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Chemical Characteristics and Biological Activity of Organic Substances Extracted from Soils by Root Exudates

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ABSTRACT

Plants have evolved with roots in close contact with the solid phase of the soil. Therefore, root exudates may be a better medium for extracting low molecular size (LMS) organic fractions than currently used alkaline solutions. Our objective was to compare the chemical and biological activity of LMS extracts using maize (*Zea mays* L.), *Picea abies*, and *Pinus sylvestris* root exudates to humic substances (HS) extracted with alkaline solution. Gas chromatographic/mass spectrometric (GC/MS) spectra revealed that the LMS fractions had a greater variety of fatty acids than the HS. Fourier transform infrared (FT-IR) spectra of LMS fractions also indicated different amounts of functional groups by comparison with HS. The possible biological role of LMS fractions with respect to HS was assessed by measuring hormone-like activity and nitrate uptake in *P. sylvestris* seedlings. The LMS fractions from agricultural soil stimulated nitrate uptake and nitrate reductase (NR) and glutamine synthetase (GS) activities, whereas those from a forest soil increased ammonium uptake, NR, and glutamate dehydrogenase (GDH) activities. The stimulation of nitrate and ammonium uptake via a NR-GS or NR-GDH metabolic pattern was consistent with the different chemical composition of the LMS fractions. This indicates LMS fractions in soil have consequential affects on the plant's capacity to adapt to different environmental conditions.

In the last decade, a large amount of information has accumulated on humic substances (HS), warranting the creation of an independent science of humic compounds (Tan, 2003). Two different concepts have emerged, one claiming humic compounds to be operational or fake compounds produced by the analytical extraction procedures and the other considering them to be natural compounds occurring in soils, rivers, lakes, oceans, and their sediments (Tan, 2003). The two opposing opinions have apparently created some confusion as to what HS are and their role in plant metabolism.

Humic substances are hydrophilic, acidic, and high in molecular weight, ranging from several hundred to thousands of daltons, and are usually obtained from soils by extraction, fractionation, and isolation procedures using caustic alkaline solution (Stevenson, 1994). Some studies have suggested that HS have only an apparent high molecular size, which can be reversibly disrupted by treating humic solutions with low concentrations of mono-, di-, and tricarboxylic acids (Dell'Agnola and Nardi, 1987; Nardi et al., 1996). More recently, numerous studies have shown that the amphiphilic properties of the organic acids in root exudates can dissociate HS into low molecular size (LMS) and high molecular size (HMS) structures (Nardi et al., 1997, 2000; Piccolo, 2002, 2003). This new interpretation may support the hypothesis that the conformational behavior of dissolved humus in the rhizosphere, and therefore also the interaction of humic components with plant-root cells, may be controlled by the presence of root-exuded or microbe-released organic acids in the soil solution (Piccolo et al., 2003).

Humic substances are also known to affect plant growth, producing a wide spectrum of physiologic effects, although the overall mechanism explaining how HS interact with the plant's physiological system is not clear (Varanini and Pinton, 2001; Clapp et al., 2001). Nardi et al. (2002a) showed that the LMS humic fraction was small enough to cross the plasma membrane and actively participate in plant metabolism. Other LMS humic fractions obtained from different substrates, earthworm feces, and forest soils showed a hormone-like activity and positive effects on some metabolic pathways (Pizzeghello et al., 2001; Quaggiotti et al., 2004).

One of the most prominent physiologic effects of HS on plants concerns N uptake and assimilation (Quaggiotti et al., 2004). For N to be assimilated by plants, after NO_3^- uptake, the nitrate must first be reduced to ammonia. This is done by two enzymes: a cytoplasmic nitrate reductase (NR), which converts the nitrate to nitrite, and a chloroplastic nitrite reductase that catalyses the reduction of nitrite to ammonium. Ammonium is toxic and does not accumulate anywhere in the plant. In higher plants, ammonium is assimilated, at low or normal levels, more through glutamine synthetase (GS) activity than the reductive amination of 2-oxoglutarate, catalyzed by glutamate dehydrogenase (GDH). Glutamate dehydrogenase serves the main physiologic purpose of deaminating glutamate, thereby supplying sufficient carbon skeletons for the tricarboxylate cycle to function (Robinson et al., 1991). This enzyme also has an important role in plant N metabolism, providing a link between the citrate cycle (via 2-oxoglutarate) and amino acid biosynthesis (via glutamate).

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Abbreviations: EC, eutric cambisol; FT-IR, Fourier transform infrared spectra; GC/MS, gas chromatographic/mass spectrometric; GDH, glutamate dehydrogenase; GS, glutamine synthetase; HMS, high molecular size; HS, humic substances; LMS, low molecular size; NR, nitrate reductase; RL, rendzic leptosol.

In previous studies (Nardi et al., 2002a), we extracted LMS organic substances from agricultural and forest soils using sterile plant root exudates. These extracts were chemically analyzed by chromatographic/mass spectrometry (GC/MS) and then tested on watercress and lettuce to determine their auxin- and gibberellin-like activities and on maize seedlings to study their influence on N metabolism.

In the present study, we analyzed the effect of extracts from agricultural and forest soils obtained by treating both soils with maize (cv. Sandek), Scotch pine, and Norway spruce exudates. These six extracts were first tested against maize seedlings (Nardi et al., 2002a), and then, as reported here, against Scotch pine seedlings. The rationale behind these studies was to seek a possible link between soil (agricultural or forest) and plant (maize or Scotch pine) that is mediated by root exudates (maize, Scotch pine, or spruce).

To gain a better understanding of the chemical characteristics of the root exudates and extracted organic fractions, the root exudates and the extracted organic fractions were chemically analyzed and compared using GC/MS (as in the previous study) and by Fourier transform infrared (FT-IR) spectra measurements. The biological activity of the organic extracts was determined by evaluating their hormone-like activity and their effects on N metabolism.

