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Rhizosphere characteristics of zinc hyperaccumulator *Sedum alfredii* involved in zinc accumulation

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ABSTRACT

A hyperaccumulating ecotype (HE) and a non-hyperaccumulating ecotype (NHE) of *Sedum alfredii* were grown in a pot experiment to investigate the chemical characteristics of the rhizosphere. The results indicated that HE accumulated more Zn in the shoot than NHE after growing in both heavily and slightly polluted soil. The water soluble Zn and mobile Zn (extractable with 1 M NH_4NO_3) fraction in both rhizosphere and bulk soils decreased considerably after growth of HE compared to NHE. However, the decreases in mobile fraction accounted for less than 8.5% of the total Zn uptake by HE indicating that HE was effective in mobilizing Zn from the non-mobile fractions. Zinc-induced root exudates reduced the soil pH (by 0.6–0.8 units) and increased dissolved organic carbon concentrations in the rhizosphere of HE compared to the bulk soil. The dissolved organic matter (DOM) from the rhizosphere of HE showed greater (1.7–2.5 times) extracting ability of Zn from various Zn minerals than those of NHE-DOM ($P < 0.05$). Results from this study suggests that rhizosphere acidification and the exudation of high amounts of DOM with great metal extracting ability might be two important mechanisms by which HE *S. alfredii* is involved in activating metal in the rhizosphere.

1. Introduction

Zinc is a ubiquitous trace metal and is found in virtually all environmental media. It is an essential micronutrient for plant growth and development as it is known to be required in several metabolic processes [1]. However, Zn toxicity occurs in soils contaminated by mining and smelting activities, in agricultural soils treated with sewage sludge, and in urban and peri-urban soils enriched by anthropogenic inputs of Zn, especially in low-pH soils [2]. Technologies currently available for the remediation of Zn contaminated soils are expensive and time-consuming, can create risks to workers, and can produce secondary wastes [3]. Phytoextraction, the use of green plants to clean up contaminated soil, has attracted attention as an environmentally friendly and low-input remediation technique. This technology makes use of hyperaccumulator plants that extract pollutants from the soil and accumulate them in their harvestable above-ground biomass [3,4]. A number of hyperaccumulators have been identified in the past three decades, and many studies have been carried out to investigate the mechanisms of uptake, transport, and detoxification of pollutants [5–7]. However, potential use of hyperaccumulators in phytoremediation is limited by a lack of knowledge of many basic plant processes [8].

Improving the efficacy of phytoextraction technique is critical for its successful application in metal contaminated soils which requires a sound understanding of the mechanisms by which hyperaccumulator plants mobilize and take up heavy metals [9]. Root–soil interactions in the rhizosphere play a key role in controlling metal bioavailability to plants. Plants can modify their rhizosphere to enhance the uptake of nutrients such as Fe, P, and Zn by different mechanisms such as acidification, decrease of redox potential, and release of root exudates [10]. Research on *Thlaspi caerulescens*, a Zn/Cd hyperaccumulator, indicates that the plant mobilizes Zn and Cd efficiently from soils [11], but this ability may not be due to rhizosphere acidification [12]. Luo et al. [13] found a slight increase in rhizosphere pH of *T. caerulescens*. While other results show that the initial soil pH can considerably affect metal uptake in *T. caerulescens* [14] and decreasing pH is an effective strategy to enhance phytoextraction [15]. In addition, hyperaccumulator species may release root exudates containing chelators with the potential to enhance heavy metal uptake, translocation and resistance. Wenzel et al. [16] found that enhanced Ni concentrations in soil solutions from depleted rhizospheres of *Thlaspi goesingense* were associated with increased levels of dissolved organic carbon. Root exudates of phytic acid and oxalic acid were both effective in mobilizing As from Fe–As and Al–As minerals in rhizosphere of *Pteris vittata* [17]. However, Salt et al. [18] have

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Table 1
Selected physico-chemical properties of the soils used in the study.

