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Chemical composition and bioactivity properties of size-fractions separated from a vermicompost humic acid

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ABSTRACT

Preparative high performance size-exclusion chromatography (HPSEC) was applied to humic acids (HA) extracted from vermicompost in order to separate humic matter of different molecular dimension and evaluate the relationship between chemical properties of size-fractions (SF) and their effects on plant root growth. Molecular dimensions of components in humic SF was further achieved by diffusion-ordered nuclear magnetic resonance spectroscopy (DOSY-NMR) based on diffusion coefficients (*D*), while carbon distribution was evaluated by solid state (CP/MAS) ^{13}C NMR. Seedlings of maize and Arabidopsis were treated with different concentrations of SF to evaluate root growth. Six different SF were obtained and their carbohydrate-like content and alkyl chain length decreased with decreasing molecular size. Progressive reduction of aromatic carbon was also observed with decreasing molecular size of separated fractions. Diffusion-ordered spectroscopy (DOSY) spectra showed that SF were composed of complex mixtures of aliphatic, aromatic and carbohydrates constituents that could be separated on the basis of their diffusion. All SF promoted root growth in Arabidopsis and maize seedlings but the effects differed according to molecular size and plant species. In Arabidopsis seedlings, the bulk HA and its SF revealed a classical large auxin-like exogenous response, i.e.: shortened the principal root axis and induced lateral roots, while the effects in maize corresponded to low auxin-like levels, as suggested by enhanced principal axis length and induction of lateral roots. The reduction of humic heterogeneity obtained in HPSEC separated size-fractions suggested that their physiological influence on root growth and architecture was less an effect of their size than their content of specific bioactive molecules. However, these molecules may be dynamically released from humic superstructures and exert their bioactivity when weaker is the humic conformational stability as that obtained in the separated size-fractions.

1. Introduction

The rising market for humic substances (HS) has attracted attention to compost as a possible economic source for the extraction of such substances instead of reliance on expensive fossil matrices, represented mainly by different kinds of mined lignite (e.g. leonardite) (Valdrighi et al., 1996). In spite to the large use of HS on plant crops since last century, only recently an increasing attention has been devoted to clarify the mechanisms by which HS influence biological activities of plants (Nardi et al., 2002).

HS were found to improve cell growth and nutrients uptake by forming soluble complexes with several ions (Pinton et al., 1999) and promoting cell energy as inducers of plasma membrane H^+-ATPase synthesis (Canellas et al., 2002). This enzyme cleaves ATP molecules and generate the electrochemical gradient that provide energy to secondary cell transporters (Morsomme and Boutry, 2000). However, studies on structural composition of HS and its relationship with plant stimulation effects have produced contradictory results. In particular, some works have shown that HS biological activity depends on their molecular dimension, being the low molecular size extract of humic matter the most active fraction (Piccolo et al., 1992; Nardi et al., 2002; Quaggiotti et al., 2004; Muscolo et al., 2007; Nardi et al., 2007). However, large molecular size HS were also found to readily act as root growth regulators (Zandonadi et al., 2007) by using cell auxin signaling (Dobbss et al., 2007).

Therefore, a renovated task is to achieve a better knowledge on the interaction between molecular distribution of HS and plant physiology. Recently, it was found that preparative high

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performance size-exclusion chromatography (HPSEC) was capable to fractionate HS into more homogeneous and chemically different size-fractions (Piccolo et al., 2002; Conte et al., 2007; Maia et al., 2008) and that diffusion ordered (DOSY) NMR spectroscopy efficiently evaluated the size of humic mixtures (Šmejkalová and Piccolo, 2008).

The aim of this work was then to relate the chemical properties of humic size-fractions separated by preparative HPSEC to the lateral root emergence of Arabidopsis and maize seedlings. The size-fractions were characterized by cross polarization magic angle spinning (CP-MAS) ^{13}C NMR and their size assessed by DOSY-H NMR. The ATP hydrolysis and H^+ transport across membrane vesicles were used as biochemical indicators of humic bioactivity.

2. Materials and methods

2.1. Isolation of humic acids (HA) from vermicompost

A vermicompost was obtained from mixture of plant residues from *Panicum maximun* Jacq. and cattle manure 5:1 (v/v). The organic residues were mixed and earthworms were added at a ratio of 5 kg earthworms (*Eisenia foetida*) per m^3 of organic residue. A bed of worms and organic residues was first prepared in a container and additional layers of organic residues were periodically placed over the pile as a function of temperature until the pile reached 50 cm. At the end of the transformation process (3 months after addition of the last organic residues), worms were removed into a pile of fresh organic residue (plant + cattle manure) placed in a corner of the container. The organic matter composition of the resulting vermicompost was: pH 7.8, 46.5 g kg^{-1} total organic carbon, and 17.3 g kg^{-1} HA carbon. HA were isolated from vermicompost and purified as reported elsewhere (Canellas et al., 2002). The HA were suspended in distilled water and titrated to pH 7.0 by automatic titrator (VIT 909 Videotitrator, Copenhagen) with a 0.1 KOH solution under N_2. The resulting potassium-humates were then passed through a 0.45 μm Millipore filter and freeze-dried.