MATERIALS AND METHODS

Study Sites, Soils, and Humic Substance Extraction

Two soils, a Eutric Cambisol (EC) and a Rendzic Leptosol (RL) (FAO–UNESCO, 1990; TypicEutrudept, Inceptisol Umbrepts Haplumbrepts, US Classification), were used for this study. The EC is developed under a field of Bermuda grass (*Cynodon dactylon* Pers.) near the Agricultural College of the University of Padova (Legnaro, Padova, Italy), and the RL comes from near Cortina d'Ampezzo (Belluno, Italy), in the mountains, and is covered by a Scotch pine forest. Soil analysis was performed following standard methods (SISS, 2000).

Humic substances were extracted from the A horizons of the EC and RL with common alkaline extraction procedures (Stevenson, 1994). The extracts were extensively dialyzed into 18 kDa cut-off Visking tubing (Medicell, London, England) against distilled water and purified of metals by elution through a cation exchange resin Amberlite IR 120 in a protonated form. The pH was then adjusted to 6.5 with 0.1 *M* KOH. The oxidimetric method (Stevenson, 1994) was used to measure the humic C content.

Root Exudate Collection and Chemical Characterization

Two commercial maize hybrids (*Zea mays* L., *cv*. Mytos and Sandek; Dekalb, Italy) and two forest tree species [*Picea abies* (*Karst.*) and *Pinus. Sylvestris* (*L.*)] were used for this study. These plant species were chosen because maize is a crop of economic relevance in lowland soil (EC), and *P. abies* and *P. sylvestris* are the main vegetation of the forest soil (RL).

The maize root exudates were collected from seedlings grown under sterile hydroponic conditions (Mench and Martin, 1991). The maize seeds were surface sterilized and germinated in Petri dishes containing nutrient agar at 28°C for 3 d to check their sterility. Six maize seedlings were transferred aseptically into a glass tube (60 mm in diameter × 300 mm), which contained distilled sterile water and a steel net to prevent the seeds coming into contact with the water. To prevent the occurrence of anaerobic conditions, sterile air was bubbled into the tube using an air pump. The plants were grown for 10 d in a phytotron (14 h photoperiod with day/night temperature 25/18°C, 65/80% relative humidity, 500 μmol m^{-2} s^{-1} light intensity). Two days before collecting the root exudates, each tube was checked for sterility. After the growth period, tube solutions were collected and filtered at 0.45 μm (Millipore, Milford, MA). The filtrate was assumed to contain soluble root exudates (Kraffczyk et al., 1984).

The forest tree seeds were surface sterilized with 3% hydrogen peroxide for 10 min and rinsed and soaked with distilled water for 1 h before sowing in inert sand as a soil substitute: 590 g of inert sand and 67 mL of distilled water were placed in Vitro Vent containers (Duchefa Biochemie BV, Haarlem, The Netherlands) and sterilized at 120°C for 20 min; 36 seeds per box were placed at equal distances on the surface of the inert sand. The seedlings were grown for 20 d in a phytotron (14 h photoperiod with day/night 20/16°C temperature, 65/80% relative humidity, 300 μmol m^{-2} s^{-1} light intensity). Two days before collecting the root exudates, the sterility of each container was checked. After the growth period, the solution in the container was collected by suction and filtered at 0.45 μm (Millipore). The filtrate was assumed to contain soluble root exudates (Kraffczyk et al., 1984). At the end of the experiments, 150 maize seedlings, 850 *P. abies* seedlings, and 1430 *P. sylvestris* seedlings, respectively producing 2700, 1250, and 2200 mL of root exudates, were analyzed.

An aliquot of root exudate was concentrated under vacuum at 40°C and analyzed for total organic C, N (SISS, 2000), protein (Bradford, 1976), and free amino acid content (Muscolo et al., 2002). For the LMW carboxylic acids, an aliquot of exudates was passed onto an anion exchange solid-phase extraction column (SAX; Alltech, Deerfield, IL). Anionic fractions were eluted with 500 m*M* H_2SO_4. Free organic acids were separated by HPLC on an HPX 87 H Aminex column (Biorad, Richmond, CA) with 6 m*M* H_2SO_4 as the mobile phase (0.4 mL min^{-1}), at room temperature and detected at 210 nm (Pecina et al., 1984). Semiquantitative results were obtained because only identified organic acids were integrated.

Extraction of Low Molecular Size Organic Fractions

To mimic the interaction between soil and plant in the rhizosphere and to use a more realistic HS extraction method, organic substances were extracted from the bulk soil not only by alkaline solution, but also using root exudates. Two grams of soils (A horizons) were gently shaken with 20 mL of water or 20 mL of root exudate at room temperature for 16 h under a N_2 atmosphere. The suspensions were centrifuged at 5000 *g* and 10°C for 30 min (Nardi et al., 2002b). The supernatants (extracts) were analyzed for total organic C, N content (SISS, 2000), and protein content (Bradford, 1976).

Gas Chromatographic/Mass Spectrometric Analysis

Two milliliters of exudates or extracts were placed in a vial (4 mL), freeze-dried using the Modulyo 4K system (Edwards, Crawley, England), and added to 200 μL of 2.2-dimethoxypropane and 20 μL of concentrated HCl. This reagent enables high yield derivation of all acidic species in the sample to produce the corresponding methyl ester (Rachelle, 1963). When the modified acids were analyzed using the GC/MS system, they showed an improvement over the original acids in detection and gas chromatographic performance. After 30

Table 1. Main characteristics of the two soils studied, Eutric Cambisol (EC) and Rendzic Leptosol (RL) (FAO-Classification 1990).†

Soil	Horizon	pH	Sand	Silt	Clay	Total $CaCO_3$	Active $CaCO_3$	OC	C/N	CEC	NO_3^-	NH_4^+
			%							cmol kg^{-1}	%	
EC	A	6.50 a‡	65 a	17 a	18 b	6 b	0.1 b	2.4 b	10.3 b	18 b	0.015 a	0.055 b
RL	Ah	7.65 b	26 b	18 a	20 a	40 a	10 a	21.0 a	18.8 a	108 a	0.011 b	0.160 a

