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Inducible defense responses in *Populus alba berolinensis* to Pb stress

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ABSTRACT

To better understand the effects of heavy metal stress on inducible defenses of woody plants, contents of nutritional substances and secondary metabolites, plus the activities of defense enzymes and protease inhibitors in the leaves of *Populus alba berolinensis* seedlings that were planted in either non-contaminated soil (control) or Pb-contaminated soil (at 300, 500 and 700 mg/kg) were studied. Our results showed that protein and soluble sugar contents in leaves 30–50 days after the Pb stress were significantly lower than those in the control group in a dose-dependent manner, whereas the lignin and flavonoid contents were significantly higher. Polyphenol oxidase (PPO) and phenylalnine ammonialyase (PAL) activities in leaves were significantly enhanced by the low Pb concentration stress (300 mg/kg) for all three sampling dates (30, 40 and 50 days after the Pb treatments), while at the two higher Pb concentrations (500 and 700 mg/kg), PPO activities were always inhibited and PAL activities presented a tendency of "promoting first and then restraining" with the increase of exposure duration. The response patterns of trypsin inhibitor (TI) and chymotrypsin inhibitor (CI) activities to Pb stress were similar to that of PPO, and presented a "low-promotion, high-inhibition" effect of Pb concentrations in leaves. These results suggest that the inducible defense responses in *P. alba berolinensis* leaves to Pb stress depend on both the Pb concentrations and the exposure duration, and the low Pb concentration at 300 mg/kg seemed to induce the strongest defense responses in the poplar seedlings, much stronger than those at the higher Pb concentrations.

1. Introduction

Heavy metal pollutants, released by various anthropogenic activities including mining, chemical fertilizer application and industrial waste disposal, have become a global problem and pose a serious threat to the whole ecosystem (Gichner et al., 2008; Laidlaw et al., 2012). Among heavy metals, lead (Pb) is one of the most phytotoxic nonessential elements present in the environment and has no known biological and physiological functions in plants (Liu et al., 2008; Izbiańska et al., 2014). Furthermore, as a redox-inactive metal, Pb is unable to catalyze generation of reactive oxygen species (ROS) such as hydrogen peroxide, singlet oxygen and superoxide via Fenton-Haber-Weiss reactions, but can indirectly cause oxidative stress through the inhibition of antioxidant enzymes or depletion of antioxidants (Izbiańska et al., 2014; Valko et al., 2016). Plants are capable of absorbing and accumulating Pb from polluted soils, and exposure to excessive Pb can cause morphological, metabolic, and physiological anomalies in plants including lipid peroxidation, cellular damage and disruption of protein functions (Zhou et al., 2015; Dar et al., 2015).

Plant resistance to insects is associated with the activation of wide array of defense responses (e.g. preexisting constitutive defenses and inducible defense responses) that serve to inhibit or disturb normal development and reproduction of herbivores (Kawazu et al., 2013; Dučaiová et al., 2016; Jiang and Yan, 2018). Heavy metal stress, as an abiotic stimulus, can induce the biosynthesis of diverse phytohormones in plants such as jasmonic acid, salicylic acid and ethylene in a pattern similar to mechanical or herbivorous insect-driven wounding (Maksymiec et al., 2005; Foroughi et al., 2014). For example, Cabot et al. (2013) reported that Cd accumulation in *Arabidopsis thaliana*-enhanced the expression of marker genes PR1 and BGL2 for the salicylic acid and the PDF1.2 for the jasmonic acid–ethylene signaling pathways. Jasmonic acid, salicylic acid and ethylene are well-known to play a vital role in triggering the plant immune signaling network. Heavy metals stress maybe, therefore, improve the inducible defense responses in plants by activating the defense signaling pathways that can induce biosynthesis of defense-related enzymes or secondary metabolites to impede the ability of herbivores to utilize ingested nutrients.

