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Enhanced Lead Phytoextraction of *Lantana camara* L. by a Biological Agent, Earthworm *Pontoscolex corethrurus*

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ABSTRACT

Phytoextraction is an emerging technology that uses plants to extract metals from contaminated soils. Improving the phytoextraction process is a primary goal of current research. The application of earthworm to soils has been proposed as a way of biologically improving phytoextraction. In this study, we conducted a greenhouse experiment to evaluate the effects of earthworms *Pontoscolex corethrurus* on *Lantana camara* growth and the phytoextraction process in Pb-contaminated soils at 500 and 1000 mg kg^{-1}. The results showed that earthworm activity significantly increased the shoot and root biomass in soils contaminated with lead (1.75 and 1.71 times higher for 500 mg Pb kg^{-1}, 1.63 and 1.27 times higher for 1000 mg Pb kg^{-1}, respectively). The lead phytoextraction by *L. camara* was also higher in the soils with earthworms, with the total lead uptake higher by factors of 2.96 and 2.03 at the two different contamination levels. In addition, positive correlations were found between lead absorption and biomass of *L. camara* in the presence of earthworm. The combination of earthworm *P. corethrurus* and plant *L. camara* suggested a considerable potential for the treatment of industrial sites polluted with lead.

Key words: Earthworms, *Pontoscolex corethrurus*, *Lantana camara*, heavy metal, phytoextraction

INTRODUCTION

Over the past few decades, the mining industry has produced a large amount of acid, metal-rich waste which has often created a serious risk to the soil environment (Sheoran and Sheoran, 2006). The nature and extent of this contamination is highly variable, depending on the nature of each mine's ore body and its associated geological strata and climate (Johnson and Halberg, 2005). It is, therefore, important to make an accurate assessment of the availability and toxicity of heavy metals in each polluted soil.

Phytoextraction is a technology that uses plants to extract metals from contaminated soils and accumulate them in harvestable parts which can then be removed from the site (Shah and Nongkynrih, 2007; McGrath, 1998). This appears to be a cost-effective, non-intrusive and environmentally friendly technique compared to conventional techniques and promises to be a phytotechnology with great potential for solving the problem of soils polluted with metals (Glass, 2000). Improving the phytoextraction process is a primary goal of current research. There are several avenues of study for increasing the efficiency of remediation. (i) Identify new hyperaccumulator species with the requisite properties such as high biomass, high metal uptake and ease of propagation. (ii) Increase the biomass of the hyperaccumulator plant by adding fertilizer or using plant growth hormones (e.g., IAA, gibberellins or cytokinin), associating them with Plant Growth Promoting Rhizobacteria (PGPR) (Ma *et al*., 2009; Monstant *el al*., 2008). (iii) Inoculate the soil with a bacterial community improving the availability of the metals (Braud *et al*., 2009) and/or with Arbuscular Mycorrhizal Fungi (AMF) (Pawlowska *et al*., 2000). (iv) Increase the metal content in the plant by the addition of chelating agents such as glycoletherdiamine tetra-acetic acid (EGTA) or NTA (nitrilotriacetic acid) (Kayser *et al*., 2000; Finzgar and Lestan, 2008; Monstant *et al*., 2008). Little is known about how the relationships between roots, microorganisms and soil fauna in the rhizosphere affect plant growth and metal uptake. Only a few studies have been carried out into microorganism-assisted metal extraction by plants. Braud *et al*. (2009) showed that inoculating the soil with free cells of *R. metallidurans* gave a five-fold increase in Cr accumulation in maize shoots and inoculating the soil with immobilized *P. aeruginosa* cells gave five-fold and three-fold increases in Cr and Pb uptake, respectively. There have been no studies on the effects of soil fauna on phytoremediation. Earthworms are important components of the rhizosphere ecosystem and can significantly increase plant production by improving soil fertility and nutrient cycling (Brown *et al*., 2004) although other interactions (e.g., between earthworms and root pathogens or beneficial soil microorganisms) may also affect plants (Scheu, 2003). Some earthworms (for example *Lumbricus terrestris, Lumbricus rubellus,* or *Aporrectodea caliginosa*) can survive in soils polluted with heavy metals and can even accumulate heavy metals such as Cd, Pb, Cu and Zn (Morgan and Morgan, 1999; Kizilkaya, 2004, 2005). Earthworms can also increase metal availability in soil by burrowing and casting and can, therefore, modify the efficiency of phytoremediation (Ma *et al*., 2002). The presence of earthworms can also increase Zn availability (Wang and Li, 2006), although the authors suggested that the main reason for the increase in Zn uptake by the plants was probably the increase in the production of dry matter stimulated by earthworms.