Soils	pH[a]	OM[b] (g kg^{-1})	Total N (g kg^{-1})	Total P (g kg^{-1})	CEC[c] (cmol kg^{-1})	Sand (%)	Silt (%)	Clay (%)	Total Zn (mg kg^{-1})	NH_4NO_3–Zn (mg kg^{-1})
Heavily polluted soil	6.82	20.8	1.40	0.81	8.95	38.9	40.6	20.5	2001	4.60
Slightly polluted soil	6.58	16.9	0.90	0.68	9.80	52.0	33.5	14.5	298	0.65

[a] 1:2.5 soil/water ratio.
[b] Organic matter.
[c] Cation exchange capacity.

shown in a hydroponics experiment that the release of citrate and histidine did not appear to be involved in Ni-hyperaccumulation in *T. goesingense*. Similarly Zhao et al. [19] found root exudates of the hyperaccumulator *T. caerulescens* do not enhance metal mobilization. To sum up, although some progress has been made towards an understanding of the rhizosphere processes associated with metal hyperaccumulation, still there is a considerable debate and uncertainty about the mechanisms by which hyperaccumulators activate and take up metal from the rhizosphere [20].

The hyperaccumulating ecotype (HE) of *Sedum alfredii* grows in the old Pb/Zn mining areas of southeast China. It has been identified as a new Zn-hyperaccumulating plant native to China which can accumulate 2.9% Zn in shoots without toxicity symptoms [21]. This plant shows extraordinary ability to absorb Zn from the soil and accumulate them in shoots under both low and high metal concentrations. To date, studies on the mechanism of metal tolerance and hyperaccumulation of *S. alfredii* were mainly focused on the uptake [22,23] and subcellular distribution characteristics [24], however, the process by which *S. alfredii* mobilizes and takes up Zn in soils is not known. The present study was conducted to (1) compare the rhizosphere characteristics of two ecotypes of *S. alfredii*; and (2) examine how these characteristics influence the availability of Zn in rhizosphere. Results of the present study will help to understand the mechanisms by which *S. alfredii* mobilizes and takes up Zn from soil and will optimize the management practices for maximum Zn phytoextraction.

2. Materials and methods

2.1. Plant material and soil characterisation

The hyperaccumulating ecotype (HE) of *S. alfredii* was collected from an old Pb/Zn mine area in Zhejiang Province, and the non-hyperaccumulating ecotype (NHE) of *S. alfredii* was obtained from a tea garden in Hangzhou, Zhejiang Province, PR China. The healthy and equal-sized plant shoots were selected and grown for two weeks in the greenhouse using a basic nutrient solution [21].

Two soils were used in the pot experiment. The heavily polluted soil was collected from the surface layer (0–20 cm) in Fuyang County of Hangzhou, Zhejiang Province, PR China. The soil has been heavily contaminated due to mining activities and not suitable for crop growth. The slightly polluted soil was collected from the topsoil (0–20 cm) in the farm of Zhejiang University, Hangzhou. Soil samples were air-dried, ground to pass through a 2-mm sieve, and stored in plastic bags until use. The soil pH was measured using a 1:2.5 soil to water ratio, organic matter content was measured by the Walkley Black method [25], cation exchange capacity was determined by an ammonium acetate method [26], soil texture was determined by using the pipette method [27]. Total Zn contents were determined by flame atomic absorption spectrometer (FAAS) (AA 6800, Shimadzu, Japan). Selected physical and chemical characteristics of this soil are shown in Table 1.

2.2. Pot experiment design

A rhizobox was designed as described by Wang et al. [28] (Fig. 1). The dimensions of the rhizobox were 140 × 140 × 200 (length × width × height in mm). It was divided into three sections, a central zone or rhizosphere zone (20 mm in width), which was surrounded by nylon mesh (300 mesh), and left and right non-rhizosphere zones (60 mm in width). Root growth was limited to the central compartment and within the nylon mesh. About 0.5 kg of soil was placed in the rhizosphere zone and 3.0 kg was placed in the non-rhizosphere zone. One week before the study, the soil equilibrated at field capacity. After pre-culturing for two weeks in hydroponic solution, three *S. alfredii* plants were transplanted in the rhizosphere zone and each treatment was replicated four times. The plants were watered throughout the study to keep the soil at

