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Microbial activity and hydrolase activities during decomposition of root exudates released by an artificial root surface in Cd-contaminated soils

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Abstract

The aim of this study was to assess the stimulatory effects of different low molecular weight organic compounds commonly present in root exudates on microbial activity and hydrolase activities, and the effects of high Cd concentrations in sandy soils collected from contaminated field plots on the stimulatory effects. Glucose, glutamic acid, citric acid, oxalic acid, or a mixture of all compounds were released by an artificial root surface in a simplified rhizosphere system. The effects were measured at <2 mm (rhizosphere soil layer) and >4 mm (bulk soil layer) distance from the root surface, 7 d after the root exudates release. Results showed that different root exudates were mineralized at different extent and had different stimulatory effects on microbial growth estimated by dsDNA content of soil, and on hydrolase activities, mostly localized in the rhizosphere soil layer. Mineralization of root exudates, microbial growth and stimulation of most of the measured hydrolase activities were drastically reduced by high Cd concentrations in soil.
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1. Introduction

Soil enzyme activities are involved in soil nutrient cycling dynamics and can catalyze the conversion of nutrient from unavailable to forms readily assimilable by plants and microorganisms. Enzyme activity is generally higher in rhizosphere than in bulk soil, as a result of a greater microbial activity sustained by exudates, actively or passively released by roots, or due to the release of enzymes from plant roots (Pinton et al., 2001). Among soil enzyme activities, hydrolase activities of rhizosphere are supposed to play an important role in plant nutrition. For example, the hydrolysis of organic phosphate monoesters by phosphomonoesterases can account for 30–80% of P taken up by plants in agricultural soils (Tarafdar and Jungk, 1987; Gilbert et al., 1999).

Because of the high complexity of the rhizosphere environment, the mineralization of the different root exudates and their stimulatory effects on microbial activity have been approached studying the effects of single low molecular weight organic molecules in simple systems mimicking the rhizosphere environment (Kozdroj and van Elsas, 2000; Badalucco and Kuikman, 2001; Falchini et al., 2003; Baudoin et al., 2003). These studies have confirmed that root exudation is the main factor controlling microbial activity and community structure in the rhizosphere. However, to our knowledge no studies have focussed on the effects of single low molecular weight organic molecules of root exudates on hydrolase activities of microbial origin in the rhizosphere.

Negative effects of heavy metals on soil enzyme activities and on microbial catabolic diversity, as compared to uncontaminated soils have been shown (Tyler et al., 1989; Reber, 1992; Wenderoth and Reber, 1999). Among heavy metals, Cd is supposed to be one of the most toxic to soil microorganisms and can reduce the activity of soil hydrolases (Renella et al., 2004b, 2005). Specific effects of high Cd concentrations on the microbial mineralization of low molecular weight root exudates and on hydrolase activity of the rhizosphere soil are unknown.

Marstorp and Witter (1999) demonstrated that the double-stranded DNA (dsDNA) content can be used to determine changes in soil microbial biomass when microbial growth is induced during glucose decomposition. This method requires

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low amounts of soil, thus being suitable for measuring microbial biomass in the rhizosphere soil. Moreover, it does not present the drawbacks of the substrate-induced respiration method (Anderson and Domsch, 1978), which only detects the glucose-responsive fraction of soil microorganisms.

We hypothesized that by increasing Cd concentrations the capacity of rhizosphere microorganisms to mineralize low molecular weight root exudates decreases. The aim of our work was to study how Cd contamination affects (i) mineralization of low molecular weigh root exudates to CO_2 and (ii) the stimulatory effects of low molecular weight root exudates on hydrolase activities of rhizosphere and bulk soil. Microbial biomass was also monitored by determining the dsDNA content so as to relate changes in mineralization rates and hydrolase activities to microbial growth. The rhizosphere environment was simulated by using an incubation unit previously described by Badalucco and Kuikman (2001) and Falchini et al. (2003). Such a system, allows creating a concentration gradient with higher concentrations close to the root surface, and sampling of soil at known distances from the root surface (Badalucco and Kuikman, 2001). The 0–2 mm and >4 mm soil distances were chosen because they represent rhizosphere and bulk soil, respectively (Scott et al., 1995; Falchini et al., 2003). Glucose, citric acid, oxalic acid and glutamic acid were chosen because they are the most aboundant among low molecular weight root exudates (Kraffczyk et al., 1984; Jones, 1998).

