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ORIGINAL PAPER

ANTIOXIDANT RESPONSE OF BAMBOO (*INDOCALAMUS LATIFOLIUS)* AS AFFECTED BY HEAVY METAL STRESS*

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Abstract

Soil concentration of trace metals is the most critical factor determining whether they have an inhibiting or a stimulating effect on to the growth and productivity of plants. The agricultural soils of the southern region of China are perniciously influenced by the excessive buildup of metals, including Cu, Zn and Pb. Since the bamboo plant is of considerable nutritional and financial importance for consumers and producers in the region, it is essential to assess how some vital enzymatic components of bamboo plants are impacted by the accentuated presence of heavy metals (HMs) in the rhizosphere. Thus, this study was conducted to determine the effects of three HMs (Cu, Pb and Zn) at four different concentrations (0, 500, 1000, 2000 mg kg^{-1}) on a single bamboo species (*Indocalamus latifolius).* For pre-experimental treatments, 2-year-old stands of the bamboo species grown in pots were inoculated with the specified amounts of HMs for 60 days. Changes in the amounts of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) showed that the bamboo species had varied reaction to heavy metal (HM) stress. HM-treated bamboo experienced a significant rise in its enzymatic activity under all HM concentrations. This was more conspicuous when *Indocalamus latifolius* grew at the 500 mg kg^{-1} HMs level. However, the antioxidant activity at the elevated levels (1000 and 2000 mg kg^{-1}) showed an erratic downward trend in comparison to the lowest HM concentration. Zn and Pb were found to be the strongest and the weakest inducer of antioxidant enzymes in the bamboo species, respectively. The malondialdehyde (MDA) content was affected differently by HM toxi-

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city. Pb triggered the highest accumulation of MDA and Zn was the heavy metal associated with the lowest MDA concentration. Overall, the results indicated that low levels of HMs led to the upregulation of the antioxidant activity in *Indocalamus latifolius*, whereas the activity of antioxidative enzymes was overwhelmed by the high levels of HMs.

Keywords: abiotic stress, SOD, MDA, Pb, peroxidase, catalase, Zn, Cu.

INTRODUCTION

Heavy metals (HMs) have recently become significant environmental pollutants worldwide that tend to disrupt the balance amongst principal life -supporting components of the planet Earth, including the pedosphere, hydrosphere, atmosphere, lithosphere and biosphere (Chen et al. 2015). The major sources through which HMs are released into the environment include extensive urbanization, mine explorations and industrial waste disposal. Moreover, the profligate use of pesticides and chemical are other main culprits for the dissemination of HMs in the environment (Adrees et al. 2015).

Due to being sessile, plants are amongst the organisms that are under constant exposure to HMs, especially in contaminated areas. Depending on their quantity and type, metals can exert both advantageous and disadvantageous effects on vital growth processes of plants. At toxic levels, they cause the excessive generation of reactive oxygen species (ROS) and overproduction of free radicals in plants, which can lead to severe abnormalities in cellular and metabolic activities (Sytar et al. 2013). However, some metals like Cu and Zn are essential for the plant growth and in fact, when present in minute amounts, they have nutritional value for plants.

Among the beneficial elements, the role of Zn in plant metabolic processes is proven. It primarily contributes to improved enzymatic activity via decreasing oxidative stress – OS (Erturk et al. 2015). But excessive concentrations of this element can generate a range of morphological and physiological anomalies in plants including impairment of enzymatic activities and nutrient disequilibrium (Li et al. 2013, Erturk et al. 2015).

Copper is an essential trace metal that contributes to the plant growth and acts as a co-factor inside plant cells. It plays an important role in the regulation of enzymes and proteins (Mostofa et al. 2015). However, any increase in a Cu concentration above biological safety levels, just like other trace metals, has serious consequences for biological functions in plants, leading to compromised efficiency of cellular metabolism and hampered protein structure (Mostofa et al. 2015).

Lead is known to be one of the major hazards to the plant growth and production. This HM is widely found in industrial areas. Lead impacts vital processes in plants via triggering the onset of serious disorders in photosynthetic properties, cell division and inhibition of enzymes responsible for seed germination (Malar et al. 2014*a*).

