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Physical and antioxidative responses of *Orthosiphon stamineus* towards various copper and lead concentrations

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ABSTRACT

Plants normally change their physiological and biochemical properties when exposed to heavy metal stress. We investigated the response of *Orthosiphon stamineus* towards different concentrations of Pb (0, 2, 5, 8 mg/L) and Cu (1, 2, 4, 5 mg/L). Heavy metals left in soil, plant physical characteristics, and the level of antioxidants in *O. stamineus* were determined. Our results showed that the tested Pb concentrations did not significantly affect stem elongation, but at 2 mg/L, Pb increased the leaf growth. Pb at 5 and 8 mg/L increased the total plant biomass. In contrast, 5 mg/L Cu treatment affected stem elongation and the root length of *O. stamineus*. The concentrations of Pb and Cu in soil were significantly reduced after the plants were harvested. Biochemically, 5 mg/L Pb had significantly increased the activity of catalase, while Cu at 5 mg/L significantly reduced the activity of superoxide dismutase and ascorbate peroxidase. Total flavonoid content increased in Pb-treated plants, but the total phenolics content decreased. Cu treatment at 2 mg/L, on the other hand increased the total phenolics content. Our results demonstrated that *O. stamineus* adapt to metal stress via physical changes, and scavenge oxygen radicals through enzymatic and non-enzymatic antioxidant productions.

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Introduction

Lead (Pb) and copper (Cu) derived from natural and anthropogenic activities are among the prevalent contaminants in the environment. Both metals are non-degradable and could produce stable complexes with organic compounds.[1] Despite the function of Cu as cofactor for many enzymes in plants, their excessive presence causes plant toxicity.[2] Pb, on the other hand has no specific function in plants, and becomes a protoplasmic poisonous element when present at significant amount.[3]

Excessive heavy metals accumulated in plants will involve in plants' physiological and biochemical reactions, thus affecting plant normal growth. Pb toxicity interferes with important processes in plants such as DNA synthesis, mitosis, photosynthesis, and seed germination.[3] It also induces leaf chlorosis as well as inhibits root and shoot growth.[4] Plants with excess amount of Cu also show similar symptoms,[5] although the effect is more pronounced on root growth. Cu normally accumulates in root tissues and only a small amount of Cu will be translocated to shoots.[6]

Heavy metal stress induces the production of reactive oxygen species (ROS). High concentrations of ROS will affect plant metabolisms.[7] Plants will react by the activation of antioxidant defense mechanisms. The first line of defense that protects plants against early events in oxidative damage involves enzymatic antioxidant activities such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT).[8]. The second line of defense involves non-enzymatic antioxidant activities such as the phenolics and flavonoids compounds to scavenge ROS and act as metal chelators.[9] Several recent reviews are available for further reading on ROS and plants under stress.[7,10]

In this study, we investigated the physical and antioxidative response of *Orthosiphon stamineus* towards different concentrations of Pb and Cu. *O. stamineus* is a medicinal plant, mainly grown in Asian countries. It can grow up to 1.5 m high with white or pale lilac flower. Due to various beneficial chemical substances such as

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flavonoid, diterpene, benzochromenes, and essential oils present in this plant, it has been widely used to treat diabetes, hypertension, gout, and kidney-related problems.[11] Flavonoid, for instance, has antioxidant, antimicrobial, antiviral, and anticancer activity[12], while diterpene has been used as an anticancer agent.[13] Besides that, a study by Sacchetti et al. [14] proved that essential oils react as antioxidants, antiradicals, and antimicrobials.

This species is also exposed to various environmental changes, including heavy metal stress. However, no study has been conducted specifically on the physical and biochemical response of *O. stamineus* towards heavy metal stress. In this study, we had three objectives: first, to determine the physical characteristics of *O. stamineus* exposed to different concentrations of Pb and Cu; second, to determine the enzymatic antioxidant activities of *O. stamineus* exposed to different concentrations of Pb and Cu, and third, to determine the total phenolics and total flavonoids contents of *O. stamineus* exposed to different concentrations of Pb and Cu.