2. Materials and methods

2.1. Soil type and characteristics

Soils (sandy-clay, Arenic Udifluvent) with 17.5% clay, 15.0% silt, 67.5% sand, were sampled from field plots at the long-term AGIR Experiment, Couhins Experimental Farm, INRA, Bordeaux (France). Field plots were contaminated in the period 1988–1990 with $Cd(NO_3)_2$ to reach Cd concentrations in the 0–20 cm depth soil layer of 0, 10, 20 and 40 mg kg^{-1} soil. Control and contaminated soils sampled had a total Cd concentrations determined by atomic absorption spectometry of 0.71, 9.8, 18.9 and 38.7 mg Cd kg^{-1}, and $pH_{(H2O)}$ values of 7.4, 7.2, 7.1 and 6.8, respectively. All the studied soils had same texture and were under maize cultivation before sampling. To study the effects of high Cd concentrations on microbial stimulation induced by different root exudates, only soils containing 0 (control soil), 20 and 40 mg Cd kg^{-1} soil were used. Soils were sampled from the 0–20 cm depth layer, transported in plastic bags to the laboratory as moist samples, sieved (<2 mm) and incubated at 40% water holding capacity (WHC) for 7 days prior to treatments with different root exudates.

2.2. Soil treatments and incubation conditions

Soil samples of 70 g moist equivalent to 63 g dry weight (dw) were placed in plastic cylinders (68 mm diameter, 20 mm height), carefully pressed by a stainless steel piston to a fixed density of 1.2 g dm^3, covered by a cellulose round filter paper (Whatman 41, 68 mm diameter) representing the root surface, and moistened with 2 ml of root exudate solutions containing glucose, citric acid, oxalic acid, glutamic acid or the four compounds, to give a rate of 300 mg of root exudates-C kg^{-1} soil. Soils treated with deionized water represented the controls. Apart from glucose, solutions were prepared using a mixture of acids and their respective sodium salts, to buffer the root exudate solutions at pH ≈ 5.5 to avoid drops in soil pH. Soils were incubated at 25 °C in the dark for 7 days in 1 l air tight jars containing 10 ml of 1 M NaOH to trap the CO_2. During the 7 days-long incubation, three root exudate additions were done on day 0, 2 and 4 of incubation. After root exudate additions, soil moisture content did not exceed 55% of WHC in all soils. At the end of incubation period, soils were sampled from <2 mm (rhizosphere soil) and >4 mm (bulk soil) distance from the root surface as described by Falchini et al. (2003), and analysed for their dsDNA content and hydrolase activities.

2.3. Analyses

The CO_2 evolution from soil was measured after 2, 4 and 7 days, before each root exudate addition by titrating residual NaOH with 0.1 M HCl, after precipitaion of carbonates by 1.5 M $BaCl_2$ (Stotzky, 1965).

Total soil DNA was extracted from 0.5 g of soil using a bead beating method (FastDNA SPIN Kit for soil, Bio 101, Inc., USA), according to the manufacturer's instructions. The amount of extracted dsDNA was measured by fluorometry (Hoefer™ DyNA Quant™ 200) using bisbenzimide-dye (Hoechst H 33258).

Acid and alkaline phosphomonoesterase activities were assayed according to Tabatabai and Bremner (1969) and phosphodiesterase activity as reported by Browman and Tabatabai (1978). Urease activity was measured using 0.1 M pH 7 phosphate buffer, as reported by Nannipieri et al. (1974). Protease activity was determined by the hydrolysis of *N*-benzoylargininamide (*N*-BAA) according to Ladd and Butler (1972). All enzyme activities were assayed at 37 °C for 1 h, with centrifugation of soil slurries at 6000*g* at 4 °C. Concentration of *p*-nitrophenol produced in the assays of acid and alkaline phosphomonoesterase and phosphodiesterase activities was calculated from a calibration curve after subtracting the absorbance of the control at 400 nm wavelength using a UV–VIS spectrophotometer Lambda 2 (Perkin Elmer). The NH_4^+ produced by urease and *N*-BAA-hydrolysing activities was extracted by 2 M KCl and quantified by a flow injection analyzer (FIAStar, Tecator, Sweden). To account for the NH_4^+ fixation by soils, NH_4^+ solutions with concentrations in the range of those released by urease and protease activities were incubated with these soils. The recovery of NH_4^+ was always greater than 98% (data not shown).

