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Effects of a humic acid and its size-fractions on the bacterial community of soil rhizosphere under maize (*Zea mays* L.)

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ABSTRACT

The effects of a humic acid (HA) and its size-fractions on plants carbon deposition and the structure of microbial communities in the rhizosphere soil of maize (*Zea mays* L.) plants were studied. Experiments were conducted in rhizobox systems that separate an upper soil–plant compartment from a lower compartment, where roots are excluded from the rhizosphere soil by a nylon membrane. The upper rhizobox compartment received the humic additions, whereas, after roots development, the rhizosphere soil in the lower compartment was sampled and sliced into thin layers. The *lux*-marked biosensor *Pseudomonas fluorescens* 10586 pUCD607 biosensor showed a significant increase in the deposition of bioavailable sources of carbon in the rhizosphere of soils when treated with bulk HA, but no response was found for treatments with the separated size-fractions. PCR–DGGE molecular fingerprintings revealed that the structure of rhizosphere microbial communities was changed by all humic treatments and that the smaller and more bioavailable size-fractions were more easily degraded by microbial activity than the bulk HA. On the other hand, highly hydrophobic and strongly associated humic molecules in the bulk HA required additional plant rhizodeposition before their bio-transformation could occur. This work highlights the importance of applying advanced biological and biotechnological methods to notice changes occurring in plant rhizodeposition and rhizosphere microbial activity. Moreover, it suggests correlations between the molecular properties of humic matter and their effects on microbial communities in the rhizosphere as mediated by root exudation.

1. Introduction

Humification of different organic materials for compost production is an useful strategy for both waste disposal and soil fertility increase. The evaluation of the effects of humified compost materials on soil–plant systems thus represents an important challenge in a future scenario of increased population, larger amounts of waste to be disposed, and progressive reduction of soil organic matter levels due to intensive agricultural activity.

Humic acids (HA) are a major component of organic fertilizers, and as heterogeneous molecules of different sizes that are self-organized in supramolecular conformations (Piccolo, 2002), they are also the most reactive ones. HA effects on plant physiology are mainly positive, and they include enhancement of biomass yields (Ayuso et al., 1996; Arancon et al., 2006), induction of lateral roots emergence and ATPase activity (Canellas et al., 2002), increase of cell respiration and membrane uptake of nutrients, and exertion of hormone-like activities (Nardi et al., 2002). Given the HA structural complexity, a structure–activity approach aimed at linking effects on plant physiology with specific humic chemical properties is a difficult task. One way to partly reduce the HA heterogeneity is to carry out a size-fractionation of humic matter and characterize the separated size-fractions by combined pyrolysis and NMR spectroscopy (Piccolo et al., 2002). The characteristics of such more homogenous humic molecules may be related to well defined soil–plant process of ecological importance.

Rhizodeposition is an important ecological process, through which plants supply microbes with readily available organic carbon sources, increase the microbial biomass surrounding the roots, and profoundly affect the activity and composition of microbial communities. On the other hand, microbes mobilize nutrients, primarily nitrogen, and render them available to plants, as described by the "microbial loop" model (Bonkowski, 2004; Paterson, 2003). A multidisciplinary approach based on different physical, chemical and molecular techniques is necessary to study plant

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rhizodeposition and its effects on soil microbial community structure. Rhizobox systems are useful tools to study root-induced changes in soil properties with a minimum disturbance (Youssef and Chino, 1989; Wenzel et al., 2001). In fact, a HA amendment to the upper and physically separated rhizobox compartment allows to evaluate its effects in the untreated lower rhizosphere compartment. The amount of bioavailable C in rhizodeposits can then be assessed by means of lux-marked biosensors such as *Pseudomonas fluorescens* pUCD607 (Standing et al., 2005; Puglisi et al., 2008), while possible changes in the structure of microbial communities are estimated by means of polymerase chain reaction (PCR) of ribosomal DNA coupled to denaturing or temperature gradient gel electrophoresis (Muyzer and Smalla, 1998).

The objective of this work was to evaluate whether a well-characterized HA and its separated size-fractions had an effect on C deposition of maize plants and on the structure of rhizosphere microbial communities. The hypotheses were: (i) HA can affect the amount of C deposited by plant roots; (ii) if rhizodeposition is affected, then the structure of microbial communities is significantly changed; (iii) it is possible to link the effects of humic matter to plant rhizosphere processes by indentifying the responsible specific molecular properties. In order to test these hypotheses, maize plants were grown in rhizobox systems amended in their upper soil compartment with a soil-extracted HA and its size-fractions separated by preparative high pressure size exclusion chromatography (HPSEC). The consequent carbon deposition in each unamended rhizosphere lower layer was assessed with the *lux*-marked *P. fluorescence* 10586 pUCD607 biosensor, while soil bacterial communities were characterized by 16S PCR–DGGE applied to soil-extracted DNA.

2. Materials and methods

2.1. Soil

An orchard loamy soil (USDA) was collected near Matera, South-East Italy. Soil samples (7 cm diameter cores) were randomly collected from the surface layer (0–20 cm) at least 2 m far from trees, sieved at 2 mm, pooled on site and stored at 4 °C for maximum 4 weeks before starting the bulk soil microcosms and rhizobox experiments. The soil had a loamy texture (2.47 g kg^{-1} clay, 3.38 g kg^{-1} silt, 4.15 g kg^{-1} sand), a pH (H_2O) of 6.7 and total organic C of 8.3 mg C g^{-1} d.w. soil.

