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Cu Accumulation in the Rhizosphere of *Lindenbergia philippensis* (Cham.) Benth. Growing in the Contaminated Sediment

[1,2]Thanchanok Kangwankraiphaisan and [2,3]Kallaya Suntornvongsagul
[1]International Postgraduate Programs in Environmental Management, Graduate School, Chulalongkorn University, Bangkok 10330, Thailand
[2]Center of Excellence for Environmental and Hazardous Waste Management, Chulalongkorn University, Bangkok 10330, Thailand
[3]Environmental Research Institute, Chulalongkorn University, Bangkok 10330, Thailand

Abstract: The association of *Lindenbergia philippensis* (Cham.) Benth. and Arbuscular Mycorrhizal Fungi (AMF) enhanced plant tolerance to high concentration of Cu by reducing the accumulation of Cu in plant root. The aim of the study was to study Cu accumulation in the zinc smelting sediment that occurred in the rhizosphere sediment and root associated with AMF. The plant was grown in the sediment containing low level of nutrients (0.86±0.03% of Total Organic Carbon (TOC), 1.15±0.40 mg kg^{-1} dried sediment of nitrate (NO_3-N) and 5.67±5.51 mg kg^{-1} dried sediment of phosphorus (P)) and high concentration of heavy metals under weak basic condition (pH 7.79-8.28). High level of Cu accumulation was detected in the rhizosphere suggesting that the biological function of the plant and AMF may limit the excess Cu from reaching into plant cells. Solid particle sheath containing Cu showed rectangular and amorphous particles was observed around rhizosphere zone by SEM-EDS. Relatively lower Cu concentration was detected in plant root with active AMF (T1) comparing to plant root with suppressed AMF (T2). Interestingly, higher Cu concentration was observed in rhizosphere of plant with active AMF whereas the Cu was lower in rhizosphere of the sample without active AMF. These results indicated that Cu in the rhizosphere is influenced by the function of AMF. The results of this study provide useful insight on Cu accumulation with the presence of association between AMF and dominant plant and may be considered as a valuable tool for phytostabilization.

Key words: Accumulation, heavy metal, rhizosphere, arbuscular mycorrhizal fungi, tolerant

INTRODUCTION

Heavy metals resulting from mining, smelting, agriculture and other heavy industries are important contaminants to the environment and living organisms. Copper (Cu) one of the heavy metals involves with several plant metabolic processes since it is an indispensible component of many oxidative enzymes involving photosynthesis and other cellular structures such as cell wall (Maksymiec, 1998). Although, Cu is one of the essential trace elements required for the survival of plants, excessive amount of Cu can cause damages to cells (Lou *et al.*, 2004; Wei *et al.*, 2008) and affect the growth and development of plant (Madejon *et al.*, 2009).

However, the adverse effects of Cu are varied depending on the type of plants as well as other physicochemical factors including Cu speciation, bioavailability of Cu and soil pH (Tao *et al.*, 2003; Chaignon *et al.*, 2009). Plant tolerance toward elevated level of Cu was reported in some studies (Lou *et al.*, 2004; Santibanez *et al.*, 2008; Wei *et al.*, 2008). The differences in plant physiological characteristics including metabolic processes, gene expression and excretion of certain substances such as exudates may contribute to the various plant responses to Cu (Hall, 2002; Jonak *et al.*, 2004). In addition to plant physiology, the mutualistic relationship between plant and the microorganisms in root-soil interface or rhizosphere was purposed as an important factor for the observed resistance to high concentration of Cu.