2.4. Data analysis

The LSD values (Tukey–Kramer test, P level <0.05) were calculated to assess the significance of differences of the means ($n=3$). Net C mineralization was calculated by subtracting the values of CO_2–C evolved from control soils from those of CO_2–C evolved from soils treated with root exudates.

3. Results

3.1. Root exudates-induced soil respiration

The mineralization of the different low molecular weight root exudates depended on the added compound and soil Cd concentration (Fig. 1). After 7 days of incubation, the percentage of organic C added to the uncontaminated (control) soil accounted for 86, 66, 52, 31 and 58% for citric acid, glutamic acid, glucose, oxalic acid and root exudates mixture, respectively (Fig. 1). In soil contaminated with 20 mg Cd kg^{-1}, mineralization of citric acid, glutamic acid, glucose, oxalic acid and the root exudates mixture accounted for 62, 42, 54, 23 and 50% of organic C added to soil, respectively (Fig. 1). In soil contaminated with 40 mg Cd kg^{-1} mineralization of citric acid, glutamic acid, glucose, oxalic acid and the root exudates mixture accounted for 51, 26, 36, 20 and 28% of organic C added to soil, respectively (Fig. 1).

3.2. dsDNA content in rhizosphere and bulk soil layers

The dsDNA content was significantly increased by the addition of all root exudates in both rhizosphere and bulk layers of the control soil, except in layers of soil treated with the mixture of root exudates, and the highest values were obtained with glucose or glutamic acid treatments (Fig. 2).

In the rhizosphere layer of soil contaminated with 20 mg Cd kg^{-1}, the dsDNA content was significantly increased only by the addition of glucose, glutamic acid and citric acid (Fig. 2), whereas in the rhizosphere layer of soil contaminated with 40 mg Cd kg^{-1}, a significant increase of dsDNA content was only observed after treatment with glucose (Fig. 2).

3.3. Hydrolase activities of rhizosphere and bulk soil layers

Different root exudates had different effects on the measured hydrolase activities. In the control soil, glucose, glutamic acid and citric acid and root exudates mixture significantly increased the alkaline phosphomonoesterase and phosphodiesterase activites in the rhizosphere soil layer (Table 1). Acid phosphomonoesterase of the rhizosphere soil layer was significantly increased only by glutamic acid (Table 1). No significant increase of phosphatase activities

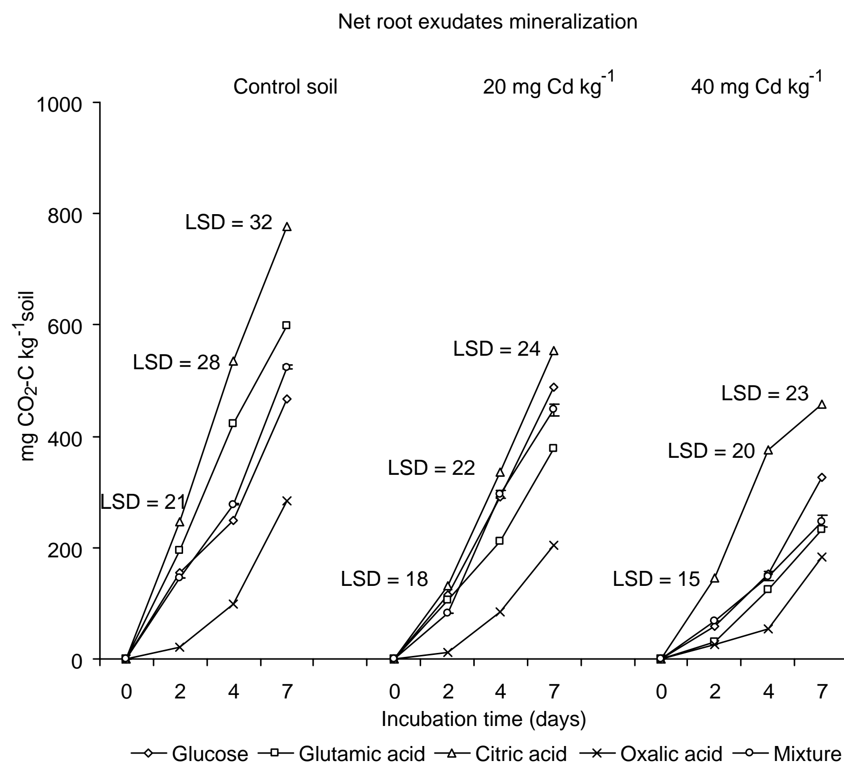

Fig. 1. Net root exudates mineralization in uncontaminated (control) soil and soils contaminated with 20 and 40 mg Cd kg^{-1}. The LSD values represent the sampling-day specific minimum significant differences of the means ($n=3$).

