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Effect of sulphur on soil Cu/Zn availability and microbial community composition

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ABSTRACT

Successful phytoremediation depends mainly on the bioavailability of copper (Cu) and zinc (Zn) in the soil. We studied the potential effects of sulphur (S) amendment on mobility of copper, zinc and microbial community composition in soil under laboratory conditions. The results showed that with S application at 20 g S kg^{-1}, soil pH decreased about 3 units and the solubility of the Cu and Zn significantly increased after 64 days of incubation. The concentration of Cu in Cu-accumulator *Elsholtzia splendens* shoots and roots increased with S treatment. Concentration of Cu in the shoots was 156.5 mg kg^{-1} under S treatment. It was 2.5 times of without application of S. PCR-denaturing gradient gel electrophoresis (PCR-DGGE) fingerprint analysis revealed that there were certain groups of acidophilic soil bacteria in the soil after addition of S. We found specific clones such as 1 (from biofilter-treating hydrogen sulfide and methanol) and 4 (from metal-rich and acidic River Tinto) in the soil with S treatment. The above results indicated that S facilitated the mobility of Cu and Zn by soil microorganism and provided a basis for further studies of S-assisted phytoremediation.

1. Introduction

Copper (Cu) and Zinc (Zn) are essential elements. Elevated levels of Cu and Zn in soil adversely affect microbially mediated soil processes. The European Union Council's directive limits for concentrations of heavy metals in arable soils indicate limits as 140 mg kg^{-1} for Cu, 300 mg kg^{-1} for Zn [1]. The strict implementation of environmental laws urges the development of cost-effective soil remediation methods. Phytoremediation, the use of plants to remove, stabilize, or detoxify pollutants, provides an effective and *in situ* alternative method of cleaning up heavy metals from contaminated soils [2,3]. It was reported that phytoremediation not only reduced the environmental risk of soil metal contamination, but also increased the activity and diversity of soil microorganism and improves soil quality [4,5].

Successful phytoremediation depends mainly on the bioavailability of Cu and Zn in the soil; however, the availability of Cu and Zn for plants is usually restricted by the complexation of metals within solid soil fractions. Chelate-assisted phytoremediation, the use of synthetic chelators, e.g. ethylenediamine tetraacetate (EDTA), has been used to artificially enhance heavy metal solubility in soil and thus increase heavy metal phytoavailability. Nevertheless, it was reported that many synthetic chelators capable of inducing phytoextraction might form chemically and microbiologically stable complexes with heavy metals and pose a threat of soil quality and groundwater contamination [6]. So far to avoid some of these constraints, the use of elemental S to decrease soil pH and increase the solubility of heavy metals in soils were suggested [7–13]. However, relatively few studies focused on the effects of S on soil microorganisms. These microbes might be crucial for S transformation in the soil.

In this study we explored element S on the microbial community composition in Cu and Zn contaminated soil. The objectives were to (1) compare the metals solubility of soil by application of S, (2) research element S on the microbial community composition in Cu contaminated soil and (3) investigate the potential ability of metal uptake and accumulation by *Elsholtzia splendens Nakai ex F. Maekawa* (*E. splendens*) through application of S. *E. splendens* is an indicator of copper mines, which is widely distributed on Cu mining wastes and Cu-contaminated soils along the middle and lower reaches of the Yangtze River, China [14–16]. Soil bacteria community analysis was carried out using activation-independent methods [17]. Partial 16S rDNA genes were amplified from soil bacterial community DNA by PCR, using primers which bind to evolutionarily conserved regions within these genes in the eubacteria. The community composition of PCR-amplified products was transformed to genetic fingerprints using DGGE.

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2. Materials and methods

2.1. Soil characterization

The study area selected is near to and downwind of a copper-zinc smelter started in 1985 in Zhujiawu county, Zhejiang province, China. This area was formerly used to cultivate cereal grass. After 1990s the site was on longer cultivated due to the reduction of crop output and contamination of the crop by Cu and Zn. A sward consisting of plants tolerant to Cu and Zn covered the ground at time of sampling. Soil was collected from the top layer (0–15 cm) of an agriculture field. According to USDA soil taxonomy [18], the Alluvial sandy loam, paddy soil that developed on the river alluvium is fluvaquents. The soil was air-dried and sieved (<0.45 mm) to remove plant materials, soil macrofauna and stones. Total content of Cu and Zn in the soil was analyzed with flame atomic absorption spectrophotometry (FAAS) by digesting 100 mg of soil in a mixture of HF–$HClO_4$–HNO_3 [19]. Soil organic material, cation exchange capacity (CEC) and pH were determined following the methods described by Lu [19]. Some selected physical and chemical properties of the soil were shown in Table 1.

