Heavy metal accumulation characteristics of Nepalese alder (*Alnus nepalensis*) growing in a lead-zinc spoil heap, Yunnan, south-western China

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A fast-growing alder species native to the eastern Himalayas, Nepalese alder (*Alnus nepalensis*), has recently received considerable attention in the restoration of contaminated lands due to its significant economic benefits and ecological functions. The bioaccumulation characteristics and phytoremediation potential of naturally regenerated Nepalese alder were evaluated in a lead-zinc spoil heap located in Lancang county, Yunnan province, south-western China. Results showed that bioaccumulation factors (BFs) of *A. nepalensis* for Zn and Pb were always >1 in slightly contaminated soils (extractable Zn, Pb of 4.2-17.9 and 3.4-13.1 mg kg^{-1}, respectively) and >1 for Cd in contaminated soils (extractable Cd 0.3- 6.8 mg kg^{-1}). By contrast, translocation factors (TFs) for Zn were <1 in all sampling plots, but >1 for Pb in soil slightly contaminated by 13.1 mg kg^{-1} extractable Pb and >1 for Cd in contaminated soils (extractable Cd 2.6-6.8 mg kg^{-1}). Significant positive correlations were found between heavy metals (HMs) in roots and extractable HMs in soils (p<0.01) and between HMs in shoots and extractable HMs in soils (p<0.05) except for Cd. Based on the accumulation capacity revealed in this study, we suggest that *A.nepalensis* is a promising tree species for phytostabilization of zinc and lead in soils slightly contaminated with Zn and Pb and for phytoextraction of cadmium in Cd-polluted soil.

Keywords: Phytoremediation, Nepalese Alder, *Alnus nepalensis*, Metal Contamination, Bioaccumulation Factor, Translocation Factor

Introduction

Contamination of soil with zinc, lead and cadmium due to residues from metalliferous mining, smelting and other anthropic activities has attracted considerable attention in the past decade (Zhao et al. 2003). Because of the high toxicity of heavy metals (HMs) and their undegradable nature, metal pollution of soils has become one of the most serious environmental problems (Lee et al. 2009). In addition, heavy metals can be transferred to the food chain from the soil and seriously affect human health (An et al. 2011). First investigated by Chaney in 1983 (An et al. 2011), phytoremediation is a promising, ecologically-friendly approach to the remediation of contaminated soil using plants to remove toxic elements from the environment or render them non-toxic (Salt et al. 1998, Suresh & Ravishankar 2004). However, most plants are sensitive to excessive HMs in soil. In nature, only a small fraction of plants that exhibit metal tolerance or super-accumulative capacity (hyperaccumulators) can survive in toxic metal-contaminated soils, and the majority are herbaceous species with small biomasses (Sarma 2011). Woody plants or trees with developed root systems and large biomasses are especially attractive for revegetation and phytoremediation in metal-polluted sites (Lee et al. 2009, Capuana 2013). Additionally, exploring native plants with phytoremediation potential is a particularly important strategy, as indigenous plants are often more dependant in terms of survival, growth and reproduction under environmental stress than exotic plants (Yoon et al. 2006).

Native to Pakistan, eastern Nepal, Bhutan, northern India, south-western China, upper Myanmar and parts of Indochina, *A.nepalensis* is a multipurpose alder species. Characterized by a series of merits such as fast growth and nitrogen fixation, its uses include commercial timber production of moderately soft wood and charcoal feedstock. Moreover, *A. nepalensis* is a chief associate in various commercial timber plantations, where it not only helps to raise the nitrogen levels of fields but also acts as a pioneer species on denuded habitats with freshly exposed soils, or rocky and eroded slopes in subtropical-to-temperate belts of the Himalaya (Sharma & Ambasht 1984). Several other *Alnus* species have been surveyed to evaluate their phytoremediation potential for metal contaminated soils. For example, Rosselli et al. (2003) found that *Alnus incana* could be considered as an excluder of Zn, Cd and Cu. Mertens et al. (2004) found that *Alnus glutinosa* showed low foliar heavy metal concentrations and might therefore be suitable for phytostabilization purposes on polluted sites. Lee et al. (2009) also reported that *Alnus hirsuta* was a better candidate than *Alnus firma* for phytoremediation of HM-contaminated soils. However, there is no information about the potential of *Alnus nepalensis* to remediate Zn-, Pb- and Cd-contaminated soils.