† CEC, cation exchange capacity; OC, organic C.
‡ Values in the same column followed by the same letter are not statistically different at $P \leq 0.05$ by Student-Newmann-Keuls test (Sokal and Rohlf, 1969).

min, the liquid phase was dried under a N flow, and 20 μL methanol was added. A portion of the final liquid phase was injected into the GC/MS for analysis. The GC/MS system was a HP 5890 II gas chromatograph coupled with a quadrupole HP 5971 A. The chromatographic separation of the analytes was obtained using the capillary column HP 50+ with the following dimensions: length 30 m, film thickness 0.5 μm, and internal diameter 0.25 mm. The column underwent the following temperature program: from 100°C × 1 min to 250°C to 5°C/min–250°C × 10 min, with injector and transfer-line temperatures of 250 and 280°C, respectively. The mass spectrometer operated in SCAN mode to detect the ions generated by electron ionization (70 eV) at the ion source temperature of 180°C, with a mass range from 50 to 320 D. The acid species were identified using certified linear standards submitted to the same derivation treatment and compared with the mass spectra reported in the NBS mass spectra library.

Fourier Transform Infrared Spectroscopy

The FT-IR spectra were obtained with a Nicolet 5PC FTIR. Mixtures containing 1 mg of sample and 400 mg of KBr, spectrometry grade, were uniformly prepared and formed into pellets under negative pressure. The spectra were recorded in the 4000 to 400 cm^{-1} wavelength range.

Hormone-like Activities of Low-Molecular-Size Organic Fractions

The auxin-like and gibberellin-like activity of the LMS organic fractions extracted by the root exudates were assessed by checking the reduction in the growth of watercress (*Lepidium sativum* L.) roots and the increase in the length of lettuce (*Lactuca sativa* L.) epicotyls (Audus, 1972). Watercress and lettuce seeds were surface sterilized by immersion in 8% hydrogen peroxide for 15 min. After the seeds were rinsed five times with sterile distilled water, 10 seeds were placed on a sterile filter paper in a sterile Petri dish. The filter paper was wetted, for the watercress, with 1.2 mL of 1 m*M* $CaSO_4$ (control) or 1.2 mL of 20, 10, 1, and 0.1 mg L^{-1} indoleacetic acid (Sigma, St. Louis, MO) for the calibration curve or 1.2 mL of 10, 5, 2.5, 1, 0.5, 0.2, and 0.1 mg C L^{-1}. The experimental design for the lettuce was the same as for the watercress except that the sterile filter paper was wetted with 1.4 mL and the calibration curve was a progression of 100, 10, 1, and 0.1, mg L^{-1} gibberellic acid (Sigma). The seeds were germinated in the dark at 25°C in a germination room. The watercress for was left for 48 h and the lettuce for 72 h. The seedlings were removed, and the lengths of the roots and epicotyls were measured (Audus, 1972).

Table 2. Composition of root exudates used to extract low-molecular-size organic fractions.

Species	pH	C	N	Proteins	Amino acids
		μg cm^{-1} root			
Z. mays (Mytos)	4.8 b†	21.67 b	16.03 a	0.0096 b	0.7132 c
Z. mays (Sandek)	5.8 a	37.08 a	9.92 c	0.0478 a	1.1678 a
P. abies	4.4 b	10.39 d	2.46 d	0.0019 c	0.9649 b
P. sylvestris	4.6 b	16.88 c	11.68 b	0.0012 d	0.6420 d

† Values in the same column followed by the same letter are not statistically different at $P \leq 0.05$ by Student-Newmann-Keuls test (Sokal and Rohlf, 1969).

Plant Material, Nitrate and Ammonium Uptake, Nitrate Reductase, Glutamine Synthetase, and NADH-Glutamate Dehydrogenase Assays

Scotch pine seeds were surface sterilized with 3% hydrogen peroxide solution for 10 min and rinsed and soaked in distilled water for 1 h before sowing on inert sand as a soil substitute: 590 g of inert sand and 67 mL of distilled water were placed in Vitro Vent containers (Duchefa Biochemie BV; Haarlem, The Netherlands) and sterilized at 120°C for 20 min; 36 seeds per box were placed at equal distances on the surface of the inert sand. The boxes were covered with transparent lids and kept for 12 d in a growth chamber under white light and long day conditions (16/8 h light/dark, 25/20°C, 70/75% humidity) and harvested. Seedlings were watered with sterilized one-half N-free Ingestad's solution, pH 5.6, every 4 d (Ingestad, 1960). After the growth period, the seedlings were gently removed from the rooting medium and adapted to a hydroponic culture in Plexiglas tanks for 24 h before the experiments were performed.

The seedlings were left for 24 h in contact with an aerated solution of the extracted LMS organic fractions (0.5 μgC/mL), classical HS (0.5 μgC/mL), or water extracts (0.5 μgC/mL) and transferred to a 100 μ*M* KNO_3 or 100 μ*M* $(NH_4)_2SO_4$ solution. The seedlings (0.5 g) were placed in beakers containing 50 mL of the conditioning nutrient solutions with nitrate or ammonium (100 μ*M*) and left for 30 min (Panuccio et al., 2001) in the growing chamber solution under the same climatic conditions as reported previously. The pH of the nutrient media remained within a physiological range (pH 5.7–6.9) throughout the period. Samples were also taken from beakers containing uptake solutions alone to check for any nitrate or ammonium depletion due to microbial activity. At the end of the uptake period, the samples were analyzed following the methods of Goldsmith et al. (1973) for nitrate and Weatherburn (1967) for ammonium. The uptake rates were calculated by measuring the depletion from the solutions. Before the experiments, the roots were examined under the microscope to check for the absence of mycorrhiza. At the end of the experiments, 3856 Scotch pine seedlings were analyzed, and the average fresh weight of the seedlings was 41.88 mg. The average fresh weight of the epicotyl was 32.95 mg, and the average fresh weight of the hypocotyl was 8.93 mg.