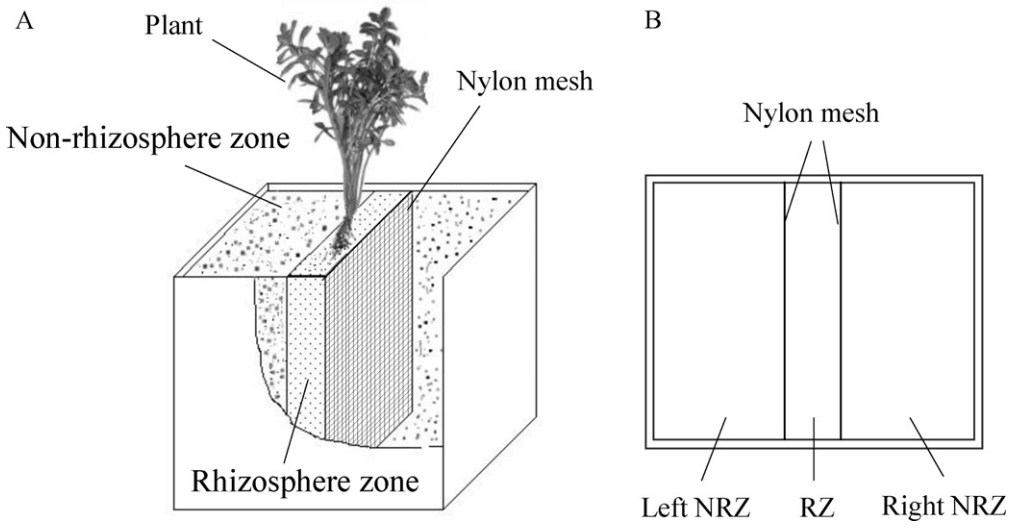

Fig. 1. Diagram of the rhizobox. Dimension: 140 × 140 × 200 = length × width × height, mm; rhizosphere zone (RZ): 20 mm in width; non-rhizosphere zone (NRZ): 60 mm in width. (A) Space view of the rhizobox; (B) planform of the rhizobox.

approximately 65% of its field capacity. The plants were allowed to grow for 14 weeks in a greenhouse with natural light and an average day/night temperature of 30/24 °C, and day/night humidity of 70/85%. At the end of the experiment, the plants were harvested and each plant was separated into root and shoot parts. These parts were washed thoroughly with tap water and finally rinsed with distilled water. Then these were oven dried for two days at 65 °C, weighed, and ground to a 60-mesh fineness for chemical analysis. Rhizosphere soil (soil in rhizosphere zone) and bulk soil (soil in non-rhizosphere zone) were separated, the field-moist soil samples were sieved through a 2-mm sieve and stored at 4 °C until use.

2.3. Extracting experiment

The dissolved organic matter (DOM) was extracted according to the method of [29] with some modification. Both rhizosphere and bulk soil were extracted with deionized water using a soil:water ratio of 1:5 (w/v) on a dry weight basis and shaker at 200 rpm for 8 h and at 20 °C on a reciprocal shaker. The suspensions were centrifuged at 10,000 × *g* for 25 min, and the supernatant was filtered through a 0.45 μm filter and subsequently through a sodium cation exchanger to remove cations (e.g., Zn^{2+}, Ca^{2+}, Mg^{2+}). The filtrates were stored at 4 °C until use. An extracting experiment was conducted to investigate the effect of DOM on Zn availability. Four Zn minerals of sulphide, orthophosphate, oxide, or carbonate with different solubility in water were selected. These minerals were sieved (<1 mm) and a mineral sample of 2.0 g was placed into 50-ml polyethylene centrifuge tubes and equilibrated with 20 ml of rhizosphere DOM. Deionized water was added to each Zn mineral as a control. The suspensions were shaken on a reciprocal shaker at 200 rpm and 25 °C for 2 h and then centrifuged at 5000 × *g* and filtered. Zinc concentrations in the filtrates were determined by inductively coupled plasma atomic emission spectrometer (ICP-AES) (ICPE-9000, Shimadzu, Japan).