Arbuscular Mycorrhizal Fungi (AMF) are normally found in rhizosphere and are crucial for plant survival under extreme environments. The AMF and other rhizosphere microorganisms receive the necessary soluble carbon based nutrients from plant and in return, they facilitate other mineral nutrient uptakes from the surrounding environment of the plants (Dandan and Zhiwei, 2007). Moreover, the rhizospheric microorganisms

Corresponding Author: Kallaya Suntornvongsagul, Environmental Research Institute, Chulalongkorn University, Bangkok, 10330, Thailand Tel: (+66) 2-218-8218 Fax: (+66)-2-218-8210

also enhance plant resistance toward pathogenic microorganisms and plant survivability under stressful conditions. The importance of AMF on the plant susceptibility to copper was revealed in many studies (Chen *et al.*, 2007; Zhang *et al.*, 2009). Evidences showed that microorganism community in the rhizosphere contributes to the changes in soil pH and speciation of Cu and therefore, creates fractionation of copper and reduce the amount of accumulated Cu in plant cells (Cloutier-Hurteau *et al.*, 2008; Zhang *et al.*, 2009). Lower level of Cu was observed in plant shoot with the presence of AMF comparing to plant without AMF indicating an important protective role of AMP under high Cu concentration (Chen *et al.*, 2007). In this study, the accumulation of Cu from the zinc smelter sediment in *Lindenbergia philippensis* (Cham.) Benth. with and without the active AMF was investigated.

MATERIALS AND METHODS

Study area: The sampling site was located in the settling pond at the zinc smelter, Tak province (Northern part of Thailand). The settling pond received sediment containing heavy metals from smelting processes. Subsequently, the leachate and the solid fractions of the sediment were separated and sent to wastewater treatment facility and secure landfill, respectively. Five plots in the settling pond (1×1 m^2 each) covered with *L. philippensis* (Cham.) Benth. was collected sediment, rhizosphere sediment and plant roots. The samples were preserved under 4°C until analysis of the characteristics of sediment, root colonization by AMF, solid sheath on the surface root and the Cu concentration in the root and the rhizosphere sediment.

Experimental setup: Sediment was collected from the settling pond. Approximately 4 kg (wet weight) of sediment was homogenized and filled into plastic pots. The 45 days old seedling *of L. philippensis* (Cham.) Benth. which were grown on the peat moss (Pindstrup) was transferred into the pots containing the obtained sediment. The experimental setup consisted of two treatments with five replicates for each treatment. The two treatments were treatment with active AMF (T1) and treatment without active AMF by applying fungicide (captan: Orthocide, Arysta LifeScience) 100 mg kg^{-1} (wet weight sediment) every week for suppressing the active AMF (T2). Addition of this captan can reduce the root colonization by AMF about 50% in the preliminary study. (O'Neill and Mitchell, 2000) demonstrated that the root colonization by AMF could be reduced to similar level as without inoculated of AMF by applying captan. The sediment in the pots was added with tap water regularly to maintain the moisture at 60-70%. The pots were rearranged randomly once a week. After 6 months of experiment, the rhizosphere sediment and plant root were collected and analyzed for the amount of Cu.

Sample analysis: The sediment, rhizosphere sediment and plant roots were dried at 80°C until weight was constant. As for the preparation for Cu concentration analysis, the dried samples were grounded in a mortar and sieved (74 μm, 200 mesh) and subjected to measure properties of the sediment and heavy metal analysis. The fresh roots were separated to measure the root colonization by AMF and solid sheath by scanning electron microscope and dispersive X-ray spectrometer (SEM-EDS).

The properties of the sediment collected from the settling pond were determined. The pH of liquid solution was measured by using the HACH® SensION™ Waterproof pH/mV/ISE Meter (HACH Company, Loveland, CO, USA). The texture from the density of the suspension was determined by the hydrometer technique (Kettler *et al.*, 2001) and the Cation Exchange Capacity (CEC) was measured in according to the U.S. EPA method, 9080 by using ammonium acetate. Total N and NO_3-N were analyzed by adding 2 N of KCl into 10 g of sediment and titrated with 0.005 N H_2SO_4 (Ryan *et al.*, 2001). The P was measured by using the sodium bicarbonate (Olsen) method (pH>7) and the ammonium heptamolybdate-ammonium vanadate was applied for determining available P (Ryan *et al.*, 2001). TOC and total N were analyzed by using a total organic carbon analyzer (TOC-SSM 5000A, Shimadzu-Module) following U.S. EPA method 9060. Total microorganisms including bacteria, actinomycetes and fungi in the sediment were determined by standard spread plate technique with serial dilutions (Maier *et al.*, 2000; Pepper and Gerba, 2005). Total Cu of the samples was determined by U.S. EPA method 3050B using 65%HNO_3 and 30%H_2O_2 and Atomic Absorption Spectrometry (AAS).

