Microbial activity related to N cycling in the rhizosphere of maize stressed by heavy metals

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Abstract: A greenhouse experiment was carried out to compare differences in potential activities of ammonification, nitrification and denitrification in rhizosphere and bulk soil in a heavy-metal-stressed system. Exchangeable fractions of Cd, Cu and Cr were all higher in the rhizosphere of maize than in bulk soil. Results showed that the mineralization of N in soil was stimulated by low concentration of Cd. Addition of Cd at low levels stimulated the ammonifying and nitrifying activity in soil, while inhibitory influences were shown at high levels. Nitrifying bacteria was proved to be the most sensitive one, whilst the effect on denitrifying bacteria was very limited. Comparing Cd, Cu and Cr(VI) at 20 mg/kg soil, Cd was the most effective inhibitor of ammonification and denitrification, while Cr(VI) had the strongest inhibitory influence on nitrifying activity. Root exudates played important roles on the different exchangeable metal fractions and bacterial activities between rhizosphere and non-rhizosphere. Nitrate was the main form of mineral N in soil, as well as the main form of N absorbed by plants, but the formation and relative absorption of ammonium were promoted in response to high Cd exposure.

Keywords: heavy metal; nitrogen cycle; rhizosphere; root exudates

Introduction

Due to anthropogenic activities, such as mining, smelting, electroplating, ore-refining and land disposal of wastes, numerous soil sites are contaminated by heavy metals. The presence of heavy metals can significantly affect the microbial populations, bacteria communities and microbial activities in soil, thus potentially altering the ecology in soil (Wilson, 1977; Bååth, 1989; Suhadolc, 2004).

As a result of fertilizer shortages and escalating costs, the soil N cycling has been attained greater importance (Berg, 1987; Ramirez-Fuentes, 2002). Researches showed net N mineralization was inhibited by the addition of heavy metals to an acidic silty loam (Chang, 1982) and by very high metal concentrations in other soils (Abdennaceur, 1998; Babich, 1985). However, Munn *et al.* (Munn, 1997) found no adverse effects on net mineralization of legume and soil organic N from heavy metals accumulated in soil after treatment with metal-contaminated sewage sludge. The soil studied by Munn *et al.* (Munn, 1997) contained maximal total heavy metal concentrations that were 50% of those associated with reduced microbial biomass at Woburn in the UK (Brookes, 1984) or that resulted in accumulation of organic matter in other soils in the UK (Chander, 1991a; 1991b). However, the information relating to physiological groups of bacteria involved in soil nitrogen cycle is still limited.

Rhizosphere, as an important interface of soil and plant, plays a significant role in the agro-environmental system (Rovira, 1967; O'Connell, 1996; Wang, 2002), in which, microbial biomass, activity and community structure are highly influenced by specific physicochemical and biological characteristics prevailing in this habitat (Sörense, 1997). Controlling or at least predicting the biological and the physiochemical conditions will encourage the development of stable rhizosphere condition with an enhanced capacity to relieve heavy metal contamination.

The present investigation is to study the effects of heavy metal stress on soil N-cycling. In a greenhouse study, using rhizoboxes, ammonification, nitrification and denitrification were investigated in bulk and rhizosphere soil in a heavy-metal-stressed system. Pollutant-induced changes in rhizosphere, heavy metal mobility and root exudates were also considered in the context of the soil N-cycle.

1 Materials and methods

1.1 Soils

The alluvial soils for test were collected from Hangzhou, located in the northern part of Zhejiang Province, Southern China, with pH 7.92(H_2O), total organic material 12.9 mg/kg, total N 0.92 mg/kg, total P 0.90 mg/kg, available Fe 51 mg/kg soil, total Cd 0.52 mg/kg soil, total Cu 18.54 mg/kg soil and total Cr(VI) 0.64 mg/kg soil. All soils were taken from the surface layer(0—20 cm) of cultivated soils. The soils were air-dried, ground and sieved to pass though 1 mm plastic mesh.

1.2 Greenhouse experiment

A pot experiment was designed to imitate a metal-polluted agro-environmental system in a similar way to that of Lin *et al.* (Lin, 2003). The previous air-dried soils were treated with 0.15 gP_2O_5/kg dry soil as a solution of Ca(H_2PO_4)$_2$, and 0.15 gK_2O/kg dry soil as a solution of K_2SO_4 respectively. Then the soils were treated with three metals separately, Cd in four levels as 2, 5, 10 and 20 mg/kg soil as a solution of $3CdSO_4 \cdot 8H_2O$, Cu 20 mg/kg soil as a solution of $CuSO_4 \cdot 5H_2O$, and Cr(VI) 20 mg/kg soil as a solution of $K_2Cr_2O_7$. In each step, after the soil and solution were mixed thoroughly, the soils were air-dried and ground to pass through a 1 mm of sieve to make sure that the fertilizer and heavy metals were well distributed in the soil.