Materials and methods

Plant materials

Matured *O. stamineus*, also known as *Orthosiphon aristatus* were transplanted using similar sizes of stem cutting in a 12.7 × 17.7 cm polybags. Voucher specimen (40212) was deposited at the herbarium of Universiti Kebangsaan Malaysia (UKM), Bangi, Malaysia. Copper (T1 = 1, T2 = 2, T3 = 4, T4 = 5 mg/L) and lead (T1 = 0, T2 = 2, T3 = 5, T4 = 8 mg/L) were applied to the soil at the early stage of growth. The range of metal concentration was selected based on our preliminary testing of soil metal concentrations that was subsequently used as control treatment. Plants were grown in a glasshouse in Universiti Teknologi Malaysia from September to December 2013. There were covered by shades and watered twice daily with tap water. During the growth, stem length and leaves number were recorded fortnightly. After 3 months, plants were harvested and physically characterized. The longest root was measured manually using a meter scale after removal from the soil. Plants were stored at −20 °C, while soil samples were kept at room temperature prior to analysis.

Determination of heavy metal content in soil

Prior to heavy metal analysis, soil samples were dried in the oven at 80 °C for 48 h. Dried samples were pulverized into powder, and approximately 0.5 g samples were subjected to acid digestion process. Nitric acid followed by perchloric acid (2:1) was added to the powdered samples. The digested sample was further diluted and aliquots were used for the estimation of Pb and Cu concentrations. The measurements of these metal elements were conducted using Atomic Absorption Spectrophotometer Model Analyst 400. Sample preparation and heavy metal analysis were conducted based on the standard method by American Public Health Association.[15]

Sample preparation for enzymatic antioxidant activity

Approximately 500 mg of plant samples were homogenized in cold extraction buffer (50 mM potassium phosphate buffer pH 7.0, 1% (w/v) polivinyl pyrolidone) using mortar and pestle. The homogenates were subjected to centrifugation at 15,000×*g* for 10 min at 4 °C and the supernatant was kept as enzyme extract.

Assay of SOD activity

SOD was determined using Superoxide Dismutase Assay Kit II (Calbiochem, SanDiego, CA, USA). This assay utilizes a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The absorbance was read at 450 nm. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

Assay of CAT activity

CAT activity was assayed in a reaction solution containing 50 mM phosphate buffer (pH 7.0), 150 mM H_2O_2, and 200 µl of enzyme extract.[16] The activity of catalase was estimated by the decrease in absorbance at 240 nm for 1 min as a consequence of H_2O_2 consumed.

Assay of APX activity

APX activity was determined as described by [17], based on the decrease in the absorbance of ascorbate at 290 nm. The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 6 mM H_2O_2 and 200 µl enzyme extract.

Preparation of aqueous extracts

Dried plant samples were pulverized using mortar and pestle and extracted with autoclaved deionized water at a 1:40 (dry weight:volume) ratio at 90 °C for 1 h. The heat-incubated homogenate was vacuum-filtered, and the filtrate was centrifuged at 9000 rpm at 4 °C for 15 min. The supernatant obtained was immediately aliquoted (500 µl each) and stored at −20 °C until used.

Determination of total phenolic content

The concentration of total phenolics in the extracts was determined using a Folin–Ciocalteu colorimetric assay.[18] A mixture of extract (0.2 mL), deionized water (0.8 mL), and Folin–Ciocalteu reagent (0.1 mL) was first incubated at room temperature for 3 min. Next, 0.3 mL of Na_2CO_3 (20% w/v) was added and the mixture was incubated at room temperature for 120 min. Absorbance of the mixture was recorded at 765 nm. A standard curve was prepared from 0 to 100 mg/L gallic acid. Total phenolic content was expressed in mg gallic acid equivalents/g dry matter.

Determination of total flavonoid content

The concentration of total flavonoids in the extracts was determined using an assay modified from a method by Zou et al. [19]. Plant extract (0.2 mL) was added to 0.15 mL of $NaNO_2$ (5% w/v) and the mixture was incubated at room temperature for 6 min. Then, 0.15 mL of $AlCl_3{\cdot}6H_2O$ (10% w/v) was added to the mixture and incubated for 6 min at room temperature, followed by the addition of 0.8 mL of NaOH (10% w/v). The mixture was incubated at room temperature for 15 min and absorbance of the mixture was recorded at 510 nm. A standard curve was prepared from 0 to 500 µg/mL quercetin dissolved in 80% ethanol and total flavonoid content was expressed in mg quercetin equivalents/g dry matter.