2.2. HA and fractionation by HPSEC

A representative soil HA was isolated from a Fulvudand soil near Vico (Italy), by standard methods reported earlier (Piccolo et al., 1999). 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 HPSEC mobile phase, 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 0.6 g L^{-1}. Preparative separation of HA was conducted with a Biosep SEC-S-2000 (300 mm × 21.2 mm internal diameter, 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, a Gilson autosampler model 231, a Gilson FC205 fraction collector, and a Gilson 116 UV detector (Gilson Inc., Middleton, WI, USA) 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 (Piccolo et al., 2002). 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 (Gilson Inc., Middleton, WI, USA) was used to automatically record all chromatographic runs. Three fractions (I–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 collected fractions were dialyzed in dialysis tubes (1000 Da cut-off) against distilled water until they were free of chloride, and freeze-dried. The preparative HA elution was repeated automatically 100 times, and, for a total injection of 300 mg, the recovery was 91, 92, and 98 mg for fractions I, II, and III, respectively. All humic samples were characterized for their elemental content using a Fisons EA 1108 Elemental Analyzer (Fisons Instruments, Milano, Italy).

2.3. On-line thermochemolysis-GC–MS and CPMAS-NMR spectroscopy characterizations

Thermochemolysis-GC–MS and cross-polarization magic angle spinning carbon-13 nuclear magnetic resonance spectroscopy (CPMAS-^{13}C NMR) analyses of the HA and its three size-fractions were carried out as described in detail by Spaccini and Piccolo (2007). 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 singular peak over the total peaks area.

2.4. Plants cultivation and microtome sampling

All experiments were carried out in the rhizobox system described by Wenzel et al. (2001). The system consists of a soil–plant compartment (130 × 64 × 50 cm) with a narrow slit at its bottom that can be vertically penetrated only by plant roots to form a root plane. In the lower compartment (130 × 30 × 105 cm) such root system is confined between a transparent acrylic window on one side and a membrane inhibiting further root penetration on the other side. Nylon membranes of 7-μm mesh width, which exclude root hairs from penetrating into the rhizosphere, were used in the experiment. The membrane separates the root plane from the rhizosphere soil compartment. The soil humidity at the beginning of the experiments was set to 50% WHC, and was then kept in the rhizoboxes by means of glass fiber strips connected to a water reservoir.

The following treatments were applied to the upper rhizobox compartment as humic solutions (three replicates per each treatment, each replicate being represented by a single rhizobox): (i) control, no organic addition; (ii) 14 mg kg^{-1} of bulk HA (HA-1); (iii) 140 mg kg^{-1} of the same HA (HA-10); (iv) 14 mg kg^{-1} of fraction I (FR-I); (v) 14 mg kg^{-1} of fraction II (FR-II); and (vi) 14 mg kg^{-1} of fraction III (FR-III). The amount of HA added to the soil in this study accounts for the upper average found in soils as dissolved organic matter (Lilienfein et al., 2004; Jokinen et al., 2006), and it is similar to that applied in other HA amendment studies (Arancon et al., 2006).

Maize (*Zea mays* L.) seeds were germinated in Petri dishes and pre-grown in the upper compartment of each rhizobox (six seeds per each box). Ten days after germination the upper and the lower compartments of each rhizobox were connected and the roots allowed penetrating through the slit. Twenty five days after germination the lower compartments, which did not receive any amendment, were sampled using a microtome (Wenzel et al., 2001), in order to obtain soil samples at specific distances from the root plane (0–2, 2–4 and 6–4 mm). Soil samples from the upper compartment of the control treatment (i.e., bulk soil without any amendment) were also sampled. Plants were sampled from each

treatment at the same development stage when the root plane fully developed in each rhizobox, and dry biomass values not significantly differed among treatments. In all experiments the rhizoboxes were kept in a Binder KBWF 240 phytotrone (Tuttlingen, Germany) in order to maintain the following controlled conditions: 16 h photoperiod at 120 μE s^{-1} m^2 with day/night temperatures 30 °C/20 °C and 60% humidity.

DNA fingerprinting was also investigated in bulk soil microcosms without plants following additions of humic solutions as described for the upper rhizobox compartment. Analyses were conducted for each microcosm made up with 100 g of soil in a three replicate experiment. Microcosms were incubated up to 60 d at room temperature and 50% WHC and aliquots of 2 g were sampled at 0, 10, 20, 30 and 60 d.

2.5. Bioassay protocol

Cells cultures and bioassays were conducted as described by Yeomans et al. (1999). Cultures of *P. fluorescens* pUCD607 (*lux* CDABE from *Vibrio fischerii*, *kan*^{r}, *amp*^{r}; Hamin-Hanjani et al., 1993) were grown in 250 mL Erlenmeyer flasks containing 100 mL of LB broth at 25 °C, and rotated at 200 rpm. Late exponential cells were harvested by centrifugation (5 min at 4000 *g*) at OD_{550} of 2.0, corresponding to a concentration of cells of around 3×10^7 mL^{-1} (Yeomans et al., 1999). The supernatant was decanted and the cells resuspended in the same volume of C-free M9 minimal medium. The process was repeated, and the final cell suspension shaken at 200 rpm in C-free M9 for 2 h at 25 °C (starvation step). Kanamycin was added at 50 μg mL^{-1} to all cultures.