Plants are equipped with enzymatic and non-enzymatic defense mechanisms to counteract the lethal effects of HMs. This can be achieved through deposition, accumulation, sequestration and compartmentalization of HMs constituents in their tissues (Lomaglioa et al. 2015). In addition, plant cells contain protective enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) that act as scavengers when the amount of cellular ROS reaches excessive levels (Sytar et al. 2013).

Bamboo (*Bambusoideae*), with more than 70 genera and approximately 1200 species, is indigenous to a wide spectrum of the world's climatic conditions, including tropical, subtropical and temperate regions of all the continents except Europe. Bamboo occupies one quarter of the Earth's total terrestrial area, making it the most pervasive genus on the planet (Jiang et al. 2013). Moreover, it covers more than 6 million hectares of land in China. Bamboo is an indispensable source of nutrition, medicine and livelihood for the majority of households in southern China (Hogarth, Belcher 2013). However, this part of China is acutely and chronically affected by toxic contamination of HMs. Zn, Pb and Cu are the elements that are frequently found in excess in the agricultural forest lands of southern China (Zhang et al. 2015).

The information available in the literature on the response of bamboo to HM stress is scant. Given the versatility and ubiquity of this plant in China, it is important to provide some insight into how some vital attributes of bamboo are impacted by HM toxicity. The selection of the bamboo species for this study was done based on its regional prevalence and dispersion.

The aim of the current investigation was to assess the impact of three HMs and their varying concentrations on some enzymatic indices of *Indocalamus latifolius*. This would provide some insight into how vital defense and antioxidant enzymes in bamboo are affected by low or extreme doses of the HMs.

MATERIAL AND METHODS

Experimental design and statistical analysis

This study was conducted under controlled greenhouse conditions at Nanjing Forestry University in China. The experiment was laid out in a completely randomized design (CRD) with a 2-way factorial arrangement and five replications. We selected the bamboo species *Indocalamus latifoliuss* for this study when it was a 2-year-old plant. The first factor was allocated to three applications of HMs (Cu, Pb, Zn) and the second factor was assigned to four different concentrations of HMs (0 mg kg^{-1} control, 500 mg kg^{-1}, 1000 mg kg^{-1} and 2000 mg kg^{-1}). Analysis of variance (ANOVA) was performed by the statistical software package R. Treatment mean differences were

compared using the Tukey's test at the $P > 0.05$ probability level. Vertical bars in the figures represent standard deviation (SD).

Pre-experimental procedures and plant sampling

For pre-experimental treatments, 2-year-old stands of *Indocalamus latifolius* grown in pots were inoculated with the specified amounts of HMs plus 300 ml distilled water, which were applied in the following time intervals: half of the previously mentioned amounts was applied in the morning and the remaining half was given in the afternoon for a period of two days. The concentrations of elements (500 mg kg^{-1}, 1000 mg kg^{-1} and 2000 mg kg^{-1}) were applied in the form of an aqueous solution where Cu, Pb and Zn were respectively used in the following formulas: ($Cu\ SO_4 \cdot 5H_2O$) – ($Pb(NO_3)_2$) – ($Zn(SO_4) \cdot 7H_2O$). In all the experiments, distilled water (0 mg kg^{-1}) sevred as the control. Plants were grown under the low or excessive concentrations of HMs for a period of 60 days. The addition of the elements to each pot at any given concentration was done simultaneously. The applied amounts of HMs during the experimental period (60 days) are summarized in Table 1. Sam-

Table 1

HM concentrations in pots

HM concentrations	500 (mg kg^{-1})	1000 (mg kg^{-1})	2000 (mg kg^{-1})
Pots	647 mg	1295 mg	2591 mg

ples (after two months) were taken from pots, transferred to a laboratory and kept in a refrigerator for the following enzymatic experiments. For specimen preparation, 0.5 g of fresh leaves was squished so that all the internal tissues were removed. For eliminating the interior tissues of the leaves, nitrogen liquid was used and then 2 mg of the resulting powder were mixed with phosphate buffer (pH 7.8) in a test tube. Then, the samples were centrifuged with 7000 rpm for 10 minutes.

