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Pb stress effects on leaf chlorophyll fluorescence, antioxidative enzyme activities, and organic acid contents of *Pogonatherum crinitum* seedlings

Xiaolong Hou[a,b,c], Hang Han[a], Liping Cai[a,c,*], Aiqin Liu[a,c], Xiangqing Ma[a,c], Chuifan Zhou[a,c], Guo Wang[b], Fanrui Meng[d]

[a] College of Forestry, Fujian Agriculture and Forestry University, Fuzhou, 350002, China
[b] College of Resources and Environment, Fujian Agriculture and Forestry University, Fuzhou, 350002, China
[c] Cross-Strait Collaborative Innovation Center of Soil and Water Conservation, Fuzhou, 350002, China
[d] Faculty of Forestry & Environmental Management, University of New Brunswick, Fredericton, E3B5A3, Canada

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ABSTRACT

Pogonatherum crinitum (Thunb.) Kunth is a lead (Pb) tolerant, perennial grass and a hyperaccumulator of Pb based on field surveys and greenhouse experiments. However, only few attempts have been made to understand the response mechanisms of *P. crinitum* to high Pb concentrations. In this study, we measured leaf chlorophyll fluorescence, antioxidative enzyme activities, organic acid contents, and Pb concentrations of *P. crinitum* grown in soil containing four concentrations of Pb (0, 500, 1000, and 2500 mg kg^{-1}, respectively) for a period of three months. Our results indicated that chlorophyll *a* and total chlorophyll concentrations were significantly lower in Pb treated plants compared to the control, while chlorophyll *b* concentrations were not significantly different from those in the control. Furthermore, Pb stress increased the photosystem II (PSII) maximum photochemical efficiency. The activities of antioxidant enzymes such as superoxide dismutase (SOD) and peroxidase (POD), and organic acid contents such as oxalic and malic acids in roots were significantly increased by Pb treatments. Furthermore, the malondialdehyde content in leaves was significantly reduced by higher Pb concentration treatments. The Pb concentration was significantly increased by Pb treatments, and transfer coefficient exceeded 1 (Pb2500 treatment). These results suggest that increase in light use efficiency, scavenging reactive oxygen by strengthening anti-oxidative enzyme activity, and complexation of Pb with organic acids may play important roles in alleviating the toxicity of Pb in *P. crinitum*.

1. Introduction

Heavy metal contamination of soils is a worldwide environmental problem (Chai et al., 2014; Khan et al., 2010; Khuzestani and Souri, 2013). As heavy metals are persistent and non-biodegradable, many efforts have been made to remove them from the environment by using various chemical and biological methods (Calisi et al., 2013; Ren et al., 2015). Lead (Pb) is a major heavy metal pollutant being found in mining areas (Li et al., 2012a,b; Wang et al., 2010), and it can accumulate in soils at very high levels (Walraven et al., 1997; Zhanet al., 2014). Pb is a non-essential heavy metal in plants, and has toxic effects and unknown biological functions on plant growth and development (Tanhan et al., 2007; Walraven et al., 2014). High Pb concentrations can inhibit photosynthesis, disrupt cell proliferation, and retard plant growth or cause plant death (Bailey et al., 2011; Liu et al., 2007; García-Rosales et al., 2012; Islam et al., 2007). Pb toxicity can also be related to oxidative stress induced in living organisms either by increasing concentrations of reactive oxygen species (ROS) or by reducing cellular antioxidant capacity (Antonova et al., 2004; Kasperczyk et al., 2015; Shakoor et al., 2014). An indicator of cellular damage due to ROS is the accumulation of malondialdehyde (MDA), a product of lipid peroxidation. Several studies indicated that antioxidant enzymes can scavenge reactive oxygen to protect plants against heavy metal toxicity (Sharma et al., 2016; Xu et al., 2011). SOD is the key protective enzyme, and it can remove excess superoxide anions in plants generated by the Haber-Weiss reaction (Nehnevajova et al., 2012). POD and CAT have the function to eliminate excess H_2O_2 in plant (Goswami and Das, 2016).