One milli Liter aliquot of starved cells was added to 100 mg samples of soil collected from rhizoboxes in order to measure *in situ* carbon depositions. After 20 min of contact, 800 μL of soil and bacteria slurry was collected and measured for light emission.

2.6. Luminometry and biosensor response data

Light output was measured using a SystemSure luminometer Model 18172 (Nova Biomedical Waltham MA, USA). In order to compare results from different experiments, bioassays were calibrated on a dilution series of glucose standard solutions in the range of concentrations between 1 and 10 mM of C content. Luminescence data were then converted from relative light units (RLU; 1 RLU equivalent to 1 mV per 10 s) to relative glucose–carbon units (RGU), following interpolation with the calibration curve of glucose standard solutions, and expressed in terms of μmol of glucose–C equivalents per g of soil.

2.7. DNA extraction from soil and genetic fingerprinting

DNA fingerprinting was performed not only on soil samples collected from rhizobox lower compartments, as described above, but also on soil samples from soil microcosms amended as in rhizoboxes, but without maize plants (bulk). For the bulk soil experiments, samples were analysed at different incubation times, in order to assess the response of microbial communities to different amendments at different sampling time. For DNA fingerprinting analyses, the 2–4 and 4–6 mm layers from rhizobox samples were pooled in a single 2–6 mm fraction.

Total DNA was extracted from 500 mg samples with a rapid and efficient method of direct lysis. Soil samples were placed into a bead beater vial and shaken in a Fast Prep 1 FP120 beater (BIO 101 Vista, CA, USA) at 5.5 m s^{-1} for 30 s. Total DNA from soil samples was isolated with a fast DNA kit for soil (BIO 101 Vista, CA, USA). Extracted DNA was analysed on 0.7% agarose gel containing 0.5 μg mL^{-1} ethidium bromide.

Soil DNA was amplified in a PCR Sprint thermocycler (Hybaid, Ashford, UK) with one of the universal primer sets for 16S rDNA, 968F-1401R (Heuer and Smalla, 1997), to obtain products of about 500 base pairs. The 968F primer was modified with a 40mer GC-clamp to better separate PCR products in the DGGE gradient gels (Muyzer et al., 1993). Each PCR mixture contained 50 ng of DNA, 1 × reaction buffer implemented with 2.5 mM $MgCl_2$, 50 pmol of each primer, 0.2 mM of each dNTP, 3 U Taq-polimerase (Euroclone) in a final volume of 50 μL. Bovine serum albumin (BSA) (4 μg) was added to minimize any inhibition of amplification by organic compounds co-extracted from soil. The PCR consisted of 3 min at 95 °C followed by 40 cycles, each one consisting of a denaturing step (10 s at 95 °C), primer annealing (20 s at 54 °C), and an extension step (40 s at 72 °C). A final extension step (10 min at 72 °C) was finally performed. Amplification products, together with a Low Range ladder (1000–80 bp, MBI Fermentas), were checked by electrophoresis on ethidium bromide stained 1.5% agarose gel run at 10 V cm^{-1} in 0.5 × TBE buffer.

PCR products were characterized by denaturing gradient gel electrophoresis (DGGE) which allows the detection of DNA bands representing dominant bacterial species in soil microbial community (Muyzer et al., 1993). DGGE was performed with the Bio-Rad Dcode system. PCR products, 8 μL, were loaded onto denaturing gradients. Bacterial amplicons were separated by a 6% polyacrilamide gel (acrylamide: bisacrylamide, 37:1) with 45% (3.15 M urea-18% v/v formamide) 60% (4.2 M urea-24% v/v formamide) top filling gradient and run for 15 h at 75 V at 60 °C in TAE buffer. Sybr Green stained gels were recorded with a Bio-Rad Gel Doc 2000 Documentation system from Bio-Rad Laboratories (Hercules, CA, USA), equipped with Quantity One software for comparison and clustering profiles.

2.8. Statistical analyses

Different effects on biosensors response were studied by means of *fixed* model analysis of variance (ANOVA) using SPSS software:

(1) Effect of sampling position (classification variable SAMP). The sampling positions were: bulk 0 d (for control only), rhizosphere 0–2 mm, rhizosphere 2–4 mm, rhizosphere 4–6 mm.
(2) Effect of treatments (classification variable TREAT). The five treatments were: control, HA-1, HA-10, FR-I, FR-II, FR-III.
(3) The interaction between the two previous effects (classification variable TREAT * SAMP).

All significant effects were confirmed by Tukey test (at $P < 0.05$) for comparison of means. Genetic fingerprints were analysed using the Quantity One software of the Bio-Rad Gel Doc image analyser system. The similarity of the electrophoretic profiles was evaluated by determining the Dice coefficients of similarity and by obtaining the unweighted pair group with average linkage method (UPGMA) relative clusters.

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 bulk 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

Table 1
Elemental analysis (%), elemental ratios, and relative distribution (%) of signal area over chemical shift regions (ppm) in ^{13}C-CPMAS-NMR spectra of humic samples.

