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Relationship between molecular characteristics of soil humic fractions and glycolytic pathway and krebs cycle in maize seedlings

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Abstract

A humic acid (HA) isolated from a volcanic soil was separated in three fractions of decreasing molecular size (I, II and III) by preparative high performance size exclusion chromatography (HPSEC). The molecular content of the bulk soil HA and its size fractions was characterized by pyrolysis-GC-MS (thermochemolysis with tetramethylammonium hydroxide) and NMR spectroscopy. All soil humic materials were used to evaluate their effects on the enzymatic activities involved in glycolytic and respiratory processes of *Zea mays* (L.) seedlings. The elementary analyses and NMR spectra of the humic fractions indicated that the content of polar carbons (mainly carbohydrates) increased with decreasing molecular size of separated fractions. The products evolved by on-line thermochemolysis showed that the smallest size fraction (Fraction III) with the least rigid molecular conformation among the humic samples had the lowest content of lignin moieties and the largest amount of other non-lignin aromatic compounds. The bulk HA and the three humic fractions affected the enzyme activities related to glycolysis and tricarboxylic acid cycle (TCA) in different ways depending on molecular size, molecular characteristics and concentrations. The overall effectiveness of the four fractions in promoting the metabolic pathways was in the order: III > HA > II > I. The largest effect of Fraction III, either alone or incorporated into the bulk HA, was attributed to a flexible conformational structure that promoted a more efficient diffusion of bioactive humic components to maize cells. A better knowledge of the relationship between molecular structure of soil humic matter and plant activity may be of practical interest in increasing carbon fixation in plants and redirect atmospheric CO_2 into bio-fuel resources.
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Keywords: Soil humic acids; Maize; Glycolytic pathway; Krebs cycle; High performance size exclusion chromatography; NMR spectroscopy; Thermochemolysis

1. Introduction

The past century has seen a marked increase in atmospheric carbon dioxide concentrations and a concomitant 'greenhouse warming' that has drawn scientific attention to the link between global carbon stocks and climate change (Cox et al., 2000). In particular, the decomposition and turnover of soil organic matter (SOM) due to intense agricultural production is recognized as an important determinant of carbon driven climate change (Briones et al., 2007). Moreover, SOM is recognized as a key factor in soil fertility since it controls the physical, chemical and biological properties of the rhizosphere (Nardi et al., 2002b; Gastal and Lemaire, 2002). In this respect, the unravelling of the biochemical and physiological events underlying the effect on plant growth of humic substances (HS), that are the major components of SOM, has become a primary goal to improve plant nutrition and, consequently increase photosynthate carbon (Nardi et al., 2002a).

The HS, heterogeneous organic compounds formed in soil as by-products of microbial metabolism on dead cell materials, were found up to now to exhibit a range of

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different effects on plant metabolism (Tan, 2003; Nardi et al., 1996, 2002a), depending on their origin, molecular size, chemical characteristics and concentration. According to a new view of their chemistry, HS are collections of heterogeneous, relatively low molecular-mass components forming dynamic associations stabilized by hydrophobic interactions and hydrogen bonds (Piccolo et al., 1996; Piccolo and Conte, 1999; Piccolo, 2001; Sutton and Sposito, 2005). These associations are capable of organizing, in suitable aqueous environments, into supramolecular structures of only apparently large molecular sizes. This novel interpretation implies that root-exuded organic acids present in soil solution may affect the stability of humic conformation and hence their effect on permeability of root membranes (Nardi et al., 1996).

In the rhizosphere, an interaction between the root system and humic matter is possible when humic molecules, present in the soil solution, are able to flow into the apoplast and reach the plasma membrane. This event occurs in the vicinity of the root surface, where the simultaneous release of protons and organic acids by both roots and microbes enables the disruption of humic macrostructures and the subsequent release of the otherwise unavailable bioactive fractions (Piccolo et al., 1992). These substances may enter into the plant, translocate from roots to shoots (Vaughan and MacDonald, 1976; Nardi et al., 1996), and affect plant growth by different mechanisms: increasing respiration (Vaughan and Malcom, 1985), enhancing mineral nutrition (Clapp et al., 2001; Varanini and Pinton, 1995, 2001), and/or stimulating hormonal activities (Vaughan et al., 1985; Visser, 1986; Nardi et al., 1988, 2000).

Visser (1987) showed that low molecular size (LMS) HS induced a more significant increase in respiration than high molecular size material in rat liver mitochondria. Moreover, in relation with concentration, they increased the efficiency of the oxidative phosphorylation process in vitro, particularly after contact time with the mitochondria for over 1 h. Khristeva et al. (1980) already showed an increase in ATP production due to HS. Following the hypothesis that an auxin-like activity may be exerted by HS on plant metabolism (Bottomley, 1914a, b), it has been elucidated that HS increase both the activity (Maggioni et al., 1987; Nardi et al., 1991) and amount (Canellas et al., 2002) of plasma membrane (PM) H^+ATPase, thereby allowing an apoplast acidification and an indirect cell elongation. Moreover, recent studies showed that LMS-HS stimulate nitrate uptake, possibly through the up-regulation of m-RNA synthesis of the major H^+ATPase form such as Mha2 (Quaggiotti et al., 2004). However, the effect of HS on the important glycolysis and respiration pathways are not yet well understood due to still insufficient experimental work that relates a detailed molecular description of humic matter to its biological activity (Vaughan et al., 1985; Chen and Aviad, 1990; Varanini and Pinton, 1995; Nardi et al., 2002a).

