Applied Soil Ecology 96 (2015) 151–158

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

# Effects of earthworms on the fungal community and microbial activity in root-adhering soil of *Lantana camara* during phytoextraction of lead

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ARTICLE INFO

*Article history:*
Received 3 February 2014
Received in revised form 22 June 2015
Accepted 15 July 2015
Available online 13 August 2015

*Keywords:*
Earthworm
Fungal community
Microbial activity
Heavy metals
*Lantana camara*
Phytoextraction

ABSTRACT

Earthworms are known to stimulate soil microorganisms and to enhance phytoextraction of metals. This study was designed to test the hypothesis that the positive effect of earthworms on phytoextraction performance results from the stimulation of soil fungi that are known to be tolerant to heavy metals in contaminated soils. It set out to assess the relationships between lead phytoextraction and the changes in soil fungi in response to earthworm activity. The experiment was performed in outdoor microcosms combining: earthworms (*Pontoscolex corethrurus*), Spanish Flag (*Lantana camara*) and soil spiked with 500 and 1000 mg Pb $kg^{-1}$. Cultivable fungi were enumerated using plate counts. PCR–DGGE analysis targeting the 28S rRNA gene was used to determine the structure of the fungal community. Total microbial activity was measured by fluorescein diacetate (FDA) hydrolysis assay. Plant Pb uptake by *L. camara* increased by about 2.5-fold in the presence of earthworms, regardless of the Pb concentration. This was significantly correlated with the increase in total microbial activity, abundance of cultivable fungi and richness index of the fungal community calculated from DGGE banding patterns. In the presence of earthworms, there was a significant positive correlation between microbial activity, plant biomass and plant Pb uptake. There was no correlation for the control without earthworms. These results provide evidence that interactions between earthworms and soil microorganisms have a positive effect on Pb-phytoextraction yield. The study of the ecological context of phytoremediation should be broadened by considering the interactions between plants, microorganisms and earthworms that affect heavy metal uptake by plants.



## 1. Introduction

Soil pollution by heavy metals (HM) is a major environmental concern as their potential (eco) toxicity can affect all living beings from soil organisms, through plants and animals to humans as a result of food chain contamination. Lead (Pb) is considered to be one of the most toxic of heavy metals and is responsible for significant reductions in biological activities in soils (Giller et al., 1998).

Phytoextraction is a cost-effective, environmentally-friendly technology based on the use of plants to extract HMs from soils, to transport them into and accumulate them in the harvestable parts of roots and shoots (Padmavathiamma Prabha, 2007). Plants exhibiting tolerance for HMs such as Indian mustard (*Brassica juncea*) (Salt et al., 1998; Papoyan et al., 2007), grass pea (*Lathyrus sativa* L.) (Brunet et al., 2009) and also Spanish Flag (*Lantana camara*) (Jusselme et al., 2012, 2013) have been used for phytoextraction of lead from contaminated sites. *L. camara* is a species that thrives in metal-polluted soils in many tropical and subtropical areas. This plant is fast growing and can grow in extreme conditions, such as long periods of drought or heavy rains. Of the three plants cited above, *L. camara* showed the highest growth on the two lead-contaminated sites in Vietnam: Bien Hoa (industrial site with lead contaminated soil) and Binh Duong (near a lead and cadmium refinery). With a higher tolerance, *L. camara* accumulated a significantly higher level of lead and cadmium than Indian mustard and grass pea. It is generally recognized that the growth and health of plants, including those used for phytoremediation, depend partly on soil quality and fertility.

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http://dx.doi.org/10.1016/j.apsoil.2015.07.011
0929-1393/

The abundance, distribution, composition and activity of soil microorganisms play an important part in soil functioning to improve plant health and yields (Epelde et al., 2008).

Fungi are largely involved in the soil functioning as decomposers, including mycorrhizal mutualists (O'Brien et al., 2005). They perform important ecological services related to nutrient cycles, water dynamics and disease suppression. In polluted soils, fungi are known to be highly tolerant to heavy metals (Baldrian, 2003) and they are able to modify heavy metal speciation (Gadd, 1993). Therefore, there is increasing interest in using fungi to assist phytoextraction of HM contaminated soils but with rather different results (Bhaduri and Fulekar, 2012; Lebeau et al., 2008; Zhang et al., 2012).

Soil microorganisms are partly influenced by soil macrofauna (Aira et al., 2009; Liu et al., 2011). It has been shown that earthworms strongly affect the composition, distribution and activity of soil microorganisms, in particular by (i) ingesting fungal spores and certain types of fungi, (ii) creating favorable microsites for fungal development, (iii) dispersing the fungi and (iv) transforming and redistributing soil organic matter (Brown, 1995; Lavelle, 2002; Scheu et al., 2002). Some earthworms such as *Lumbricus terrestris*, *Lumbricus rubelles* and *Aporrectodea caliginosa* can survive in HM polluted soils (Morgan and Morgan, 1999; Kızılkaya, 2004). They increase the mobility and availability of HMs by burrowing and casting (Sizmur et al., 2011a, 2011b, 2011c). Although there is a large body of literature on the impact of earthworms and microorganisms on HMs in soils, only a few studies have addressed the question of using earthworms to assist phytoextraction of HMs (Wang and Li, 2006; Yu et al., 2005) and these have not considered the role of microorganisms.