Humic samples	C (%)	N (%)	H (%)	O (%)	C/N	H/C	O/C
Bulk HA	39.5	2.3	3.1	55.1	17.5	0.08	1.4
Fraction I	26.1	1.8	2.6	69.5	14.4	0.10	2. 7
Fraction II	34.3	2.1	3.4	60.1	16.0	0.10	1.7
Fraction III	21.5	1.2	2.5	74.8	18.4	0.12	3.5

	Alkyl-C (0–50)	AlkylC-O (50–110)	Aromatic-C (100–160)	Carboxyl-C (160–190)	Ketonic-C (190–230)	HI/HB[a]
Bulk HA	21.0	36.6	29.3	11.7	1.4	0.99
Fraction I	26.5	39.6	24.5	9.2	0.14	0.96
Fraction II	25.8	40.8	24.0	9.4	0.03	1.01
Fraction III	25.1	42.5	24.3	7.6	0.38	1.02

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

O/C ratios were 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. This is in line with literature findings based on HPSEC isolations of size-fractions (Conte et al., 2006, 2007).

The carbon distribution in humic samples was obtained by the CPMAS-^{13}C NMR spectra. The spectra (not shown) revealed 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 typical 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 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). An 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 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 Vander-Hart, 1999). The aromatic region (110–160 ppm) revealed a broad band around 130 ppm that is 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 bulk HA to 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 also shown in Table 1, where signal integrations are reported. The size-fractions were 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

Table 2
Relative abundance (%) and composition[a] of main termochemolysis products released from bulk HA and size-fractions.

Identified products	Bulk HA	Fraction I	Fraction II	Fraction III
Lignin				
p-Hydroxyphenyl	19.8 (±2.46)	8.8 (±2.98)	7.3 (±0.68)	4.4 (±0.24)
Guaiacyl	15.1 (±1.42)	8.1 (±1.61)	10.6 (±1.85)	6.6 (±0.37)
Syringyl	5.6 (±1.47)	2.2 (±0.44)	3.1 (±0.72)	1.5 (±0.58)
Cinnamic acids	3.2 (±0.98)	1.9 (±0.88)	2.9 (±0.59)	1.3 (±0.72)
Average sum	*43.9*	*21.1*	*24.1*	*14.0*
Other aromatic compounds	12.6 (±2.01)	15.9 (±4.61)	7.3 (±4.79)	16.2 (±4.95)
Average sum of aromatics	*56.6*	*37.1*	*31.4*	*30.3*
Alkyl				
Saturated fatty acids	10.4 (±1.81) C_{13}-C_{31}(C_{16})	11.0 (±1.83) C_9-C_{31}(C_{16}.C_{18})	26.7 (±2.47) C_{10}.C_{28}(C_{16}.C_{18})	17.6 (±3.03) C_{30}-C_{31}(C_{16})
Unsaturated fatty acids	2.2 (±0.33) C_9.C_{16}.C_{18}	1.3 (±0.43) C_{16}.C_{18}	12.0 (±3.22) C_7.C_{16}.C_{18}	1.0 (±0.43) C_{16}.C_{18}
Alkanes/alkenes	6.2 (±3.15) C_{16}-C_{30} (C_{19})	16.2 (±5.41) C_{16}-C_{30} (C_{19})	9.0 (±1.77) C_{16}-C_{30} (C_{19})	21.0 (±2.73) C_{16}-C_{30} (C_{19})
α.ω-Alkane dioic acids	1.4 (±0.32) C_5-C_{11}(C_9)	3.1 (±0.47) C_5-C_9(C_9)	4.5 (±0.56) C_6-C_{22}(C_{16})	2.5 (±1.16) C_6-C_{30}(C_6)
Sterols	0.6 (±0.06)	0.1 (±0.02)	0.2 (±0.6)	0.0 (±0.01)
Terpenoids	0.3 (±0.12)	0	0.3 (±0.02)	0
Average sum	*21.3*	*31.9*	*43.9*	*42.3*
Hydrophilic				
Protein derivatives	15.2 (±0.96)	21.8 (±0.24)	16.3 (±1.77)	14.2 (±1.62)
Polysaccarides derivatives	6.6 (±2.22)	9.2 (±2.68)	4.9 (±2.67)	11.0 (±1.11)
Average sum	*21.8*	*31.0*	*21.2*	*25.2*
Total average	*99.7*	*100.0*	*96.6*	*97.9*

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

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 2. The majority of compounds derived from higher plants, and was represented by lignin, waxes and aliphatic biopolymers, as already found for other bio- and geochemical materials (Guignard et al., 2005).

The original content of lignin products in HA was spread over the three size-fractions by 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. However, the sum of average content of lignin plus other aromatic compounds showed that aromaticity was larger in the bulk HA and decreased in fractions with molecular size (Table 2). Among the alkyl products, also the saturated fatty acids, the alkanes/alkenes, and the α,ω-alkanedioic acids were found to be more present in the size-fractions than in bulk HA, while the unsaturated fatty acids showed slightly less relative content, except for fraction II. Sterols decreased in relative importance passing from the bulk HA to the fractions of progressively decreasing size. Terpenoids were detected only in bulk HA and fraction II. The average sum of alkyl compounds revealed a decreasing trend with molecular size of fractions (Table 2). Among the hydrophilic components detected by on-line thermochemolysis, protein derivatives were more important in the TIC of size-fractions, while polysaccharides derivatives were more prominent in fractions I and III.