Glycolysis is of crucial importance in plants because it is the predominant pathway that "fuels" plant respiration. Moreover, a significant proportion of the carbon entering the plant glycolytic pathway and tricarboxylic acid (TCA) cycle is not oxidized to CO_2, but it is used in the biosynthesis of numerous compounds such as secondary metabolites, isoprenoids, amino acids, nucleic acids, and fatty acids. The biosynthetic role of glycolysis and respiration is particularly important in actively growing autotrophic tissues (Plaxton, 1996).

The objectives of this work were: (i) to characterize by pyrolysis-GC-MS and NMR spectroscopy a soil humic acid (HA) and its three size fractions (I, II and III) separated by preparative high performance size exclusion chromatography (HPSEC), (ii) to test different concentrations of such soil humic materials on maize (*Zea mays* L.) seedlings, in order to evaluate their effects on metabolism through the measurement of enzymatic activities involved in glycolytic and respiratory processes. The enzymatic activities studied here and related to the glycolysis pathway were: glucokinase, phosphoglucose isomerase, PPi-dependent phosphofructokinase, pyruvate kinase, while those involved in respiration process were: cytrate synthase, malate dehydrogenase, and the cytosolic form of $NADP^+$-isocitrate dehydrogenase.

2. Materials and methods

2.1. Soil humic matter and separation of size fractions

A HA from a Fulvudand soil of the volcanic caldera of Vico, near Rome, Italy, was isolated by standard methods as reported elsewhere (Piccolo et al., 2002). The HA was titrated to pH 7.2 with a 0.5 M KOH solution in an automatic titrator (VIT 90 Videotitrator, Radiometer, Copenhagen) under N_2 atmosphere and stirring. After having reached the constant pH 7.2, the solution containing potassium humate was left under titration for 2 h, filtered through a glass microfibre filter (Whatman GF/C), and freeze-dried.

The mobile phase for HPSEC, a $NaCl/NaN_3$ ($2.89\,g\,l^{-1}$/$0.3\,g\,l^{-1}$) solution, was used to dissolve the HA to reach a concentration of $600\,mg\,l^{-1}$. Preparative separation of HA was conducted through a Biosep SEC-S-2000 (300 mm × 21.2 mm i.d.) column preceded by a Biosep SEC-S-2000 Guard Column (78.0 mm × 21.2 mm i.d.) by Phenomenex. A Gilson 305 pump (Gilson Inc., Middleton, WI, USA), a Gilson autosampler model 231, a Gilson FC205 fraction collector, and a Gilson 116 UV detector set at 280 nm were used to automatically isolate humic fractions in continuous. The nominal molecular-weight range of the preparative column was calibrated with polystyrene sulphonates of known molecular weights. The HA and standard solutions were injected with a Rheodyne rotary injector equipped with a 5 ml loop and the elution run at a $1.5\,ml\,min^{-1}$ flow rate. A Unipoint Gilson Software was used to automatically record all chromato-

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graphic runs. Three Fractions (I, II and III) were collected during the HPSEC elution of HA separation: I, between 26 and 38 min; II, between 38 and 50 min; III, between 50 and 98 min. The preparative HA elution was repeated automatically 100 times and out of 300 mg of injected matter, the recovery was 88.5, 93, and 106 mg for Fractions I, II, and III, respectively. The collected fractions were dialyzed in dialysis tubes (1000 Da cut-off) against distilled water until chloride-free, and freeze-dried. All humic samples were characterized for their elemental content using a Fisons EA 1108 Elemental Analyzer (Fisons Instruments S.p.A., Rodana, MI, Italy).

2.2. On-line thermochemolysis-GC-MS

Thermochenolysis-GC-MS was carried out by a Pyrojector II pyrolyser mounted on-line on a PerkinElmer Autosystem XL gas chromatograph (PerkinElmer Inc., Wellesley, MA, USA), coupled to a PerkinElmer Turbomass Gold Mass Spectrometer. About 1 mg of dried humic sample was placed in a quartz sampling capillary tube and moistened with 10 µl of tetramethylammonium hydroxide (TMAH) (25% w/w in methanol) and left to dry for 24 h. The tube containing the sample was attached to a sampling probe for solids by means of a spring hook, rapidly inserted in the injection head of the pyrolyser and analyzed at 600 °C. Helium was used as a carrier gas. Thermochemolytic products were then separated in the GC through a fused-silica capillary column (Restek Rtx©-5MS, 30 m length × 0.25 mm i.d. × 0.25 µm film thickness) using helium as carrier gas with a flow rate of 1.6 ml min^{-1}. During thermochemolysis, the oven temperature was kept at 50 °C, then it was heated at a rate of 7 °C min^{-1} to 300 °C, and held at this temperature for 10 min. The mass spectrometer operated in full scan mode in the range of m/z 50–600 and by an electron impact ionization energy of 70 eV with a cycle time of 1.0 s. All pyrolysis-TMAH GC-MS analyses were conducted in triplicates. The relative abundance (%) of each compound was calculated as the ratio of the area of each single peak over the total area of peaks.