2.2. Elemental composition

The elemental composition of humic materials was evaluated by a CHN Perkin Elmer autoanalyzer (14800). The oxygen content was obtained by difference and the ash content by incineration of 50 mg HA for 700 °C during 8 h. 50 mg of the bulk HA were mineralized with a solution of concentrated $HNO_3/HClO_4$ until fuming, transferred in 20 mL volumetric flasks, and brought to volume with bi-distilled water. This solution was analysed by atomic-absorption spectrometry (Perkin-Elmer AAS Analyst 700) and no trace of metal species (Fe, Mn, Cu, Al) and K was found.

2.3. HA fractionation by preparative HPSEC

The HPSEC mobile phase consisted of a 10 mM CH_3CO_2Na, 5 mM KCl and 1 mM CH_3CO_2H milli-Q water solution adjusted to pH 7.0 with 100 mM KOH. The same solution was used to dissolve the potassium-humates to a concentration of 600 mg L^{-1}. The humic solution was filtered through glass microfibre filters (Whatman GF/C) and loaded into a rheodyne rotatory injector, equipped with a 5 mL sample loop. The HPSEC system consisted of a Gilson autosampler model 231, a Gilson 305 pump, a preparative Biosep SEC-S-2000 (600 mm × 21.2 mm id) column, preceded by a Biosep SEC-S-2000 guard column (78.0 mm × 21.2 mm id) both from Phenomenex (USA), a Gilson 116 UV detector set at 280 nm, and a Gilson FC205 fraction collector, to automatically collect humic fractions in continuous. The elution flow-rate was set at 1.5 mL min^{-1} and all chromatographic runs were automatically recorded by a Unipoint Gilson Software. The six isolated size-fractions (SF) were first freeze-dried to reduce their volume, resuspended in a 5 mL of deionized water, dialyzed (Spectra/Por 6 dialysis tube, 1kD MW cut-off) against deionized water, and freeze-dried again. Out of 642 injections of HA solution (1926 mg), the weight measured for the six isolated size-fractions (SF1–SF6) was 492.6, 168.6, 369.1, 567.9, 61.0, 136.6 mg, respectively, for a total recovery of 93% (1798 mg) of initial HA weight.

2.4. Solid state NMR spectroscopy

Cross polarization magic angle spinning (CP-MAS) ^{13}C NMR spectra were acquired with a Bruker AVANCE™ 300, equipped with a 4 mm Wide Bore MAS probe, operating at a ^{13}C frequency of 75.475 MHz. Humic samples (100–200 mg) were packed in 4 mm zirconia rotors with Kel-F caps and spun at 13 ± 1 kHz. A ^1H Ramp sequence was used during a contact time of 1 ms to account for possible inhomogeneity of the Hartmann-Hahn condition. Scans (2000) with 3782 data points were collected over an acquisition time of 25 ms, and a recycle delay of 2.0 s. The Bruker Topspin 1.3 software was used for spectral collection and elaboration. All free induction decays (FID) were transformed by applying a 4 k zero filling and a line broadening of 75 Hz. Spectra were integrated in the chemical shift (ppm) intervals 188–162 (carbonyls of ketones, quinones, aldehydes and carboxyls), 162–112 (aromatic and olefinic carbons), 112–93 (anomeric carbons), 93–48 (C–O and C–N systems, as in alcohols, ethers, and aminoacids), and 48–0 ppm (sp^3 alkyl carbons). The relative areas of alkyl (48–0 ppm) and aromatic (162–112 ppm) components were summed to represent the proportion of hydrophobic carbons in humic samples (degree of hydrophobicity, HB). Similarly, the sum of relative areas in intervals related to polar groups (188–162 and 93–48 ppm) indicated the degree of carbon hydrophilicity (HI). Both HB and HI values were used to calculate the HB/HI ratio.

2.5. Diffusion-ordered spectroscopy (DOSY)

Solution–state DOSY-NMR spectra were obtained on a Bruker Avance 400 MHz instrument operating at a proton frequency of 400.13 MHz, equipped with a 5 mm Bruker inverse broadband probe. All spectra were elaborated by Bruker Topspin 1.3 (Bruker Biospin). Five milligrams of HA was dissolved in 0.75 mL of deuterated water (D_2O) and transferred to 5 mm NMR quartz tubes fitted with Doty Susceptibility plugs. ^1H NMR spectra were referenced to the chemical shift of solvent, resonating at 4.8 ppm and ^1H 90° pulse was calibrated using HOD signal. 2-D–DOSY diffusion–ordered spectra were obtained using a stimulated echo pulse sequence with bipolar gradients (STEBPGP) provided by watergate 3-9-19 pulse train with gradients for presaturation of water signal. Scans (320) were collected using 2.5 ms sine–shaped pulses (5 ms bipolar pulse pair) ranging from 0.674 to 32.030 G cm^{-1} in 32 increments, with a diffusion time of 100–160 ms, and 8 K time domain data points. Apodization was made by multiplying data with a line broadening of 1.0 Hz, spike suppression factor of 1.0, maximum interactions number set to 100, noise sensitivity factor of 2, and number of components set to 1. A mono-exponential decay without entropy minimization was applied during data processing. Diffusion coefficients of seven standard compounds of known molecular weight were measured in order to express diffusion as a function of molecular weight, from which diffusion data were approximated to molecular sizes. The selected standards: CH_3OH (32.0 Da), catechol (110.1 Da), caffeic acid (180.2 Da), catechin (290.0 Da), bromocresol green (698.0 Da), and two polystyrene sulfonates of 1100 and 6780 Da, were dissolved and acquired as for the HA samples.