Antioxidant enzyme activity

The levels of antioxidant components (SOD, POD, and CAT) were measured in the laboratory to assess the defense response of the bamboo plant to HM stress. Superoxide dismutase enzyme activity (SOD, EC 1.15.1.1) was quantified according to the method described in ZHANG (1992). Based on the photo diminution of nitroblue tetrazolium (NBT), the assays were performed employing NBT 0.1 g 1000 ml^{-1} in the reaction mixture with riboflavin (Rib) 0.01 g 100 ml^{-1} and methionine MET 1 g 50 ml^{-1} and EDTA 2.1 g 100 ml^{-1}. For the preparation of soluble 0.2 ml NBT, 0.2 ml MET, 0.2 ml EDTA, 0.2 ml Rib, 3.1 ml 7.0 buffer and 0.1 ml of the sample were poured into a test tube. Then, the test tubes were exposed to light for 10 to 20 minutes. After the color of the aqueous solution turned to blue, a tube was transferred to a 470 nm spectrophotometer to calculate the SOD content. The estimation of antioxi-

dant activities of peroxidase dismutase (POD, E.C. 1.11.1.7) was done according to ZHANG (1992). It was based on the variations in absorbance of 470 nm wavelengths by the bamboo species, which had been exposed to HM stress. For this purpose, we used 4 ml 2-metroxyphenol + 0.2 ml H_2O_2 + 20 µl sample + 0.8 ml pH 7 phosphate. After the solution changed the color to red, it was transferred to a spectrophotometer (470 nm) for the subsequent measurements. Then the POD data were recorded three times every 30 s and the POD value was revealed by ΔA470 min^{-1} FW g^{-1}. The catalase CAT (EC 1.11.1.6) activity was evaluated based on H_2O_2 catalysis at 240 nm. Therefore, solutions of 1 ml Tris - HCL ,1.6 ml water and 0.1 ml sample plus 0.2 ml H_2O_2 were prepared, transferred to a spectrophotometer at 240 nm and assayed two or three times according to AEBI (1984).

Determination of lipid peroxidation (LP)

The MDA content of leaves was calculated as an indicator of plant LP activities using the method proposed by DUAN et al. (2005). Fresh leaves weighing 0.2 g were subjected to homogenization in a solution containing 10 ml of 10% trichloroacetic acid (TCA). Afterwards, the homogenized leaf suspension was spun in a centrifuge with the force gravity at 10.000 × g for 10 minutes. Then, 2 ml of 0.6% thiobarbituric acid (TBA) in a mixture with 10% TCA were added to 2 ml of the homogenate extract. After this, the compound was maintained in boiling water for a period of 30 minutes. This was subsequently followed by swift cooling of the compound from the heat via exposing it to a dry ice bath. Afterwards, the sample was subjected to a second round of centrifugation at 10.000 g for 10 minutes. After the completion of this process, the MDA content (mmol g^{-1} FW) was determined by measuring the supernatant absorbance at 450, 532, and 600 nm using a spectrometer.

The formula used for the calculation was: C (mmol g^{-1} FW) = 6.45(A532-A600) - 0.56 A450.

RESULTS

Effects of HMs on POD activity

The POD activity significantly increased with the Cu treatment under the exposure to 500 mg kg^{-1} and 1000 mg kg^{-1} (Figure 1). The greatest increase occurred with Cu where the POD content of *Indocalamus latifolius* rose by 32%, 29% and 9% under the exposure to 500 mg kg^{-1}, 1000 mg kg^{-1} and 2000 mg kg^{-1}, respectively, when compared with their controls. The corresponding increases for POD levels under Pb stress were 21%, 6% and 6%. Zinc caused the POD amount to rise by 27%, 24% and 15% under 500, 1000 and 2000 mg kg^{-1}, respectively, when compared to the control. At the augmented HMs level (2000 mg kg^{-1}), the greatest POD decline was observed