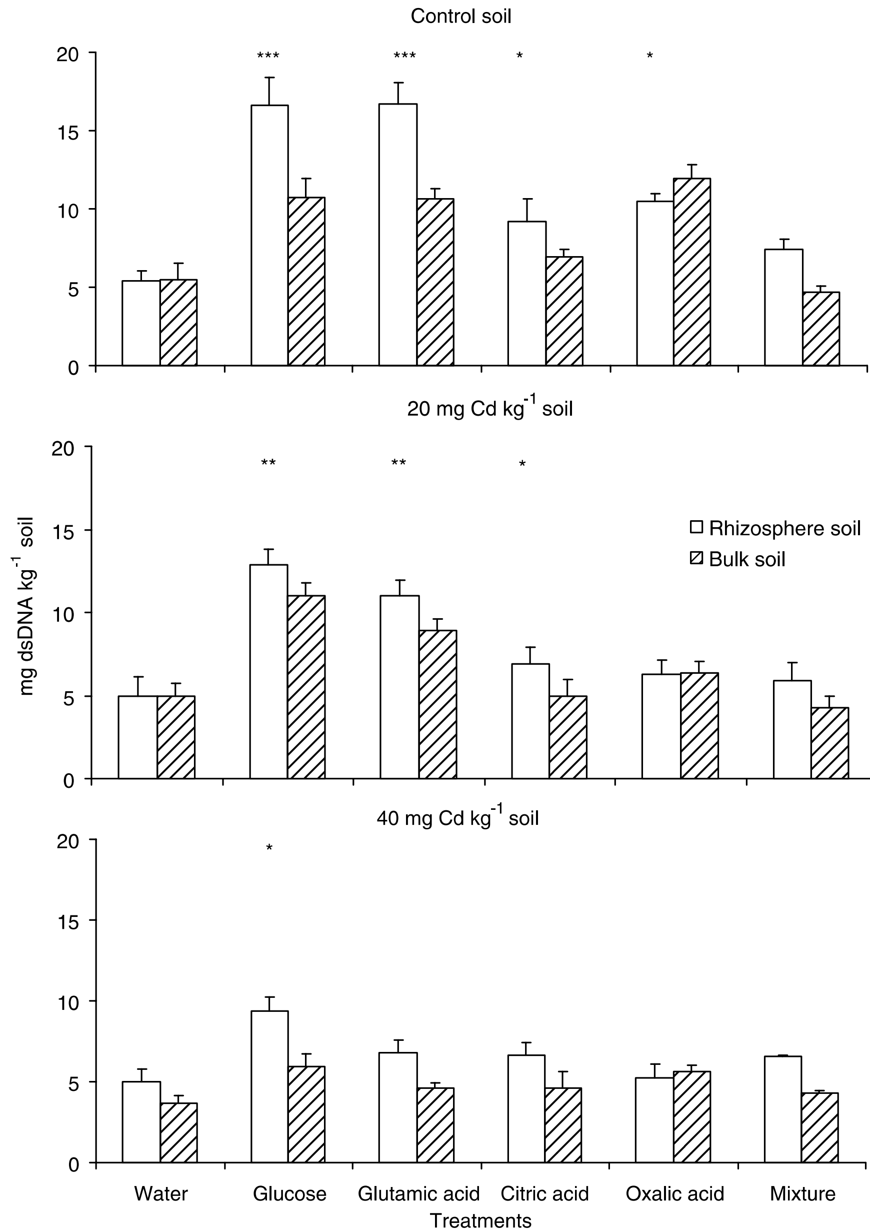

Fig. 2. The dsDNA content of the rhizosphere and bulk soil layers of uncontaminated (control) soil, and soils contaminated with 20 and 40 mg Cd kg^{-1}. The error bars represent the standard deviation of the means ($n=3$). Symbols *, ** and *** indicate significant differences at P level <0.05, 0.01 and 0.001, respectively, between rhizosphere soils treated with water and root exudates.

were observed in the bulk soil (Tables 1 and 2). Urease activity in both rhizosphere and bulk soil layers was significantly stimulated by glucose, glutamic acid, citric acid and the root exudates mixture, whereas protease activity was not significantly stimulated by any root exudate addition (Table 2).

