Synergistic and Antagonistic Effects of Zinc Bioaccumulation with Lead and Antioxidant Activities in *Centella asiatica*
(Kesan Sinergistik dan Antagonistik oleh Bioakumulasi Zink dengan Plumbum dan Aktiviti Antioksidan *Centella asiatica*)

G.H. ONG, C.K. YAP*, M. MAZIAH & S.G. TAN

ABSTRACT

This study was carried out by using Centella asiatica *grown using a hydroponic system under laboratory conditions to study synergistic and antagonistic effects of Zn bioaccumulation with added Pb and the changes in antioxidant activities in leaves and roots of* C. asiatica. *The antioxidant activities included superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX). The treatments Zn (2 ppm) + Pb (0.4 ppm) and Zn (4 ppm) + Pb (0.6 ppm) increased the accumulation of Zn in leaves by 14.06 and 16.84%, respectively, but decreased by 7.36% uptake in roots (Zn 4 ppm + Pb 0.6 ppm). This showed that Pb and Zn acted synergistically to Zn accumulation in leaves but antagonistically in roots. CAT and SOD activities in leaves were increased when Zn was added together with Pb. In roots, CAT, APX and SOD activities were increased but GPX was decreased. Owing to their sensitivities to Zn with Pb, SOD and CAT could be used as biomarkers to monitor the toxicity of Pb and Zn exposure in the leaves and roots of* C. asiatica.

Keywords: Antagonistic; antioxidant activities; Centella asiatica*; synergistic*

ABSTRAK

Kajian ini telah dijalankan dengan Centella asiatica *secara hidroponik dalam keadaan makmal untuk mengkaji kesan sinergistik dan antagonistik Bioakumulasi Zn ditambah dengan Pb dan perubahan dalam aktiviti antioksidan dalam daun dan akar* C. asiatica. *Aktiviti antioksidan termasuk superokside dimustase (SOD), katalase (CAT), peroksidase askorbat (APX) dan peroksidase guaiacol (GPX). Rawatan Zn (2 ppm) + Pb (0.4 ppm) dan Zn (4 ppm) + Pb (0.6 ppm) menunjukkan pengumpulan Zn dalam daun sebanyak 14.06 dan 16.84% masing-masing tetapi menurun sebanyak 7.36% dalam pengambilan akar (Zn 4 ppm + Pb 0.6 ppm). Ini menunjukkan bahawa Pb dan Zn bertindak secara sinergistik untuk pengumpulan Zn dalam daun tetapi antogonistik dalam akar. Aktiviti CAT dan SOD dalam daun meningkat apabila Zn ditambah bersama dengan Pb. Dalam akar, CAT, APX dan SOD telah meningkat tetapi GPX telah menurun. Disebabkan sentiviti kepada Zn dengan Pb, SOD dan CAT boleh digunakan sebagai penanda biologi untuk memantau ketoksikan pendedah Pb dan Zn dalam daun dan akar* C. asiatica.

Kata kunci: Aktiviti antioksidan; antagonistik; Centella asiatica*; sinergistik*

INTRODUCTION

Pb contamination is resulted from exhaust fumes of automobiles, chimneys of factories using Pb, mining or smelting of Pb ores, fertilizers and pesticides (Eick et al. 1999). In the environment, Pb tends to accumulate in the surface ground layer and its concentration decreases with soil depth (de Abreu et al. 1998). Pb is a non-essential metal and is considered as a protoplasmic poison which is cumulative, slow acting and subtle. Pb toxicity symptoms such as restricted growth have been observed on plants at 1.0 mM Pb supply (Sinha et al. 2006). Pb can be accumulated in plants and animals and transferred to humans through the food chain.

The abilities to absorb and accumulate heavy metals are different for different plant species, varieties and tissues. The feasibility of using crop species or varieties with low heavy metal accumulation capabilities decreasing the risk of heavy metals entering the food chain from contaminated soil should be studied. In this study, we focused on *Centella asiatica* (family: Umbelliferae) which had been widely used in folk medicine for hundreds of years (Brinkhaus et al. 2000). It is also listed as one of the useful medicinal herbs by WHO (1999). In Malaysia, it is used for rapidly healing small wounds, chaps and scratches and surgical wounds.

