. T = treatment; C = concentration; TxC = interaction.

Table 7. Nitrogen, nitrate and protein content of maize plantlets treated with brassica (B), ricinus (R), linen (L) and digestate (D) humic-like (HL) substances.

		Roots			Leaves		
		N	NO_3^-	protein	N	NO_3^-	protein
		%	mmol g^{-1} fw	mg g^{-1} fw	%	mmol g^{-1} fw	mg g^{-1} fw
T	CTR	4.81B*	63.48B	1.16E	3.60B	179.25B	3.01D
	B-HL	9.54A	99.63A	3.37A	7.13A	310.98A	7.92A
	R-HL	4.77B	55.65C	1.67C	3.66B	132.98C	4.45C
	L-HL	4.85B	55.09C	1.42D	3.64B	132.84C	4.81C
	DG-HL	4.78B	54.75C	2.67B	3.58B	138.44C	6.75B
C	0	4.81*a*	63.48*a*	1.15*c*	3.60*a*	179.25*a*	3.01*c*
	0.1	4.77a	53.90b	1.90*a*	3.62*a*	143.05*b*	4.63*b*
	1	4,82a	53.75b	1.82*b*	3.60*a*	136.83*b*	5.36*a*
TxC	CTR	4.81a	63.48a	1.16d	3.60a	179.25a	3.01d
	B-HL 0.1	4.79a	49.78c	1.64c	3.57a	163.04b	3.39d
	B-HL 1	4.76a	49.84c	1.73b	3.56a	147.94c	4.53c
	R-HL 0.1	4.73a	55.21b	1.81b	3.66a	135.81c	4.27c
	R-HL 1	4.82a	56.09b	1.53c	3.65a	130.16c	4.64c
	L-HL 0.1	4.76a	56.22b	1.48c	3.71a	130.12c	4.23c
	L-HL 1	4.95a	53.96b	1.36d	3.58a	135.55c	5.38b
	D-HL 0.1	4.78a	54.39b	2.68a	3.56a	143.24c	6.61a
	D-HL 1	4.77a	55.11b	2.67a	3.60a	133.65c	6.90a

*Values in the same column followed by the same letter are not statistically different at $P<0.05$ as per the LSD test.

Table 8. Mean values for treatment (T), concentration (C) and TxC interaction of fresh and dry weight (FW, DW), and nitrate reductase (NR), glutamine synthetase (GS) and glutamate synthase (GOGAT) activities in roots of 14 d-old maize plantlets treated with brassica (B), ricinus (R), linen (L) and digestate (D) humic-like (HL) substances.

		Root			Leaf		
Factor		NR	GS	GOGAT	NR	GS	GOGAT
		nmol g^{-1} min^{-1}	mmol g^{-1} min^{-1}	μmol g^{-1}	nmol g^{-1} min^{-1}	mmol g^{-1} min^{-1}	μmol g^{-1}
T	CTR	1.336A	0.058D	8.30D	1.734C	0.049D	7.489E
	B-HL	1.092B	0.094C	13.38C	1.891B	0.116B	17.953B
	R-HL	1.021C	0.123B	18.74B	1.489D	0.113B	17.174C
	L-HL	1.368A	0.054D	8.56D	1.993A	0.094C	13.926D
	D-HL	1.336A	0.170A	25.71A	1.770C	0.141A	21.129A
C	0	1.336*b*	0.058*c*	8.30*c*	1.734*b*	0.049*c*	7.489*c*
	0.1	1.375*a*	0.103*b*	15.91*b*	1.713*b*	0.124*a*	18.849*a*
	1	1.011*c*	0.118*a*	17.29*a*	1.866*a*	0.107*b*	16.242*b*
TxC	CTR	1.336c	0.058e	8.30g	1.734d	0.049e	7.849g
	B-HL 0.1	1.155e	0.068d	10.35e	1.860bc	0.131b	19.876c
	B-HL 1	1.037f	0.118c	16.42d	1.918b	0.104c	16.029d
	R-HL 0.1	1.407b	0.129b	19.52b	1.231e	0.132b	19.751c
	R-HL 1	0.538g	0.114c	17.97c	1.811c	0.090d	14.596e
	L-HL 0.1	1.465a	0.051f	8.19g	2.041a	0.099c	14.933e
	L-HL 1	1.216d	0.058e	8.93f	1.918b	0.086d	12.920f
	D-HL 0.1	1.424ab	0.170a	25.58a	1.717d	0.139a	20.836b
	D-HL 1	1.248d	0.169a	25.86a	1.822c	0.142a	21.422a

*Values in the same column followed by the same letter are not statistically different at P<0.05 as per the LSD test. CTR = control.

Table 9. Mean values for treatment (T), concentration (C) and TxC interaction of chlorophyll a, b and total carotene and SPAD of maize plantlets treated with brassica (B), ricinus (R), linen (L) and digestate (D) humic-like (HL) substances.

		Leaf			
Factor		Chl a	Chl b	Total Carotene	SPAD
		mg g^{-1}			
T	CTR	0.681C	0.312C	0.651C	28.318C
	B-HL	0.802B	0.365B	0.798B	35.894A
	R-HL	0.289D	0.309C	0.667C	27.511D
	L-HL	1.008A	0.451A	0.939A	29.433C
	D-HL	0.608C	0.278D	0.593D	32.883B
C	0	0.681*a*	0.312*b*	0.651*b*	28.318*c*
	0.1	0.663*a*	0.374*a*	0.814*a*	30.582*b*
	1	0.689*a*	0.324*b*	0.673*b*	32.273*a*
TxC	CTR	0.681d	0.312c	0.651d	28.318d
	B-HL 0.1	0.734c	0.325c	0.725c	34.638b
	B-HL 1	0.863b	0.401b	0.862b	37.011a
	R-HL 0.1	0.197g	0.401b	0.857b	28.062d
	R-HL 1	0.404f	0.195e	0.430f	26.125e
	L-HL 0.1	1.014a	0.447a	0.954a	28.609d
	L-HL 1	1.000a	0.457a	0.917a	30.729c
	D-HL 0.1	0.691d	0.299c	0.674d	31.567c
	D-HL 1	0.525e	0.258d	0.511e	34.200b

*Values in the same column followed by the same letter are not statistically different at $P<0.05$ as per the LSD test. CTR = control.