The question arises whether the beneficial effect of earthworms on the performance of phytoextraction is the result of the stimulation of soil microorganisms. To address this question, a mesocosm experiment was designed with plants (*L. camara*), earthworms (*Pontoscolex corethrurus*) and soil spiked with Pb at 500 and 1000 mg Pb $kg^{-1}$. The study measured the phytoextraction performance and changes, in response to lead pollution and earthworm activity in: (i) microbial activity by fluorescein diacetate (FDA) hydrolysis assay, (ii) the abundance of the cultivable fungal community and (iii) the diversity and structure of the fungal community using PCR–DGGE targeting the 28S rRNA gene. The microbial analysis focused on the root-adhering soil (RAS) which can be defined as the soil that remains attached to the roots after gentle shaking or mechanical stirring. This area of the rhizosphere was described by Lavelle (2002) as a functional soil unit.

## 2. Materials and methods

### 2.1. Soil collection, experimental design and root-adhering soil (RAS) sampling

The experiments were performed in microcosms planted with *L. camara* and had a completely randomized block design with two types of treatment: a variable level of lead pollution and the presence or not of earthworm. The soils were spiked by spraying with lead acetate at three concentrations (0, 500 and 1000 mg Pb $kg^{-1}$) following the draft OECD earthworm reproduction toxicity protocol (OECD, 2000).

The microcosms (25 cm diameter × 30 cm high) were prepared by weighing 10 kg of dry soil. The soil used in this study was collected from the uncontaminated upper layer (0–20 cm depth) of the soil at the Phu An Ecomuseum. This was a sandy soil (39.9%) and its composition has been described by Jusselme et al. (2012).

*L. camara* seedlings were planted in 5L plastic pots in a mixture of soil, manure and coconut fiber as described by Jusselme et al. (2012). After one month of growth, similar sized seedlings (40 cm high and 20 cm diameter) were transferred to microcosms (one seedling per pot) after their roots had been washed with ultra-pure water.

The microcosms were left for seven days before earthworms were added. The earthworms were collected from Pb-free grassland in Dong Nai (Vietnam). They were acclimated for one week in plastic boxes containing uncontaminated soil collected at the Phu An Ecomuseum. Ten adult earthworms weighing a total of about 10 g were sampled and introduced into each microcosm. The microcosms were placed outside the laboratory and incubated for one month.

At the end of the experiment, the shoots were removed by cutting the plants close to the soil surface, oven-dried and weighed. The root system was carefully removed by hand at the laboratory, oven-dried and weighed. The shoots and roots were then analyzed for lead. The RAS was collected, air-dried, sieved (<2 mm) and kept at 4 °C for further microbial analysis. The RAS was also analyzed for available lead.

### 2.2. Analysis of available lead in soil

RAS samples (20 g) were oven-dried at 70 °C for 48 h and weighed. The available lead was determined using a two-step sequential extraction procedure, as described by Maiz et al. (2000). Subsamples (5 g) were mixed in 20 mL of 0.01 M $CaCl_2$ and stirred for 24 h at room temperature. The suspensions were centrifuged at 3000 × *g* for 30 min and the supernatants were collected to determine the mobile fraction. The residues were then rinsed twice with ultra-pure water and re-suspended in 20 mL of diethylene triamine pentaacetic acid (DTPA) extraction solution. After centrifugation, the supernatants were collected and filtered to determine the mobilizable fraction. In the rest of this article this fraction is called the "available lead". The lead concentrations in the two extracts were measured by inductively coupled plasma optical emission spectrometry (ICP-OES) using a Jobin-Yvon JY24. The wavelength was selected using the "profile" function to give the highest sensitivity for lead (220–353 nm) without interference. Quantitative analyses were performed using calibration curves for the appropriate concentration ranges determined using a certified PlasmaCal single element standard solution (SCP Science, Canada). All calibration standards were prepared using the same matrix as that used for the soil extracts. To check the analytical precision, the samples were analyzed in triplicate. The limit of detection for lead was 2.5 μg $L^{-1}$.

### 2.3. Lead analysis

The shoots and roots were first washed thoroughly in deionized water to remove soil particles and then in 1 mM H-EDTA (pH 5.0) at 4 °C for 30 min. They were oven-dried at 70 °C for 48 h and weighed. Subsamples (1 g) of dried plant tissue were digested in a mixture of $HNO_3/HClO_4$ (1:1.5) and the lead concentration was determined by atomic absorption spectrophotometry, AAS (Varian Spectra AA220, Lab Recyclers, USA) according to Thompson and Wood (1982). A certified plant material (i.e., NIST 1570a) was used to ensure the quality of plant digestion analyses. The recovery of lead was within the certified limits.

### 2.4. Enumeration of cultivable fungi

Root-adhering soil (5 g) was shaken in 50 mL of a 0.9% (w/v) NaCl sterile solution for 30 min at room temperature. Serial 10-fold dilutions were made to $10^{-3}$ in triplicate from this initial suspension. Plates containing Sabouraud culture medium (peptone 10 g $L^{-1}$, glucose 40 g $L^{-1}$, agar 20 g $L^{-1}$) were