2.3. CPMAS-NMR spectroscopy

The carbon distribution in the humic samples was evaluated using cross-polarization magic angle spinning carbon-13 nuclear magnetic resonance spectroscopy (CPMAS-^{13}C-NMR). Spectra were obtained with a Bruker AV300 instrument (Bruker, Rheinstetten, GE) operating on the carbon-13. The rotor spin rate was set at 13000 HZ. A contact time of 1 ms, a recycle time of 1.5 s and an acquisition time of 20 µs were used. All experiments were conducted with CP pulse sequence with 1 H RAMP to take into account the inhomogeneity of the Hartmann–Hahn condition at high rotor spin rates. CPMAS-NMR spectra were done on triplicates for each sample.

2.4. Plant material

Maize seeds (*Z. mays* L. var. DK 585) were soaked in distilled water for one night in running water and germinated for 60 h, in the dark, at 25 °C on a filter paper wet with 1 mM $CaSO_4$ (Nardi et al., 2002a). Then, seedlings were raised in hydroponic conditions with 450 ml of a Hoagland no. 2 modified solution (Hoagland and Arnon, 1950) in growth chamber for 14 days at the following conditions: 14 h light at 27 °C and 60% relative humidity, and 10 h dark, at 21 °C and 80% relative humidity. At day 12 plants were treated with the different humic samples at different concentrations: 0 (Control), 0.5, 1, and 5 mg C l^{-1}. The treatment lasted 48 h. The enzymatic analyses were carried out on leaves and roots.

2.5. Enzyme extraction and assay conditions

2.5.1. Enzymatic activities related to the glycolysis

For the extraction of the glucokinase (GK E.C. 2.7.1.1) enzyme, leaves were homogenized (1:5 w/v) using a pre-chilled mortar and pestle with 100 mM HEPES-NaOH buffer pH 7.7, containing 10 mM $MgCl_2$, 0.4 mM Na_2EDTA, 100 mM Na-ascorbate, 1% (w/v) PVP (polyvinylpyrrolidone), 1% (w/v) bovine serum albumine (BSA) and 5 mM GSH (gluthatione reduced form) (Muscolo et al., 2000). The extracts were filtered through four layers of gauze and centrifuged at 20,000g for 20 min at 4 °C. The GK activity was measured by coupling glucose phosphate production with NAD reduction by glucose-6-phosphate (G6P) dehydrogenase. The assay contained 50 mM bicine-NaOH (pH 8.5), 5 mM $MgCl_2$, 1 mM ATP, 1 mM glucose, 1 mM NAD, 1 IU ml^{-1} G6P dehydrogenase and 50 µl extract. The assay was initiated with glucose, and the enzyme activity was measured at 30 °C, by monitoring the change in absorbance at 340 nm using JASCO V-530 UV/VIS spectrophotometer (Dohelert et al., 1988).

For the phosphoglucose isomerase (PGI EC 5.3.1.9) enzyme, leaves were homogenized (1:5 w/v) using a pre-chilled mortar and pestle with 100 mM HEPES-NaOH buffer pH 7.7, containing 10 mM $MgCl_2$, 0.4 mM Na_2EDTA, 100 mM Na-ascorbate, 1% (w/v) PVP (polyvinylpyrrolidone), 1% (w/v) and 5 mM GSH (Gluthatione reduced form) (Muscolo et al., 2000). The extracts were filtered through four layers of gauze and centrifuged at 20,000g for 20 min at 4 °C. For PGI assay, 75 µl 20 mM β-NADP–-Na_2–salt in distilled water, 75 µl 80 mM fructose-6-phosphate-Na_2 in 0.2 M Tris, pH 9.0 and 20 µl glucose-6-phosphate-dehydrogenase (from yeast diluted to 30 U ml^{-1} with 0.2 M Tris, pH 9.0) were added to 530 µl 0.2 M Tris adjusted with 0.1 M HCl to pH 9.0. The reaction was started by adding 50 µl extract after a lag time of 20 min at 30 °C by monitoring the change in absorbance at 340 nm using JASCO V-530 UV/VIS spectrophotometer to evaluate the $NADP^+$ reduction (Nowotny et al., 1998).

To extract the PPi-dependent phosphofructokinase (PPi-PFK E.C. 2.7.1.90) enzyme, leaves were homogenized

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(1:2.5 w/v) with: 100 mM HEPES/NaOH (pH 8) containing 5 mM DTT, 1 mM magnesium acetate, 2 mM EDTA, 2% PVP (w/v) and 1% glycerol (v/v) The homogenate was filtered through a gauze and centrifuged for 30 min at 15,000 g (Maciejewska and Bogatek, 2002). The enzymatic activity of PPi-PFK was determined in the reaction medium of a total volume of 1.2 ml containing: 100 mM HEPES/NaOH pH 8,0 2.0 mM magnesium acetate, 1 mM fructose-6-phosphate, 0.16 mM NADH, 0.32 U aldolase, 0.62 U triose phosphate isomerase/glycerol-3-phosphate-dehydrogenase (Sigma), and 0.1 ml of enzyme extract (Smyth et al., 1984). The reaction was started by addition of 1 mM pyrophosphate. Enzyme activities were measured at 25 °C.