Data analysis

Experiments were carried out in triplicates. Data were analyzed using Microsoft Excel 2007 and reported as mean ± standard error. Student's *t* test was used at the 0.05 level of probability for comparison of the set of means.

Results and discussion

Heavy metal removal in soils

Pb and Cu concentrations left in the respective soils were quantified using AAS (Figure 1). Almost 100% reductions of Pb had been achieved after Pb-treated plants were harvested. For Cu-treated plants, the highest reduction of Cu from the soil was determined in 5 mg/L Cu treatment (93.2%). It was noticeable that, the higher the concentrations of heavy metals applied, the higher percentages of metals were removed from the soil. These results were consistent with the previous findings on the capability of *O. stamineus* to reduce the amount of metals in plant medium containing sewage sludge.[20] High removal of heavy metals from the soil might be due to active absorption of soluble metals by the root systems. These metals could be transported to aerial parts of the plant with the aid of transport proteins or immobilized in the vacuoles.[21]

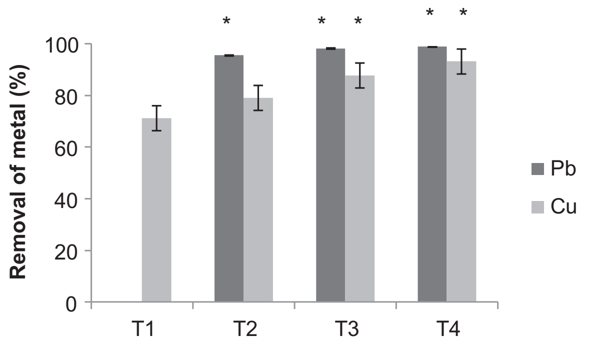

Figure 1. Removal of Pb and Cu from soil after the harvest of *O. stamineus* Data are means ± standard errors (n = 3). An asterisk denotes significant difference between the mean values of treatment, compared to T1 as determined by using Student's *t* test at $p < 0.05$.

Physical characteristics of metal-treated O. stamineus

The growth of *O. stamineus* was monitored by measuring the length of stems and counting the number of normal leaves fortnightly. The percentages of increment in stem length and leaves number were computed when the plants have been harvested (Figure 2). The highest concentration of Cu, 5 mg/L had significantly contributed to the lowest percentage of stem elongation (28.7%

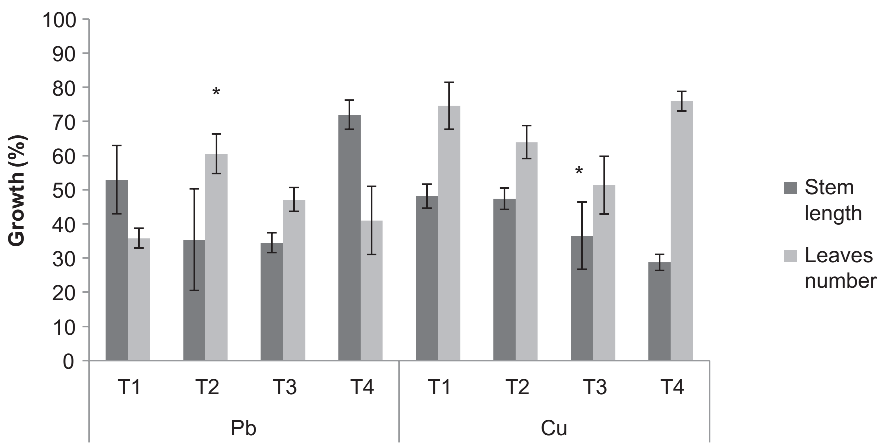

Figure 2. Growth percentage of *O. stamineus* in Pb and Cu treatments based on stem length and leaves number. Data are means ± standard errors (n = 3). An asterisk denotes significant difference between the mean values of treatment, compared to T1 as determined by using Student's *t* test at $p < 0.05$.