The root colonization of AMF was determined by the gridline intersection method following Brundrett *et al.* (1996). Approximately 10 g of fresh roots were suspended in 0.1% sodium pyrophosphate and sonicated to remove adherent rhizosphere sediment particles. The freshly cleaned roots were prepared and the root surface was cleared with 10% KOH and stained in trypan blue solution in lactoglycerol over night. The root colonization was observed by light microscope.

The selected small pieces of fresh roots with rhizosphere samples were completely dried in desiccators until without moisture. Then the dried samples were coated with carbon before subjected to scanning electron microscopy with an energy dispersive, X-ray spectrometer (SEM-EDS) JEOL, model JSM-5410LV and 5800LV (Japan) to study the solid particle sheath (Wullstein and Pratt, 1981; Kodama *et al.*, 1994).

Statistic analysis: All data are reported as means±standard deviation. The significant differences were accepted when the values of alpha level are less than 0.05. The statistical model of SPSS 16.0 (LEAD Technologies Inc., Chicago, IL) was used for all statistical tests.

RESULTS

Sampling site: The analysis of the obtained settling pond sediment revealed that the sediment was weak basic, (pH 7.79-8.28) with small amount of nutrients and microorganisms and contained high heavy metal concentrations (Table 1). *L. philippensis* (Cham.) Benth. was the dominant species and two species of AMF (Suntornvongsagul *et at.*, 2011) was found associated with its root (Fig. 1a, b).

Cu accumulation in the rhizosphere and root

Concentration of Cu in the rhizosphere and root of the settling pond: The concentration of Cu accumulated in the rhizosphere sediment and plant root at the settling pond was measured. The detectable Cu concentration was 403.70±158.24 mg kg^{-1} of dried sample and 104.05±37.37 mg kg^{-1} of dried sample for rhizosphere sediment and plant root tissue, respectively. Statistical analysis using paired t-tests of Cu concentrations suggested that the Cu concentration in rhizosphere sediment was significantly higher than the level observed in plant root ($p<0.05$).

Solid particle sheath in the rhizosphere: Solid particle sheath was found cover the root surface at the rhizosphere (Fig. 2). The dense solid particles occurred in the rhizosphere might result from root exudates which contain the complex polysaccharide forming a layer of mucilage, enzymes, ethylene, sugars, organic acids, amino acids, root cap (Chaboud and Rougier, 1990; Xu *et al.*, 2007) and dead cell lysates (Marschner, 2002).

With the presence of the exudates, Cu might be trapped into the substances and integrated in the solid

Table 1: Physical and chemical characteristics of the contaminated sediment

Characteristic	Values
pH	7.79-8.28
NO_3-N (mg kg^{-1} of dried sediment)	1.15±0.40
P (mg kg^{-1} of dried sediment)	2.50±0.70
TOC (%)	0.86±0.03
CEC (meq 100 g^{-1} of dried sediment)	4.00±0.44
Zn (mg kg^{-1} of dried sediment)	23.211±3.745
Pb (mg kg^{-1} of dried sediment)	11.744±2.943
Cd (mg kg^{-1} of dried sediment)	481.61±237.80
Cu (mg kg^{-1} of dried sediment)	734.50±69.91
Bacteria (CFU g^{-1} dried sediment)	$3.36\times10^4\pm6.00\times10^3$
Fungi (CFU g^{-1} dried sediment)	$6.80\times10^2\pm5.30\times10^1$
Actinomycetes (CFU g^{-1} dried sediment)	$10^3\pm7.77\times10^2$