In soil contaminated with 20 mg Cd kg^{-1}, alkaline phosphomonoesterase and phosphodiesterase activities of the rhizosphere soil layer were significantly stimulated by glucose and citric acid, whereas acid phosphomonoesterase activity was not stimulated by any root exudate addition (Table 1). Urease activity was stimulated by glucose, glutamic acid, citric acid and root exudates mixture to different extent, whereas protease activity was not stimulated by any root exudate addition (Table 2). No stimulation of hydrolase activities was observed in the bulk soil layers by any root exudate treatment (Tables 1 and 2).

In soil contaminated with 40 mg Cd kg^{-1}, alkaline phosphomonoesterase and phosphodiesterase activities of the rhizosphere soil layer were significantly stimulated by glucose and citric acid, whereas acid phosphomonoesterase was not stimulated (Table 1). Urease and protease activities were not significanlty stimulated by any root exudate addition (Table 2). No significant stimulation was observed for all hydrolase activities in the bulk soil layer (Tables 1 and 2).

Table 1
Alkaline phosphomonoesterase, acid phosphomonoesterase and phosphodiesterase activities in the rhizosphere and bulk soil of the control soil and Cd-contamianted soils

Soils	Alkaline phosphomonoesterase (mg *p*-nitrophenol kg^{-1} soil*h^{-1})		Acid phosphomonoesterase (mg *p*-nitrophenol kg^{-1} soil*h^{-1})		Phosphodiesterase (mg *p*-nitrophenol kg^{-1} soil*h^{-1})	
	Rhizosphere	Bulk	Rhizosphere	Bulk	Rhizosphere	Bulk
Deionized H_2O						
Control soil	6802	7101	4183	4940	1045	1161
20 mg Cd kg^{-1}	6135	6538	3939	2988	998	990
40 mg Cd kg^{-1}	4808	4712	4043	3642	879	864
Glucose						
Control soil	8629*	5410	4735	4410	2908***	1155
20 mg Cd kg^{-1}	8696*	7167	3702	3979	1309*	728
40 mg Cd kg^{-1}	8099*	4461	3860	2671	1838*	1751
Glutamic acid						
Control soil	8109*	6112	5618*	3429	1863**	1156
20 mg Cd kg^{-1}	6962	5380	4195	2188	990	935
40 mg Cd kg^{-1}	5308	4845	5079	3388	1247	813
Citric acid						
Control soil	9254*	8221	3456	4672	1611*	905
20 mg Cd kg^{-1}	8323*	6116	2930	3322	1481*	835
40 mg Cd kg^{-1}	7864*	4101	2798	2358	2385*	2127*
Oxalic acid						
Control soil	6199	5880	2711	3355	774	983
20 mg Cd kg^{-1}	7166	7579	3812	690	690	657
40 mg Cd kg^{-1}	4644	4728	2195	714	714	842
Mixture						
Control soil	5918	5642	5632*	4370	1565	1144
20 mg Cd kg^{-1}	7002	6220	3076	3134	1158	923
40 mg Cd kg^{-1}	4863	4687	3763	3943	1284	1090

Symbols *, **, ***, indicate significant differences at P level <0.05, 0.01 and 0.001, respectively, between soils treated with deionized H_2O and soils treated with root exudate soloutions.

4. Discussion

Our results confirm that the incubation units used created a low molecular weight organic compounds gradient from the root surface, as reported in a previous study based on the release of ^{14}C-labelled compounds (Falchini et al., 2003). In fact, the stimulation of soil microorganisms was mostly observed within the 0–2 mm soil portion (rhizosphere soil) whereas the bulk soil was only negligibly affected by the organic compounds; this low effect was likely due to the repetead amendments.

Mineralization of the studied low molecular weight root exudates to CO_2 can only occurr through intracellular metabolic pathways such as glycolysis and Krebs cycle (Bremner and Kuikman, 1994); the use of these compounds by microorganisms in neutral sandy soils is generally rapid (Owen and Jones, 2001). The increase of hydrolase activities, particularly in the rhizosphere soil layer upon root exudate additions, is related to actively growing microorganisms, as deducible by the increase of dsDNA content of this soil layer during root exudates mineralization (Fig. 2). The rapid increase of hydrolase activities may suggest that most of the measured enzyme activity was due to both intracellular and pericellular enzymes. It has been shown that both intracellular and pericellular phosphohydrolases were increased in *Pseudomonas sp.* by the addition of glucose (Schmidt et al., 1987). If this hypothesis is true, the present soil hydrolase assays also measure the contribution of intracellular enzyme activities. The distinction between intracellular and the stabilized extracellular enzyme activity is essential to better understand the meaning of the enzyme activity measurements in soil (Nannipieri et al., 2003).