Furthermore, high Pb exposure can lead to a decrease in chlorophyll (Chl) content and performance (Chettri et al., 1998; Li and Zhang, 2015). To cope with these adverse effects, heavy metal tolerant plants and hyperaccumulators may possess various morphological and physiological mechanisms to detoxify heavy metals (Huang and Wang, 2010; Yan et al., 2006). Complexation of heavy metals by organic acids has been indicated in several hyperaccumulator species (Brooks et al.,

* Corresponding author at: College of Forestry, Fujian Agriculture and Forestry University, Fuzhou, 350002, China.
E-mail address: fjclp@126.com (L. Cai).

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1981; Boominathan and Doran, 2003). Low molecular weight organic acids play an important role in the bioavailability of heavy metals as modified by root exudates (Debela et al., 2013; Montiel-Rozas et al., 2016).

Phytoremediation is a plant-based, cost-effective technology to detoxify or stabilize soils contaminated with heavy metals. Perennial plants that are heavy metal tolerant, fast growing, and have high biomass are good candidates for phytoremediation (Cho et al., 2009; Gisbert et al., 2006; Visioli and Marmiroli, 2013). A previous study showed that *Pogonatherum crinitum* (a perennial grass species) is a Pb hyperaccumulator screened from over 300 plant species growing in mining wastelands (Chang, 2005). Moreover, *P. crinitum* can grow normally in Pb-Zn mining areas with soil Pb concentrations of up to 17496 $mg\ kg^{-1}$ (Chang, 2005; Hou et al., 2012a). It can accumulate large amounts of Pb in aboveground and belowground parts of the plant. For example, Pb concentration of shoots was as high as 5091 $mg\ kg^{-1}$ and Pb bioconcentration factor was 10.18 under 500 $mg\ L^{-1}$ Pb treatment (Hou et al., 2012b, 2013a, 2017).

Due to its great potential to accumulate Pb, *P. crinitum* can be used in phytoremediation to remove Pb in mining wasteland (Chang, 2005; Hou et al., 2012b, 2013b, c; Wang et al., 2010). However, to date, only a few studies have focused on the mechanisms of tolerance of hyperaccumulators to heavy metals such as Pb (Li et al., 2009; Ribeiro de Souza et al., 2012). Thus, it is important to examine the physiological responses of *P. crinitum* to high Pb concentrations.

The objective of this study was to use greenhouse cultivated plants to assess the physiological responses of *P. crinitum* under Pb stress. The specific objectives include: (i) assessing effects of Pb stress on the functionality of *P. crinitum* seedlings' photosynthetic apparatus, measured by chlorophyll fluorescence; (ii) examining the Pb stress effects on chlorophyll content, antioxidative enzyme activities (e.g. SOD, POD and CAT), and malondialdehyde (MDA) contents in *P. crinitum* seedlings; and (iii) exploring the role of organic acid complexation of Pb in *P. crinitum* seedlings for metal detoxification.

2. Materials and methods

2.1. Plant material

Pogonatherum crinitum (Thunb.) Kunth seeds were soaked in distilled water for 24 h, and then placed in germinating beds which comprised of 9 cm diameter Petri dishes, filled with disinfected silver sand. Care was taken to ensure sufficient space between the seeds. Each Petri dish contained approximately 20 seeds. The dishes were incubated at 25 °C and at 70% relative humidity in an artificial climate chamber, with light period and intensity being 14 h/d and 7.3 $W\ m^{-2}$, respectively. After seven days, each individual seedling was transplanted into separate polyvinyl chloride plastic cups (10 cm in diameter and 15 cm in depth), filled with disinfected silver sand. After 7 days when seedlings reached about 15 cm in height, they were kept in a greenhouse for three months with air temperature ranging from 18 to 25 °C (mean daily minimum and maximum, respectively), and a relative humidity of around 55%.