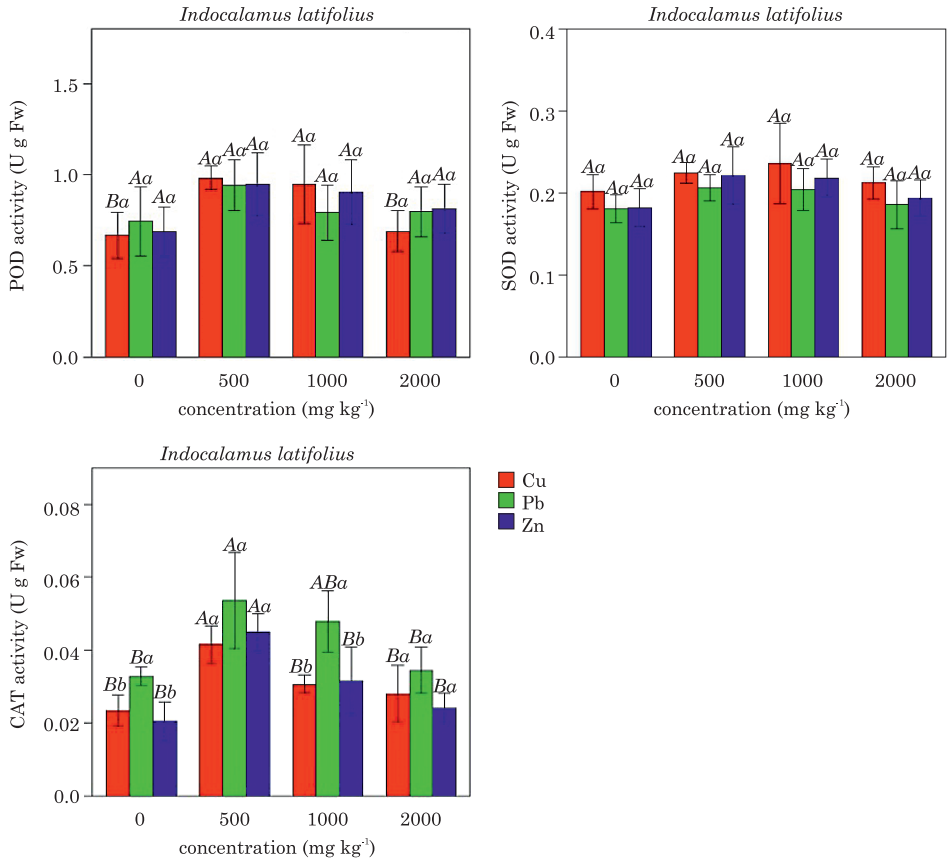

Fig. 1. Effects of HMs on antioxidant levels of *Indocalamus latifolius*. The capital letters denote statistical significance between different HMs across various concentrations, and the small letters denote statistical significance between HMs at each concentration. Vertical bars represent ± SD (n = 5)

under the Pb treatment, which was 1.23-fold lower than the highest value observed at 500 mg kg^{-1}.

Effects of HMs on SOD activity

As shown in Figure 1, the activity of SOD in all treatments remained unchanged. Across the mean of HMs concentrations, *Indocalamus latifoliuss* showed roughly 1.11-, 1.10- and 1.15-fold increase in its SOD amount under the Cu, Pb and Zn treatments, respectively, as compared to the control. Also, when averaged across HMs types, the SOD level raised by approximately 14% by 500, 1000 and 2000 mg kg^{-1} relative to the control, respectively.

Effects of HMs on CAT activity

The results revealed that there was a significant rise in the CAT activity when the bamboo species was treated with Pb at 500 and 1000 mg kg^{-1}. However, at the lowest HMs concentration (500 mg kg^{-1}), the CAT activity was enhanced more noticeably than in response to the other concentrations (Figure 1). When averaged across HM types, the greatest CAT level (0.053 U g^{-1} FW) was observed at the low concentration of 500 mg kg^{-1}, whereas the minimum value of the CAT content (0.024 U.g^{-1} FW) was detected at the extreme concentration of 2000 mg kg^{-1}. Across the mean of HMs concentrations, the CAT content was about 1.29-, 1.27- and 1.40-fold higher than the control under the Cu, Pb and Zn treatments, respectively. Moreover, when averaged across HMs types, the CAT level increased by approximately 45%, 30%, 11% over the control by 500, 1000 and 2000 mg kg^{-1}, respectively.