2.4. Chemical analysis

Plants were digested using EPA Method 3050A for the Hot Block Digestion System (Environmental Express, Mt. Pleasant, SC) and metal concentrations in the digest were determined using flame atomic absorption spectrophotometer (FAAS) (AA 6800, Shimadzu, Japan). Soil pH was measured using a 1:2.5 soil to water ratio. Rhizosphere and bulk soil were evaluated for water-soluble and NH_4NO_3-extractable Zn in 1:2.5 soil to water ratio obtained after shaking (1 h), centrifuging (15 min at 4000 × *g*) and filtering (0.45 μm syringe filter). The concentrations of Zn were determined using ICP-AES (ICPE-9000, Shimadzu, Japan). The concentrations of dissolved organic carbon (DOC) were measured using a TOC-5050A TOC analyzer (Shimadzu, Japan) [29].

Data were analyzed statistically using analysis of variance (ANOVA). Means of significant difference were separated using least significant difference (LSD, $P < 0.05$).

3. Results and discussion

3.1. Plant growth and uptake of Zn

During the 14-week growth, no visual symptoms of Zn toxicity were observed for the HE *S. alfredii*, whereas the growth of the NHE *S. alfredii* was inhibited significantly in heavily polluted soil. The shoot and root biomass of the HE *S. alfredii* was not affected by soil Zn concentration. However, the shoot and root biomass of NHE *S. alfredii* was reduced by 46.9% and 54.3% when soil Zn increased from 298 to 2001 mg kg^{-1} (Table 2). The shoot and root biomass of the HE *S. alfredii* grown in heavily polluted soil were 1.8 and 2.2 times higher than the NHE, respectively. The Zn hyperaccumulator *S. alfredii* can tolerate Zn levels up to 2000 mg kg^{-1} in the soil without reduction in dry matter yield, which is close to those of *Thlaspi caerulescens* [5] and *Arabidopsis halleri* [30]. After growing in heavily and slightly polluted soil for 14 weeks, the Zn concentrations in HE *S. alfredii* shoot were 8990.5 and 6040.8 mg kg^{-1}, and those in the roots were 1108.0 and 809.5 mg kg^{-1}. The corresponding values for the shoot and root of NHE *S. alfredii* were 340.5 and 88.5 mg kg^{-1}, and 1175.8 and 198.0 mg kg^{-1}, respectively (Table 2). Zinc concentrations in HE *S. alfredii* shoots were 8.1 and 7.5 times of those in the roots whereas in NHE *S. alfredii* the shoot/root ratios of the Zn concentrations were 0.29 and 0.45, respectively. These indicate that HE *S. alfredii* was much more efficient in translocating Zn from the roots to the shoots than NHE under both low and high metal levels. Comparing the two ecotypes of *S. alfredii*, after growing in heavily and slightly polluted soil for 14 weeks, HE *S. alfredii* accumulated significantly ($P < 0.05$) more Zn in the shoot (23.82 mg pot^{-1} and 15.65 mg pot^{-1}) than NHE *S. alfredii* (0.39 mg pot^{-1} and 0.24 mg pot^{-1}) from the two soils (Table 2). These results agree with the previous findings [22–24] and suggest that HE *S. alfredii* have a great potential for removal of Zn from contaminated soil.

Table 2
Plant biomass, Zn concentration and accumulation of *S. alfredii* after growing for 14 weeks in two Zn-contaminated soils containing 298 and 2001 mg kg^{-1} Zn.