2.2. Experimental design and treatments

The experimental designs were employed with 2.00 g elemental sulphur (S) and 100 g soil was applied and mixed thoroughly. Totally, nine treatments including eight different sample time with S addition and one treatment without S treatment were set. The sample time was after 1, 15, 24, 31, 38, 43, 50, 64 days of incubation. All treatments were carried out in triplicate. During the experiment, soil moisture content was adjusted regularly by weight to about 80% of water holding capacity. The soil suspensions incubated in a greenhouse with average temperatures of 28 °C at a shaking rate 180 rpm. The water content of the soil in the pots was maintained at 80% WHC (control by weight) by a daily supply of distilled water.

2.3. Copper and zinc extraction experiments and pH determine

Soil availability of Cu and Zn was used to investigate the effect of sulphur on the contaminated soil and changes of bacteria community composition in the soil. Soil availability of Cu and Zn was extracted with NH_4NO_3 (1:10, w/v) in triplicate [19]. The soil suspensions were centrifuged at 4000 rpm for 10 min and filtered. Content of Cu and Zn in each filtrate was determined by FAAS (Thermo Element MKII-M6). Soil pH was measured with a glass electrode using a 1:2.5 soil-to-water ratio.

2.4. PCR-DGGE

Total soil DNA was extracted by placing approximately 500 mg of soil in tubes containing lysing matrix and then using the Fast DNA™ SPIN Kit for Soil (Qbiogene, Carlsbad, CA, USA) following the manufacturer's protocol. PCR amplification for DGGE analysis, details of the DGGE protocol, gel staining have been described elsewhere. Briefly, PCR amplicons were generated using the generally conserved 16S rDNA gene primer pair F338 and R518 for the amplification of bacterial 16S rDNA genes. The GC-clamp described by Muyzer et al. [17] was added to the forward primers to facilitate the DGGE. For DGGE analysis, 400 ng of PCR product generated from each sample was separated on an 8% acrylamide gel with a linear denaturant gradient range of 35–60% using the Bio-Rad D-GENE System. Gels were stained with SYBR Green I and bands were visualized using a Bio-Rad Gel Doc 1000 and Molecular Analyst software (Bio-Rad Laboratories).

Table 1
Selected physical and chemical properties of the soil used in the experiments

Soil type[a]	Alluvial sandy loam
O-M[a] (%)	3.94
CEC[b] (cmol kg^{-1})	4.14
pH (H_2O:soil = 2.5:1)	7.80
T-Cu[c] (mg kg^{-1})	317 ± 31
T-Zn[c] (mg kg^{-1})	1040 ± 87
T-Pb[c] (mg kg^{-1})	58 ± 9

[a] O-M, organic matter.
[b] CEC, cation exchange capacity.
[c] T-Cu, T-Pb, total copper, total zinc, total lead.

2.5. Cloning and DNA sequencing

The bands were recovered from DGGE gels for further sequence analysis. Briefly, bands were excised from DGGE gels, transferred to 500 μl microcentrifuge tubes, and then macerated and mixed with 50 μl of Tris–HCl buffer (pH 8.0) [20]. The mixtures were incubated overnight at 4 °C to elute the DNA from the acrylamide matrix. The eluted PCR products were again amplified by PCR as described above and then purified using Qiaquick PCR Clean-up columns (Qiagen, Valencia, CA). The purified products were cloned into the plasmid vector pGEM-T easy (Promega). Plasmid clones were grown overnight in Luria-Bertani broth amended with ampicillin and identified based on blue-white screening, and plasmid DNA was purified by using Qiagen mini-prep kits as specified. To confirm the identity of each clone, purified plasmid DNA was used as template for PCR using the same F338GC-R518 primer pair, and the resulting product was analyzed by DGGE alongside the original total-community PCR products. Products were analyzed on an Applied Biosystems 377 DNA sequencer. Sequences recovered from excised bands were analyzed for chimeric character by using the Ribosomal Database Project II (RDP II) Chimera Check program (http://rdp.cme.msu.edu/html/). The closest known microorganisms represented by recovered sequences were identified with the Sequence Match program of RDP II.