In the present study, the metal accumulation capacity of *A. nepalensis* growing in a lead and zinc mining spoil heap in Lancang county of Yunnan province (south-western China) was evaluated. The potential application of *Alnus nepalensis* for phytoremediation of Zn-, Pb- and Cd-contaminated soils is also discussed.

Materials and methods

Site description

The sample site is approximately 3 x 5 km and is located in the tailing of Munai mine in Lancang county, Yunnan province, south-western China, where zinc and lead mining has been carried out using traditional Chinese methods for approximately 600 years (Chen et al. 1997). An *A. nepalensis* population has developed and is dominant in the lead-zinc spoil heap. Several shrub, grass and forb species, such as *Bidens pilosa, Cynodon dactylon, Ageratina adenophora, Arthraxon hispidus, Pseudocyclosorus esquirolii* and *Sphenomeris chinensis,* are growing in addition to *A.nepalensis*. Five sampling

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Tab. 1 - Heavy metal contents in the soil (mg kg^{-1}, mean ± SE, n=6) from the tailing of Munai mine in Lancang county of Yunnan province, south-western China. P1 to P5 represent five sampling plots with different distances (200 m, 500 m, 1700 m, 2800 m and 3800 m) to the lead-zinc smelting facility. Different letters in the same column indicate significant differences at p<0.05 level, according to the LSD test.

Plot	Zn		Pb		Cd	
	Total	Extractable	Total	Extractable	Total	Extractable
P1	6285.3 ± 1172.8 [a]	1417.1 ± 479.3 [a]	36845.5 ± 10895.1 [a]	12148.0 ± 1707.0 [a]	81.8 ± 43.0 [a]	32.4 ± 19.0 [a]
P2	4147.7 ± 1108.3 [b]	900.7 ± 105.6 [b]	32323.5 ± 17071.1 [a]	5544.4 ± 1032.5 [b]	35.9 ± 17.4 [b]	13.8 ± 4.3 [b]
P3	6351.4 ± 1565.7 [a]	392.9 ± 80.9 [c]	15189.7 ± 20029.1 [b]	2699.9 ± 838.1 [c]	25.7 ± 14.9 [bc]	6.8 ± 1.3 [bc]
P4	415.8 ± 206.4 [c]	17.9 ± 2.6 [d]	186.1 ± 13.9 [c]	13.1 ± 1.5 [d]	12.0 ± 6.3 [c]	2.6 ± 1.2 [c]
P5	212.2 ± 56.1 [d]	4.2 ± 2.8 [d]	40.5 ± 36.3 [c]	3.4 ± 2.0 [d]	5.9 ± 3.1 [d]	0.3 ± 0.2 [d]

plots (P1-P5) were selected within the study area. The distances from sampled plots to the lead-zinc smelting facility were 200 m, 500 m, 1700 m, 2800 m and 3800 m, and these distances constituted the gradient of HM concentrations in soils involved in our study.

Sample collection and analysis

At each of the sampling plots, 6 to 10 naturally regenerated *A. nepalensis* seedlings (approximately 2-3 years old) were randomly chosen. Their roots, shoots (stems + leaves) and rhizosphere soils (depth 10-25 cm, 500 g) were collected in May 2010.