Plant parameters	Heavily polluted soil		Slightly polluted soil	
	HE	NHE	HE	NHE
Shoot biomass (g pot^{-1})	2.65 a	1.46 b	2.59 a	2.75 a
Root biomass (g pot^{-1})	0.36 a	0.16 b	0.39 a	0.35 a
Shoot Zn (mg kg^{-1})	8990.5 a	340.5 b	6040.8 a	88.5 b
Root Zn (mg kg^{-1})	1108.0 a	1175.8 a	809.5 a	198.0 b
Shoot Zn (mg pot^{-1})	23.82 a	0.39 b	15.65 a	0.24 b
Root Zn (mg pot^{-1})	0.40 a	0.17 b	0.32 a	0.09 b

Means followed by the same letter for a given soil are not significantly different at $P < 0.05$.

3.2. Mobile Zn in the rhizosphere of HE S. alfredii

The water soluble Zn in soil was determined before and after growth of *S. alfredii* (Fig. 2a). The two soils with different initial water soluble Zn concentrations showed significant decrease in both rhizosphere and bulk soil after growth of HE *S. alfredii* ($P < 0.05$), and the decreases in the rhizosphere (58% for heavily polluted soil and 65% for slightly polluted soil, respectively) was much greater than bulk soils. In contrast to HE *S. alfredii*, heavily polluted soil showed no change in water soluble Zn concentration after growth of NHE *S. alfredii*, however, water soluble Zn concentration in the rhizosphere of slightly polluted soil showed a distinct increase after growth of NHE *S. alfredii*, this may be due to the increased release of root exudates and less uptake of the soluble-Zn in rhizosphere of NHE *S. alfredii* (Fig. 2a). Extraction with 1 M NH_4NO_3 has been used as a standard method for the estimation of mobile (soluble + exchangeable) heavy metals in soils [12]. For NHE *S. alfredii*, no significant changes were found for the two soils in different compartments (Fig. 2b). However, for HE *S. alfredii* the concentrations of mobile Zn in both rhizosphere and bulk soils for the two soils decreased as compared to their initial values (before HE transplantation), and the decreases in the rhizosphere was much greater than bulk soils ($P < 0.05$) (Fig. 2b). These results agree with the study on Zn hyperaccumulator *T. caerulescens* [11,12]. In this study, since the roots were mainly confined to the rhizosphere soils, the decreases in mobile Zn in the bulk soils suggested that Zn move from the bulk soil to the rhizosphere soils, this may due to the fast depletion of the soluble-Zn in the rhizosphere of HE *S. alfredii*. Although the mobile Zn fraction in soils decreased considerably after planting HE *S. alfredii*, the decreases in this fraction in