2.6. Effect of sulphur on E. splendens uptake of Cu and Zn

The experimental designs were employed with 20.0 g sulphur and 1 kg soil was applied and mixed thoroughly in triplication. Seeds of *E. splendens* were soaked in distilled water for 30 min and then germinated in commercial potting mixture. Seedlings of the species were watered daily with a nutrient solution for 3 weeks. Uniform seedlings were then selected and transplanted to porcelain pots each containing 1.0 kg of the Cu and Zn contaminated soil and 20.0 g sulphur, and treated with necessary amounts of fertilizer at a rate of 1.0 g urea, and 0.2 g potassium. All plants were grown in a greenhouse with average day and night temperatures of 32 and 16 °C, respectively, and were irrigated slightly above field capacity moisture. After 60 days of incubation, the plants were harvested. Plant roots were firstly washed in tap water to remove soil and then washed with deionized water. Plant shoots and roots were dried at 105 °C for 30 min and then 75 °C for 24 h prior to the Cu and Zn analysis.

2.7. Statistical analysis

All data were analyzed using Microsoft Excel and SPSS 11.0. A probability level of 0.05 was considered to be statistically significant.

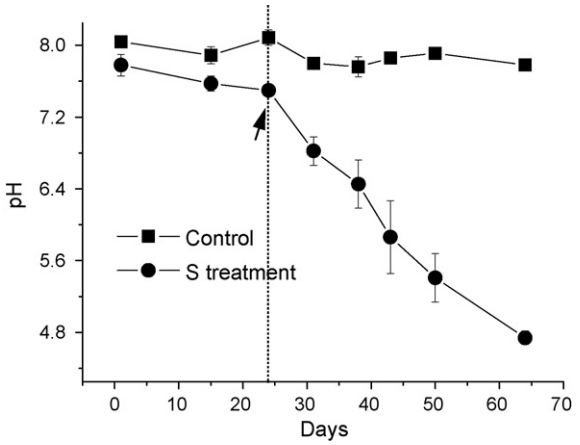

Fig. 1. Changes in soil pH after S application during 64 days of incubation.

3. Results

3.1. Soil pH

In the present experiment, S application caused the soil pH to decrease about 3 units within 64 days. However, no significant decrease was found under S treatment in the initial 24 days. After 24 days of incubation, soil pH decreased (Fig. 1). Significant correlation was found between soil pH and days of incubation ($r = -0.995$) from day 24 to day 50. When the experiment was end, soil pH was the lowest (pH 4.74). During the incubation, no significant difference was found without application of S.

3.2. Soil extractable Cu and Zn

In the initial 31 days, no significant increase was found on soil extractable Cu and Zn under S treatment. However, soil extractable Cu and Zn increased after 31 days of incubation. Concentrations of Cu and Zn in the soil were 10.3, 152.6 mg kg^{-1} after 64 days incubation. It is 3.7 and 16.8 times of 1-day incubation, respectively (Fig. 2).

3.3. DGGE analyses of S addition to soil microorganisms

By the optimization of DGGE running conditions, we found that DGGE profiles of triplicate samples were highly reproducible. Hence, by running formal DGGE, we decided to use two randomly selected samples. Meanwhile, PCR-DGGE protocol has been proved to be sensitive and highly reproducible in analyzing microbial community compositions in various treatments. In addition, DGGE profiles of the randomly selected replicates were highly reproducible, as shown in Fig. 3. Although lots of bands were detected in all samples, apparent differences in the bacteria community with S treatment were readily observed after 30 days of incubation. As shown in Fig. 3, number of bands in soil with S was significantly decreased as compared to that of bulk soil.