The soils were then transferred to plastic rhizoboxes. Each rhizobox had two compartments separated by a 300 mesh nylon screen, the inside one designed as growth chamber holding with 0.5 kg soil, which was sown with three germinated seeds of maize, the outside one containing 1.0 kg soil, with no crops planted, the surfaces of the soil inside and outside the nylon screen were level. Each treatment was replicated three times. After four-week growth in a green house, the rhizoboxes were dismantled, and the soils in each compartment were sampled separately for analyses(Fig.1).

1.3 Water content and inorganic N pools

The gravimetric water content of the soil was measured as the weight loss after 24 h at 105℃. The pools of NO_3^- and NO_2^- were measured by a bioassay technique for small soil samples as described by Binnerup and Sørensen (Binnerup, 1992), except that 10 kPa acetylene(C_2H_2) was added to inhibit any N_2O reduction (Højberg, 1996). NH_4^+ was measured photometrically by reaction with salicylate and free chlorine in the presence of sodium nitroprusside as catalyst (Verdouw, 1977) after extraction of soil samples (0.1 g) in
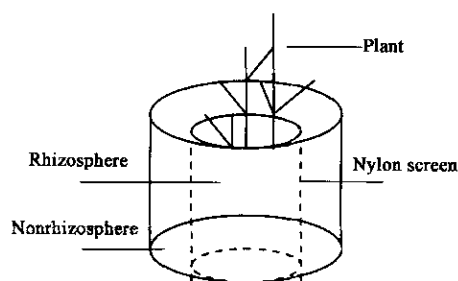
Fig.1   Sketch diagram of rhizobox.

1 ml 2 mmol/L KCl for 5 min.

1.4   Analyses of microbial activities related to N-cycle

Ammonification, nitrification and denitrification of soil samples were determined by the methods proposed by Li et al. (Li, 1995). Ammonifying activity was estimated by the ammonia released during the incubation period, expressed as mg NH_4^+ -N per kg soil. Briefly, 20 g fresh soil and 2 ml ammonifying bacteria culture was added into 500 ml conical flask, then incubated at 28℃ for 10 d. The ammonifying bacteria culture medium consisted of 5 g K_2HPO_4, 2.5 g $MgSO_4 \cdot 7H_2O$, 2.5 g $CaCl_2$, 0.05 g $Fe_2(SO_4)_3$ and 0.05 g $MnSO_4$ in 1 L distilled water at pH 7.2 adjusted with KOH.

Nitrifying activity was determined by the disappearance of nitrite (%) during incubation, firstly 10 g soil was thoroughly mixed with 90 ml distilled water, then 1 ml 10% soil solution was transferred into 150 ml conical flask which contained 30 ml nitrifying bacteria culture, and incubated for 15 d at 28℃. The nitrifying bacteria culture medium consisted of 1.0 g $NaNO_2$, 0.75 g K_2HPO_4, 0.25 g $NaH_2PO_4 \cdot 7H_2O$, 1.0 g Na_2CO_3, 0.03 g $MgSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4$ and 3.0 g $CaCO_3$ in one liter distilled water.

Denitrifying activity was measured by the decreasing of nitrate, expressed as %. During that period, 15 g soil and 10 ml 50 mg/L KNO_3 was added into 50 ml bottle, then it was incubated anaerobicly for three days at 28℃.

1.5   Exchangeable fraction of heavy metal analysis

Exchangeable fraction of heavy metals in soil was determined immediately. 10 g fresh soil samples were shaken at 30℃ for 1 h with 50 ml of 0.1 mol/L HCl, then centrifuging for 10 min at 2000 r/min. When that, the supernatant was analyzed using a Perkin Elmer Analyst 100 Flame Atomic Absorption Spectrophotometer.

1.6   Root exudates collection and characterization

Seeds of maize were surface-sterilized and germinated. When their roots grew to 3—4 cm in length, the seedlings were transplanted in 4 L Stamm-Nahrlcsurg culture solution (refreshed every five days).

