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Bioaccumulation and detoxification mechanisms for lead uptake identified in *Rhus chinensis* Mill. seedlings

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ABSTRACT

A greenhouse experiment was conducted to assay the bioaccumulation and tolerance characteristics of *Rhus chinensis* Mill. to lead (Pb). The effects of exposing R. chinensis Mill seedlings to increasing Pb concentrations (0, 250, 500, 100 mg kg-1) in the soil were assessed by measuring Pb accumulation, subcellular distribution, ultrastructure, photosynthetic characteristics, antioxidative enzyme activity, malondialdehyde content, and phytochelatin content. The majority of Pb taken up by R. chinensis Mill was associated with the cell wall fraction in the roots, where the absorption of Ca increased to maintain cell wall stability, and Pb deposits were found in the intercellular space or in the cell wall structures. In leaves, Pb was primarily stored in the cell wall, while it was compartmentalized into the vacuolar structures in the stem. Pb concentrations adversely affected the morphology of *Rhus chinensis* Mill cellular substructures. Furthermore, increased Peroxidase (POD) and catalase (CAT) activity was observed in plants grown in Pb-amended soil, and this may have led to reduced ROS to maintain the function of the membrane. Changes in phytochelatin levels (PCs) that were observed in Pb treated plants suggest that PCs formed complexes with Pb in the cytoplasm to reduce Pb^{2+} toxicity in the metabolically active cellular compartment. This mechanism may allow for the plant to accumulate higher concentrations of toxic Pb and survive for a longer period of time. Our study provides a better understanding of how *Rhus chinensis* Mill detoxifies Pb.

1. Introduction

There is an abundance of high quality lead (Pb) / zinc (Zn) mines in the heavy metal mining areas in southern China. Bulk mining of these mines with outdated mining techniques and poor management has resulted in wastewater discharge and tailing stacking, polluting the soil around the mining areas with heavy metals. High soil Pb concentrations around these mines have resulted in gradual deterioration of the surrounding environment, with some areas now too toxic to support vegetation. As of now, Pb pollution has become the main limiting environmental factor for revegetation in these Pb /Zn mining areas.

Previous studies documenting plants capable of growing in Pb-Zn mine wastelands identified *Rhus chinensis* Mill (*Anacardiaceae*), a small deciduous tree, as one of the few woody plants that can grow in Pb-Zn polluted areas. *Rhus chinensis* Mill is the pioneer plant for revegetation of the Pb-Zn mine wastelands in southern China (Zhou et al., 2016) because of its strong adaptability, rapid growth, resistance to drought and ability to grow in environments with limited nutrients, as well as its high root tillering ability. *Rhus chinensis* Mill is Pb-tolerant, and also has some capacity to absorb and accumulate Pb. The Pb content can reach up to 546 mg kg^{-1} in leaves and 352 mg kg^{-1}in the roots (Shi et al., 2012). Therefore *Rhus chinensis* Mill is a woody plant that has the potential for phytoremediation of heavy metals. In addition, compared to hyperaccumulators, trees have the advantages of having large biomass, well-developed root systems, perennation and strong accumulation ability, meaning that they are better able to extract heavy metals from the soil (Shi et al., 2012). In recent years, an increasing number of studies have investigated the use of trees for remediation of heavy-metal polluted land (Estrella-Gómez et al., 2009, Fu et al., 2011). A hitherto little studied topic is the mechanisms of tolerance and detoxification of woody plants under heavy metal stress, which has restricted the application and widespread adoption of *Rhus chinensis* Mill for phytoremediation of land contaminated with heavy metals, including Pb and cadmium.