3.2. Response of *P. fluorescens* pUCD607

The linear regression obtained with glucose standards was used to convert RLU values into RGU (relative glucose–carbon units), defined as a mol of available C per g of soil. The choice of glucose as standard is justified by the evidence provided by Yeomans et al. (1999), who showed that in the first weeks of maize growth the *P. fluorescens* pUCD607 biosensor response to root exudates was very similar to that of a glucose standard.

Biosensor responses for each treatment at each sampling position are reported in Fig. 1, together with the results of Tukey's test for comparison of means for the TREAT * SAMP effect. The treatment effect (TREAT) and the interaction between treatment and sampling position effect (TREAT * SAMP) were statistically significant for the biosensor response (F values of 46.91 and 12.61, respectively). In particular, the HA-10 treatment (Fig. 2) had the largest biosensor response (1.39 RGU), followed by the HA-1 treatment (0.88 RGU). The three HA size-fractions and control had significantly lower RGU values (FR-I: 0.59; FR-II: 0.57; FR-III: 0.56; control: 0.48), with no significant differences among them (Tukey's test for comparison of means on the TREAT effect).

In the control treatment, where no organic matter was added to the rhizobox upper compartments, RGU units for luminescence in rhizosphere samples were slightly larger (though not statistically significant) than in bulk soil samples. The rhizosphere samples of the rhizoboxes treated with HA at both 14 and 140 mg kg^{-1} rates had RGU values significantly larger than those treated with HA size-fractions. Furthermore, RGU values in the first three layers were almost twice in the HA-10 treatment than in the HA-1 treatment. No differences were observed between control soil and soils added with the three humic size-fractions.

3.3. DNA fingerprinting

Bulk soil microcosms without the presence of plants and roots were analysed for soil bacterial community structure at different incubation times up to 60 d. This period was more than twice the period of soil incubation in the rhiobox.

Replicates were separately incubated, corresponding DNA was extracted, PCR amplified and DGGE analysed immediately at the end of each experiment. A very high degree of reproducibility among replicates at each incubation time was found for all treatments and control. In fact, the cluster analysis of the electrophoretic profiles showed Dice indices of similarity with values between 0.9 and 1.0, indicating that any difference found among profiles should not be attributed to the variability among replicates. Fig. 2 is an example of the reproducibility of the replicates, as it clearly indicates that the three bulk soil microcosm replicates amended with 14 mg (HA-1) humic acids kg^{-1} soil and sampled after 10 d (a), 20 d (b), 30 d (c), 60 d (d) show almost identical profiles at each sampling (Dice coefficients > 0.95). Moreover, in order to evaluate the changes of microbial community during incubation time, regardless of the treatment, control samples at different incubation times were compared. A similarity index $\geqslant 0.85$ indicated a remarkable stability of the microbial community in soil microcosms (data not shown).

Given the large number of samples, there was no way to load all replicates onto one gel. Although it is possible to compare results from different gels, this approach might bear some pitfalls strictly related to the general consideration that gel electrophoresis, in particular under denaturing conditions, depends on experimental variables that make it not perfectly reproducible. Thus, once we

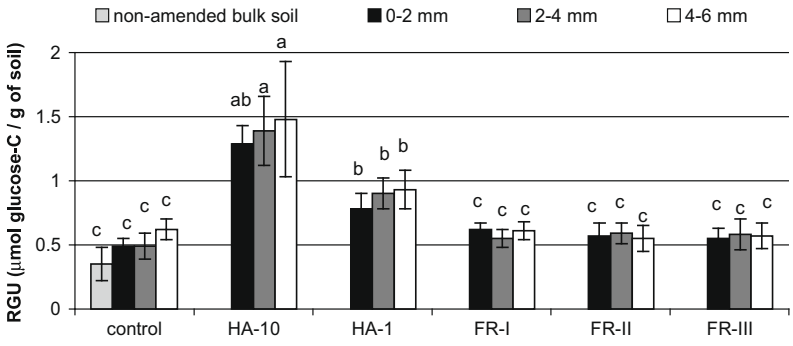

Fig. 1. Response of *P. fluorescens* pUCD 607 in samples from rhizoboxes subjected to different treatments. Data are expressed in terms of relative carbon units (RGU), which are µmol of carbon equivalents per g of soil (means ± standard deviations, $n = 3$). Results of Tukey's test for comparison of means for the TREAT * SAMP effect are reported as minor letters above each bar.