To assay the nitrate reductase (NR), 1 g of freshly excised seedlings was thoroughly homogenized with a mortar and pestle in 6 mL of ice-cold buffer containing 1 m*M* Na_2 DTA, 10 m*M* hydrochloride monohydrate, 25 m*M* potassium phosphate, and 3% BSA (pH 7.8). The debris was filtered through four layers of gauze and centrifuged at 4°C at 17 000 *g* for 15 min. The NR activity was assayed as per Lewis et al. (1982).

For the glutamine synthetase (GS) assay, 1 g of freshly excised seedlings was homogenized in 10 mL of ice-cold buffer

Table 3. Low molecular weight organic acid composition of root exudates in the two maize (*Z. mays* L.) cultivars and *P. abies* Karst. and *P. sylvestris* L. seedlings.

	Organic acids, nM cm^{-1} root					
	Oxalic	Citric	Tartaric	L-Malic	Succinic	Fumaric
Z. mays (Mytos)	6.074 c†	5.614 a	44.944 a	2.465 b	n.d.‡	1.905 a
Z. mays (Sandek)	2.862 d	4.939 b	5.734 d	29.709 a	n.d.	0.368 c
P. abies	30.121 a	n.d.	19.828 c	2.118 c	1.374 a	0.206 d
P. sylvestris	18.466 b	5.890 a	22.868 b	n.d.	1.468 a	0.510 b

† Values in the same column followed by the same letter are not statistically different at $P \leq 0.05$ by Student-Newmann-Keuls test (Sokal and Rohlf, 1969).
‡ Not detected.

(pH 7.2) containing 50 m*M* imidazole, 1 m*M* Na_2 EDTA, and 10 m*M* hydrochloride monohydrate. The debris was filtered through four layers of gauze and centrifuged at 4°C at 17 000 *g* for 10 min. The GS activity was assayed as suggested by Rhodes et al. (1975).

The NADH-glutamate dehydrogenase was assayed by homogenizing 1 g of freshly excised seedlings in 5 mL of ice-cold buffer containing 50 m*M* KH_2PO_4 and K_2HPO_4 (pH 7.5). The debris was filtered through four layers of gauze and centrifuged at 4°C at 1000 *g* for 5 min (pellets discarded) and then at 30 000 *g* for 10 min (pellets discarded). The supernatants from the first centrifugation were treated with 10 μL of 10% lubrol for 10 min. The GDH activity was assayed according to Robinson et al. (1991).

All enzymatic data were the means of five replicates, and the standard deviations did not exceed 5%.

RESULTS

The main chemical properties of the two soils are given in Table 1. The EC is characterized by a moderate soil texture grade, a neutral pH, and a good level of organic C and cation exchange capacity. The RL, developed on a carbonate substrate, shows the chemical characteristics of the Dolomite soils typical of the mountains of Northern Italy. It is characterized by a subalkaline soil reaction and a high level of total and active carbonate. The environmental conditions and the presence of the Scotch pine litter are the main reasons for the high level of organic C and the high C/N ratio. The NO_3^- and NH_4^+ contents of the two soils were also determined by studying N uptake and assimilation. The NO_3^- content was 0.015% in the EC and 0.011% in the RL, whereas the NH_4^+ levels were 0.055 and 0.16%, respectively.

Table 2 shows the main characteristics of the root exudates used to remove the organic fractions from the soils. The two maize hybrid exudates revealed higher amounts of C, N, and protein than those of the two forest tree species. The concentrations of oxalic and succinic carboxylic acids were always higher in the *P. abies* and *P. sylvestris* than in the two maize hybrid root exudates (Table 3). The two forest tree exudates differed in that the *P. abies* had a high content of oxalic and L-malic acids, whereas the *P. sylvestris* contained citric acid. The two maize hybrid root exudates also differed: Mytos had a high tartaric acid content, whereas Sandek had a high L-malic acid content.

The LMS organic fractions obtained by treating the RL with maize or forest tree root exudates were always richer in C content than the fractions extracted from the EC (Table 4). The N and protein content showed the same trend (Table 4). GC/MS analysis (Table 5) revealed the presence of $C_{11}H_{23}COOH$ and $C_{13}H_{27}COOH$ in the classical HS (alkaline extraction) from both soils. The water extract revealed the presence of $C_{13}H_{27}COOH$ and $C_{15}H_{31}COOH$ for EC and $C_{11}H_{23}COOH$, $C_{13}H_{27}COOH$, $C_{14}H_{29}COOH$, and $C_{15}H_{31}COOH$ for the RL. The LMS organic fractions obtained by treating the soils with the maize exudates revealed $C_{11}H_{23}COOH$, $C_{13}H_{27}COOH$, and $C_{14}H_{29}COOH$. The two hybrids differed in that the Mytos extracted $C_{15}H_{31}COOH$ and $C_{16}H_{33}COOH$ from the EC, whereas the Sandek extracted only $C_{15}H_{31}COOH$. The *P. abies* extracted $C_{11}H_{23}COOH$ and $C_{13}H_{27}COOH$ from both soils, but it extracted $C_{14}H_{29}COOH$ from EC and $C_{15}H_{31}COOH$ from RL. The fraction extracted from the EC by *P. sylvestris* revealed $C_{13}H_{27}COOH$ and $C_{17}H_{35}COOH$, whereas the extract from the *P. sylvestris* and the RL contained $C_{14}H_{29}COOH$, $C_{15}H_{31}COOH$, $C_{16}H_{33}COOH$, and $C_{17}H_{35}COOH$.