After 20 d growth, the plants were subjected to different metal treatments: half seedlings treated with 1 ml 4 gCd/L(as $3CdSO_4 \cdot 8H_2O$) per day for 5 d, until the final Cd concentration of culture solution reached 5 mg/L; half plants treated with 1 ml 4 gCu/L(as $CuSO_4 \cdot 5H_2O$) per day for 5 d, until the final concentration of culture solution reached 5 mgCu/L. After 2 d growth, the metal solution was removed and the plants were replaced with deionized water for 4 h to collect the root exudates, then, the solution was freeze-dried and concentrated to 10 ml for analyses. The total carbohydrate in root exudates was determined by the same method proposed by Dubis et al. (Dubis, 1956).

2   Results and discussion

2.1   Metal mobility in rhizosphere and non-rhizosphere of maize

Exchangeable fraction is generally taken granted as then active fraction of heavy metal in soil environment, it may present a intensive potential impact on soil nutrient cycle.

Results showed that all the exchangeable fraction of Cd in rhizosphere was higher than those in bulk soil, and the enhancement of exchangeable Cd fraction in rhizosphere increasingly raised with the addition of Cd pollutants (Fig.2).
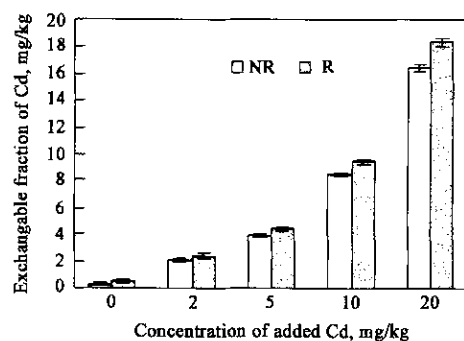

Fig.2   Changes of exchangeable fraction of Cd in rhizosphere and non-rhizosphere of maize as a function of Cd addition

The exchangeable metal fraction in soil varied with different metal species(Fig.3). Comparing the exchangeable fractions of three metals, Cr was the lowest one, typically showing its strong adsorption in soil(Chen, 2000a), while Cd was found to be the highest one, displaying its relatively high mobility in soil. However, all of the exchangeable heavy metal fractions were higher in rhizosphere than those in non-rhizosphere.
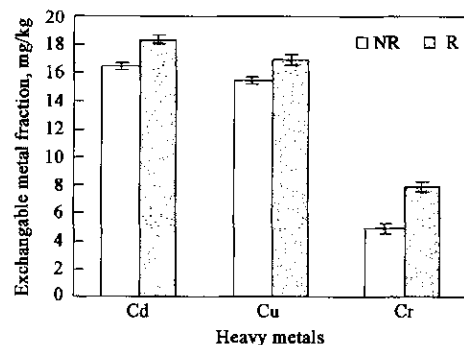

Fig.3   Changes of exchangeable metals in rhizosphere and non-rhizosphere of maize grown in various metal contaminated soil at the addition of 20 mg/kg in soil

2.2   Microbial characteristics of soil N cycle

The important physiological groups of bacteria involved in N-cycle, ammonifying, nitrifying and denitrifying activity were investigated to assess the influence of heavy metals on soil N-cycle. It shows in Table 1 that the ammonifying activities in bulk soil under the treatment of 2 mg/kg and 5 mg/kg Cd were stronger than that of control, whereas a slight inhibition was observed at 10 mg/kg Cd treatment. Similar trendency was also found in nitrifying bacteria, demonstrating a stimulatory effect of heavy metals on soil microorganism at low Cd level. The results were also reported by Hu et al. (Hu, 1990) about that low concentration of Cd, Pb could enhance the activity of urease in red soil. In comparison with different microbe groups in bulk soil, nitrifying activity was proved to be the most sensitive one to Cd pollution, however, the effect of Cd on denitrifying bacteria activity was very limited in this experiment. The changes of microbial activities in bulk soil influenced by various heavy metals were listed in Table 2. Cr and Cu stimulated ammonifying and denitrifying activity, whereas inhibitory influences were showed on nitrifying activity.

Since the effects of plant root on biological, chemical, and physical properties of soil are considered to be of agronomic and ecological interest(Rovira, 1979; Youssef, 1988), the behavior of heavy metal pollutants had been studied in the rhizosphere. It was found that microbial activities were all enhanced in rhizosphere. The enhancement

of ammonification and nitrification in rhizosphere was increased with the Cd concentration until 10 mg/kg, and then a decline was observed at 20 mg/kg(Table 1). In comparison with different metal species, Cr(VI) was proved to induce the most remarkable enhancement of ammonification, nitrification and denitrification in rhizosphere. It might be induced by the positive reduction of Cr(VI) to Cr(III) in rhizosphere(Chen, 2000b), and the relatively sufficient existing of organic matter in root zone, which intensified the fixation of Cr.