To extract the pyruvate kinase (PK E.C. 2.7.1.40) enzyme, leaves were homogenized (1:5 w/v) using a pre-chilled mortar and pestle with 100 mM HEPES-NaOH buffer pH 7.7, containing 10 mM $MgCl_2$, 0.4 mM Na_2EDTA, 100 mM Na-ascorbate, 1% (w/v) PVP (polyvinylpyrrolidone), 1% (w/v) and 5 mM GSH (gluthatione reduced form) (Muscolo et al., 2000). The extracts were filtered through four layers of gauze and centrifuged at 20,000 g for 20 min at 4 °C. The supernatants were used for enzymatic analysis of PK. 50 µl 3 mM β-NADH-Na_2-salt in 0.1 M TEA pH 7.75, 50 µl 52 µM adenosine 5′-diphosphate-Na_2 in 0.1 M TEA (pH 7.75), 50 µl 0.15 M $MgSO_4$-$6H_2O$ and 0.15 M KCl in 0.1 M triethanolamine (TEA) pH 7.75, 50 µl L-lactic dehydrogenase diluted to 225 U ml^{-1} with 0.1 M TEA (pH 7.75), and 50 µl extract were added to 450 µl 0.1 M (TEA), adjusted with 0.1 M NaOH to pH 7.75. The reaction was started after a lag time of 10 min at 30 °C by adding 50 µl 0.225 M 2-phosphoenolpyruvate-Na-H_2O in 0.1 M TEA (pH 7.75) (Nowotny et al., 1998).

2.6. Enzymatic activity related to respiratory pathway

For the assay of cytrate synthase (CS EC 1.11.1.6), leaves were homogenized using 100 mM TRIS HCl buffer pH 8.2, containing 5 mM β-Mercaptoethanol (Sigma), 1 mM Na_2EDTA, 10% glycerol. Leaves were filtered and centrifuged as reported (Bergmeyer et al., 1986). All steps were performed at 4 °C. CS enzyme was assayed adding 50 µl of oxalacetic acid 0.17 mM, 50 µl acetyl-CoA 0.2 mM, and 10 µl extract, to 3 ml of Tris–HCl 0.1 M (pH 8.0). This activity was measured spectrophotometrically at 25 °C, by monitoring the reduction of acetyl-coenzyme A (CoA) to CoA, at wavelength 232 nm (Bergmeyer et al., 1986).

For assaying $NADP^+$-isocitrate dehydrogenase ($NADP^+$-IDH EC 1.1.1.42), leaves were homogenized using 100 mM TRIS HCl buffer pH 8.2, containing 5 mM β-mercaptoethanol (Sigma), 1 mM Na_2EDTA, 10% glycerol. The extracts were filtered and centrifuged as reported (Bergmeyer et al., 1986). All steps were performed at 4 °C. About 50 µl of crude extract was added to 2.85 final volume of a reaction mixture containing 88 mM imidazole buffer (pH 8.0), 3.5 mM $MgCl_2$, 0.41 mM β-NADP-Na salt and 0.55 mM isocitrate-Na salt. The assay was performed at 25 °C following the formation of NADPH at 340 nm, using JASCO V-530 UV/VIS spectrophotometer (Goldberg and Ellis, 1986).

For the assay of malate dehydrogenase (MDH EC 1.1.1.37), leaves were homogenized using 100 mM TRIS HCl buffer pH 8.2, containing 5 mM β-Mercaptoethanol (Sigma), 1 mM Na_2EDTA, 10% glycerol. The extracts were filtered and centrifuged as reported (Bergmeyer et al., 1986). All steps were performed at 4 °C. The 3.17 ml assay contained 94.6 mM phosphate buffer (pH 6.7), 0.2 mM β-NADH-Na_2-salt, 0.5 mM oxalacetic acid, and 1.67 mM $MgCl_2$. MDH activity was assayed at 25 °C, following the formation of NAD^+ at 340 nm, using JASCO V-530 UV/VIS spectrophotometer (Bergmeyer et al., 1986).

2.6. Statistical analysis

All enzymatic data were the means of five replicates, and the standard deviations did not exceed 5%. The results obtained were processed statistically with the Student–-Newman–Keuls test (Sokel and Rohlf, 1969).

3. Results

3.1. Molecular characteristics of humic samples

The elemental analyses of HA and its size separates (Table 1) show how the compositional elements and their ratios were differently distributed when the HA was separated in its size fraction during HPSEC elution. In particular, while the carbon, nitrogen, and hydrogen content as well as the C/N ratios were reduced in the size separates in comparison to HA, the oxygen content was complementary increased in the fractions. Moreover, the H/C and the O/C ratios resulted larger in the fractions, especially in Fraction III, than in HA, thereby suggesting that the size fractions were progressively more hydrophilic than the bulk HA.

The carbon distribution in the humic samples were shown by the CPMAS-^{13}C-NMR spectra (Fig. 1). The spectra showed signals in the alkyl-C (0-50 ppm) and the N–C and O–C (50–110 ppm) regions. The former region is composed by carbons in $(CH_2)n$- and terminal CH_3 groups of plants lipid compounds, such as waxes and aliphatic bio-polyesters. Plant woody tissues were also indicated by the 56 ppm shoulder of methoxy groups on aromatic rings of

Table 1
Elemental analysis of humic samples

Humic samples	C	N	H	O	C/N	H/C	O/C
HA	39.48	2.26	3.13	55.13	17.47	0.08	1.40
Fraction I	26.07	1.81	2.57	69.55	14.43	0.10	2.67
Fraction II	34.33	2.14	3.39	60.13	16.04	0.10	1.75
Fraction III	21.51	1.17	2.49	74.83	18.44	0.12	3.48

The data are the means of three replicates and the standard deviations always were ⩽ 0.2.