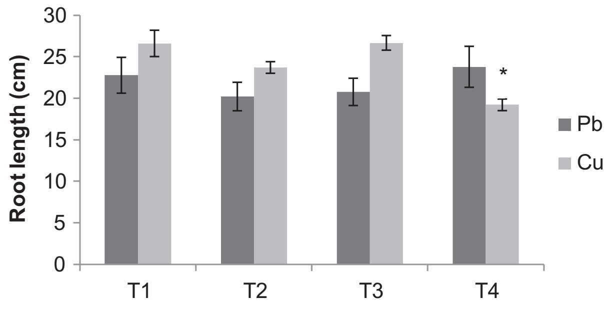

Figure 3. Root length of *O. stamineus* in Pb and Cu treatments. Data are means ± standard errors (n = 3). An asterisk denotes significant difference between the mean values of treatment, compared to T1 as determined by using Student's t test at $p < 0.05$.

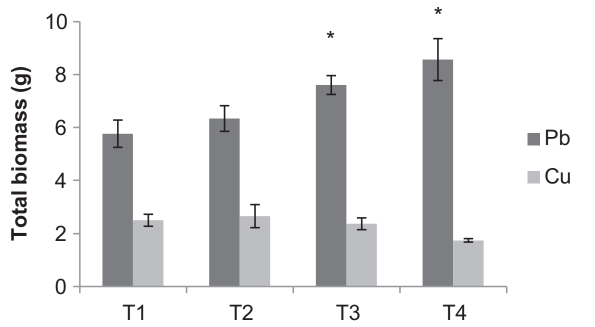

Figure 4. Total biomass *O. stamineus* in Pb and Cu treatments. Data are means ± standard errors (n = 3). An asterisk denotes significant difference between the mean values of treatment, compared to T1 as determined by using Student's t test at $p < 0.05$.

stem length increment). This indicates the distortion of stem elongation. On the other hand, stem elongation for Pb-treated plants were not significantly affected by the tested Pb concentrations. For number of leaves, Pb at 2 mg/L increased the normal leaves number to 60.5% from the time of planting to the time of harvest. High percentage of leaves number increment could be one of the responses showed by the plants towards foreign elements. However, the increment percentages of leaves numbers were reduced when the plants were treated with 5 and 8 mg/L of Pb. At both concentrations, the symptoms of necrosis were visible as a signal of Pb toxicity.[22] Cu treatments, on the other hand did not affect the percentage of normal leaves growth at all.

The longest roots for plants from each treatment were measured (Figure 3). Our results showed that the root length of *O. stamineus* remained unaffected with different concentrations of Pb. On the other hand, the highest concentration of Cu (5 mg/L) had significantly reduced the root length which was 30% less than the control. These results further confirmed that Cu disrupts the development of plant cells as reported previously. Cu inhibit cell elongations, thus stem growth and root length were affected.[23]

Total biomass from the leaves, stems, and roots of *O. stamineus* were determined (Figure 4). We found that 5 and 8 mg/L Pb treatments significantly increased the total biomass of *O. stamineus*, with 31.9 and 48.6% higher than T1, respectively. Although Pb reduced plant biomass in many previous cases, the increased biomass in plants exposed to Pb has also been reported. Pyroligneous acid, fertilizer containing Pb had been shown to promote the growth of rockmelon, when used at appropriate amounts.[24] Other than that, hydroponically grown *Sesbania exaltata*, with increasing amount of supplied Pb showed increasing root and shoot biomass during the earlier stage of exposure, although the trend changed after sometime.[25] Hyperaccumulator species, *Thlaspi caerulescens* promotes root growth when exposed to Zn and Cd.[26] However, the mechanism underlying this situation is still unclear. One of the reasons that promote positive growth is due to microbial activities in the soil and roots [27], besides unique responses of hyperaccumulators when exposed to certain level of heavy metals. Cu treatment, however, did not significantly affect the total fresh weight of *O. stamineus*.

Enzymatic antioxidant activities in O. stamineus *in Pb and Cu treatments*

The SOD, APX, and CAT activities varied in Pb- and Cu-treated plants (Table 1). In Pb-treated plants, the changes in SOD and APX activities at different Pb concentrations were not significant. However, the activity of CAT significantly increased at almost three-fold in 5 mg/L Pb. Our results showed that CAT activities were not significantly affected by different Cu treatments. However, SOD activities were significantly reduced in 5 mg/L Cu, with 2.4-fold lower than T1. Similarly, APX activities were

Table 1. Enzymatic antioxidant responses of *O. stamineus* towards Pb and Cu stress.