It was considered that the diffusible water-soluble root exudates and insoluble root cell debris were the primary

Table 1 Ammonifying, nitrifying and denitrifying activities in rhizosphere and non-rhizosphere of maize in Cd contaminated soil

	Ammonification, mg NH_4^+ -N/kg		Nitrification, %		Denitrification, %	
	NR	R	NR	R	NR	R
CK	71.47	80.11	88.04	98.07	23.22	41.84
2 mg Cd/kg	74.65	93.25	90.90	99.02	21.80	42.10
5 mg Cd/kg	79.24	95.71	91.98	99.82	22.38	36.16
10 mg Cd/kg	70.94	97.01	84.93	97.64	23.15	33.11
20 mg Cd/kg	69.40	86.13	74.41	77.36	22.78	42.44
$LSD_{0.05}$	6.531	4.664	5.777	1.965	1.661	2.324
$LSD_{0.01}$	9.289	6.635	8.217	2.794	2.363	3.305

Notes: NR- non-rhizosphere; R- rhizosphere; CK- control treatment

Table 2 Effects of various metals on ammonifying, nitrifying and denitrifying activities in rhizosphere and non-rhizosphere of maize at the addition of 20 mg/kg soil

Heavy metal	Ammonification, mg NH_4^+ -N/kg		Nitrification, %		Denitrification, %	
	NR	R	NR	R	NR	R
CK	71.47	80.11	88.04	98.07	23.22	41.84
Cd	69.40	86.13	74.41	77.36	22.78	42.44
Cu	73.84	85.12	78.65	97.62	25.85	43.58
Cr	76.35	96.55	64.76	91.03	27.21	52.33
$LSD_{0.05}$	5.569	4.584	7.923	3.072	2.602	3.207
$LSD_{0.01}$	8.103	6.670	11.529	4.470	3.786	4.667

Notes: NR—non-rhizosphere; R—rhizosphere; CK—control treatment

nutrient source for most of the microbial activities in rhizosphere(Kraffczyk, 1984; Rovira, 1967). Table 3 shows that the total carbohydrate in root exudates were 7.6, 12.8 and 20.3 mg/g root for the control, Cu and Cd, respectively. The remarkable increase of total hydrocarbons in root exudates suggested that membrane of root cell had been stimulated by Cd and Cu contaminants (Lin, 2000), consequently increased the carbon allocation in soil and supported the soil microbes in rhizosphere. Organic acids were also important constituents of root exudates and were of particular importance due to their metal chelating properties for mobilization of mineral nutrients(Jones, 1996; Gao, 2003; Qin, 2004). Our former investigations had conducted that the species of organic acid varied widely in response to Cd and Cu stress, and the quantity of organic acids increased significantly under heavy metal treatments (Chen, 2000b). The increased leaching of organic acid could improve the carbon allocation in rhizosphere, however the increased leakage of organic acid also improved the mobility of heavy metal in rhizosphere, especially in heavily contaminated metal soils(Fig.1), thus intensified the stress of metal pollutants on microbe in rhizosphere. Furthermore, as the metal concentration getting up, the color of root became dark, and a notable inhibition on root growth was observed in the high Cd treatment(20 mg/ kg), damaging the root cell plasmalemma, and causing increased leakage of ions and other toxic solutions from root cell (De Vos, 1991), consequently strengthen the toxic impacts of heavy metals on soil microbe.

2.3 Mineral N-cycle in soil

As notable impacts of heavy metals were shown on soil microorganism, concern had been raised that metal contamination should consequently influence the mineralization of organic N in soil. Changes of ammonium, nitrate and nitrite in soil are displayed in Fig.4 and Fig.5. Total amounts of ammonium, nitrate and nitrite were measured as a synthetically signal of the mineralization of N in soil. Results showed that the concentration of mineral N in bulk soil increased at the first four Cd levels, then declined under the treatment of 20 mg/kg Cd in soil. It was also found that the amounts of ammonium in bulk soil were enhanced with the increase of the concentration of Cd. As the main form of mineral N in soil, the amounts of nitrate in bulk soil increased with the Cd concentration initially, and then an

inhibitory effect was observed under the treatment of 20 mg/ kg. And a shortness of mineral N was found in rhizosphere due to the plant root absorption.