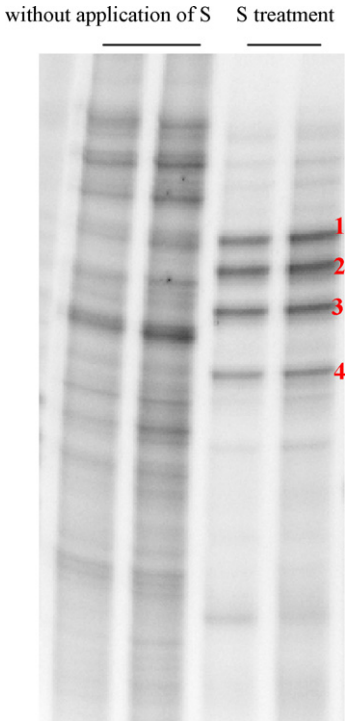

Fig. 3. DGGE separation patterns of PCR amplified 16S rDNA fragments from soil (after 1 month) with or without S treatments.

The closest matches of the obtained sequences to the known species were determined by comparison with the RDP II database (Table 2). The majority of the sequences were most closely related to the known soil organisms or environmental clones. Specific clones such as 1 (from biofilter-treating hydrogen sulfide and methanol) and 4 (from metal-rich and acidic River Tinto) were found in the soil with S treatment.

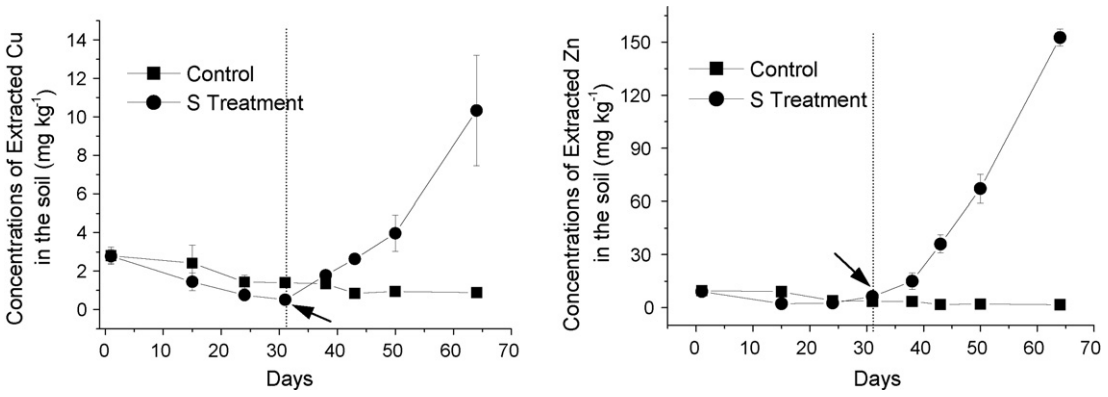

Fig. 2. Data indicated that the resulted in changes of soil NH_4NO_3 extractable Cu after treated by S. Without S application is given for comparison. The mean of three replicates is shown with their standard deviations.

Table 2
Closest match of cloned DGGE bands to known species

Site and clone name	Best match to known species	Sab score
1	Uncultured yard-trimming-compost bacterium clone (from biofilter-treating hydrogen sulfide and methanol) AY095397	0.907
2	*Bacillus subtilis* 16S ribosomal RNA AY833569	0.921
3	*Bacillus mojavensis*; KL-198 AB021191	0.915
4	Uncultured gamma proteobacterium (from metal-rich and acidic River Tinto) AY475206	0.963

3.4. Plant uptake of Cu and Zn

The analysis of plant material indicated that *E. splendens* were effective in shoot and root uptake of Cu from the contaminated soil after S treatment (Table 3). The effects of the application of S on the plant uptake of Cu and Zn from the soil were different. For Cu, S was almost effective on facilitating root and shoot uptake of Cu. For Zn, S could not be effective on facilitating shoot uptake of Zn. Concentration of Cu in the shoots of *E. splendens* was 156.5 mg kg^{-1} under S treatment. It is 2.5 times of without application of S. Responses of plant root to Zn uptake were similar after the addition of S, however, concentration of Cu in the roots is 5.0 times of that of without application of S. In addition, plant dry matter biomass was not affected by the application of S.

4. Discussion

Recent studies have shown that low bioavailability of soil Cu may be rate limiting for Cu absorption by plants [21,22]. In present study, the use of S, which removes metals from their binding sites in the soil, has been proposed as a solution to this problem. It was indicated that S could facilitate the bioavailability and plants uptake of Cu and Zn (Table 3, Fig. 2).