Comparing the FT-IR spectra of the root exudate samples showed differences in the appearance of new bands and the relative intensities of some bands, indicating different relative amounts of functional groups. The FT-IR spectra of the Mytos and Sandek root exudates (Fig. 1a and 1b) featured the following common bands: 3400 cm^{-1} (phenolic, alcoholic, and carboxylic acids OH stretching), 2900 cm^{-1} (symmetric and asymmetric stretching of CH_2), 1649 cm^{-1} (aromatic C = C vibrations, C = O stretching of amide I, and asymmetric stretching of COO^-), 1540 cm^{-1} (C = C aromatic ring stretching) more intense in Mytos root exudate, 1412 cm^{-1} (vibrational modes of CH_2) more intense in Sandek

Table 4. Composition of low-molecular-size organic fractions extracted by treating the Eutric Cambisol and Rendzic Leptosol with root exudates.†

	pH	C	N	Protein
		μg mL^{-1}		
EC + water	8.4 a‡	46.9 g	21.6 g	0.019 h
EC + KOH	3.2 c	336 b	n.d.	n.d.
EC + Mytos	8.6 a	72.1 e	60.6 d	0.325 c
EC + Sandek	8.7 a	78.8 e	22.5 fg	0.172 f
EC + *P. abies*	8.5 a	53.3 f	22.9 f	0.325 c
EC + *P. sylvestris*	8.7 a	75 e	31.6 e	0.019 h
RL + water	8.4 a	153.3 d	14.6 h	0.363 b
RL + KOH	5.8 b	757.9 a	n.d.	n.d.
RL + Mytos	8.6 a	274.6 c	90.9 a	0.402 a
RL + Sandek	7.9 a	284.4 c	77.9 c	0.248 e
RL + *P. abies*	8.8 a	239.8 c	87.2 ab	0.287 d
RL + *P. sylvestris*	8.4 a	259.2 c	81.50 bc	0.134 g

† EC, Eutric Cambisol; n.d., not detected; RL, Rendzic Leptosol.
‡ Values in the same column followed by the same letter are not statistically different at $P \leq 0.05$ by Student-Newmann-Keuls test (Sokal and Rohlf, 1969).

Table 5. GC/MS analysis of fatty acids in Eutric Cambisol and Rendzic Leptosol organic fractions mobilized by maize (*Z. mays* L., cultivar Mytos and Sandek) root exudates or forest (*P. abies* Karst. and *P. sylvestris* L.) root exudates (excluding the fatty acids already existing in the exudates).

	EC	RL
KOH	$C_{11}H_{23}COOH$; $C_{13}H_{27}COOH$	$C_{11}H_{23}COOH$; $C_{13}H_{27}COOH$
Water	$C_{13}H_{27}COOH$; $C_{15}H_{31}COOH$	$C_{11}H_{23}COOH$; $C_{13}H_{27}COOH$; $C_{14}H_{29}COOH$; $C_{15}H_{31}COOH$
***Z. mays* (Mytos)**	$C_{11}H_{23}COOH$; $C_{13}H_{27}COOH$; $C_{14}H_{29}COOH$; $C_{15}H_{31}COOH$; $C_{16}H_{33}COOH$	$C_{11}H_{23}COOH$; $C_{13}H_{27}COOH$; $C_{14}H_{29}COOH$
***Z. mays* (Sandek)**	$C_{11}H_{23}COOH$; $C_{13}H_{27}COOH$; $C_{14}H_{29}COOH$; $C_{15}H_{31}COOH$	$C_{11}H_{23}COOH$; $C_{13}H_{27}COOH$; $C_{14}H_{29}COOH$; $C_{15}H_{31}COOH$
P. abies	$C_{11}H_{23}COOH$; $C_{13}H_{27}COOH$; $C_{14}H_{29}COOH$	$C_{11}H_{23}COOH$; $C_{13}H_{27}COOH$; $C_{15}H_{31}COOH$
P. sylvestris	$C_{13}H_{27}COOH$; $C_{17}H_{35}COOH$	$C_{14}H_{29}COOH$; $C_{15}H_{31}COOH$; $C_{16}H_{33}COOH$; $C_{17}H_{35}COOH$

root exudate, 1380 cm^{-1} (COO- stretching and OH deforming and stretching of phenolic C-O), 1232 cm^{-1} (C-C stretching), 1149 cm^{-1} (C-O-C asymmetric and symmetric stretching of esters and carbohydrates), 1079 cm^{-1} (C-O-H stretching of alcohols), and 1034 cm^{-1} (C-O stretching of secondary alcohols, aromatic ethers, and polysaccharides). The Sandek exhibited a peak at 1724 cm^{-1} assigned to C = O stretching of esters and carboxylic acids. The *P. abies* and *P. sylvestris* exudates produced a FT-IR spectra (Fig. 1c and 1d) characterized by the following peaks: 3400 cm^{-1} (phenolic, alcoholic, and carboxylic acids OH stretching); 2900 cm^{-1} (symmetric and asymmetric stretching of CH_2), which was present as a shoulder in *P. sylvestris*; 1656 cm^{-1} (amide I), present only in *P. abies* root exudates; 1636 cm^{-1} (aromatic C = C vibrations, C = O stretching of amide I, and asymmetric stretching of COO^-); 1399 cm^{-1} (vibrational modes of CH_2), which was present as a shoulder in *P. abies* root exudates; 1386 cm^{-1} (COO- stretching and OH deforming and stretching of phenolic C-O); 1080 cm^{-1} (C-O-H stretching of alcohols); and 1047 cm^{-1} (C-O stretching of secondary alcohols and of polysaccharides). The last two absorptions seemed to be much more intense in the *P. sylvestris* root exudates, which also showed a band at 919 cm^{-1} (vibrational modes of alcoholic C-O-H). A strong band at 836 cm^{-1} (aromatic C-H out of plain bending mode) was also evident in the *P. abies* root exudate spectrum.