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guaiacyl and siringyl units of lignin structures (Hatcher, 1987). The most dominant resonance in the 50–110 ppm region is mainly assigned to monomeric units in oligo- and poly-saccharidic chains of plant woody tissues (Vane et al., 2001). The intense signal around 72 ppm corresponds to the overlapping resonances of C2, C3 and C5 carbons in the pyranoside structure of cellulose and hemicellulose, whereas the signals at 106 ppm (sharp), 65 ppm, and 82–85 ppm (shoulders) are assigned to the anomeric C1 carbon and the C6 and C4 carbons, respectively (Atalla and VanderHart, 1999). The aromatic region (110–160 ppm) revealed a broad band around 130 ppm

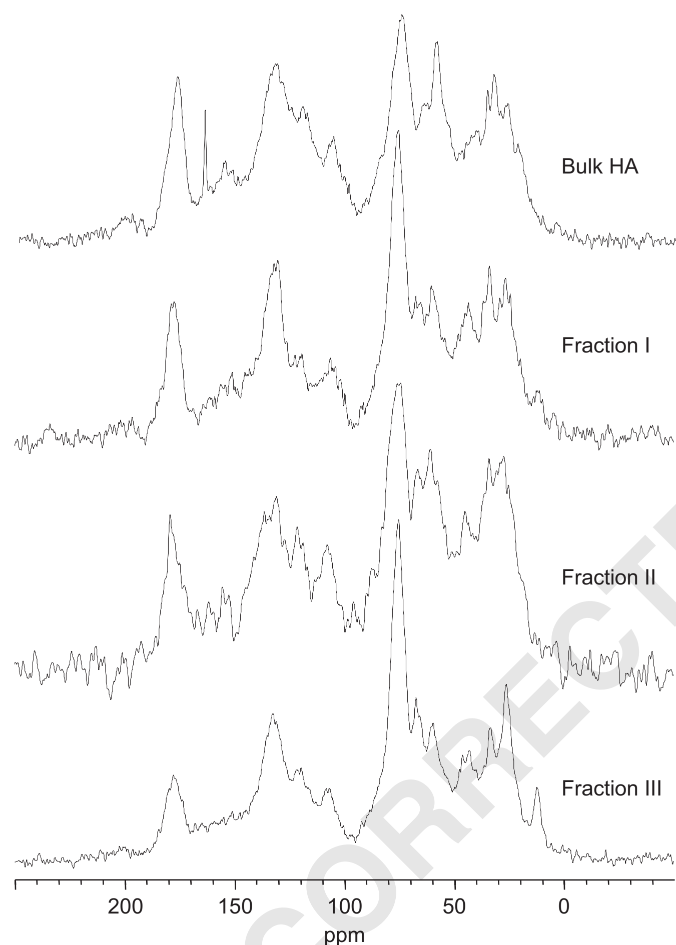

Fig. 1. CPMAS-^{13}C-NMR spectra of humic acid (HA), and three humic fractions with decreasing molecular weight (I, II, III).

that may be related to *p*-hydroxy phenyl rings of cynnamic units in both lignin and suberin biopolymers (Hatcher et al., 1995). A prominent signal for quaternary carbons at 172 ppm is currently assigned to carboxyl groups.

The spectra showed a general redistribution of the different classes of carbon components when passing from the bulk HA to the size fractions. The major feature was the progressive relative increase of signals for alkyl and carbohydrate carbons with decreasing size of fractions. The relative carbon distribution is shown in Table 2, where signal integrations are reported. The size fractions became richer in alkyl carbon (0–50 ppm) than the bulk HA, but its content somewhat decreased with decreasing size of fractions. Similarly, the carbohydrate carbon, mainly represented in the 50–110 ppm interval, increased significantly in the size separates and especially in the fraction of lowest size. Conversely, the content of aromatic carbon (100–160 ppm) and carboxyl carbon (160–190 ppm) was lower in size fractions than in bulk HA. This relative distribution suggests that Fraction III contained more hydrophilic carbon than the rest of humic samples, as it is also indicated by the slightly increasing HI/HB value for this fraction. These NMR results are in line with the findings by elemental analyses that also showed the largest oxygen content for Fraction III.

The classes of compounds identified in total ion chromatograms (TIC) derived from the on-line thermochemolysis of humic samples, as well as their relative content, are shown in Table 3. The majority of compounds derived from higher plants, and were represented by lignin, waxes and aliphatic biopolymers, as already found for other bio- and geochemical materials (Grasset et al., 2002; Guignard et al., 2005).

The original content of lignin products in HA was spread over the three size fractions during the HPSEC treatment, with the least amount found in the smallest size fraction III. For the rest of the identified products, a shift of relative content was observed from the bulk HA to the fractions. In particular, aromatic compounds (including some polyaromatic hydrocarbons), which are not immediately related to lignin, were less prominent in Fraction II than in bulk HA. Among the alkyl products, also the saturated fatty acids, the alkanes/alkenes, and the α,ω-alkanedioic acids were found to be enriched in size fractions in respect to bulk HA, while the unsaturated fatty acids showed slightly less relative importance, except for Fraction II. Sterols decreased in relative importance passing from the bulk

Table 2
Relative distribution (%) of signal area over chemical shift regions (ppm) in ^{13}C-CPMAS-NMR spectra of humic samples