	SOD (U/g)	CAT (µmol H_2O_2 decomposed/min/mg protein)	APX (nmol ascorbate oxidized/min/mg protein)
	Pb	Pb	Pb
T1	0.927 ± 0.033	0.625 ± 0.040	3.173 ± 0.453
T2	1.170 ± 0.146	0.916 ± 0.093	2.446 ± 0.489
T3	1.311 ± 0.140	1.860 ± 0.155*	3.434 ± 0.656
T4	1.109 ± 0.067	0.932 ± 0.115	2.659 ± 0.767
	Cu	Cu	Cu
T1	29.571 ± 3.690	12.657 ± 2.311	21.920 ± 5.629
T2	23.231 ± 7.600	5.881 ± 0.714	30.176 ± 1.310
T3	17.062 ± 0.830	9.996 ± 2.250	35.868 ± 8.102
T4	12.379 ± 1.578*	11.115 ± 3.377	6.836 ± 1.952*

Notes: An asterisk denotes significant difference between the mean values of treatment, compared to T1 as determined by using Student's t test at $p < 0.05$. For SOD, 1 U is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

Table 2. Total phenolic and flavonoid contents of *O. stamineus* in Pb and Cu treatments.

	Total phenolics (mg GAE/g)	Total flavonoids (mg quercetin/g)
	Pb	Pb
T1	47.265 ± 0.584	0.341 ± 0.033
T2	46.229 ± 0.891	0.340 ± 0.006
T3	40.516 ± 1.573*	0.498 ± 0.042*
T4	40.438 ± 0.916*	0.357 ± 0.007
	Cu	Cu
T1	4.480 ± 0.837	0.093 ± 0.009
T2	8.372 ± 0.128*	0.073 ± 0.001
T3	2.765 ± 0.156	0.099 ± 0.002
T4	3.730 ± 0.639	0.087 ± 0.004

Note: An asterisk denotes significant difference between the mean values of treatment, compared to T1 as determined by using Student's *t* test at $p < 0.05$.

significantly 3.2-fold lower in 5 mg/L Cu compared to T1. Overall, the APX level was quite low, indicating that *O. stamineus* preferably activated their catalase pathway to scavenge ROS during metal stress. In stress response, ROS will be converted to H_2O_2 by SOD prior to the activity of CAT or APX to dismutate H_2O_2 to water and oxygen.[28] At certain amounts of heavy metals, the activities of these antioxidant enzymes might be inhibited as reported previously in other plants.[29,30]

Non-enzymatic antioxidant content of O. stamineus *in Pb and Cu treatments*

Analysis of ethanol extracts of *O. stamineus* treated with different Pb and Cu concentrations revealed different trends of total phenolics and total flavonoids content (Table 2). The total phenolics markedly decreased in Pb-treated plants. The highest concentration of Pb, 8 mg/L revealed 1.17-fold decrease in total phenolics, when compared to T1. Total flavonoids increased significantly at 5 mg/L Pb treatment, a 1.5-fold higher than T1. Despite the increase in total flavonoids and the decrease in total phenolics for Pb-treated plants, the activities were more pronounced in the latter, whereas, for Cu-treated plants, a 1.9-fold increase in total phenolics was recorded in 2 mg/L. The production of antioxidant compounds are normally triggered by heavy metal stress, but it can be inhibited at certain level of stress.[31]

Conclusion

Our study suggests that plants, specifically *O. stamineus* were actively responding towards Pb and Cu stress by expressing different visible physical characteristics. On top of that, biochemical adaptation mechanisms including the activation or inhibition of enzymatic antioxidant activities and the production of non-enzymatic antioxidants, such as total phenolics and total flavonoids are also important for plants to combat stress. Both provide resistant mechanisms against heavy metal stress, alongside with other biochemical pathways that help them survive metal stress.

Disclosure statement

No potential conflict of interest was reported by the authors.

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