Our results concerning glucose- and glutamic acid-induced microbial growth in the control soil were comparable to those reported by Marstorp (1996), whereas to our knowledge no similar data exist for low molecular weight organic acids. Lower mineralization and microbial growth rates upon additions of oxalic acid could be due to its lower availability due to greater adsorption by soil mineral constituents as compared to glucose and glutamic acid (Darrah, 1991a, b; van Hees et al., 2003). A greater diffusion of glucose and glutamic acid as compared to oxalic acid under similar incubation conditions was reported by Falchini et al. (2003).

The decrease of C mineralization and microbial growth in the presence of high Cd concentrations (Figs. 1 and 2), could be due to the delay in the mineralization of root exudates, which is a common feature of heavy metal contaminated soils (Haanstra and Doelman, 1984; Nordgren et al., 1988) and/or to lower amounts of root exudates metabolized by soil microorganisms due to formation of Cd-root exudates

Table 2
Ureae and protease activities in the rhizosphere and bulk soil of the control soil and Cd-contamianted soils

Soils	Urease (mg NH_4^+–N kg^{-1} soil*h^{-1})		Protease (mg NH_4^+–N kg^{-1} soil*h^{-1})	
	Rhizosphere	Bulk	Rhizosphere	Bulk
Deionized H_2O				
Control soil	28	23	176	180
20 mg Cd kg^{-1}	23	23	175	159
40 mg Cd kg^{-1}	26	18	152	156
Glucose				
Control soil	159***	56*	165	176
20 mg Cd kg^{-1}	145***	39	134	154
40 mg Cd kg^{-1}	38	14	171	182
Glutamic acid				
Control soil	118**	54*	132	150
20 mg Cd kg^{-1}	43*	29	190	191
40 mg Cd kg^{-1}	16	18	143	159
Citric acid				
Control soil	138***	71**	111	117
20 mg Cd kg^{-1}	67**	22	158	162
40 mg Cd kg^{-1}	34	14	174	170
Oxalic acid				
Control soil	42	36	166	177
20 mg Cd kg^{-1}	61	45	136	144
40 mg Cd kg^{-1}	20	17	192	195
Mixture				
Control soil	83*	53*	212	209
20 mg Cd kg^{-1}	39	29	182	153
40 mg Cd kg^{-1}	27	14	163	129

Symbols *, **, ***, indicate significant differences at P level <0.05, 0.01 and 0.001, respectively, between soils treated with deionized H_2O and soils treated with root exudate soloutions.

complexes. Low molecular weight organic acids and amino acids can form Cd-complexes (Krishnamurti et al., 1997), which are not readily mineralizable by soil microorganisms (Brynhildsen and Rosswall, 1989; Renella et al., 2004a). However, reduced microbial mineralization and growth rates in Cd-contaminated soils were also observed after treatment with glucose which has no chelating ability, suggesting that the lower metabolic efficiency (i.e. diversion of metabolic energy from biosynthesis to maintenance) of Cd-stressed soil microorganisms was probably the major mechanism responsible for the lower mineralization and synthesis. Similar conclusions were drawn by also Renella et al. (2005).

Differences in enzyme activities in Cd-contaminated soils could be also due to changes in the composition of soil microorganisms (Wenderoth et al., 2001; Chander and Joergenesen, 2001). However, the studied hydrolases are produced by a wide fraction of soil microorganisms and adverse effects of heavy metals on soil enzyme activities without major changes in the composition of the soil microbial communities have been previously reported (Kandeler et al., 2000; Renella et al., 2004b).

In conclusion, our work confirmed that sugars, carboxylic and amino acids at concentrations commonly present in root exudates may have different stimulatory effects on soil microorganisms and hydrolase activities in the rhizosphere. High Cd concentration decreased the mineralization of low molecular weight root exudates, and phosphatase and urease activities, thus reducing soil fertility and potential nutrient availability to plants. Further studies are needed to better elucidate whether reduced mineralization in the rhizosphere of contaminated soils is due to lower amounts of root exudates metabolized by soil microorganisms due to formation of Cd-root exudates complexes or to direct toxic effects of Cd to rhizosphere microorganisms.

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