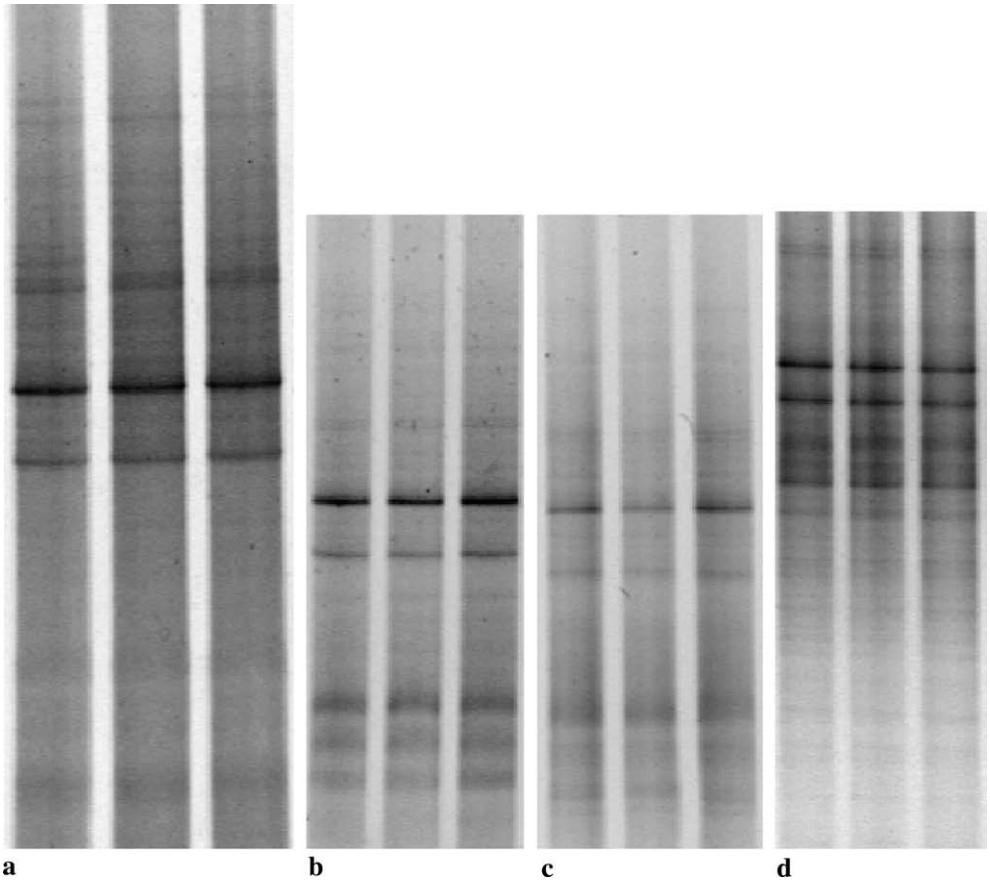

Fig. 2. 16S rDNA-DGGE electrophoretic profiles. Three bulk soil microcosm replicates amended with 14 mg (HA-1) humic acids kg^{-1} soil. Samples collected after 10 d (a), 20 d (b), 30 d (c), 60 d (d). See M & M for more details.

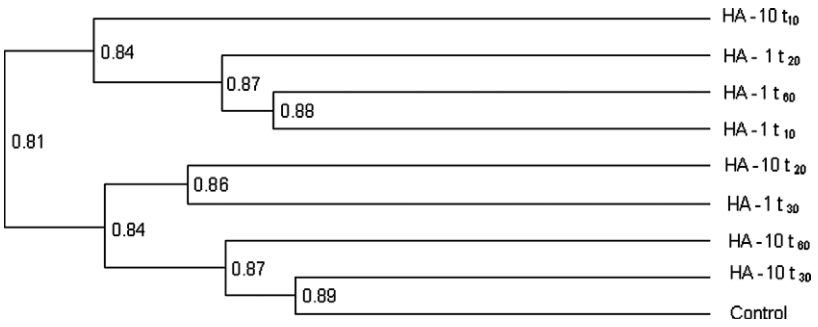

Fig. 3. Cluster analysis of 16S rDNA-DGGE electrophoretic profiles. Bulk soil microcosms amended with 140 mg (HA-10) and 14 mg (HA-1) humic acids kg^{-1} soil; samples collected after 0–60 d. See M & M for more details.

verified the reproducibility of our replicates, we decided to avoid these potential drawbacks to better detect the effects of the treatments under investigation by loading onto a single summarizing gel only one sample per treatment. This approach was performed for bulk soil incubations as well as for rhizoboxes.

The effects of the two rates of bulk HA (140 and 14 mg) on soil bacteria diversity are reported in Fig. 3. The rather similar Dice coefficients for controls and amended microcosms (0.81–0.89) indicate that the unfractionated HA did not particularly affect the structure of bacteria in soil microcosms, as the size-fractions instead did.

The effect of time on the structure of the bacterial communities was evaluated for each amendment. Concerning this, Fig. 4 reports the cluster analysis of genetic fingerprints relative to soil microcosms amended with the three HA size-fractions. Dice coefficients of similarity indicated that controls (t_o samples) clustered separately (0.54–0.65) in comparison with the profiles corresponding to amended microcosms incubated up to 60 d, which tightly clustered together (0.84–0.92; 0.79–0.88; 0.87–0.91, for FR-I, FR-II, FR-III, respectively). Soil bacteria were therefore affected by the humic size-fractions, since changes in community structure occurred rapidly and persisted during time. However, no significant difference was observed among the size-fractions.