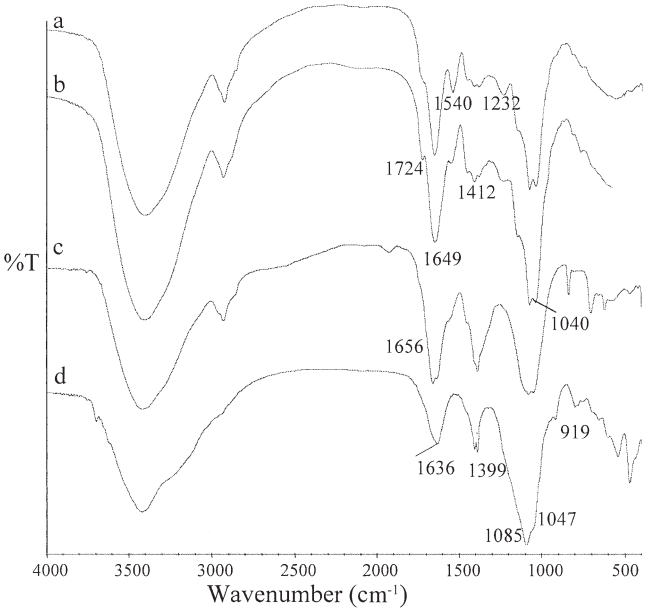

Fig. 1. FT-IR spectra of Mytos (a), Sandek (b), Norway spruce (c), and Scotch pine (d) root exudates.

P. abies and *P. sylvestris* root exudates were characterized by a different number of functional groups. The *P. abies* had a higher content of carboxylic groups, whereas the *P. sylvestris* had a higher content of alcoholic groups.

The FT-IR spectra of aqueous soil extracts (Fig. 2) were similar, but significant differences were evident in the peaks of relative intensity. The most prominent band was located at 1640 cm^{-1} (aromatic C = C vibrations, amide I, and asymmetric stretching of COO^-) in the RL aqueous soil extract spectrum (Fig. 2a) and at 1418 cm^{-1} (vibrational modes of CH_2) in the EC aqueous soil extract spectrum (Fig. 2b), whereas both bands at 1079 cm^{-1} and 1041 cm^{-1} seemed to be more intense in EC soil than in the RL soil. These results account for the higher amounts of carboxylic acids, which are to be expected in the soil solution of a forest soil.

The FT-IR spectra obtained on extracts obtained from soils treated with root exudates showed different peaks of relative intensity from those of the aqueous soil extracts. There was an increase in the absorption at 1078 cm^{-1} assigned to the vibrational modes of the alcoholic C-O-H in the EC + *P. sylvestris* and EC + *P. abies* (Fig. 3b and 3c), whereas in the RL + *P. sylvestris* and RL + *P. abies* (Fig. 4b and 4c) the peak relative increase at 1418 cm^{-1} accounts for the greater aliphatic component in the forest tree root exudates. A strong increment in the peak at 1420 cm^{-1} was also observed in the EC + Sandek (Fig. 5b), together with a decrease in the absorption at 1078 and 1040 cm^{-1}. The EC + Mytos (Fig. 5c), however, showed a decrease in aliphatic component (smaller peak of relative intensity at 1420 cm^{-1}). The RL + Mytos (Fig. 6b) showed a decrease in all absorptions in the range between 1617 and 1047

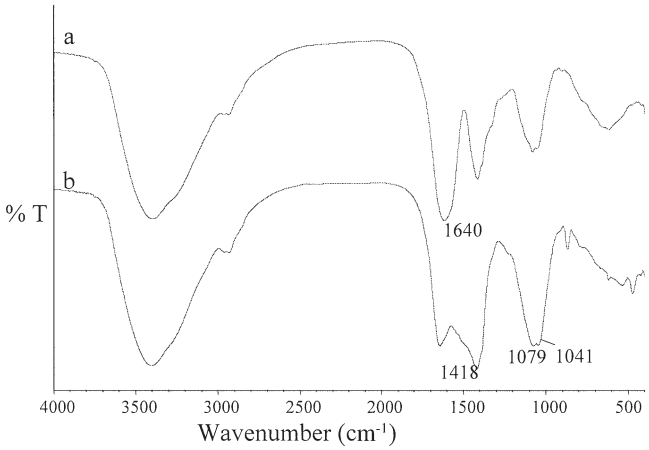

Fig. 2. FT-IR spectra of aqueous soil extracts from the Eutric Cambisol (EC) (a) and Rendzic Leptosol (RL) (b).

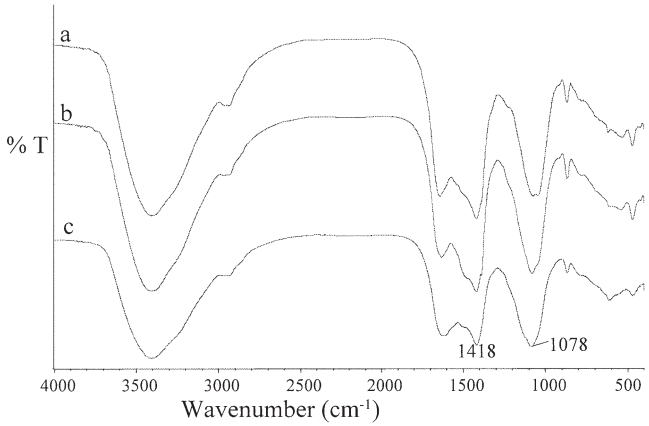

Fig. 3. FT-IR spectra of aqueous soil extract from Eutric Cambisol (EC) (a) and extracts obtained by treating EC with *P. sylvestris* (b) and *P. abies* (c) root exudates.