From each sampling plot, 6 plant samples were selected for analysis. The shoots (stems + leaves) and roots of the whole *A. nepalensis* seedlings were washed thoroughly under tap water (roots were scrubbed with a brush carefully) and then placed in an 80 °C oven to be dried to constant weight. All dried root and shoot samples were ground separately by a mini-vegetation grinder (FZ102, Tianjing City Test Instrument Co. Ltd., China), and 0.5 g shoot and 0.5 g root powder of each sample were then subsampled by the usual method of coning and quartering. These representative subsamples were digested by HNO_3 + $HClO_4$, and the HM ion concentrations were determined by flame atomic absorption spectrometry (FAAS) using a Z2000 polarized Zeeman atomic absorption spectrophotometer (Hitachi, Japan). Quantification was carried out with a calibration curve using a graded series of diluted lead (GSW08619: 0, 0.1, 0.2, 0.4, 0.8, 1.2 µg ml^{-1}), zinc (GSW08620: 0, 0.1, 0.2, 0.4, 0.8, 1.2 µg ml^{-1}) and cadmium (GSW08612: 0, 0.1, 0.2, 0.4, 0.8, 1.2 µg ml^{-1} - National Research Center for Certified Reference Materials, China). A standard reference material of poplar leaves (GBW07604 - National Research Center for Certified Reference Materials, China) was carried through the digestion and analyzed as part of the quality control protocol (accuracies within 100 ± 20 %). A hollow cathode lamp was operated at 4 mA for Cd and 5 mA for Pb and Zn, and the analytical wavelengths were set at 217.0, 213.9 and 228.8 nm for detection of Pb (II), Zn (II) and Cd (II), respectively (AOAC 1984).

Rhizosphere soil samples corresponding to the above plants analyzed were also chosen for measuring the concentrations of Zn, Pb and Cd (6 samples for each plot, a total of 30 samples were analyzed). Soil samples were air-dried, ground to fine powder in a mortar, passed through a 2 mm sieve, and divided into two portions by quartering. One portion was directly used for the determination of extractable soil Zn, Pb and Cd, and 5.0 g soil was weighed and put into a 100 ml Erlenmeyer flask. Then, 50 ml of 0.1 M HCl was added, and the solution was shaken for 2 h at 20 °C and filtered into a volumetric flask. Concentrations of Zn, Pb and Cd were determined by FAAS as described above. The other portion was further put through a 0.15 mm sieve for the analysis of soil total HMs. For each soil sample, 0.2 g soil was digested in 5 ml of *aqua regia* (ultrapure mixture of concentrated HNO_3/HCl, 1/3 v/v) in a teflon crucible and diluted with 50 ml distilled water, and then their total HM content was analyzed by FAAS (SEPA 1997).

Translocation factors (TF = C_{shoot}/C_{root}, *i.e.*, the ratio of HM concentration in shoot to root) were calculated to quantify the translocation of HMs from root to shoot, and bioaccumulation factors (BF = C_{shoot}/C_{soil}, *i.e.*, the ratio of HM concentration in shoot to the extractable HMs in the soil) were calculated to quantify the HM accumulation ability of *A. nepalensis* from soil to shoot (Lorestani et al. 2011).

Statistical Analysis

Departure from the normal distribution was tested for all variables by Shapiro-Wilk's test, and Levene's test was used to verify the equality of variances. HM ion concentration data were subjected to one-way ANOVA to test for differences among the five sampling plots. The threshold for statistical significance was set at α=0.05. The relationships among extractable soil metals and total soil metals, plant metal concentrations, BF and TF were determined by Pearson's correlation analysis. Statistical analysis was carried out using the SPSS software package (version 17.0).

Results

HM content in soil

Concentrations of HMs in the soils investigated are summarized in Tab. 1. Three plots, P1, P2 and P3, were seriously polluted by Zn, Pb and Cd, and 2 plots, P4 and P5, were slightly contaminated, except for Cd. Extractable HMs in the proportion of the total soil HMs ranged from 2.0 to 22.5 % for Zn, 8.5 to 17.2 % for Pb and 4.8 to 39.3 % for Cd. In addition, a significant, positive correlation between soil total and extractable HMs was observed (Tab. 2).