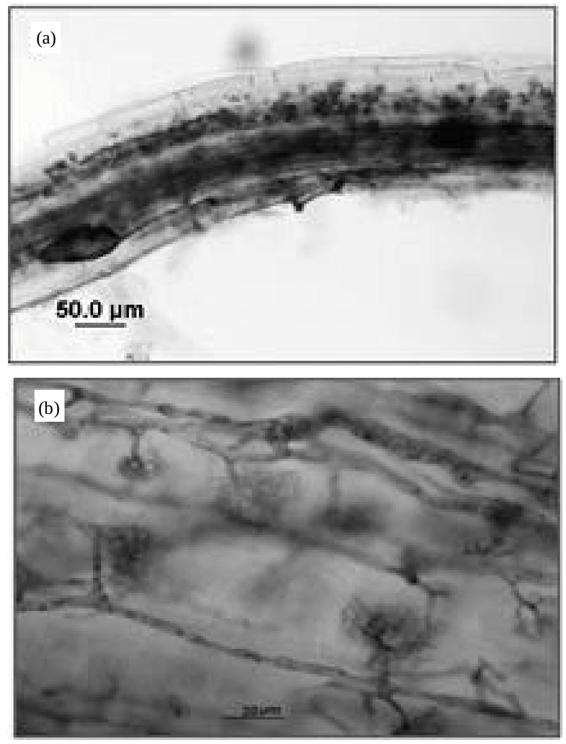

Fig. 1(a-b): Image of (a) Vesicle (20x) and (b) Arbuscue (40x) of AMF in the *L. philippensis* (Cham.) Benth. root observed by light microscope

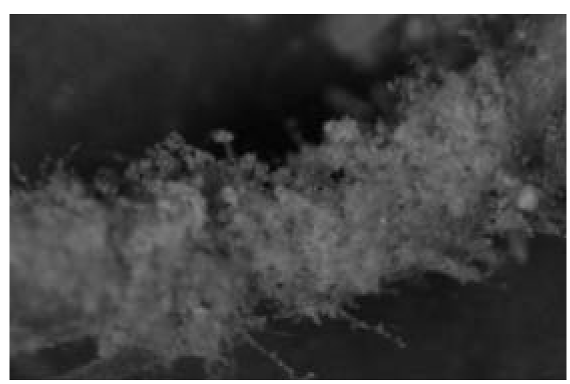

Fig. 2: Plant root of *L. philippensis* (Cham.) Benth. with rhizosphere sediment observed with light stereo microscope (20x)

particle sheath. In order to elucidate this, samples possibility were analyzed by SEM-EDS and the results indicated Cu contributed to 1.89% of element in the solid sheath (Fig. 3a, b).

Role of AMF on Cu accumulation: Higher concentration of Cu in plant root was detected in T2 in which the AMF were relatively inactive comparing to T1 (Fig. 4). In T1 and T2, the detectable Cu concentration was 177.69±49.11 and 381.39±140.70 mg kg^{-1} of dried sample, respectively. On the contrary, higher accumulation of Cu was found in the rhizosphere sediment of T1 (437.75±36.63 mg kg^{-1} of dried sample) comparing to T2 (309.74±33.85 mg kg^{-1} of dried sample). In summary, samples with active AMF accumulated less Cu in root tissue, whereas more Cu was detected in their rhizosphere sediment comparing to the samples without active AMF. These results suggested that the lower Cu concentration observed in T1 may due to the boundary effect created by the active AMF in the rhizosphere sediment.