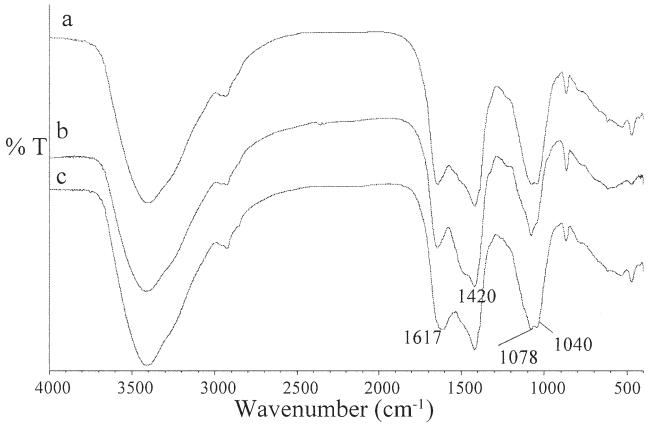

Fig. 5. FT-IR spectra of aqueous soil extract from Eutric Cambisol (EC) (a) and extracts obtained by treating EC with Sandek (b) and Mytos (c) root exudates.

cm^{-1}, whereas the peak of relative intensity assigned to the polysaccharides (1040 cm^{-1}) seemed to be accentuated in the RL + Sandek (Fig. 6c).

As for plant metabolism, only the LMS organic fractions extracted by treating the soils with root exudates exhibited auxin- and gibberellin-like activity, whereas the water extracts, alkaline solutions (HS), and root exudates showed no such activity (Table 6). The extracts from the EC and maize exudates had a higher auxin-like and lower gibberellin-like activity than those obtained from the RL (Table 6). In contrast, the extracts from the RL and forest tree species root exudates showed high auxin- and gibberellin-like activity (Table 6). The effects of the LMS organic fractions on N metabolism in Scotch pine seedlings are shown in Table 7. The extracts from EC and RL stimulated N uptake and NR activity, but to different extents. The fractions from EC enhanced nitrate uptake and NR and GS activities, whereas those from RL increased ammonium uptake and NR and GDH activities. The LMS organic fractions from RL and *P. abies* and from RL and *P. sylvestris* root exudates exhibited the greatest increase in ammonium uptake and GDH activity, whereas the seedlings grown in the extracts from the EC and maize exudates had higher NR-GS activities. The classical HS and water extracts influenced neither the N uptake nor its assimilation.

DISCUSSION

Many processes occur at the root/soil interface as direct and indirect consequences of plant demand to improve the biological properties of the rhizosphere. The main chemical changes that can influence a plant's mineral nutrition are changes in ionic concentration and pH; enzyme excretion; and phenol, amino acid, and organic acid exudation (Gregory and Hinsinger, 1999).

Concerning the organic acid extrusion, in our study, *P. abies* and *P. sylvestris* released exudates into the soil that were endowed with a higher oxalic and succinic acid content than maize; the same trend was demonstrated by Jones (1998). As for the GC/MS data, classic HS has only $C_{11}H_{23}COOH$ and $C_{13}H_{27}COOH$, despite alkaline solutions extracting 80% of the humic substances (Stevenson, 1994), whereas extracts obtained by root exudates had a wider variety of fatty acids (i.e., $C_{14}H_{29}COOH$, $C_{16}H_{33}COOH$, $C_{17}H_{35}COOH$), although their HS content is smaller. Despite the low humic extraction yield, organic acids have an important role in the rhizosphere because they are able to mobilize bioac-

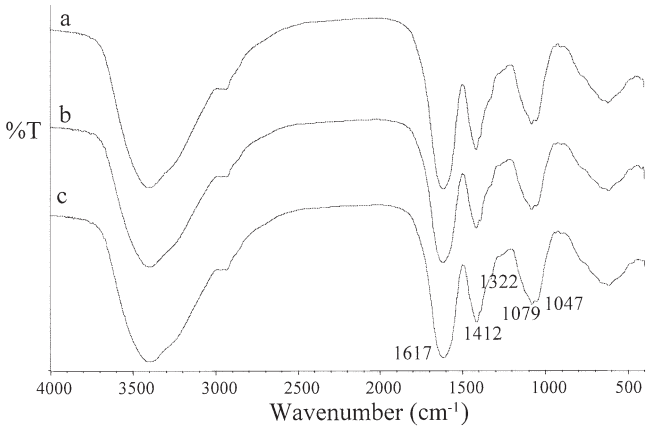

Fig. 4. FT-IR spectra of aqueous soil extract from Rendzic Leptosol (RL) (a) and extracts obtained by treating RL with *P. sylvestris* (b) and *P. abies* (c) root exudates.

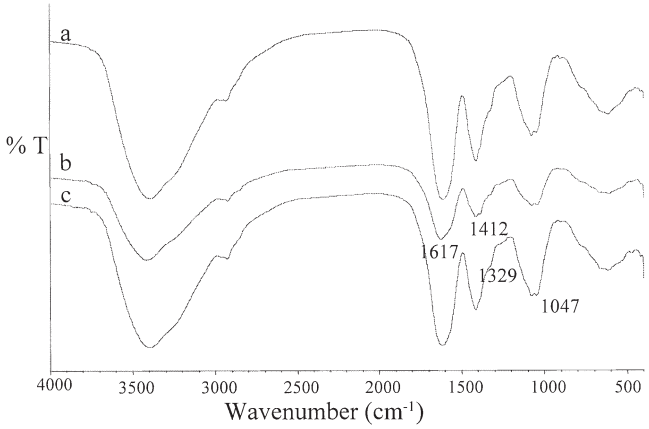

Fig. 6. FT-IR spectra of aqueous soil extract from Rendzic Leptosol (RL) (a) and extracts obtained by treating RL with Mytos (b) and Sandek (c) root exudates.

Table 6. Auxin-like (IAA) and gibberellin-like (GA) activity of low molecular size (LMS) organic fractions mobilized by treating soils (Eutric Cambisol, EC, and Rendzic Leptosol, RL) with root exudates.