Effects of HMs on LP

Effects of HMs on MDA content

The MDA content significantly increased in our bamboo species at the extreme concentration of Pb (2000 mg kg^{-1}) – Figure 2. The MDA amount of

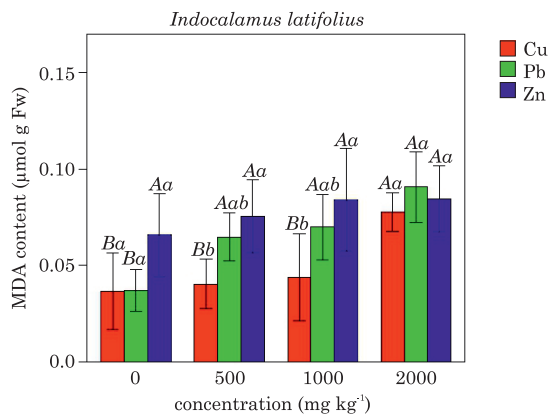

Fig. 2. Effects of HMs on MDA content of *Indocalamus latifolius*. The capital letters denote statistical significance between different HMs across various concentrations, and the small letters denote statistical significance between HMs at each concentration. Vertical bars represent ± SD (n = 5)

Indocalamus latifoliuss showed an approximately 1.32-, 1.50- and 1.19-fold rise over the control under the Cu, Pb and Zn treatments, respectively. Also, when averaged across HMs types, the MDA amount rose by about 23%, 29%, and 45% of the control under 500,1000 and 2000 mg kg^{-1}, respectively. Pb was the HM that induced the highest accumulation of MDA under the various concentrations relative to its control and Zn was the HM that was associated with the lowest release of MDA amounts.

DISCUSSION

Antioxidant enzymatic activities

One of the best biochemical mechanisms in plants for combating OS is the activation of key antioxidant enzymes including SOD, POD and CAT (Zayneb et al. 2015). These enzymatic scavengers may act synergistically to negate or attenuate the toxicity arising from OS. SOD is the first constituent of this defensive system that plays an important part in the production and activities of antioxidants via converting O2 •− into hydrogen peroxide (H_2O_2), which is subsequently broken down to water and oxygen by CAT and POD (Zayneb et al. 2015, Mostofa et al. 2015, Kang et al. 2015).

Overall, in our experiment, it was evident that with the application of any level of HM stress, there was an increase in the antioxidant enzyme activity of *Indocalamus latifolius* as compared to the control, where no HMs were applied. However, this increase in the plant enzymatic activity was more pronounced when it was treated with the lowest HMs concentration (500 mg kg^{-1}).

The protective antioxidant response of various plants when facing HMs stress has been reported in several studies, which mainly include the increased SOD and POD activities under Co, Ni, Cu, Zn and Cd stress in spinach (Pandey et al. 2009) or the activation of SOD, POD and CAT under Cu stress in bean (Yurekli, Porgali 2006) as well as the elevated levels of SOD and POD in *Pisum sativum* under Cu, Pb, Zn and Cd stress (Malecka et al. 2001). On the other hand, the functional capacity of antioxidants in plants depends on the efficacious manufacturing of antioxidant defense enzymes in the cells (Terzi, Yildiz 2015). It is suggested that cellular antioxidant levels are sometimes not adequate enough to reduce high amounts of ROS accumulated in cells as a result of stress (Kang et al. 2015).

As mentioned previously, we observed that amounts of antioxidants in the bamboo species tend to decrease when higher concentrations of HMs (1000 or 2000 mg kg^{-1}) are used relative to the lower HMs level (500 mg kg^{-1}). The drop in the content of antioxidants can be ascribed to the failure in the survival efforts made by plants to cope with the excessive accumulation of HMs. Thus, when HMs levels inside cells surpass the threshold level of a plant's defense system, HMs actively enter various plant tissues and organs, resulting in an inevitable exposure of the plant to OS and the excessive buildup of ROS (Ali et al. 2015). These events overwhelm the capacity of plant antioxidant enzymes (Zayneb et al. 2015). Therefore, accumulation of elevated levels of superoxide radical leads to the initiation of destructive processes in cellular compounds, impairing the transmission of stress signals that are responsible for triggering antioxidant genes.