The effects of rhizodeposition of maize plants grown in rhizoboxes treated in their upper compartments with HA and its size-fractions were evaluated by 16S PCR–DGGE in soil samples collected from the lower compartment of rhizoboxes, which did not receive the HA amendments. As for the microcosm studies,

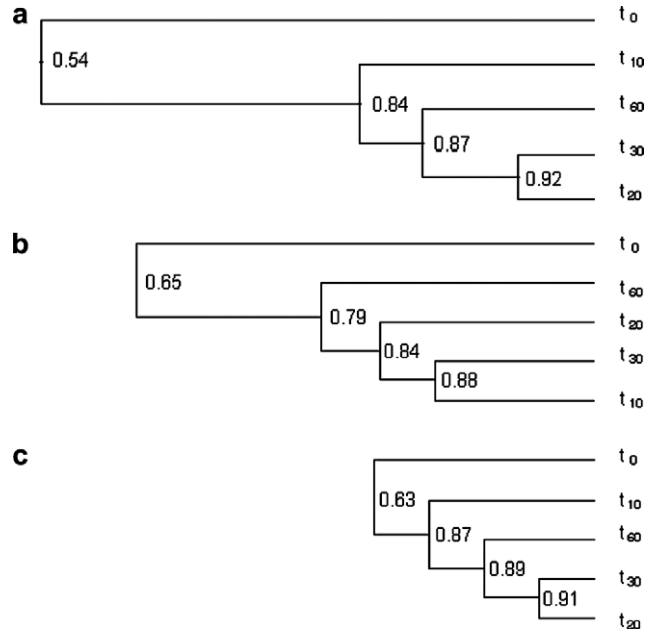

Fig. 4. Cluster analysis of 16S rDNA-DGGE electrophoretic profiles. Bulk soil microcosms amended with of 14 mg of each HA size-fraction kg^{-1} soil; samples collected after 0–60 d. (a) FR-I, (b) FR-II, (c) FR-III.

cluster analysis of genetic fingerprints (not shown) indicate that reproducibility of replicates was large. Fig. 5 reports the effects of organic amendments on bacterial fingerprinting of rhizosphere soil samples at distances from the root plane of 0–2 and 2–6 mm. Within the same treatment, the community structure did not change significantly as a consequence of the radial distance from the root plane, although the profiles for FR-II and FR-III were slightly overlapped. With the only exception of the HA-10 treatment, all treated samples clustered separately (Dice coefficients 0.69–0.37) as compared to the controls, thus suggesting a significant change of soil bacterial community structure as a consequence of humic treatments mediated by plant growth and rhizodeposition.

4. Discussion

Among the three hypotheses tested in this work, the first two, i.e., a stimulating activity of humic acids on maize plants rhizodeposition and a consequent change in rhizosphere microbial communities, were confirmed. Concomitantly, interesting suggestions and conclusions arose from the third hypothesis regarding the existence of a structure–activity relationship between physicochemical properties of humic acids and their effects on the plant-bacteria interactions occurring in the rhizosphere. In the rhizobox systems employed here, humic acids are added only in the upper compartment, while measures were carried out in the lower compartment where no humic acids were added. Previous evidences (Wenzel et al., 2001; Cattani et al., 2006) have shown also that pH changes in the rizosphere samples are neglectable. The measured effects are thus mediated by plant physiology and no direct effects of humic acids are involved.

In rhizoboxes treated with bulk HA, the biosensor response to rhizosphere soils was in fact larger than that to the control soil, whereas the three layers at different distances from the roots plane had similar RGU values. A dose effect was also observed, since the soil treated with larger HA amount (HA-10) showed a greater biosensor response. The similar RGU values found for the three soil slices at different distances may indicate that carbon-rich root exudates tend to move in the soil solution by passive diffusion. In particular, this may be likely for sugars compounds, while organic acids are usually more sorbed and thus have lower diffusion coefficients (Kuzyakov et al., 2003). Only the bulk humic materials (HA-1 and HA-10) stimulated the deposition of easily available carbon compounds from roots to rhizosphere, whereas no differences from control were observed in soils treated with the three size-fractions.

Application of DNA fingerprinting methods enabled the detection of diverse members of soil bacterial consortia, even including bacteria that are not cultivable. DNA fingerprinting was also obtained for bulk soil microcosms to test both the reproducibility of this molecular approach and the stability of the microbial community in unamended soil samples. The comparison of genetic fingerprints between soils treated with the two HA rates failed to show variation in the structure of bacterial communities, while changes were induced by amendments with the separated size-fractions. DNA fingerprinting of treated and control soil from the lower rhizobox compartment, where 25-d-grown maize roots could have mediated the effect of organic amendments on soil bacteria, revealed that HA and its three size-fractions had a different influence on the structure of rhizospheric bacterial community. In particular, the bulk HA, which had poorly responded in bulk soil microcosms, had larger effects in the lower rhizobox compartment through rhizodeposition processes, at least the lower rate. This result is in