Pb is one of the most phytotoxic metals, and plants can have varying metal sensitivity to heavy metals based on their physiological characteristics and genotype. Lead can damage cell membrane cells and change the chloroplast ultrastructure, inhibit leaf/root growth and

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photosynthesis, and eventually lead to plant death (Shi et al., 2012; Gupta et al., 2012; Li et al., 2016). Previous studies of herbaceous plants that are heavy metal tolerant or have hyperaccumulation properties, such as *Salvinia minima* (Estrella-Gómez et al., 2009), *Phytolacca americana* L. (Fu et al., 2011) and *Sedum alfredii* (Huang et al., 2012), classified Pb tolerance and detoxification mechanisms into two major types: i) inactive mechanisms that prevent Pb ions from entering the plant cells, such as changes in the cell wall, or increased ability to pump Pb ions out of cells (Li et al., 2016); ii) internal tolerance mechanisms that involve synthesis of organic ligands of Pb ions, such as cysteine, glutathione, phytochelatin and metallothionein that can bind to Pb and reduce its toxicity (Pourrut et al., 2011). Although both strategies can reduce Pb toxicity in plants, high levels of exogenous Pb can lead to excessive Pb^{2+} accumulation, where Pb can enter the protoplasm and trigger oxidative stress and the production of reactive oxygen ($O_2^{\bullet-}$ and $OH^{\bullet}$). High levels of reactive oxygen can damage plant cell membranes. Plants that are Pb-tolerant often respond to Pb exposure by inducing production of enzymes, such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), to eliminate Pb (Gupta et al., 2012). Suppressing antioxidant enzyme activity leads to an excess of reactive oxygen species (ROS), which oxidize and destroy the plant cellular membrane. Furthermore, Pb can damage cellular ultrastructures, such as chloroplasts, mitochondria, cell nuclei and cell walls and tissues and organs. Lead-induced damage can abolish organelle function, and affect normal physiological functions, including photosynthesis, respiration, protein synthesis and cell division (Huang et al., 2012), thereby disturbing the physiological-biochemical functions of the plant.

Quantifying the physiological and biochemical properties of *Rhus chinensis* Mill grown in Pb-treated soil is important for understanding the physiological response of *Rhus chinensis* Mill under heavy metal stress. In order to achieve this, we measured subcellular distribution of Pb, phytochelatin characteristics, the antioxidant index, the integrity of the cellular membrane system, lipid peroxidation of cell membrane and changes in the ultrastructure in Pb-exposed *Rhus chinensis* Mill plants. We aimed to examine the toxicity of Pb, the absorption and transportation mechanisms of Pb, and detoxification mechanisms against Pb. Our results are significant for understanding the biological mechanisms for the remediation and control of woody plants in Pb-polluted soil.

2. Methods

2.1. Plant material and lead treatment

The seeds of *Rhus chinensis* Mill were collected from Fujian Agriculture and Forestry University, Fujian, China. After washing by tap-water, the seeds were soaked in 98% concentrated sulfuric acid for 90 min in order to increase the germination rate by removing the hard seed coat and wax on the surface of the seed. Seeds were then cleaned using running water and soaked in cold water for 24 h at room temperature. Afterwards, two layers of 11 cm qualitative filter paper were used as germination substrate, and germination was conducted in a constant temperature incubator (25 °C, without illumination). After 15 days, when the root length reached 2 cm, healthy seedlings were transferred to a greenhouse for cultivation in an organic substrate in November 2015. After one month, when the plants were up to ~30 cm tall and had 5–7 leaves, seedlings with the identical growth stage, measured by having the plant height of 14 ± 2 cm. were selected for stress experiments conducted in pots. The selected seedlings were transferred into individual round plastic pots (15 cm diameter x 25 cm height) with vent holes at the bottom. Each pot contained 1.5 kg of red soil loaded into the pot by stratified compaction to ensure consistent soil density. The soil used in the experiment was collected from 0 to 30 cm depth near Fujian Agriculture and Forestry University, and the soil properties were as follows: organic matter 0.87 g kg^{-1}, total N 0.13 g kg^{-1}, total P 0.154 g kg^{-1}, total Ca 3.11 g kg^{-1}, total Fe 21.29 g kg^{-1}, total Mg 2.55 g kg^{-1}, total Na 0.84 g kg^{-1}, total K 2.14 g kg^{-1}, available P 1.14 mg kg^{-1}, available K 112.5 mg kg^{-1}, and zero Pb detected.