The stress signal transduction in plant tissues is prohibited as some ions are blocked or replaced in important cellular groups by the excessive accu-

mulation of HMs (Dazy et al. 2009). It is reported that CAT and POD in the radish plant exhibited a downward trend in a time-specific manner under 200 mg kg^{-1} Cu stress, which suggests that CAT and POD were unable to extinguish H_2O_2 and residual free radicals left over from SOD scavenging activities (Sun et al. 2010). This phenomenon is confirmed by many researchers working on different plants and HMs where the diminished SOD, POD or CAT activities under Cr stress in mung bean (Jabeen et al. 2016), under Cu stress in turfgrass (Zhao et al. 2010) and under Pb stress in *Sesbania grandiflora* (Malar et al. 2014*a*) and water hyacinths *Eichhornia crassipes* Mart. (Malar et al. 2014*b*) are observed.

The data presented here show that the production of the enzymatic antioxidants i.e. SOD, POD and CAT in the bamboo species is disrupted by any sharp or high increase in the HM concentration. This may result in the infliction of damage to cell membranes and instability of their structural lipids.

Lipid peroxidation (LP) activities

LP causes disturbance in the efficiency of biological membranes and also increases their permeability. This results in amino acid oxidation and severe leakages of ions such as K^+ through membranes, eventually leading to cell death (Li et al. 2013). The plant's response to excessive levels of HMs obtained from MDA measurements shows precipitous induction of LP, which is an indication of the occurrence of cellular OS damage (Gonzalez et al. 2015, Zayneb et al. 2015). Hence, when there is a shortage of antioxidants, structural membrane lipids are attacked by ROS and MDA values demonstrate the extent of the destruction (Kang et al. 2015).

The results obtained in our study indicated that as the amount of HMs increased, the MDA content of bamboo species showed a rising trend. This can be attributed to the shortage of adequate antioxidant enzymes that are exhausted due to the growing amounts of ROS. The lack of effective and efficient production of antioxidant enzymes can be the reason as to why ROS enhances in large quantities in cells, which is associated with augmented levels of cellular free radicals, leading to the destruction of plasma membranes and increased LP in cell membranes of the bamboo species. The extent of the MDA increase depends on the tolerance or sensitivity of a plant species to cell damage induced by ROS concentrations (Gonzalez et al. 2015). In addition, the excess of HMs can directly lead to the enhanced production of LP, interferring with the membrane permeability and hindering the efficiency of enzymatic activities such as H+ -ATPase (Lomaglioa et al. 2015).

A number of experiments on the influence of various HMs on different plants e.g. Cd on sunflower, *Helianthus annuus* L. (Abd-Allah et al. 2015), Cu on turfgrass (Zhao et al. 2010), Zn on wheat plants (Li et al. 2013), corroborate the results of our study in terms of enzymatic antioxidant reactions in plants faced with HMs stress.

CONCLUSIONS

The results of the current work suggest that HMs (Cu, Pb, and Zn) at a low concentration (500 mg kg^{-1}) led to an increase in the amount of protective antioxidants in *Indocalamus latifolius*. However, higher HMs concentrations (1000 and 2000 mg kg^{-1}) resulted in enhanced lipid production. Amongst the elements tested in our experiment, Pb was found to be the strongest stimulator of MDA in *Indocalamus latifolius*.

The above findings provide a suggested outline for future work. Taking the results of our study into account, it is likely that there are considerable differences among different bamboo plants in response to HMs stress. Therefore, there is a great need to examine and evaluate various bamboo species by exposing them to different HMs in order to identify the species with a high degree of tolerance to HM stress. This will make it possible to find potential candidates for phytoremediation strategies. Measuring a tissue metal content is recommended for future research, as it can give some useful information about specific tolerance mechanisms that are employed by HM-stressed bamboo species.

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