Lantana camara L., which is an new plant owing to its remarkable capacity to extract lead and cadmium from polluted soils in Vietnam (Huynh, 2009) has been suggested as a model species for research on phytoextraction of metals. Moreover, this plant has a rapidly growing and developing very fast. They can also grow in extreme conditions and are able to endure long periods of drought or heavy rains. Finally, its multicolored flowers allow integration into a floral arrangement of a landscaping project (Huynh, 2009). However, the species *Pontoscolex corethrurus* (Oligochaeta, Glossoscolecidae) which is an endogenous tropical earthworm, is very common in wetlands in Vietnam. They live in soils with low content of organic matter and have important effects on the soil structure through its burrowing and casting activity (Topoliantz and Ponge, 2005). Moreover, this species plays an important role on the rates of mineral N availability for plants, the assimilation of phosphorous and in the recycling of other nutrients and could, therefore, be involved in phytoextraction of metals (Pashanasi *et al*., 1996).

This study sets out to determine the impact of the *Pontoscolex corethrurus* earthworm on *Lantana camara* growth and the phytoextraction process in soil artificially contaminated with lead at different levels.

MATERIALS AND METHODS

Soil collection and analysis: Unpolluted soil was collected from the A1 horizon in the grounds of the Phu An Ecomuseum, Binh Duong Province, southeastern Vietnam (11°10'N and 106°40'E).

The climate is tropical and rainfall is confined to the period from December to April. The mean annual temperature is 26.5°C (Huynh, 2009). The soil was sieved to <5 mm, air-dried and mixed to obtain homogenous soil samples. Experimental pots (microcosms) were filled with 10 kg of dry soil. The soil was artificially contaminated with lead $(CH_3COO)_2.3H_2O$ at 500 mg Pb kg^{-1} and 1000 mg Pb kg^{-1} Dry Weight (DW). The soil's field capacity was determined using five additional microcosms by weighing the pots after draining the soil for 24 h. The experiment was conducted in the same site, Phu An Ecomuseum and carried out during the dry season of March to April 2008.

Experimental design: The seedlings (about 200) of *L. camara* were planted in plastic pots (V = 5 l) in a mixture of grey soil, manure and coconut fiber (1:1:1). After one month, seedlings of similar size (40 cm high and 20 cm diameter) were transferred to the microcosms and one seedling for each pot. Before transfer, roots were washed in ultra-pure water to eliminate plant growth substrate.

Earthworms, *P. corethrurus* were hand-collected from grassland in Bien Hoa, Dong Nai Province that had not been contaminated with heavy metals. The earthworms were kept in the Bien Hoa soil in plastic boxes for one week to monitor their health before starting the experiments. Ten adult earthworms (environ 10 g, corresponding to a density of 100 g m^{-2}) were put into each pot according to treatments. This abundance is higher than average abundance of 20 g m^{-2} observed in the field (Lavelle, 1978). However, it is little lower than the 127 g m^{-2} used for *Millsonia anamala* by Blouin *et al.* (2006).

The experiment was carried out using three processes combined with three levels of lead contamination in five replicates for each. The processes were soil+plant (P), soil+earthworm (E) and soil+plant+earthworm (PE). The levels of lead contamination were control without lead (nPb), soil contaminated with 500 mg Pb kg^{-1} (LPb) and soil contaminated with 1000 mg Pb kg^{-1} (HPb). The experiment was carried out for one month.

Harvesting and soil sampling: After one month, the shoots were removed by cutting above the soil surface. The earthworms were removed from the soil, weighted and kept in 90% alcohol for further analysis (Velasquez *et al.*, 2007). The soil from each pot was kept in laboratory conditions during about one week to separate the aggregates. Before the aggregates were separated, root systems were carefully removed by hand. Root aggregate soil was defined as the soil that remained attached to the roots after gentle shaking them by hand (Hinsinger, 1998). The root aggregates in the rhizosphere were sieved and air-dried.

Lead analysis: The shoots and roots were washed thoroughly in deionized water to remove soil particles. They were then oven-dried at 70°C for 48 h and their dry weights recorded. Subsamples of dried plant tissue were digested in a mixture of $HNO_3/HClO_4$ and the lead concentration in the sample was determined by AAS (atomic absorption spectrophotometry, V arian Spectra AA220).

Statistical analysis: Differences between the contamination levels and processes (nPb-P, nPb-PE, LPb-P, LPb-PE, HPb-P and HPb-PE) were analyzed by one-way ANOVA (STATGRAPHICS, Centurion XVI, Sigma Plus, France) followed by Fischer's PLSD-test to establish the significance of the differences among means at the $p<0.05$ level.