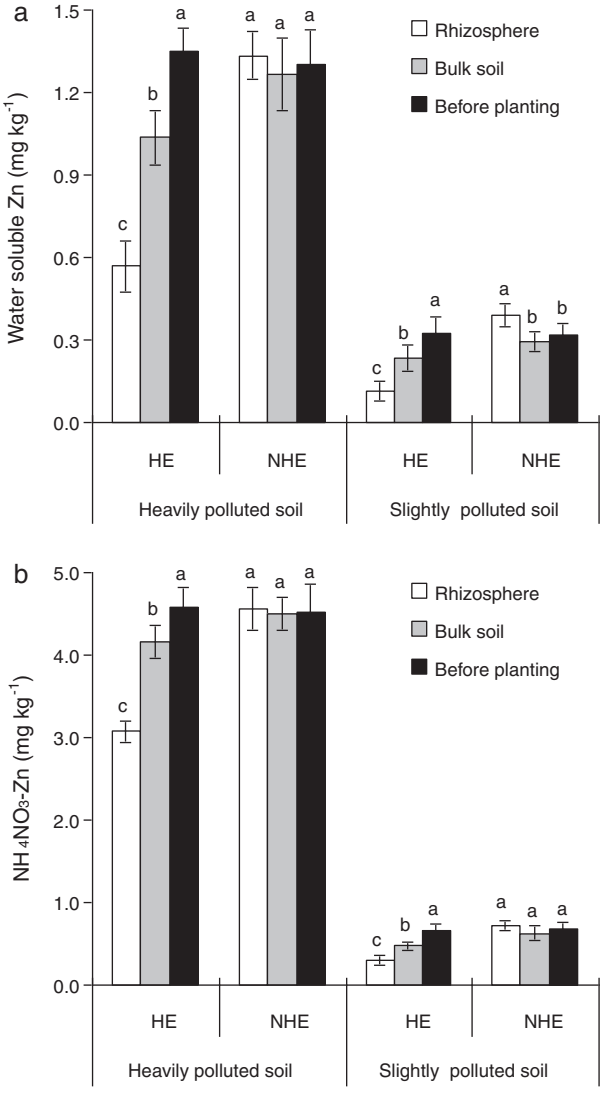

Fig. 2. Water soluble (a) and NH_4NO_3-extractable (b) Zn concentrations in the rhizosphere and bulk soil of *S. alfredii* after 14 weeks of growth in two Zn-contaminated soils. Bar represents standard deviations of four replicates. Bars sharing the same letter code among different compartments for a given ecotype are not significantly different at $P < 0.05$.

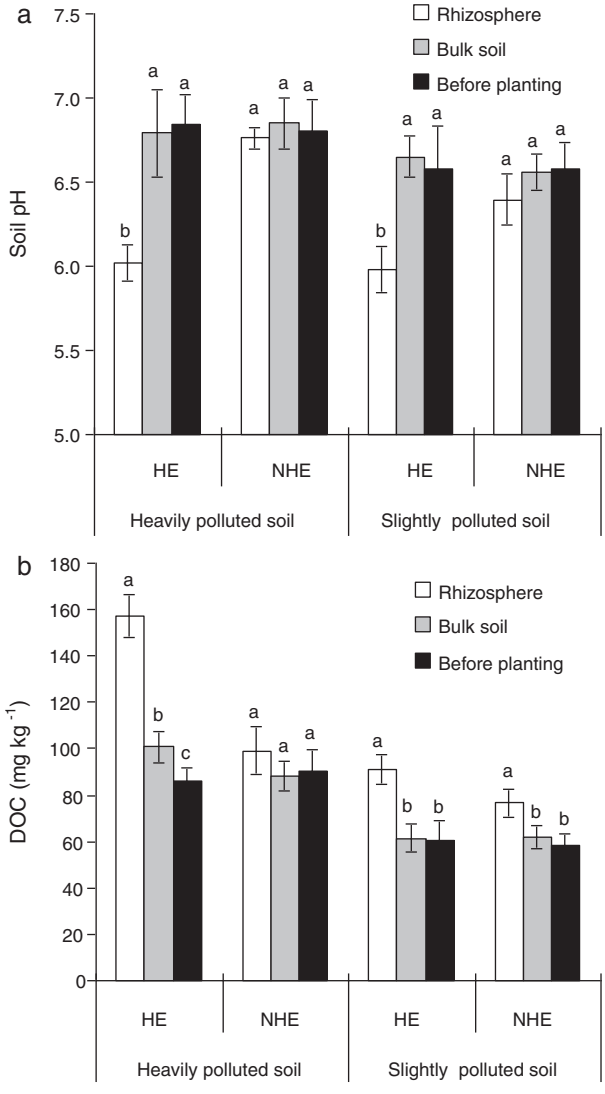

Fig. 3. The pH (a) and concentrations of dissolved organic carbon (b) in the rhizosphere and bulk soil of *S. alfredii* after 14 weeks of growth in two Zn-contaminated soils. Bar represents standard deviations of four replicates. Bars sharing the same letter code among different compartments for a given ecotype are not significantly different at $P < 0.05$.

both rhizosphere and bulk soils accounted for only 3.8–8.4% of the total uptake in the shoots. Therefore, more than 90% of the total Zn uptake must have come from the non-mobile fractions. The capacity of a soil to sustain the concentration of a particular ion in solution during plant growth depends upon the balance between the two opposite processes: solubilization of the ion from soils and its uptake by plants [10]. The results of the present study suggest that rapid Zn-uptake system in the roots of HE *S. alfredii* deplete soluble-Zn at a rate faster than that at which Zn is replenished to the soil solution and HE *S. alfredii* is highly efficient at mobilizing Zn which is not soluble initially.