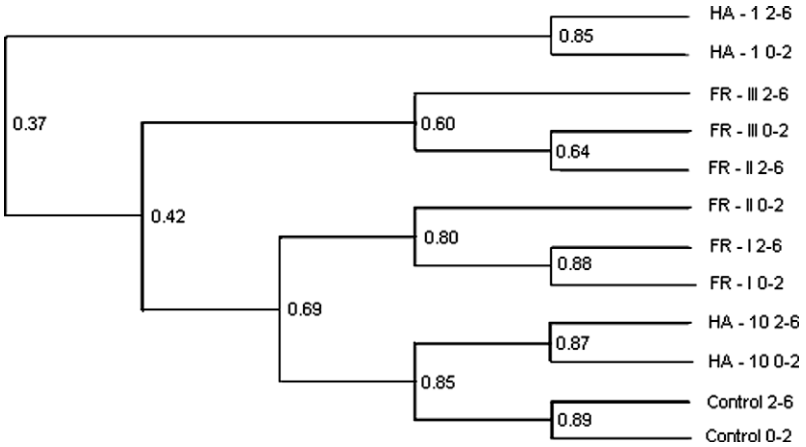

Fig. 5. Cluster analysis of 16S rDNA-DGGE electrophoretic profiles. Rhizospheric soil samples from lower compartments of rhixoboxes amended with HA and their size-fractions. HA-1: 14 mg HA kg^{-1} soil; HA-10: 140 mg HA kg^{-1} soil; FR-I: 14 mg of size-fraction I kg^{-1} soil; FR-II: 14 mg of size-fraction II kg^{-1} soil; FR-III: 14 mg of size-fraction III kg^{-1} soil. 0–2: microtome sampling of rhizospheric soil at a distance of 0–2 mm from root plane; 2–6: microtome sampling of rhizospheric soil at a distance of 2–6 mm from root plane. See M & M for more details.

accordance with the greater biosensor response for HA-1 and HA-10 treatments and supports the hypothesis that the increased rhizodeposition induced by bulk HA had also a larger effect on the rhizosphere microbial communities.

Although difficult to be correlated by statistical means, rhizodeposition and community structure results can be interpreted in the light of pyrolsyis-GC–MS and CPMAS-NMR characteristics of HA and its three size-fractions. These showed distinct differences between the molecular composition of HA and its three fractions (Tables 1 and 2). ^{13}C-CPAS-NMR spectra revealed a larger content of aromatic, carboxylic and ketonic carbon compounds in HA (Table 1). Consequently, GC/MS analysis of thermolytic products showed that the amount of lignin compounds in HA was larger than in the three fractions. Some of the identified compounds (p-hydroxyphenyl, guaiacyl, syringyl and cinnamic acid) have known effects on plant physiology (Yu et al., 2003), thus suggesting a significant structure activity relationship between the molecular characteristics of HA and the effects on roots rhizodeposition processes. Separated size-fractions were progressively more hydrophilic than the bulk HA. The larger hydrophobic character of the latter was conducive of a stronger conformational stability, with its molecular components remaining less accessible to metabolization by soil microorganisms. Molecular fingerprinting results for the bulk microcosms amended with HA and its size-fractions indicated that soil microorganisms hardly utilized the bulk HA, most probably because of its structural complexity and stability.

These evidences suggest that bulk HA is less degraded by soil microorganisms, and hence maintains a larger stimulation on plants rhizodeposition. Conversely, it is very likely that solubilization, extracellular degradation, and uptake of the three size-fractions occurred more easily, thus making these humic amendments available for heterotrophic bacteria. This increased degradation might be explained by changes in pH and solubility of organic molecules, increased microbial activities, and cooperative degradation among different bacterial species, which are phenomena typically induced by plant root rhizodeposition. Therefore, the structural and chemical complexity of added humic materials appears to be a key factor for their utilization by heterotrophic microbial community.

Previous findings have shown that the same HA and size-fractions as those studied here had a stimulatory effect on maize plants, increasing the enzyme activities related to glycolysis and tricarboxylic acid (TCA) cycle (Nardi et al., 2007). Plants grown for 10 d in presence of HA in the upper compartment of the rhizoboxes may have released more organic carbon in the rhizosphere as a consequence of the induction of such enzymatic activities. This release was still evident in the lower compartment of the rhizoboxes 15 d later, when plants were still exposed to HA in the upper compartment. An explanation of this process is that roots should first exudate sufficient organic acids to disrupt the strong humic associations, from which bioavailable humic components are then released and become accessible to microbes for transformation (Piccolo et al., 1999; Piccolo, 2002). Rather than specific differences in molecular composition between HA and its size-fractions, it is the difference in strength of molecular conformation that may explain the results. In fact, a greater root input is needed for disaggregating the bulk HA, thus implying a larger presence of bioavailable carbon that stimulated the biosensor response.

This study confirms the hypothesis that HA amendments affect the rhizodeposition of maize plants, and, concomitantly, the structure of rhizosphere microbial communities. In particular, we showed that HA with a large conformational stability increased rhizodeposition by plants, and consequently the microbial community structure. The main hypothesis, in accordance with the microbial loop model of Bonkowsky (2005), is that in the presence of hardly bioavailable organic materials, plants stimulate a larger microbial activity. This may have important consequences on the management of soil fertility and agricultural exploitation of humified organic matter.

Our results also show the importance of applying different methods to assess the effects of organic matter on soil rhizosphere. Technologically advanced methods such as biosensors and DNA fingerprinting gave more valuable results than classical approaches based on microbial counts, which usually fail to detect significant changes in the rhizosphere. Results obtained here by biosensors and DNA fingerprinting are complementary. The former method provides a quantitative assessment of carbon species readily available to the common *P. fluorescens* soil bacterium, while fingerprinting methods well distinguished among different sample treatments, as a result of induced changes in the structure of soil microbial communities.

Acknowledgements

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