This studies was focus on the sources of Pb uptake, its transportation within the plant and physiological changes due to Pb toxicity (An et al. 2004; Peng et al. 2005), but lack of information about the effects of Pb on the uptake of Zn. Zn is one of the essential micronutrients for plants (Broadley et al. 2007) and its concentration in vegetable foodstuffs were found to be in the range of 25.2 - 50.0 mg/kg. However, 300 to 500 mg/kg of Zn is considered toxic to plants (Miransari 2011). Therefore, it is important to know the optimum range of Zn concentration to plant. The current study focused on the effects of increasing

both Zn and Pb concentrations rather than increasing Zn concentration alone.

In nature, compounds of heavy metals can act either additively, synergistically or antagonistically (Wu et al. 1995). These toxic heavy metals will trigger the production of reactive oxygen species (ROS) and inducers in the antioxidant system of a plant as a mechanism to protect itself (Foyer & Noctor 2005; Foyer et al. 2009; Parra-Lobato et al. 2009) The effects of Pb to Zn uptake and their actions on antioxidant enzymes to protect plants in various compartments of the plant cell are unclear. The synchronous actions of antioxidant activities are parts of the system that protects plants against ROS in various compartments of the plant cell (Singh et al. 2006). Thus, the objective of this study was to determinate the effects of Pb exposure on the bioaccumulation of Zn and on antioxidant activities in different parts of *C. asiatica*.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

Young stems (1-2 months) of *C. asiatica* obtained from the University Agriculture Park (UAP), Universiti Putra Malaysia were planted in a greenhouse. The hydroponic experiment was carried out in the greenhouse with light density around 2500 Lux in modified Hoagland nutrient solution (Tang et al. 2009).

The plants were subjected to Zn and Pb at different concentrations as mentioned in Table 1. In the experiment, different concentrations were used for different heavy metal levels based on phytotoxicity of the trace metals where Pb is more toxic than Zn (Kopittke et al. 2010). Hence, Zn1 (2 ppm), Zn2 (4 ppm), Zn3 (6 ppm), Pb1 (0.4 ppm), Pb2 (0.6 ppm) and Pb3 (0.8 ppm) were used with nominal concentrations of Zn1 (1.85 ± 0.08 ppm), Zn2 (3.85 ± 0.17 ppm), Zn3 (5.90 ± 0.12 ppm), Pb1 (0.42 ± 0.03 ppm), Pb2 (0.59 ± 0.01 ppm) and Pb3 (0.79 ± 0.02 ppm). The toxicity tests were carried out for 20 days in green house conditions. During this period, the solutions were changed every 10 days. The pH of the nutrient solutions were adjusted to be around pH5.8. Five plants per tray were used for each treatment. Two replicates were carried out for each treatment and the whole experiment was repeated twice.

TABLE 1. Laboratory treatments and their abbreviations

No	Treatments	Metals added
1.	C	No metals added
2.	Zn1	Zn (2 ppm)
3.	Zn2	Zn (4 ppm)
4.	Zn3	Zn (6 ppm)
5.	ZnPb1	Zn (2 ppm) + Pb (0.4 ppm)
6.	ZnPb2	Zn (4 ppm) + Pb (0.6 ppm)
7.	ZnPb3	Zn (6 ppm) + Pb (0.8 ppm)

At harvest, the roots were immersed into 20 mmoll^{-1} Na$_2$-EDTA for 15 min to remove the metals adhering to the root surface due to long term direct contact with treatment solution (Yang et al. 2002). The leaves and roots were separated and thoroughly washed three times with de-ionized water.

ACCUMULATION HEAVY METAL ANALYSIS

The harvested plants were separated into two different parts namely leaves and roots. The sample digestion was done following the method of Yap et al. (2010). The separated plant tissues were then dried in an oven for 72 h at 60ºC to constant dry weights. About 0.5 g of dried plant tissue parts were weighed using an analytical balance. Ten mL of concentrated nitric acid (AnalaR grade, BDH 69%) were added to a digestion tube to digest the plant tissues. After that, the digested samples were left to be cooled down and were topped up (diluted) to 40 mL with double de-ionized water. The solution was filtered through Whatman No. 1 filter paper into acid-washed pill box and stored in a safe place until metal concentration determination.

All the samples stored in acid-washed pill boxes were analyzed using an air-acetylene Perkin-Elmer™ flame atomic absorption spectrophotometer model AAnalyst 800. Blank determination was carried out for calibration of the instrument. All data obtained from the ASS were presented in µg/g dry weight basis.