3.3. Changes in soil mobile Zn, pH and dissolved organic carbon before and after growth of S. alfredii

Fig. 3a shows that at the end of the experiment, no significant changes in pH were observed in both rhizosphere and bulk soil of NHE *S. alfredii* for two soils. However, the rhizosphere soil of HE *S. alfredii* was reduced 0.6–0.8 pH units compared to the bulk soil and soil before planting, and the reduction in heavily polluted soil was greater than those in slightly polluted soil. These results agree with Gonzaga et al. [20] who found that arsenic-induced root exudates reduced soil pH by 0.74–0.92 units in the rhizosphere of As hyperaccumulator *P. vittata*. Soil pH is considered to be one of the most important chemical factors controlling the availability of heavy metals [31]. Reduction in rhizosphere pH increases the chemical activity of most metals, thereby increasing Zn uptake by HE *S. alfredii*. This suggests that rhizosphere acidification is an important mechanism for mobilizing metals in soil by HE *S. alfredii*. However, Bernal and McGrath [32] and McGrath et al. [12] found that the Ni hyperaccumulator *Alysum murale* and the Zn hyperaccumulator *T. caerulescens* did not cause a significant decrease in the pH of the rhizosphere, suggesting that the increased metal uptake is not simply a function of low rhizosphere pH. Luo et al. [13] found that *T. caerulescens* rhizosphere soil had higher pH than non-rhizosphere soils. These conflicting results indicated that different hyperaccumulators have different mechanisms for mobilizing metals in the soil. The reduction in pH was probably due to increased release of root exudates, which was consistent with the greater DOC concentrations in the rhizosphere of HE *S. alfredii*.

The DOC concentrations in the rhizosphere of HE *S. alfredii* were greater than those in the bulk soil and initial values (before plant-

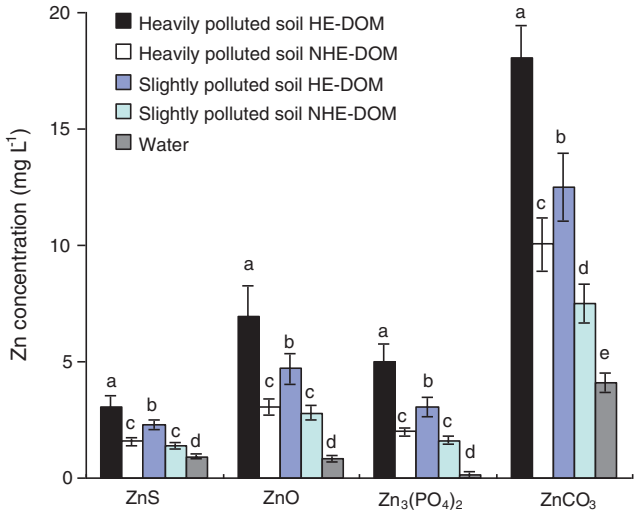

Fig. 4. The extracting ability of dissolved organic matter from the rhizosphere of hyperaccumulating ecotype (HE-DOM) and non-hyperaccumulating ecotype (NHE-DOM) of *S. alfredii* grown in heavily and slightly polluted soil. Bar represents standard deviations of three replicates. Bars sharing the same letter code among different DOM for a given mineral are not significantly different at $P < 0.05$.

ing) in two soils. For NHE *S. alfredii*, the DOC concentration in the rhizosphere of slightly polluted soil was increased as compared to bulk soil, but no difference was found in heavily polluted soil (Fig. 3b). The DOC concentration in the rhizosphere of HE *S. alfredii* was increased by 55.9% for heavily polluted soil and 48.4% for slightly polluted soil, respectively, as compared to the bulk soil. Greater Zn concentrations in the soil resulted in greater DOC for HE *S. alfredii*, indicating Zn induced plant release of root exudates. Fitz and Wenzel [33] proposed that hyperaccumulators may enhance metal solubility in the rhizosphere via root exudation, consequently increasing plant metal uptake. Our experiment seemed to support this hypothesis since HE *S. alfredii* exudated about 25–56% more DOC in the rhizosphere than NHE *S. alfredii* and we found high correlation between rhizosphere DOC and plant Zn accumulation in HE *S. alfredii* ($r = 0.891$). Despite the fact that DOC accounts for a small portion of total organic carbon, it significantly affects nutrient and contaminant mobility, microbial activity and soil properties [34]. The reaction of DOC in the rhizosphere of HE *S. alfredii* with Zn is highly expected and has the potential to influence Zn adsorption and mobility, thus further experiments were conducted to investigate the extractability of Zn from different Zn mineral by rhizosphere DOM.