Accumulation and distribution of HMs in A. nepalensis

The distribution of the metals within *A. nepalensis* is presented in Fig. 1. In general, HMs were accumulated mainly in roots, and concentrations of HMs in the roots decreased as extractable HMs in the soil decreased (Fig. 1 and Tab. 1). The highest concentration of HMs in roots occurred in sampling plot P1, where the concentrations of Zn, Pb and Cd were 573.9, 3550.1 and 94.7 mg kg^{-1}, respectively. The accumulation and distribution of Zn and Pb in shoots were similar to those in roots. The highest concentrations of Zn (262.8 mg kg^{-1}) and Pb (219.2 mg kg^{-1}) also occurred in severely polluted plot P1 and decreased as soil extractable HMs decreased. However, the accumulation of Cd in the shoots differed from that of Zn and Pb. The highest accumulation of Cd occurred in P3, which had a moderate extractable Cd concentration in soil compared to the other

Tab. 2 - Pearson's correlation coefficients between extractable soil metals and total soil metals, plant metal concentrations, bioaccumulation factors (BFs) and translocation factors (TFs). (*): p<0.05; (**): p<0.01.

Metal	Total metal	Shoot	Root	BF	TF
Extractable Zn	0.764*	0.940*	0.940**	-0.58	-0.577
Extractable Pb	0.920**	0.850*	0.990**	-0.723*	-0.531
Extractable Cd	0.997**	-0.364	0.983**	-0.772*	-0.685*

plots, and the accumulation of this metal was lower in plots with higher (P1 and P2) and lower (P4 and P5) soil extractable Cd (Fig. 1). Furthermore, significantly positive correlation between plant metal accumulation and soil extractable HM was observed, except for Cd in the shoot (Tab. 2). The BFs and TFs of *A. nepalensis* for Zn, Pb and Cd are given in Tab. 3. The *A. nepalensis* BFs for Zn and Pb were always >1 in P4 and P5 and >1 for Cd in P3, P4 and P5, which suggested that *A. nepalensis* acts as an accumulator of Zn and Pb in soils with lower levels of extractable HMs but not in severely polluted soils, and as a Cd accumulator in even moderately Cd-polluted soils. Moreover, there is a significantly negative correlation between BF and soil extractable HMs. The TF of *A. nepalensis* for Zn was <1 in all sample plots, but >1 for Pb in P4 and for Cd in P3 and P4. A negative correlation between TF and soil extractable HMs was also observed (Tab. 2).