To avoid possible contamination during metal analysis, all equipment and glassware were acid washed in 10% nitric acid solution. They were soaked in acid solution for 2 to 3 days and later rinsed with double distilled water before use. The recovery of Zn and Pb were 85.86 and 95.47%, respectively, compared to DOLT-3 Dogfish-liver CRM value.

ANTIOXIDATIVE ENZYMES

The enzyme extraction of leaves and roots was done following the method of Mishra et al. (2006). About 0.2 g of (leave and root) fresh tissues was homogenized in an ice-cooled mortar with 5 mL of 100 mM potassium phosphate buffer (pH7.0) containing 0.1 mM EDTA and 1% (w/v) polyvinylpyrrolidone. The homogenate was transferred to 1.5 mL Eppendorf tube and centrifuged at 15000 g for 15 min at 4°C (Mishra et al. 2006). The supernatant was used for enzyme determination. All enzymes activities were measured using UV-spectrophotometers.

The activity of superoxide dismutase (SOD) was assayed by measuring the inhibition of the photochemical reduction of nitrobluetetrazolium (NBT) at 560 nm for 3 min (Beauchamp & Fridovich 1971). The assay mixture contained 1.5 mL of 50 mM phosphate buffer (pH7.8), 0.3 mL of 130 mM methionine, 0.3 mL of 750 µM NBT, 0.3 mL of 0.1 mM EDTA, 0.3 mL of 20 µM riboflavin, 0.05 mL of enzyme extract and 0.25 mL of deionized H$_2$O in a total volume of 3 mL. Riboflavin was added last and the tubes were shaken and then illuminated for 15 min.

The assay of catalase (CAT) activity was done based on the method of Aebi (1984) at 240 nm for 3 min. The assay mixture contained 0.2 mL of tissue extract, 1.5 mL of 50 mM phosphate buffer (pH7.8), 1.0 mL of deionized H_2O and 0.3 mL of 0.1 M H_2O_2. H_2O_2 was added at the end. Guaiacol peroxidase (GPX) activity was determined following the oxidation of guaiacol by the method of Hemeda and Klein (1990) with modifications at 470 nm for 3 min. The assay mixture contained 2.9 mL of 50 mM phosphate buffer with pH6.0, 1.0 mL of 2% H_2O_2 and 0.1 mL of the enzyme extract. H_2O_2 was added at the end.

Ascorbate peroxidase (APX) activity was determined by the method of Nakano and Asada (1981). The assay mixture contained 3 mL of reaction mixture containing 1.8 mL of 50 mM phosphate buffer (pH7, containing 0.2 mM EDTA-Na_2), 0.1 mL of 7.5 mM ascorbic acid, 1 mL of 300 mM H_2O_2 and 0.1 mL of enzyme extract. H_2O_2 was added at the end. The change of absorbance was monitored at 290 nm by using an UV-spectrophotometer. Total protein assay was carried out based on the Bradford (1976) method at 595 nm. The Bradford reagent was prepared by dissolving 0.1 g of Coomassie Brilliant Blue G-250 in 50 mL of 95% ethanol and 100 mL of 85% phosphoric acid. The total protein content was determined as mg of BSA equivalent by using the equation obtained from the standard curve of BSA.

For antioxidant activity determination, the plant samples were stored in an ice box (5ºC) after being harvested to ensure optimal enzyme activities. All the solutions for antioxidant enzymes were freshly prepared to ensure optimal antioxidant enzyme reactions.

STATISTICAL ANALYSIS

Statistical analyses were done by using the statistical software, SPSS software version 17.0 for Windows for analysis of variance (ANOVA), SNK and Post hoc test. Besides that, the STATISTICA version 8 software was also used to determine the correlation coefficient (Zar 1996).

RESULTS

The results can be categorised into three groups which were Zn1, Zn2 and Zn3 with Pb added based on the Zn accumulations. Zn accumulations in leaves showed significant increase ($p<0.05$) for treatment Zn1 and Zn2 with Pb when compared with control and treatment of Zn alone (Figure 1). The roots showed a significant decrease ($p<0.05$) in Zn accumulation in treatment Zn2 with Pb when compared with control and treatment of Zn alone in Figure 1.

Figure 2 shows the levels of antioxidant enzymes in leaves caused by different treatments. The CAT of Zn1 with Pb showed a significant increase ($p<0.05$) when compared with control and treatment with Zn alone. For SOD, only treatment Zn3 with Pb showed a significant increase ($p<0.05$). APX and GPX did not show any significant changes ($p<0.05$) of enzymes level when compared to treatment with Zn alone and control.