RESULTS AND DISCUSSION

The 100% survival rate observed for the plants growing in lead contaminated soil after one month, the absence of visible damage to the leaves and the significant increase in growth confirmed the high metal tolerance of *Lantana camara* (Verbenaceae). The average shoot and root dry biomass was significantly ($p<0.05$) higher for soil contaminated with lead than for the controls (Fig. 1). These results were confirmed those of Epelde *et al.* (2008) for the hyperaccumulator plant *Thlaspi caerulescens* which, when exposed to Cd and Zn showed higher biomass and values of photosynthetic pigments than those observed in controls. The lower lead concentration (500 mg kg^{-1}) gave a higher biomass (g DW pot^{-1}) than the higher concentration (1000 mg kg^{-1}). However, the ratio between Dry Weights (DW) of the stems and the dry weights of the leaves was also higher for lead contaminated soil, indicating that, while the biomass of the stems was significantly higher for lead contaminated soil, the biomass of the leaves did not depend on the contamination.

For plants cultivated with earthworms, the shoot and root biomass was significantly higher ($p<0.05$) than in the absence of earthworms (Fig. 1). Most of earthworms were still alive (>90%) and the soils had been completely burrowed by earthworms after one month of experiment. This indicated that *P. corethrurus* had a high tolerance to lead ions in these experimental conditions. This tolerance could be due, as suggested by Morgan and Morgan (1999) to the ability of earthworms to accumulate metal in their tissues and to detoxify using mechanisms such as binding and storing the lead in metallothionein.

In uncontaminated soils, the effect of earthworms on the plant growth was not significant. However, several mechanisms by which earthworms modify plant growth directly and indirectly have been studied. Blouin *et al.* (2006) determined five reasons which could be invoked to explain the positive effects of earthworms. At first, earthworm increased mineralization of organic matter in the soil, which increases nutrient availability (e.g., phosphorus availability). They modified the soil structure to change water and oxygen availability for plants and stimulated plant growth via stimulation of microbial activity. The other effect important of earthworms on the plant was

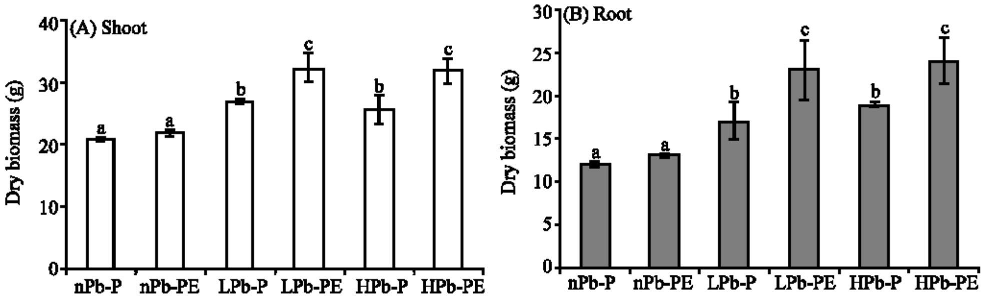

Fig. 1: (A) Shoot and (B) root biomass at the end of the phytoextraction experiment (g DW/plant/pot) of *L. camara* in control non-polluted and metal polluted. For each biomass, values followed with the different letters are significantly different ($p<0.05$) according to Fischer's PLSD-test (STATGRAPHICS, Centurion XVI, Sigma Plus, France). Values correspond to Means±Standard error; $n = 3$ per treatment, nPb-P: without Pb, Plant; nPb-PE: without Pb, plant+earthworm; LPb-P: with 500 mg Pb kg^{-1} of soil, plant; LPb-PE: with 500 mg Pb kg^{-1} of soil, plant+earthworm; HPb-P: with 1000 mg Pb kg^{-1} of soil, plant; HPb-PE: with 1000 mg Pb kg^{-1} of soil, plant+earthworm

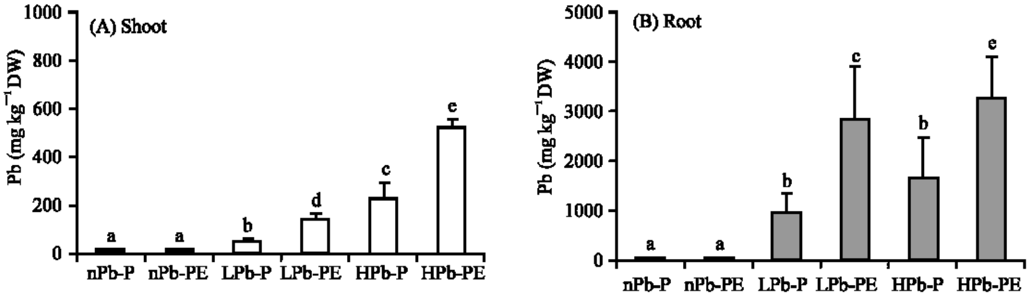

Fig. 2: Metal concentrations (mg kg^{-1} DW) in the shoot and root of *Lantana camara* in control non-polluted and metal polluted. For each metal concentration, values followed with the different letters are significantly different ($p<0.05$) according to Fischer's PLSD-test (STATGRAPHICS, Centurion XVI, Sigma Plus, France). Values correspond to Means±Standard error; n = 3 per treatment, nPb-P: without Pb, Plant; nPb-PE: without Pb, plant+earthworm; LPb-P: with 500 mg Pb kg^{-1} of soil, plant; LPb-PE: with 500 mg Pb kg^{-1} of soil, plant+earthworm; HPb-P: with 1000 mg Pb kg^{-1} of soil, plant; HPb-PE: with 1000 mg Pb kg^{-1} of soil, plant+earthworm