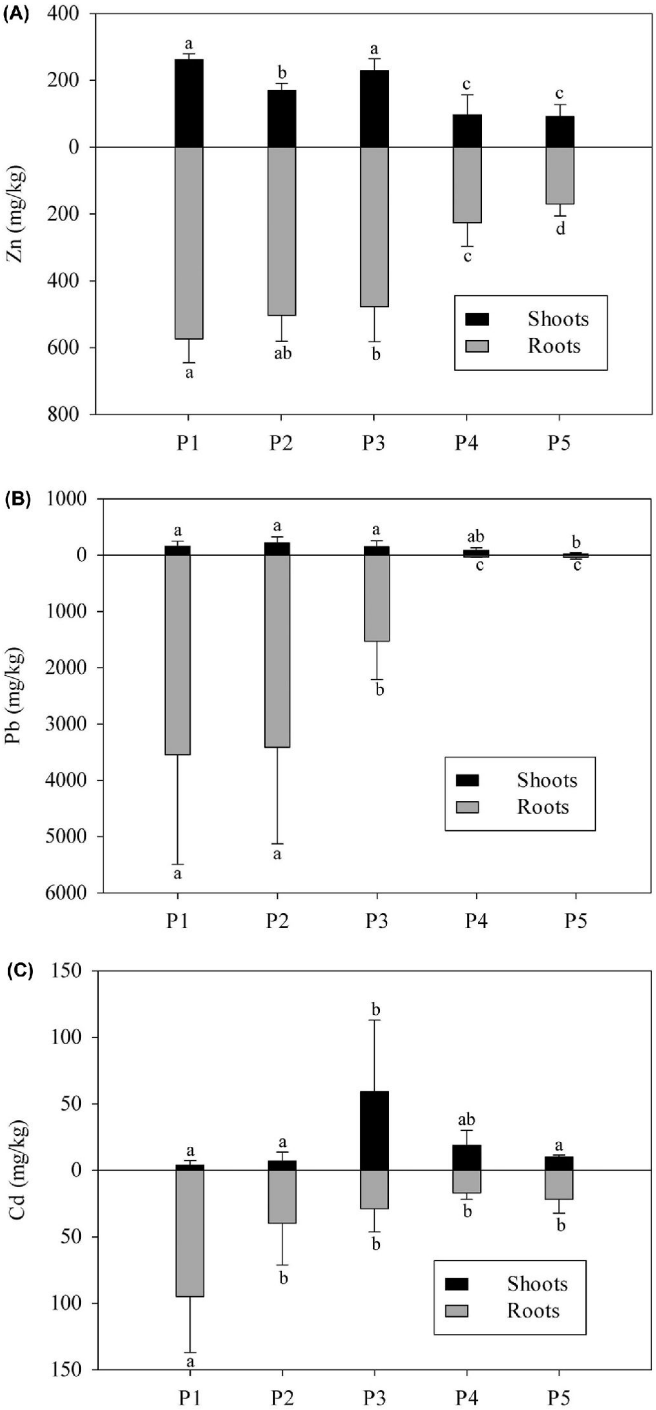

Fig. 1 - Zn (A), Pb (B) and Cd (C) accumulation in shoots and roots of *A.nepalensis* from the tailing of Munai mine in Lancang county, Yunnan province, south-western China. P1 to P5 are five sampling plots with different distances (200 m, 500 m, 1700 m, 2800 m and 3800 m) to the lead-zinc smelting facility. Vertical bars represent the mean ± SE (n = 6). Different letters above or below bars indicate significant differences among the five sampling plots analyzed at $p<0.05$ level, according to the LSD test.

Discussion

A considerable variation in soil and plant metal concentrations both within and between the sampling plots was observed, reflecting the heterogeneous nature of the polluted area and high individual variability within plants (Vogel et al. 2005, Unterbrunner et al. 2007). Our results indicated that metals were mainly accumulated in the roots of *A. nepalensis*, particularly in those plots with high metal concentrations (Fig. 1), which suggested strong metal retention during HM transportation from roots to shoots that might be interpreted as a mechanism that assists the plant with metal stress tolerance. Lee et al. (2009) reported that *Alnus hirsuta* and *Alnus firma* absorbed Zn, Pb, Cd, Cu and Cr, accumulating the metals mainly in roots, and Rosselli et al. (2003) found that *Alnus incana* showed a promising ability of excluding Zn, Cd and Cu from its above-ground tissues. Similar results were also reported in other woody genera, such as *Salix* for Pb, Zn, Cd and Cu (Landberg & Greger 1996, Regvar et al. 2010), *Fraxinus* for Zn, Cd and Cu (Rosselli et al. 2003), and *Eucalyptus* for Pb, Zn and Cu (Yang et al. 2010).

Tab. 3 - Bioaccumulation factors (BFs) and translocation factors (TFs) of Zn, Pb and Cd in *A.nepalensis* from the tailing of Munai mine in Lancang county, Yunnan province, south-western China (mean ± SE, n = 6). P1 to P5 represent five sampling plots with different distances (200 m, 500 m, 1700 m, 2800 m and 3800 m) to the lead-zinc smelting facility. Different letters in the same column indicate significant differences at $p<0.05$ level, according to the LSD test.

Plot	BF			TF		
	Zn	Pb	Cd	Zn	Pb	Cd
P1	0.20 ± 0.05^{a}	0.01 ± 0.01^{a}	0.07 ± 0.07^{a}	0.44 ± 0.07^{a}	0.05 ± 0.02^{a}	0.02 ± 0.02^{a}
P2	0.19 ± 0.06^{a}	0.04 ± 0.02^{a}	0.58 ± 0.55^{a}	0.37 ± 0.12^{a}	0.08 ± 0.04^{a}	0.45 ± 0.43^{ab}
P3	0.62 ± 0.20^{a}	0.05 ± 0.03^{a}	8.63 ± 9.00^{b}	0.46 ± 0.07^{a}	0.11 ± 0.11^{a}	1.51 ± 0.77^{b}
P4	3.53 ± 0.87^{a}	6.50 ± 3.32^{b}	8.39 ± 4.88^{b}	0.47 ± 0.20^{a}	2.33 ± 1.66^{b}	1.27 ± 1.05^{b}
P5	25.87 ± 17.96^{b}	8.85 ± 7.58^{b}	20.56 ± 5.82^{c}	0.90 ± 0.29^{b}	0.35 ± 0.19^{c}	0.54 ± 0.15^{ab}