For CAT in roots (Figure 3), treatments Zn1 and Zn2 with Pb showed significant increases ($p<0.05$) when compared with treatments Zn1 and Zn2 alone. APX also showed a similar trend with significant increase ($p<0.05$) in Zn 1 with Pb when compared with treatment Zn1 alone. For SOD, treatments Zn2 and Zn3 with Pb showed significant increases ($p<0.05$). Lastly, GPX showed the opposite trend with significant decreases ($p<0.05$) in Zn1 and Zn2 with Pb when compared with treatment with Zn alone and control.

DISCUSSION

Zn ACCUMULATION IN LEAVES AND ROOTS

From Figure 1, the accumulation in roots was higher than in leaves at about 8.90%. This was supported by the results of Soares et al. (2001), Singh and Sinha (2005) and Tang et al. (2009). This was because the roots were the first organ in plant to come in contact with the metals and the roots adhere to the soil at all time, thus increasing the chances of metal accumulation in roots. Furthermore, the large surface area of roots dues to root hairs elevates the adsorption and absorption of metals in roots (Yap et al. 2010). Roots also function as the site of water and nutrient uptake of plants by osmosis. Therefore, all the metals uptake of the plant must pass through the roots before reaching the other parts (Clemens et al. 2002).
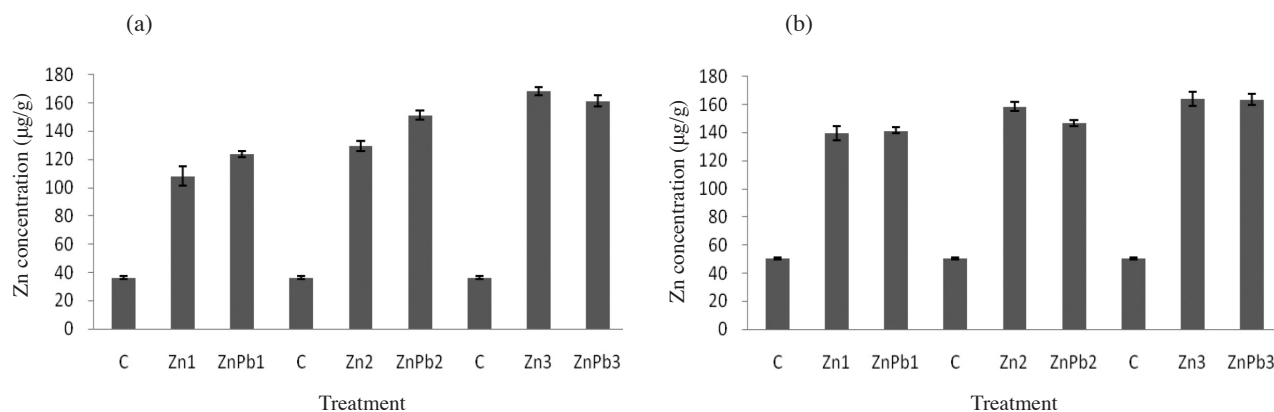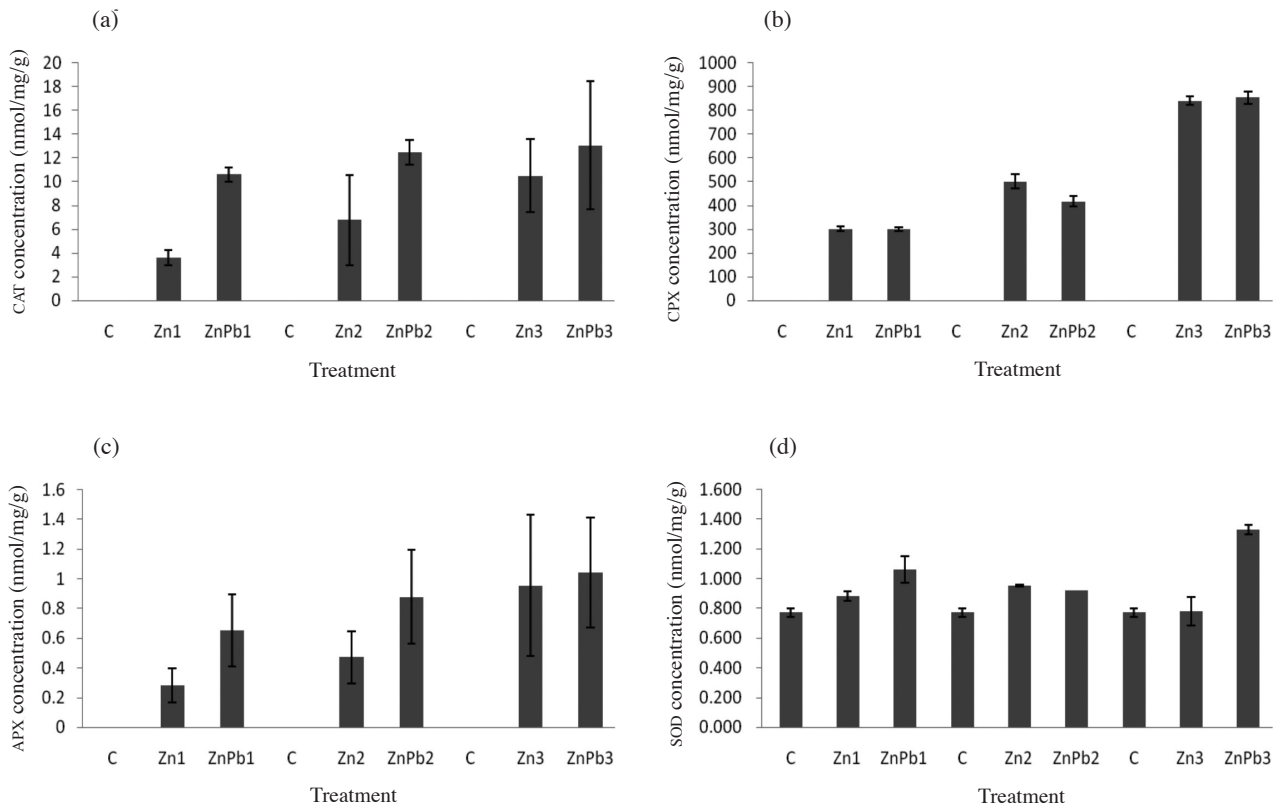