Before the experiment, the soil was air-dried, mixed by hand and passed through a 2 mm screen, and 450 mL deionized water was added to the 0 mg Pb kg^{-1} treatment. For the experimental treatments, Pb was added in the form of $Pb(NO3)_2$ solution (450 mL) at rates of 250, 500, 750 and 1000 mg Pb kg^{-1} (corresponding to actual concentrations of 0, 278.8, 573.9, 777.2, 1103.5 mg Pb kg^{-1}). The soil moisture content was maintained at 30%. Through equilibrating for one month after putting the soil in the pots, a 30-day pot experiment was conducted between December 2015 and January 2016. There were five treatments with different soil Pb loadings (including a control treatment with no added Pb) and four replicates of each treatment, with one *Rhus chinensis* Mill seedling per pot. During the experiment, the daytime temperature was 20–25 °C and the night-time temperature was 10–15 °C.

2.2. Quantification of Pb and nutrient concentrations

Rhus chinensis Mill plants treated with 0, 250, 500, 750, 1000 mg kg^{-1} Pb for 30 days were harvested to measure l Pb accumulation. Roots of intact plants were washed with distilled water for metal analysis and immersed in 20 mM Na-EDTA for 15–20 min to remove excessive Pb adhering to the root surface. Then, plants were dried at 105 °C and digested with an acid solution containing 13 M HNO_3 and 1 M $HClO_4$. For quality control, 10 repeated measurements were carried out with standard Pb solution provided by the Institute of Geophysical and Geochemical Exploration, China (Qiao et al., 2015). Pb, Fe, Zn, Ca, K, Na, Mg concentrations in the digests were determined using ICP-OES.

2.3. Subcellular distribution of Pb in Rhus chinensis Mill root, stem and leaf material

Subcellular fractions of *Rhus chinensis Mill* leaves, stems and roots were isolated as described by Li et al. (2016). Frozen plant materials (0.5000*g*) were homogenized using a chilled pestle and mortar with 20 mL of pre-chilled extraction buffer containing 50 mM Tris–HCl (pH 7.5), 250 mM sucrose, and 1.0 mM dithioerythritol. The homogenate was transferred to a 50-mL centrifuge tube and centrifuged at 300*g* for 10 min at 4 °C, and the pellet was collected as the cell wall fraction (F1). The filtrate was centrifuged at 2000*g* for 10 min, and the pellet was the nucleus-rich fraction (F2). The supernatant was then centrifuged at 10,000 *g* for 30 min. The pellet was designated the mitochondrial fraction (F3) and the supernatant as the soluble fraction (F4). All homogenizations and subsequent fractionations were performed at 4 °C (Nishizono et al., 1987). Pb concentrations in the fractions were determined by ICP-OES after digestion with HNO_3 and $HClO_4$.

2.4. Transmission electron microscopy and energy dispersive X-ray analysis (EDS)

Rhus chinensis Mill plants grown for 30 days in soil amended with 0, 250, and 1000 mg kg^{-1} Pb were imaged using transmission electron microscopy (TEM). Small Sections (1–3 mm long) from the middle of the third leaf from the top of the plant were fixed in 4% glutaraldehyde (v/v) in 0.2 M sodium phosphate buffer (pH 7.2) for 6–8 h, and post-fixed in 1% OsO_4 for 1 h and in 0.2 M sodium phosphate buffer for 1–2 h. Dehydration was performed in a graded ethanol series (50%, 70%, 85%, 90%, and 95%) followed by a wash in acetone, and then samples were infiltrated and embedded in Spurr's resin (Basile et al., 1994). Ultra-thin sections (80 nm) were prepared and mounted on copper grids for imaging with a transmission electron microscope (Tecnai G2 Spirit) at an accelerating voltage of 60.0 kV. Subsequently, the energy spectra of the prepared sections were measured using a JEM-