Humic samples	Alkyl-C (0–50)	Alkyl C-N and C-O (50–110)	Aromatic-C (100–160)	Carboxyl-C (160–190)	Ketone-C (190–230)	HI/HB[a]
HA	21.0±0.4	36.6±0.8	29.3±1.0	11.7±1.2	1.40±0.2	0.99±0.1
Fraction I	26.5±0.3	39.6±0.8	24.50±0.8	9.2±0.5	0.14±0.07	0.96±0.1
Fraction II	25.8±0.4	40.8±0.7	24.0±0.8	9.4±0.4	0.03±0.01	1.01±0.1
Fraction III	25.1±0.4	42.5±0.8	24.3±0.7	7.6±0.4	0.38±0.1	1.02±0.1

[a]Hydrophilic carbons/hydrophobic carbons = [(50-110) + (160-230)]/[(0-50) + (100-160)].

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Table 3
Relative abundance (%) and composition[a] of main thermochemolysis products released from HA bulk and size fractions

Identified products	HA	Fraction I	Fraction II	Fraction III
Lignin				
p-hydroxyphenyl	19.82 (±2.46)	8.81 (±2.98)	7.37 (±0.68)	4.46 (±0.24)
Guaiacyl	15.17 (±1.42)	8.16 (±1.61)	10.67 (±1.85)	6.66 (±0.37)
Syringyl	5.68 (±1.47)	2.25 (±0.44)	3.14 (±0.72)	1.57 (±0.58)
Cinnamic acids	3.28 (±0.98)	1.96 (±0.88)	2.92 (±0.59)	1.38 (±0.72)
Other aromatic compounds	12.65(±2.01)	15.93(±4.61)	7.34(±4.79)	16.25(±4.95)
Alkyl				
Saturated fatty acids	10.46 (±1.81)	11.07 (±1.83)	26.77 (±2.47)	17.66 (±3.03)
	C_{13}-C_{31}(C_{16})	C_9-C_{31}(C_{16}.C_{18})	C_{10}.C_{28}(C_{16}.C_{18})	C_{30}-C_{31}(C_{16})
Unsaturated fatty acids	2.23 (±0.33)	1.30 (±0.43)	12.08 (±3.22)	1.09 (±0.43)
	C_9.C_{16}.C_{18}	C_{16}.C_{18}	C_7.C_{16}.C_{18}	C_{16}.C_{18}
Alkanes/alkenes	6.21 (±3.15)	16.21 (±5.41)	9.04 (±1.77)	21.02 (±2.73)
	C_{16}-C_{30} (C_{19})	C_{16}-C_{30} (C_{19})	C_{16}-C_{30} (C_{19})	C_{16}-C_{30} (C_{19})
α.ω-alkane dioic acids	1.46 (±0.32)	3.13 (±0.47)	4.59 (±0.56)	2.56 (±1.16)
	C_5-C_{11}(C_9)	C_5-C_9(C_9)	C_6-C_{22}(C_{16})	C_6-C_{30}(C_6)
Sterols	0.62 (±0.06)	0.19 (±0.02)	0.23 (±0.6)	0.02 (±0.01)
Terpenoids	0.33 (±0.12)	0	0.36 (±0.02)	0
Hydrophilic				
Protein derivatives	15.24 (±0.96)	21.80 (±0.24)	16.30 (±1.77)	14.25 (±1.62)
Polysaccarides derivatives	6.62 (±2.22)	9.20 (±2.68)	4.91 (±2.67)	11.01 (±1.11)

[a]Total range varying from Ci to Cj; compounds in parentheses are the dominant homologous.

HA to the fractions of progressively decreasing size. Terpenoids, were detected only for bulk HA and Fraction II. Among the hydrophilic components detected by on-line thermochemolysis, protein derivatives were more relatively important in the TIC of size fractions, while polysaccharides derivatives were more prominent in Fraction III.

3.2. Effects of humic samples on maize seedlings

The effects of bulk HA and its size fractions (I, II, III) on the enzymatic activities related to the glycolytic pathway of maize seedlings are reported in Table 4. The bulk HA did not particularly influence the enzyme activities when in a $0.5\,mg\,C\,l^{-1}$ solution, with exception of PK that showed a 29% increase in respect to control. However, larger HA concentrations significantly enhanced all enzyme activities, reaching more than 100% increase for all enzymes at $5\,mg\,C\,l^{-1}$, except for PK. The size fractions had a different effect on the glycolytic enzymes. Fraction I at $0.5\,mg\,C\,l^{-1}$ increased significantly all enzymatic activities up to 80% in respect to control, while the stimulation was reduced to no more than 22% when the same fraction was used at $1\,mg\,C\,l^{-1}$. However, at the concentration of $5\,mg\,C\,l^{-1}$, Fraction I decreased every enzyme activity as compared to control. A similar behaviour was observed for Fraction II. This fraction generally ensured a significant increase in enzyme activities for both 0.5 and $1\,mg\,C\,l^{-1}$ concentrations, while the activities were again reduced to values lower than control at the largest concentration. The stimulation of Fraction III on the enzyme activities was significantly larger than control for both 0.5 and $1\,mg\,C\,l^{-1}$ concentrations, whereas, contrary to the first two size fractions, it was still 20–63% larger than control at the largest concentration ($5\,mg\,C\,l^{-1}$). Based on the measured enzyme activities, at the highest concentrations used here,