FIGURE 1. Concentrations (mean ± SD, µg/g dry weight) of Zn in (a) leaves and (b) roots for toxicity testing ($n=3$)

(a)

(b)

(c)

(d)

Note: CAT, GPX and APX for the control (C) line were blank due to the reading having been subtracted by the particular enzymes but SOD had its own control for the calculation. For the concentrations of SOD, CAT and APX, their actual values were multiplied by 1000
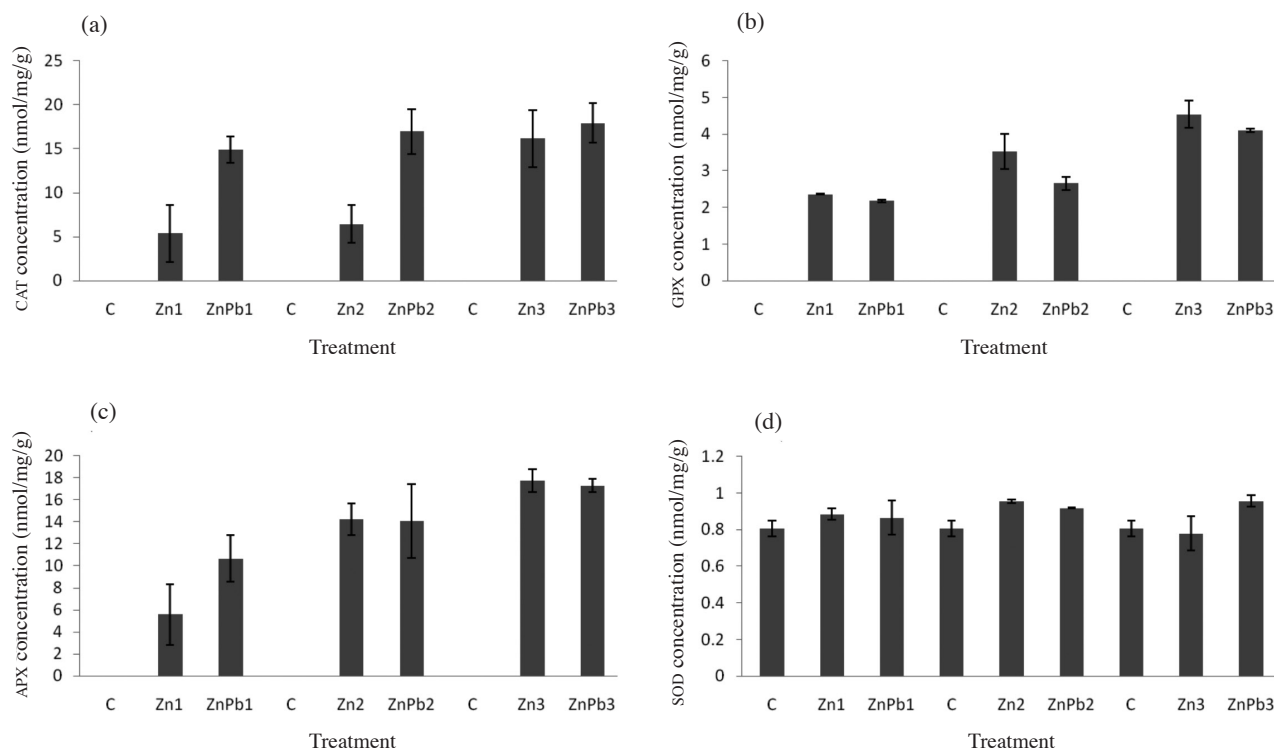
FIGURE 2. Concentrations (mean ± SD, nmol/mg/g) of antioxidant enzymes (a) CAT, (b) GPX, (c) APX and (d) SOD in leaves of *Centella asiatica* from different treatment of Zn ($n=2$)