dispersal of biocontrol agents and reduction of fungal and bacterial root diseases. Lastly, it included the stimulation of beneficial symbionts. The lack of effect on the growth of *L. camara* in the presence of *P. corethrurus* could be explained by the absence of pathogens in this experiment or the special metabolism of the plant that makes it useful for remediation. Besides, the low volume pots that could limit beneficial effect of earthworms on soil structure could also explain it.

AAS analysis of the mineral content of the roots and shoots of the controls showed that they were lead-free (Fig. 2). However, plants grown in lead contaminated soils accumulated an average of 946.83 and 1645.90 mg of lead per kg of dry root material for 500 mg kg^{-1} and 1000 mg kg^{-1} lead, respectively. The lead concentration in the shoots was significantly lower than in the roots, regardless of the concentration of lead. However, the ratio of lead translocated to the aerial parts (TF = concentration in the shoots/concentration in the roots) was 0.06 (500 mg kg^{-1}) and 0.15 (1000 mg kg^{-1}) which is significantly dependent on the concentration of lead in the soil. At first, *Lantana camara* accumulated lead in its roots and then the lead was transferred towards the aerial parts where it was also accumulated in large quantities. For both concentrations of lead in the soil, the shoot-to-root lead concentration ratio was less than 1. This is a lower value than expected for hyperaccumulator plants which typically have a shoot-to-root metal concentration ratio of more than 1 (McGrath and Zhao, 2003). However, the potential of *Lantana camara* (Verbenaceae) for lead extraction was only discovered very recently and so there are few studies of its metal tolerance and phytoextraction potential. Further studies on *L. camara* physiology, currently underway, may explain this difference in the shoot-to root lead ratio.

Inoculating the soil with earthworms resulted in a positive increase in the quantity of accumulated lead in the roots and in the shoots (Fig. 2). This increase was significant for both concentrations of lead. In the presence of earthworms, the total lead uptake in the shoots and roots was 3-fold and 2-fold higher at lead concentrations of 500 mg kg^{-1} and 1000 mg kg^{-1}, respectively. These results agreed with those of Wang and Li (2006) who reported increased Zn phytoextraction by ryegrass and Indian mustard when the soil was inoculated with the earthworm *Pheretima* sp. This increase of uptake heavy metal by plant could be due, as suggested by Yu *et al.* (2004) to influence of earthworm presence on metal availability in soil by mixing deep soils, humus and biological material in the earthworm gut. Furthermore, lead has been shown to have low mobility in soil (less than Cd and Zn) and to form organic complexes which make it unavailable for plants (Orlowska *et al*., 2002). Ma *et al*. (2002) demonstrated that the concentration of available Pb was increased by up to 48.2% by earthworm inoculation and (Chen and Wong, 2002) suggested that earthworm burrowing and feeding activities increased metal bioavailability.

Moreover, this study shows that the efficiency of *L. camara* phytoextraction depends on the concentration of lead in the soil. Similarly, the Zn uptake by *T. caerulescens* increased linearly according to Zn concentration in the 1-1000 mmol m^{-3} range but concentrations greater than 1000 mmol m^{-3} resulted in toxicity and decreased Zn uptake (Shen *et al*., 1997). In this study, the total lead values (shoot and root) and the shoot-to-root lead concentration ratio were higher at 1000 mg Pb kg^{-1} than those at 500 mg Pb kg^{-1}. This result suggested that, even at this higher concentration, lead was favorable for the growth of *L. camara*.

CONCLUSION

In conclusion, *Lantana camara* proved its capacity to grow well in soil contaminated with a high lead concentration. Efficient phytoextraction needs hyperextracting plants to remove heavy metals from polluted soils but also a high shoot-to-root for accumulating metals in the harvestable parts (Salt *et al*., 1998). The remediation efficiency also depends on the amount of aboveground biomass and the bioavailability of metals (Khan *et al*., 2000). This study shows that *P. corethrurus* earthworms survived in lead polluted soils and that their activities improved plant growth and phytoextraction of Pb. They also had a positive effect on the root biomass and TF. These results clearly demonstrate that the association of *P. corethrurus* and *L. camara* has considerable potential as an efficient lead phytoextractor. Studies are now being carried out to gain a better understanding of the relationships between plant and earthworm and improve their remediation efficiency.

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