In addition to the organic defenses induced by heavy metal stresses, the accumulated heavy metals can also effectively protect plants against herbivores, as postulated by the elemental defense hypothesis (Rascio and Navariizzo, 2011; Boyd, 2012). Compared to the organic defense, elemental defense offers more advantages; for example, the toxic elements are directly absorbed from the soil and cannot be biochemically degraded by most herbivores (Boyd, 2012; Kazemi-Dinan et al., 2014). Furthermore, according to the joint effect hypothesis, elemental

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defense can interact with organic defenses in an additive or synergistic fashion, which might lead to an enhanced overall plant defense even at the concentrations far below the hyperaccumulator level so that the elemental defense could also be efficient in non-hyperaccumulator plants (Martos et al., 2016). However, heavy metal accumulation requires extra expenditure on uptake, transport and sequestration, as well as increasing basal metal requirements, which might result in reducing plant investments in energy-demanding organic defenses (Fones and Preston, 2013). For example, as demonstrated by Tolra et al. (2001), Zn stress decreased defensive glucosinolates concentrations in Zn hyperaccumulator *Thlaspi caerulescens*. Consequently, trade-off hypothesis was postulated for organic defenses and elemental defenses, in which plants defended by toxic inorganic ions would reduce its organic defense level (Boyd, 2012). In contrast to the trade-off hypothesis, as demonstrated by Kusznierewicz et al. (2012), the biosynthesis of organic metabolites in the white cabbage was enhanced in a dose-dependent manner following its exposure to Cd or Zn. The relationship between organic defense compounds and elemental concentrations in plants thus tightly depends on the species, the metal as well as the metal exposure time.

In recent years, the accumulation, phytotoxicity and detoxification of heavy metals in various plants have been studied extensively (Soudek et al., 2016; Redovniković et al., 2017; Seneviratne et al., 2017), but little is known about the inducible defense responses in woody plants to heavy metal stresses. In this study, we analyzed the inducible defense responses in *P. alba berolinensis* seedlings to Pb stress by measuring the contents of nutritional substances (proteins and soluble sugars) and secondary metabolites (lignin and flavonoids), plus the activities of defense enzymes [phenylalnine ammonialyase (PAL) and polyphenol oxidase (PPO)] and protease inhibitors [trypsin inhibitor (TI) and chymotrypsin inhibitor (CI)] in leaves of *P. alba berolinensis* seedlings that grew in either non-contaminated soil (control) or Pb-contaminated soil (300, 500, 700 mg/kg).

2. Materials and methods

2.1. Plant materials and experimental design

The field pot experiment was carried during May to September, 2016at the Ping Shan Forest Nursery of Heilongjiang province, China. In early May, cuttings of similar size were collected from *P. alba berolinensis* and planted in 10-l pots (23 × 23 × 25 cm) each containing 5 kg of 1:1:1 mixture of sand, turf soil and native soil. The concentration of Pb in the mixed soils was analyzed, and found that the total Pb concentration is 4.42 mg/kg. Two months later, the soils in the seedling pots were amended with aqueous solutions of lead acetate at following designed concentrations of 0 (water control), 300, 500 and 700 mg/kg Pb kg^{-1} soil, respectively. The Pb concentration used in present experiments was chosen based on the soil environmental quality standards for normal plant growths in China (GB15618–1995). On the 30th, 40th and 50th day of Pb treatments, 12 plants with similar size from each treatment or control group (per sampling date) were harvested and divided into 3 replicates. Leaves from these samples were collected, and then stored at −80 °C in an ultra-low temperature freezer for future analysis.

2.2. Activities of defense enzymes and protease inhibitors

The PAL in the polar leaves was extracted and assayed according to the method described by Zucker (1965) with modifications. Assay mixture in a final volume of 5 ml contained 0.1 ml enzyme, 3.9 ml of 50 mM TBS buffer (pH 7.8) and 1 ml of 20 mM L-phenylalanine. The reaction was carried out at 40 °C for 30 min and immediately stopped by addition of 0.2 ml of 6 M HCl solution. The increase in absorbance was measured at 470 nm and results were expressed at units min^{-1} mg^{-1}. The PPO was extracted and assayed according to Soliva et al. (2000) with modifications. The assay mixture contained 100 mM pyrocatechol, 100 mM phosphate buffer and enzyme extract in a total volume of 3 ml. The change of absorbance at 420 nm was measured every minute at 30 °C, and the results reported were based on ΔOD g fresh $weight^{-1}$ min^{-1}.