Although the experiment showed short-term effects after 60 days of incubation, we observed S-induced phytoextraction practice involving *E. splendens*. It is well known that many plants retain Cu in their roots via sorption and precipitation with only minimal transport to the aboveground harvestable plant portions. In the present experiment, the concentrations of Cu and Zn in the shoots and roots were higher after S application than without (Table 3). There are a number of reasons for this result in high plant uptake and accumulation of these Cu and Zn; however, the main reason is most probably that the increase of solubility of Cu and Zn leads to an improvement in metal bioavailability.

The availability and mobility of metal ions increased due to the soil pH decreased at the end of the experiment. It was indicated that pH decreased at the day of 24 but the availability of Cu and Zn increased at the day of 31. Since Cu and Zn are a relatively immobile metal in soils due to complexation with organic matter, sorption on oxides and clays, and precipitation as carbonates, hydroxides and phosphates [3,23,24], therefore the concentration of extractable Cu did not show any increase without an appropriate decrease in soil pH (before 31 days of incubation) (Fig. 2). The day of 31 was the critical point of soil pH decrease and concentrations of Cu and Zn increase. The main reason for decrease of soil pH is most probably that soil bacteria transformed S and produced H^+ [8,9,13].

Table 3
Addition of S to plant *E. splendens* enhances Cu accumulation in tissues (shoot and root). The mean of three replicates is shown with their standard deviations

S treatment		Plant biomass (g) (shoot + root)	Shoot (mg kg^{-1})	Root (mg kg^{-1})
Cu	CK	52.2 ± 5.1	62.9 ± 0.8	251.3 ± 95.6
	S	48.4 ± 7.5	156.5 ± 18.4	1245.8 ± 287.0
Zn	CK	–	154.8 ± 33.7	491.1 ± 65.3
	S		194.9 ± 26.9	594.2 ± 50.8

After S addition in soil, the difference on the soil bacteria diversity in bulk soil and S treatments was distinct with PCR-DGGE approach after 31 days of incubation (Fig. 3). Two possible reasons might explain the phenomena. First, it is suggested that role of soil microorganism can use S as energy source, then shift the abundance of soil bacterial groups, especially some microbe could tolerate acidic environment and high concentration of heavy metals [25,26]. It has been shown that S had significant effect on soil microorganism community composition after 31 days of incubation. Second, there probably exist some specific microorganisms such as acidophilic soil bacteria in the soil, which that can oxidize S and change it to SO_4^{2-} when soil pH has decreased. These reactions may be catalyzed mainly by microorganisms [27]. It was evidenced by Ding et al. [28] who found some bacteria were able to oxidize S and reduce soil pH. DGGE and DNA sequence analyses demonstrated that there were some specific clones such as 1 (from biofilter-treating hydrogen sulfide and methanol) [28] and 4 (from metal-rich and acidic River Tinto) [29] in the soil with S treatment (Table 2). In addition, it was observed that there were some Gram-positive bacteria (2, 3). It was reported that these bacteria could synthesize extracellular polymeric substances, a mixture of polysaccharides, mucopolysaccarides or proteins capable of binding potentially toxic metals and entrapping precipitated metal sulfides and oxides [30,31].

Low bioavailability of soil Cu and Zn may be rate limiting for phytoremediation. In present study, it was indicated that S facilitated the mobility of Cu and Zn by soil microorganism and provided a basis for further studies of S-assisted phytoremediation. Although the application of S can increase uptake of some metals by plants by enhancing metal mobility, there is also a potential risk of movement of metals into the groundwater; therefore, it is essential that the potential risks when using S for phytoremediation should be evaluated before stepping forward for commercial application.

5. Conclusions

Soil pH decreased about 3 units and the solubility of the Cu and Zn was significantly increased after 64 days of incubation. Concentration of Cu in the shoots was 156.52 mg kg^{-1} under S treatment. It is 2.5 times of without application of S. PCR-DGGE fingerprint analysis revealed that there are certain groups of acidophilic soil bacteria in the soil after addition of S. We found specific clones such as 1 (from biofilter-treating hydrogen sulfide and methanol) and 4 (from metal-rich and acidic River Tinto) in the soil with S treatment. The above results indicated the S could facilitate the mobility of Cu and Zn by soil microorganism and provided a basis for further studies of S-assisted phytoremediation.

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