3.4. The extracting ability of Zn by dissolved organic matter from the rhizosphere of S. alfredii

To date, although many workers have dealt with the adsorption characteristics of dissolved organic matter (DOM) on soil constituents and its effect on metal adsorption and solubility in the soil solution [35,36], there is a lack of information concerning the influence of DOM from the rhizosphere of hyperaccumulating species on heavy metal mobility [15,16]. In the present study, the effect of DOM from the rhizosphere of hyperaccumulator on Zn mobility was investigated by extracting experiment and the results are shown in Fig. 4. The DOM from the rhizosphere of HE *S. alfredii* showed much greater extracting ability of Zn than DOM from the rhizosphere of NHE *S. alfredii* ($P < 0.05$). For example, the Zn concentrations extracted with DOM from the rhizosphere of HE *S. alfredii* grown in heavily polluted soil were 3.06, 6.97, 4.98, 18.04 mg L^{-1} for Zn-sulphide, Zn-oxide, Zn-phosphate and Zn-carbonate, respectively, which were 1.7–2.5 times of the NHE-DOM (Fig. 3). DOM consists of several types of low molecular weight organic compounds, such as polyphenols, simple aliphatic acids, amino acids and sugar acids, which can strongly bind to heavy metals such as Cu, Pb, Cd, Zn, and Ni and thus increase their solubility [37]. Different Zn minerals have different solubility in water (30 °C), the addition of DOM from the rhizosphere of *S. alfredii* significantly increased the solubility of four Zn minerals as compared to water, which may be due to the formation of soluble metal–organic complexes [35]. It is important to note that the Zn concentrations extracted with DOM from the rhizosphere of HE *S. alfredii* grown in slightly polluted soil were much greater than those of NHE-DOM from heavily polluted soil ($P < 0.05$), although this two kinds of DOM has almost the same DOC concentrations.

The main sources of DOM in soils are plant litter, soil humus, and root exudates [38]. It has been reported that As hyperaccumulator *P. vittata* releases various low molecular weight organic acids via root exudation [17]. The presence of these acids may significantly influence metal mobilization in the rhizosphere. In our study, the DOM from the rhizosphere of HE *S. alfredii* showed greater extracting ability of Zn than those of NHE *S. alfredii* even when the DOC concentrations are almost the same. These results reveal that DOM composition is the probable factor affecting the solubility of Zn. Thus further study should be conducted to elucidate the characterisation of the component organic compounds (e.g., organic acids, amino acids and sugar acids) in the DOM from rhizosphere of *S. alfredii*.

4. Conclusions

The present study confirmed that the hyperaccumulating ecotype of *S. alfredii* was more efficient in Zn accumulation in the shoot than non-hyperaccumulating ecotype, after growth in heavily and slightly polluted soils. The water soluble Zn and mobile Zn fraction in both rhizosphere and bulk soils decreased considerably after growth of HE *S. alfredii* compare to NHE *S. alfredii*, however the decreases in mobile fraction accounted for less than 8.5% of the total Zn uptake by HE *S. alfredii*, this result indicated that HE *S. alfredii* was effective in mobilizing Zn from the non-mobile fractions. It is suggested that rhizosphere acidification and the exudation of high amounts of DOM with great metal extracting ability might be two important mechanism involved in the metal activation in rhizosphere of hyperaccumulating ecotype of *S. alfredii*.

Acknowledgments

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