Hence, the excess metals that were not further transported upwards by the plant would be accumulated in the roots. The high accumulation of heavy metals in roots also resulted from the complexation of heavy metals with sulphydryl groups causing lesser translocations of metals to the shoots (Singh & Sinha 2005). These deductions were supported by the results of Street et al. (2009) and of Rout & Das (2009) in which the roots accumulated more metals than the shoots in plants.

Our findings showed an increase of Zn accumulation in the leaves of Zn1 and Zn2 when Pb was added. This increment was mostly due to the synergistically response between Zn and Pb since Zn uptake was increased by the addition of Pb. The Zn accumulation for Zn1 was increased from 108.26 to 123.49 µg/g while for Zn2 the increase was from 129.52 to 151.36 µg/g. This was supported by the study of Israr et al. (2011); in which an accumulation 1873 µg/g of Zn was observed with the addition of Pb when compared with the treatment with only Zn alone which showed an accumulation of 1180 µg/g in *S. drummondii* seedlings. Sinha et al. (2006) reported that with an increase in Pb supply, the concentration of Pb and Zn was increased in various parts of cabbage. The effects of different concentrations of Zn and Pb combinations showed a synergistic relationship at low concentrations (Aery & Rana 2007). For treatment

Zn3, it did not show any significant changes due to the toxicity exert by Zn with Pb beyond the tolerance range. Hence, the uptake of Zn by the plant was controlled by a physiological mechanism where excess Zn in the environment was not up taken into the plant. Therefore, increases of Zn concentrations in the solution did not increase the uptake of Zn by the plant.

In contrast, the Zn accumulation in roots showed a decrease (antagonistically) when Pb was added in treatment Zn2. Pb is a major contaminant and notorious for its lack of soil mobility primarily due to metal precipitation as insoluble phosphates, carbonates and hydroxides (Blaylock & Huang 1999). This affects Zn uptake into plants since Pb reduces the mobility of Zn by forming precipitates. The effects of different concentrations of Zn and Pb combinations showed an antagonistic relationship in high concentrations as also reported by Aery and Rana (2007). For treatment Zn1, it did not show any significant changes due to low Pb concentrations which reduced the formation of precipitate. This was supported by Wong et al. (1986) where Pb did not appear to have any effect on the total uptake and distribution of Zn in the roots of plants when it occurred in low concentrations. Zn3 also did not show any significant changes due to higher concentrations of Zn which overcame the Pb precipitation. This showed that Zn had greater mobility in plants when present at

Note: CAT, GPX and APX for the control (C) line were blank due to the reading having been subtracted by the particular enzymes but SOD had its own control for the calculation. For the concentrations of SOD, CAT and APX, their actual values were multiplied by 1000

FIGURE 3. Concentrations (mean ± SD, nmol/mg/g) of antioxidant enzymes (a) CAT, (b) GPX, (c) APX and (d) SOD in roots of *Centella asiatica* from different treatment of Zn (*n*=2)

high concentrations (Meers et al. 2007; Zheljazkov et al. 2006). The decreases of Zn accumulation in roots in Zn2 with Pb addition were supported by Israr et al. (2011) in which the accumulation of Zn decreased from 10360 μg/g (Zn) to 8000 μg/g (Zn + Pb) in *S. drummondii* seedlings.