Table 3 Total hydrocarbon in maize root exudates

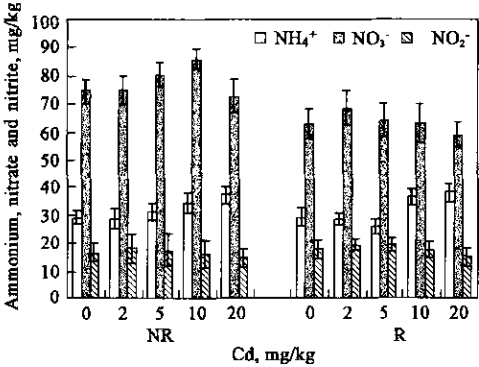| Treatment	Total carbohydrate, mg/g root
CK	7.6
5 mg Cd/L	20.3
5 mg Cu/L	12.8

Note: CK—control treatment

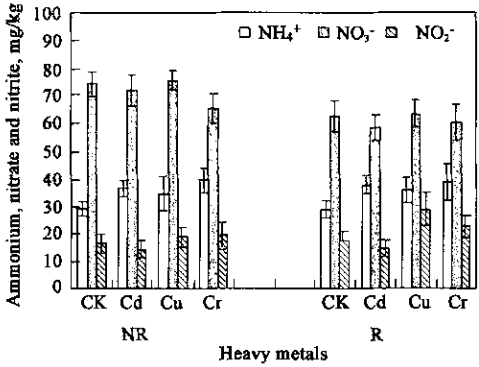Fig.4 Changes of mineral N in non-rhizosphere and rhizosphere of maize grown in Cd contaminated soil

Fig.5 Changes of mineral N in non-rhizosphere and rhizosphere of maize grown in various metal contaminated soil at the addition of 20 mg/kg in soil

pH in root zone is dependent on the absorption of plant

root. It had been found that if plants absorbed nitrogen in the form of NH_4^+, plant root would absorb more cations to excrete H^+, whereas if they absorbed nitrogen in the form of NO_3^-, they would absorb more anions to excrete HCO_3^- (Nye, 1981). Since higher pH of the rhizosphere was detected in the pot experiment, it probably meant that the plants were absorbing more anions than cation and were excreting HCO_3^- to maintain electrical neutrality across the root-soil interface. Therefore, nitrate should be the main form of nitrogen took by plant. With the Cd level getting high, the pH contrast between rhizosphere and non-rhizosphere became weaker, suggesting relatively more uptake of ammonium in rhizosphere. Since the amounts of ammonium in rhizosphere were even higher than that in bulk soil at high Cd levels(10 mg/kg, 20 mg/kg), it was reasonable to conclude that the formation of ammonium in rhizosphere was promoted in response to high Cd exposure.

Being affected by varied metals, the amounts of ammonium in bulk soil were increased at the addition of 20 mg/kg of Cu, Cd, Cr, respectively. A negative effect of metals on the amounts of nitrate was displayed both in Cr (VI) and Cd treatments, but not in Cu treated one. The total soil N mineralization increased in turn of Cd, Cr(VI), Cu.

3　Conclusions

Biochemical process in soil could be significantly affected by environmental pollutants, consequently, lead to a further influence on soil nutrient status. The addition of low Cd pollutant could stimulate the microbial activity in soil, while inhibitory influences were shown at high levels. In comparison with different microbe groups, nitrifying activity was proved to be the most sensitive one, while the effect on denitrifying activity was very limited. In comparison with Cd, Cu and Cr(VI) at 20 mg/kg soil, Cd was proved to be the most effective inhibitor of ammonification and denitrification, while Cr(VI) had the strongest inhibitory influence on the nitrifying activity. Plant root plays an important role on biochemical process of N-cycle in metal-contaminated soil. All of the microbial activities were improved in rhizosphere. The addition of low Cd pollutant increased the enhancement of microbial activity in rhizosphere due to the increased secretion of carbohydrate and organic acids in root exudation, which increase the carbon allocation in rhizosphere soil. Whereas a decline of the enhancement in rhizosphere was observed in high Cd treatments owing to critical inhibition on root growth and metal activation in rhizosphere, which strengthen the toxic impact of metal pollutants on soil microbes. Considering the improvement in rhziosphere, Cr (VI) was proved to be the most effective one. Similar influence was also displayed on soil N minerliazation showing that the mineralization of soil N in bulk soil could be stimulated by low metal pollutant. Total amount of mineral N in rhizosphere was lower than that in non-rhizosphere due to the plant uptake of N. The characterization of mineral N dynamics also indicated that nitrate was the main form of mineral N in soil, whilst the formation of ammonium was promoted in response to high Cd exposure.

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