Table 4
Glucokinase (GK), phosphoglucose isomerase (PGI), PPi-dependent phosphofructokinase (PFK), pyruvate kinase (PK) enzyme activities (% of the control) in *Zea mays* leaves treated with humic acid (HA), and three humic fractions with decreasing molecular weight (I, II, III), used at concentrations ranging from 0 (control) to $5\,mg\,C\,l^{-1}$

Humic samples	Concentration ($mg\,C\,l^{-1}$)	Enzyme activities[a]			
		GK	PGI	PFK	PK
HA	0	100d	100c	100b	100c
	0.5	109c	108bc	101b	129ab
	1	125b	114b	106b	121b
	5	194a	218a	219a	135a
Fraction I	0	100c	100c	100c	100c
	0.5	180a	179a	165a	180a
	1	122b	110b	113b	122b
	5	89d	89d	102c	101c
Fraction II	0	100c	100c	100b	100b
	0.5	166a	138b	166a	169a
	1	146b	184a	181a	164a
	5	69d	58d	69c	52c
Fraction III	0	100c	100b	100c	100c
	0.5	148a	166a	149ab	171a
	1	150a	159a	137b	165ab
	5	120b	151a	163a	152b

[a]Values in the same frame followed by the same letter are not statistically different at $P = 0.05$ as by the Student–Newman–Keuls test. The values of control were: GK = $9.7\,\mu M$ NADPH g fresh weight^{-1}min^{-1}; PGI = $30.6\,\mu M$ NADPH g fresh weight $^{-1}$min^{-1}; PFK = $3.62\,\mu M$ NADPH g fresh weight $^{-1}$min^{-1}; PK = $29.2\,\mu M$ NADPH g fresh weight $^{-1}$min^{-1}.

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Table 5
Cytrate synthase (CS), malate dehydrogenase (MDH), and isocitrate NADP+-isocitrate dehydrogenase ($NADP^+$-IDH) enzyme activities (% of the control) in *Zea mays* leaves treated with humic acid (HA), and three humic fractions with decreasing molecular weight (I, II, III), used at concentrations ranging from 0 (control) to $5\,mg\,C\,l^{-1}$

Humic samples	Concentration ($mg\,C\,l^{-1}$)	Enzyme activities[a]		
		CS	IDH	MDH
HA	0	100c	100c	100c
	0.5	116b	126b	101c
	1	170a	131ab	124b
	5	157a	144a	153a
Fraction I	0	100c	100c	100c
	0.5	179a	122b	152a
	1	158b	148a	123b
	5	103c	105c	103c
Fraction II	0	100c	100b	100c
	0.5	149a	155a	121b
	1	127b	166a	135a
	5	55d	60c	61d
Fraction III	0	100c	100d	100d
	0.5	151ab	139b	155b
	1	163a	119c	129c
	5	146b	175a	210a

[a]Values in the same frame followed by the same letter are not statistically different at $P = 0.05$ as by Student–Newman–Keuls test. The values of control were $CS = 19.3\,\mu M$ g fresh weight $^{-1}min^{-1}$, $IDH = 10\,\mu M$ NADPH g fresh weight $^{-1}min^{-1}$; $MDH = 106\,\mu M$ NADP g fresh weight $^{-1}min^{-1}$.

the effect of humic fractions on the respiratory pathway was in the order: III > HA > I > II.

Changes in the activities of CS, $NADP^+$IDH, and MDH enzymes of the Krebs cycle, with humic samples are shown in Table 5. The bulk HA significantly stimulated the activity of CS and $NADP^+$IDH at $0.5\,mg\,C\,l^{-1}$, but not that of MDH. The stimulation increased significantly at $1\,mg\,C\,l^{-1}$ for all enzymes, and especially for CS. However, a further increase in concentration was effective only on MDH (53% over control). Fraction I, at $0.5\,mg\,C\,l^{-1}$, had a significant effect on all enzymes, especially CS and MDH. However, while these enzymes did not change their activity when Fraction I was used at $1\,mg\,C\,l^{-1}$, the $NADP^+$IDH activity was further increased at this concentration. For all enzymes, the activities were not larger than control at the concentration of $5\,mg\,C\,l^{-1}$. A similar effect was shown by Fraction II, that even strongly inhibited enzyme activities, as compared to control, when used at the $5\,mg\,C\,l^{-1}$. Conversely, Fraction III showed a stimulating effect on each enzyme activity at every concentration. In particular, the $5\,mg\,C\,l^{-1}$ concentration produced an increase of 46% in CS, 110% in MDH, and 75% in $NADP^+$IDH. Based on the measured enzyme activities at the highest concentrations tested in this work, the effect of humic fractions on the respiratory pathway was again in the order: III > HA > I > II.

4. Discussion

The reported data on the increase of GK, PGI, PPi-PFK, and PK in maize seedlings treated with Fraction III and HA, indicate a large demand of ATP. This suggests that these humic materials positively affect a wide range of different physiological processes, that require an over-working of the glycolytic pathway to produce the pyruvate that feeds into different metabolic pathways (Alisdair et al., 2004). Moreover, several observations from studies of mammalian cells suggest that glycolitic enzymes may physically concentrate at sites of demand for ATP or other glycolytic intermediates (Fernie et al., 2004).