	IAA activity†	GA activity†
EC + water	n.d.‡	n.d.
EC + KOH	n.d.	n.d.
EC + Mytos	5.60 d§	2.60 d
EC + Sandek	9.60 a	0.78 e
EC + *P. abies*	8.70 ab	0.40 f
EC + *P. sylvestris*	0.31 f	0.70 e
RL + water	n.d.	n.d.
RL + KOH	n.d.	n.d.
RL + Mytos	4.50 e	2.90 c
RL + Sandek	6.40 c	7.80 b
RL + *P. abies*	8.50 b	3.10 c
RL + *P. sylvestris*	8.80 ab	9.90 a

† Concentration (mg L^{-1}) of indoleacetic or gibberellic acid of equivalent activity to 1 mg C L^{-1} LMS organic fractions.
‡ Not detected.
§ Values in the same column followed by the same letter are not statistically different at $P \leq 0.05$ by Student-Newmann-Keuls test (Sokal and Rohlf, 1969).

tive molecules such as palmitic acid and linoleic acid (Nardi et al., 2002b). Linoleic acid is important because it is considered a precursor of jasmonic acid, an essential bio-molecule (Kontos and Spyropoulos, 1996), and because palmitic acid stimulates acetil-CoA carboxylase and plays a key part in the thylakoid membrane structure (Burke et al., 1998). Comparing the FT-IR spectra showed that there were higher aliphatic components and larger alcoholic and carboxylic functional groups in *P. abies* and *P. sylvestris* root exudates, whereas the aromatic component was greater in the Mytos and Sandek root exudates. Results also showed that aqueous soil extracts were similar, although the solution from the forest soil featured a large amount of carboxylic acid. The FT-IR spectra on extracts obtained by treating soils with root exudates showed different peaks of relative intensity from those of the aqueous soil extracts, meaning that significant changes were induced in soil solutions by extraction with root exudates.

Table 7. Percentage of NO_3^- and NH_4^+ uptake and nitrate reductase (NR), glutamine synthetase (GS), and glutamate dehydrogenase (GDH) activities in *P. sylvestris* seedlings treated with low-molecular-size organic fractions extracted from Eutric Cambisol (EC) and Rendzic Leptosol (RL) by maize (*Z. mays* L., cultivar Mytos and Sandek) or forest (*P. abies* Karst. and *P. sylvestris* L.) root exudates.

Treatment	NO_3^-†	NH_4^+‡	NR§	GS¶	GDH#
EC + water	100 c††	100 c	100 c	100 b	100 d
EC + KOH	110 c	100 c	110 c	92 b	106 d
EC + Mytos	125 b	96 c	112 c	97 b	96 d
EC + Sandek	150 a	105 c	142 b	126 a	104 d
EC + *P. abies*	125 b	90 c	127 b	119 a	80 e
EC + *P. sylvestris*	150 a	95 c	172 a	109 a	84 e
RL + water	100 c	100 c	100 c	100 b	100 d
RL + KOH	105 c	108 c	115 b	63 d	110 d
RL + Mytos	115 b	125 b	132 b	95 b	131 c
RL + Sandek	140 a	140 a	136 b	102 b	143 c
RL + *P. abies*	120 b	150 a	117 b	85 c	160 b
RL + *P. sylvestris*	160 a	160 a	164 a	97 b	190 a

† NO_3^- control = 100 = 1583 nmol NO_3^- min^{-1} g^{-1} fresh weight (f wt).
‡ NH_4^+ control = 100 = 8333 nmol NH_4^+ min^{-1} g^{-1} f wt.
§ NR control = 100 = 3.71 nmol g^{-1} f wt.
¶ GS control = 100 = 114.63 nmol min^{-1} g^{-1} f wt.
# GDH control = 100 = 26.05 nmol min^{-1} g^{-1} f wt.
†† Values in the same column followed by the same letter are not statistically different at $P \leq 0.05$ by Student-Newmann-Keuls test (Sokal and Rohlf, 1969).

To better characterize the signal factors that root exudates are able to extract from the soil, we studied the hormone-like activity of the different extracts and their effects on N metabolism in Scotch pine seedlings. The extracts from the RL and forest tree species root exudates showed high auxin- and gibberellin-like activity, whereas the extracts from the EC and maize exudates showed a more limited hormone-like activity. This pattern is evidence that root exudates, in their various forms, can regulate the rhizosphere's hormone activity to create a successful interaction between plant microorganisms and the soil (Frankenberger, 1995).

As for N metabolism, many forest trees are known to use N from the soil in the form of ammonium because a little growth can be seen in soils with nitrate, whereas Scotch pine responds differently to nitrate and ammonium transport in relation to soil type (Gosz, 1981). In Scotch pine grown in the presence of extracts from the agricultural soil (EC), nitrate uptake and NR and GS activities were stimulated by comparison with the control, whereas GDH activity was inhibited. When the Scotch pine was exposed to the forest soil (RL) extract, the N metabolism stimulation regarded NR and GDH activity, whereas the GS was inhibited. In forest species, GDH has an important role in ammonium assimilation and when the plant is under stress. Under such conditions, GDH is a much more stable enzyme than GS (Schlee et al., 1994), and this is confirmed by the major role played by GDH in ammonia detoxification within the plant cells (Schlee et al., 1994). In conclusion, as regards the forest soil, the Scotch pine mainly uses the NR-GDH pathway for N assimilation but seems to prefer the NR-GS pathway in the agricultural soil.

In a previous article (Nardi et al., 2002b), we showed that agricultural soil (EC) extracts stimulated N assimilation in maize seedlings far more than extracts from forest soil (RL). The positive effects on N metabolism in maize seedlings grown in agricultural soil extracts may be related to signals existing in the said soil, but not in forest soil.

The results of this study, together with those previously reported, suggest that many different regulatory signals affect rhizosphere interactions. These signals may represent the highest level of evolved response in rhizosphere communities. Organic substances in root exudates may be a mechanism that enables plants to interact with microorganisms and the soil.

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