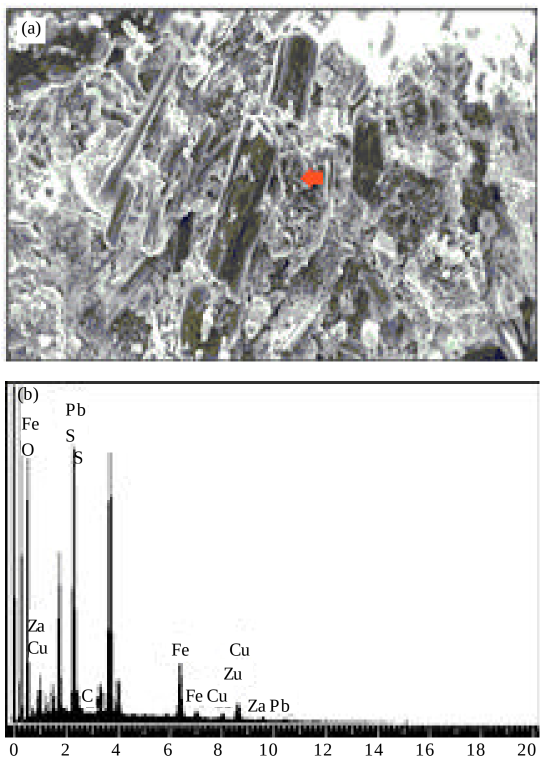

Fig. 3(a-b): (a) SEM photograph (magnified 500x) and (b) elemental analysis by EDS of the rhizosphere sediment

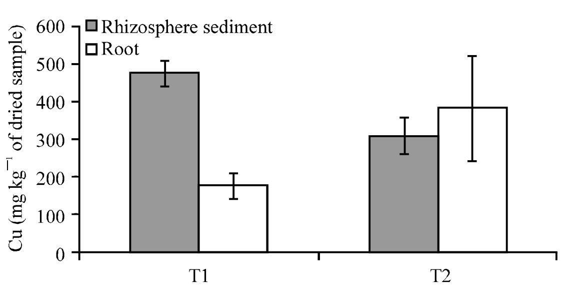

Fig. 4: Comparison of Cu concentrations (mean value) of the control (T1) and control with captan (T2) of rhizosphere sediment and plant root. Bar and error bars represent mean (n = 5) and standard deviation values

DISCUSSION

In this study area, *L. philippensis* (Cham.) Benth. was found to be the dominant species suggesting its ability to tolerate and grow under high Cu concentration. These results can be explained by Cu being accumulate in the rhizosphere zone which is the biological function may protect the excess Cu into the plant cell.

The solid particle sheath covering the plant root may contribute to plant tolerance toward Cu since part of the Cu in the soil environment was immobilized into the solid sheath as indicated by Cu content in the solid sheath. In addition to the effect of exudates, the association between plant and microorganisms may be a crucial factor as well (Sung *et al.*, 2006; Cardon and Whitbeck, 2007). Several study showed that certain microorganisms which obtain the nutrients from root exudates (Chaboud and Rougier, 1990; Xu *et al.*, 2007), may affect root metabolic processes increasing its resistance to extreme stresses (Bais *et al.*, 2006).

The role of AMF on plant resist to heavy metals was previously described in several studies. Andrade *et al.* (2009) and Wu *et al.* (2010) demonstrated that AMF could protect and increase plant tolerance against heavy metals. Nevertheless, Zhang *et al.* (2009) and Wei *et al.* (2008) observed that AMF may increase Cu accumulation in root cell wall in some plant species.

In this study, samples with active AMF revealed less Cu accumulation in root tissue together with elevated level of Cu rhizosphere sediment comparing to the samples without active AMF. This suggested that, in this case, active AMF increase plant tolerance to Cu by reducing the amount of accumulated Cu in root cell by increasing the binding of Cu to the rhizosphere sediment.

CONCLUSION

In this study, the association of *L. philippensis* (Cham.) Benth. and AMF revealed increase in plant tolerance to high concentration of Cu by reducing the accumulation of Cu in plant root. This suggested the protective role of AMF on this plant against Cu and may be considered as a valuable tool for the study of phytostabilization of heavy metals. In order to understand the mechanisms in which these AMF contribute to the plant protection, more insight on the interaction between microorganisms and the rhizosphere environment should be studied in future work.

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