Accumulation and exclusion are two basic strategies by which plants respond to elevated concentrations of HMs (Baker 1981). A TF (C_{shoot}/C_{root}) greater than 1 is common in metal accumulator species, whereas the TF is lower than 1 in metal excluder species (Baker 1981, Vogel et al. 2005). The TFs (C_{shoot}/C_{root}) and BFs (C_{shoot}/C_{soil}) of *A. nepalensis* for Zn and Pb were lower than 1 in soils severely polluted with Zn and Pb (P1, P2 and P3), indicating that exclusion might be its tolerance strategy. Stoltz & Greger (2002) showed that most of the plant species growing on mine tailings exhibited restricted translocation of metals to the shoot. Rosselli et al. (2003) also reported that *Alnus incana* could be considered a heavy metal excluder and may have a mechanism for avoiding metal uptake by stabilizing HMs in the rhizosphere or keeping them in their roots. Accumulation and translocation of Cd in *A. nepalensis* were somewhat complex; the TF and BF were greater than 1 in P3 and P4, which had moderate Cd concentrations compared to the other sampling plots, but were lower than 1 under more intensively Cd stressed soils (P1 and P2), which indicated that *A. nepalensis* may use different tolerance strategies under different levels of Cd stress. The relatively high BF and TF of *A. nepalensis* for Cd make it a suitable woody candidate for phytoextraction in Cd-contaminated soils. In addition, *A. nepalensis* accumulated relatively high concentrations of Zn, Pb and Cd in its roots (Fig. 1), which suggested that it might be a good candidate for phytostabilization of these metals.

Phytoremediation, mainly *via* the mechanisms of phytoextraction and phytostabilization, is a promising and practical technology to remove HMs from polluted environments (Grabisu et al. 2002). HM hyperaccumulators, which have the ability to absorb and translocate HMs from soil to shoot, have been considered the most promising candidates in phytoextraction. Unfortunately, the majority of plants in nature are sensitive to excessive HMs in the soils. Moreover, the slow growth and small biomass of many HM hyperaccumulators have been reported to limit their application in remediating HM-polluted soils (Gaur & Adholeya 2004). In phytostabilization, HM ions are absorbed and confined to roots and to rhizospheric soil, thus inhibiting the mobility of metal ions, restricting their leaching into groundwater and reducing metal bioavailability for entry into the food chain (Susarla et al. 2002, Conesa et al. 2006, Yoon et al. 2006). Woody plants, especially fast-growing species with large biomasses, have shown advantages and potential for application in phytostabilization (Pulford & Watson 2003, Yoon et al. 2006). In the present study, *A. nepalensis* showed a relatively high capacity to accumulate Zn, Pb and Cd in roots when growing in 5 plots with intensively polluted soils and significant Cd accumulation in shoots when growing in moderately contaminated soils, which suggested its potential for application in the phytoremediation of mining areas.

Conclusions

Adding to a series of desirable properties of *A. nepalensis*, the promising capacity of this alder species to tolerate and accumulate heavy metals was revealed for the first time in this study. *A. nepalensis* can accumulate relatively high concentrations of Zn, Pb and Cd in its roots. The BFs for Zn and Pb that were greater than 1 indicated this plant's phytostabilization strategy and capacity in slightly contaminated soils. Remarkably, its ability to phytostabilize and phytoextract Cd was verified by BFs and TFs both greater than 1, even in moderately Cd-contaminated soils. We therefore suggest that *A. nepalensis* is a potential candidate for use in the phytoremediation of Zn-, Pb- and Cd-polluted soils in its indigenous range.

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