Extraction and determination of TI and CI were performed according to the method described by Meng et al. (2017) with modifications. Leaf tissues were homogenized in an ice bath with extracting solution. The extracting solution for TI and CI was 50 M Tris–HCl buffer at pH 7.8 containing $CaCl_2$ and 50 M Tris–HCl buffer at pH 8.0 containing $CaCl_2$, Vitamin C, alpha-phenylthiourea and polyvinyl pyrrolidone, respectively. The homogenate was centrifuged at 12000 rpm for 10 min (at 4 °C), and the supernatant was used to test the activities of TI and CI. The TI extract was reacted with trypsin solution at 25 °C for 60 min and CI extract was reacted with chymotrypsin solution at 25 °C for 20 min, and then substrate-indicator mixture [benzoylarginine ethyl ester for TI and benzoyltyrosine ethyl ester for CI] was added. The changes of absorbance at 256 nm for TI reaction mixture and at 253 nm for CI reaction mixture were measured every minute.

2.3. Contents of secondary metabolites and nutritional substances

Extraction and identification of flavonoid compounds from the poplar leaf samples were performed according to the method described by Zhishen et al. (1999) with modifications. Leaf tissues were extracted with 50 mL of 80% aqueous methanol on a shaker for 24 h and then followed by a ultrasonic extraction for 2 h. The extracts were centrifuged for 15 min at 14000 rpm. 1 ml of supernatant was added to a test tube containing 4 ml of water, and then added 0.5 ml of 50% $NaNO_2$. After 6 min, 0.5 ml of 10% $AlCl_3$ was added, and the mixture was allowed to stand for a further 6 min. After that, 4 mL of 4% NaOH was added. Absorbance was measured at 510 nm, and results were expressed as milligrams of rutin equivalents per g of fresh leaf weight.

Lignin compounds in the leaves were extracted and identified according to the method described by Ren et al. (2007). Briefly, 1 g of leaf tissues were homogenized using mortar and pestle in 4 ml of 80% methanol, and then were centrifuged for 10 min at 4500 rpm. The residues were washed by successive stirring and centrifugation: three times with 80% methanol, three times with1:2 mixtures of ethanol and n-hexane(*V*/V), and then were dried in an oven (60 °C, overnight). All dry tissues were placed into a 10 ml screw-cap centrifuge tube with 1 ml of glacial acetic acid containing 25% acetyl bromide, and heated at 70 °C for 30 min. After that, the reaction was immediately stopped by addition of 0.9 ml of 2 M NaOH solution, and then added 5 ml of glacial acetic acid and 0.1 ml 7.5 M Hydroxylammonium chloride. The mixture was again centrifuged for 5 min at 4500 rpm, and the absorption of the supernatant after diluting with glacial acetic acid was determined at 280 nm. Lignin content was expressed as OD g^{-1} fresh leaf weight.

Leaf tissues were homogenized using mortar and pestle in 50 mM Tris–HCl buffer (pH 7.5), and then were centrifuged for 10 min at 4000 rpm. The supernatants were used to determine the protein content according to Bradford (1976), using Coomassie Brilliant Blue G-250 as dye and albumin as a standard.

The soluble sugars in the leaves were extracted and identified according to the method described by Nelson (1944). Briefly, 0.5 g of leaves were grinded in 80% ethanol and then were boiled for 10 min. After centrifugation at 5000 rpm for 10 min, 1 ml supernatant was added to a test tube containing 4 mL of anthrone reagent, and heated for 10 min. Absorbance was measured at 630 nm, and results were expressed as mg soluble sugars g^{-1} fresh leaf weight.

2.4. Statistical analysis

To compare the contents of nutritional substances and secondary metabolites, and the activities of defense enzymes and protease