The respiratory process continues with mitochondrial reactions of the TCA cycle, that converts phosphoenolpyruvate to malate and/or pyruvate in the cytosol. These organic acids are then taken up into the mitochondria, and subsequently interconverted to produce energy, reducing power and carbon structures (Carrari et al., 2003). Both bulk HA and Fraction III were found to positively affect the main enzymes of the TCA cycle and, in particular, the CS, considered the most important enzyme of TCA cycle, since it catalyzes the reaction which regulates the rate of respiratory pathway (Alisdair et al., 2004). We investigated the cytosolic form of NAD^+ isocitrate dehydrogenase because it is normally assumed to be the enzyme that is primarly responsible for isocitrate to α-ketoglutarate conversion in krebs cycle. In fact, the NAD^+IDH enzyme is considered to be a key step in the generation of 2-oxoglutarate for ammonium assimilation and amino acid biosynthesis in higher plants (Chen and Gadal, 1990; Palomo et al., 1998).

The most positive effect of Fraction III on both metabolic pathways studied here, is in line with previous studies which indicated that low molecular size HS (LMS-HS) were particular active in stimulating plant metabolism (Vaughanet al., 1985). The effectiveness of the LMS-HS was previously attributed to a combination of low molecular size and large content of aromatic, carboxyl and phenolic carbons (Piccolo et al., 1992; Nardi et al., 2000). However, while the activity of LMS fractions is confirmed here, the additional molecular information collected on the tested fractions, suggest that the stimulation of plant metabolism may be more precisely attributed to specific classes of compounds.

First of all, it is evident that Fraction III is more hydrophilic and richer in carbohydrates than the rest of size-separates (Tables 1-3, Fig. 1). This implies that the larger the hydrophobic components in humic samples, the lower becomes the activity of HS on plant physiology. The less metabolically active Fractions I and II were found to generally contain larger amounts of hydrophobic long-carbon-chain alkyl compounds as well as lipids, such as sterols and terpenoids (Table 1–3, Fig. 1). These hydrophobic compounds are likely to produce strongly associated supramolecular structures when the humic materials are suspended in water, thereby reducing the free flow to

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plant roots of more hydrophilic components, which remain trapped into the hydrophobic cage (Piccolo, 2001). The reduced activity of Fractions I and II was more pronounced at their larger concentrations, thereby implying that the more active hydrophilic constituents were increasingly trapped by more strongly associated hydrophobic components. Conversely, the most hydrophilic Fraction III maintained its enzymatic stimulation even at the largest concentration, suggesting that its larger water hydration ensured a sufficient diffusion of active humic components to the maize cells.

Second, while the content of non-lignin aromatic compounds were found largest in the most active Fraction III, and smallest in the least active Fraction II, the lignin-derived aromatic moieties (*p*-hydroxyphenil, guaiacyl, syringyl, cinnamic) were in lowest amount in Fraction III (Table 3). Although, on-line thermochemolysis cannot distinguish whether these moieties are single monomers, it is more plausible to assume that they derive from still undegraded di- or oligo-meric lignin molecules. Conversely, it is likely that other non-lignin aromatic compounds are monomeric, being either the end products of lignin depolymerization in soil or simple compounds of microbial origin. Both the low content of residual lignin moieties and the large amount of other non-lignin aromatic compound in Fraction III may together contribute to make its conformational structure less rigid than size fractions I and II, thereby further weakening the hydrophobic cage and favouring the diffusion of active humic components to the maize cells.

The positive effect of HA, that was second only to Fraction III, may be explained again with the stability of its conformational structure. HA was composed of all the three separated fractions and may have still retained a certain general hydrophilicity, mainly due to its relative large content of oxygenated and carboxylic carbons and low content of alkyl carbon (Table 2). This may confer to the HA conformation in water a larger degree of hydration than that reached by its more hydrophobic size fractions I and II. Such conformational flexibility may still allow to exert on plant activities a similar effect as that shown by Fraction III, when separated from the HA. This is in line with the repeated finding that plant root exudates are able to dissociate humic supramolecular structures into smaller fractions, which may deliver bio-active molecules to plants or activate stimulation mechanisms (Conte and Piccolo, 1999; Piccolo, 2001; Nardi et al., 2002b).

In conclusion, this work confirms the larger efficiency of a humic fraction of LMS in regulating plant metabolism. Our findings show that the biological activity of HS may be attributed to the relative content of specific classes of humic components, such as larger amount of hydrophilic molecules (mainly carbohydrates) and lower content of residual lignin moieties. However, the humic composition seems to be reflected into a conformational structure that conveys the largest biological activity. This activity appears to be due to a specific arrangement of humic molecules in solution, such as in the bulk HA and in the smallest size fraction, where the distribution of hydrophilic components within a hydrophobic environment, maintains a sufficient degree of conformational flexibility to allow the interaction of active humic molecules with root cells.

While these results indicate a preliminary route to reach a structure-activity relationship between humic matter and plant biological activity, the conclusions may be hardly generalized without further evidence for a wide range of plant species. Many complex issues still remain unclear in the organization and regulation of plant glycolysis (Plaxton, 1996) and in the mechanism by which HS affect plant metabolism. Nevertheless, these results indicate that the positive role exerted by HS on plant metabolism may be reflected in increased plant growth and, hence, in enhanced photosynthate C sequestration by plants. It may then be envisaged that HS, in addition or in alternative to genetically modified crops, could be of practical interest in increasing the flux of photosynthate C into economically important plant end products such as starch, triglycerides and proteins.

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