2.2. Experimental design and growth conditions

The experiment was conducted using homemade plastic culture boxes (diameter 27.0 cm; height 30.0 cm) in a greenhouse. The Pb concentrations used in the experiment were 0, 500, 1000, and 2500 $mg\ kg^{-1}$ (marked Pb0, Pb500, Pb1000, and Pb2500, respectively) measured by dry soil mass, which is in line with the soil conditions typically found in mining wasteland in southern China. The different Pb concentrations were applied using $(CH_3COO)_2Pb{\cdot}3H_2O$ solution in a culture medium consisting of a 4:1 mixture of yellow soil [pH in water: 4.6, organic matter: 3.54 g/kg, clay content: 23.25%; clay/powder ratio: 0.67; cation exchange capacity: 10.23 cmol/kg, total N, P, K: 0.25, 0.08, 30.04 g/kg, respectively; available N (determined using the Illinois Soil Nitrogen Test), P (sodium bicarbonate extraction, colorimetric determination), K (ammonium acetate extraction, flame photometer determination): 42.9, 1.93, 94.1 mg/kg] and clean sand to 24 cm depth (culture medium dry mass was 8 kg). The $(CH_3COO)_2Pb{\cdot}3H_2O$ contents added in the substrate were calculated with the following formula: mass of $(CH_3COO)_2Pb{\cdot}3H_2O$ = Pb concentration of substrate × dry mass of substrate × 379.2/207.2 (379.2 and 207.2 is the molecular mass of $(CH_3COO)_2Pb{\cdot}3H_2O$ and Pb, respectively). The pH of the final culture medium was 5.6. Finally, clean sand of 3 cm depth was placed on the top of the growing medium as a buffer layer. Seedlings were transplanted on July 15th, 2012. Seedlings of 15 cm in height were selected for physiological assessment. These seedlings were replanted after cleaning their root system using distilled water. One seedling in each box was considered as a replicate, and each Pb treatment had five replicates.

2.3. Analytical methods

2.3.1. Measurement of leaf chlorophyll fluorescence

Leaf chlorophyll fluorescence was measured on 15th of August, September and October on the 4th or 5th fully expanded leaf of each plant using a chlorophyll fluorometer (OPTI-sciences OS-30P$^+$, USA). Before measuring chlorophyll (Chl) fluorescence parameters, leaves were put in a dark-adapted state for 30 min, using light exclusion clips. The following chlorophyll fluorescence yields (FYs) were measured: minimum and maximum Chl FY in the dark-adapted state (F_0 and F_m, respectively). Using these parameters, the following ratios were calculated: (i) PSII potential activity, $F_v/F_0 = (F_m - F_0)/F_0$; (ii) PSII maximum photochemical efficiency, $F_v/F_m = (F_m - F_0)/F_m$. F_0, F_m, F_v/F_m and F_v/F_0 are important chlorophyll fluorescence characteristics that are widely used in plant stress physiology studies (Marques and Araújo do Nascimento, 2013; Rau et al., 2007). An increase in these values reflects higher light use efficiency in plants (Hazrati et al., 2016).

2.3.2. Measurement of chlorophyll concentration and antioxidative enzyme activities

Biochemical analyses were performed on August 15, 2012, one month after plants being transplanted into treatment soil. About 4–5 pieces of leaves from the upper part of seedlings were picked for the measurement of chlorophyll concentration and antioxidative enzyme activities. Chlorophyll *a*, chlorophyll *b* and total chlorophyll concentration were measured using dimethyl sulfoxide according to Hiscox and Israelstam (1979). Superoxide dismutase (SOD) activity was measured using the inhibition of photochemical reduction of nitrobluetetrazolium (NBT) chloride method (Giannopolitis and Ries, 1977). The samples were illuminated by a filament lamp for 20 min and the change in absorbance was measured at 560 nm. Peroxidase (POD) extraction buffer contained 50 μM phosphate (pH 7.8), 25 μM guaiacol, 200 μM H_2O_2, and the change in absorbance of the samples was measured at 470 nm (Zhang, 1990). For catalase (CAT), the enzyme activity was assayed according to the change in absorption at 240 nm (Sheldon and Pelt, 2013). Leaf samples were ground up in an extraction buffer containing 100 μM potassium phosphate (pH 7.0), 0.5% TritonX-100, and 1% polyvinylpyrrolidone (PVP), using a pre-chilled mortar. The supernatant was centrifuged twice at 15,000 rpm for 20 min at 4 °C and the enzymatic activities were determined using an ultraviolet-visible spectrophotometer (UV-2600, Shimadzu, Japan).

2.3.3. Measurement of malondialdehyde concentration

In the middle of August (growth for one month), 2–3 pieces of leaves from the upper part of seedling were picked for the measurement of malondialdehyde (MDA) concentration according to Heath and Packer (1986). Approximately 0.5 g of samples were homogenized in 1.5 mL of 5% trichloroacetic acid (weight/volume). The homogenate was centrifuged at 3000 rpm for 10 min, and then the supernatant was

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