CHANGES IN ANTIOXIDANT ENZYME ACTIVITIES IN Zn WITH Pb AND Zn ALONE TREATMENTS

The accumulation of metals in plants depend on some physiological processes which required the cells to conserve intracellular heavy metal ions in non toxic forms (Cobbett 2000). Pb was considered toxic to plants; therefore the plant would undergo a series of mechanisms to reduce the toxicity effects of Pb (Sharma & Dubey 2005). As expected, the enzyme activities were increased when higher concentrations of metals were added into the nutrient solution. Different cell compartments may activate different defence systems to reduce excessive ROS (Sharma et al. 2012). The results suggested that the plant's ability to induce antioxidant enzymes appeared to be crucial for its protection from metal stress.

The leaves and roots showed similar results for the SOD enzyme. The main response of plants to Pb and Zn was to generate SOD to accelerate the dismutation of O_2^- to H_2O_2 (Sarvajeet & Narendra 2010). The metalloenzyme SOD is the most effective intracellular enzymatic antioxidant which is ubiquitous in all aerobic organisms and in all

subcellular compartments prone to ROS mediated oxidative stress (Mittler 2004). SOD is important in plant stress tolerance and provides the first line of defence against the toxic effects of elevated levels of ROS. SOD is the main response and is directly affected by oxidative stress caused by heavy metal. This reaction has a 10000 fold faster rate than spontaneous dismutation (Mittler 2004). For each of these reactions, two molecules of H_2O_2 are produced causing high accumulation of it in plants. Increases of SOD due to Pb in *Oryza sativa* were reported by Verma and Dubey (2003).

CAT is able to scavenge H_2O_2 to water and molecular oxygen and is localized in mitochondria and peroxisomes which can be found in large quantities in leaves and roots (Kuk et al. 2003; Starzynska et al. 2003). The change in CAT level in leaves showed a similar trend as Zn accumulation in the leaves for treatment Zn1 (Figure 1). When Zn accumulation increased due to interaction with Pb, the CAT level also increased to overcome the oxidative stress. The CAT level increased even though the accumulation of Zn in roots showed decreases in Figure 1. This was because high precipitate of Pb occured in the roots causing high toxicity to the plant. This was supported by Yan et al. (2010) who reported that the accumulation of Pb occurred mainly in roots. Hence, the CAT level was increased due to the increase of H_2O_2 production after activation of SOD caused by Pb. Excessive Zn and Pb concentration cause oxidative stress which in turn increases the reactive oxygen

species (ROS) within the subcellular compartments (Mittler et al. 2004).

APX was also involved in the scavenging of H_2O_2 in water-water and ASH-GSH cycles (Neill et al. 2002) and utilized ASH as the electron donor. Hence, higher levels of APX in roots were required to alleviate the higher production of H_2O_2 caused by the accumulation of Zn and Pb (Neill et al. 2002). This indicated that the high toxicity caused by the direct exposure of Pb with Zn activated the enzyme APX to reduce ROS production in roots. The toxicity of Pb was higher compared to Zn (Kopittke et al. 2010). Hence, the addition of Pb would cause an increase of the APX level even though there were not significant changes in root accumulation of Zn for treatment Zn1. In leaves, the APX levels were not significant due to the lesser amount of Pb being transported from the roots. It was also reported by Yan et al. (2010) that the accumulation of Pb occurred mainly in the roots. Increases of APX due to Pb were also reported by Verma and Dubey (2003) in *Oryza sativa*.

Zn accumulation in roots for treatment Zn1 and Zn2 (Figure 1) showed similar trend where addition of Pb led to decreasing of accumulation. This shows GPX in roots exhibiting a contrasting reaction compared with other enzymes in roots. This was significant due to Pb at a concentration of 10^{-5} to 2×10^{-4} M producing about 50% inhibition in many enzymes (Sharma & Dubey 2005) which caused the decrease in GPX activity when the toxic effects exerted on the roots was beyond the limit of tolerance. Another explanation of the current finding is that antioxidant enzymes activities was decreased due to the blocking of essential functional groups in biomolecules or the displacement of essential metal ions from biomolecules by heavy metals (Schützendübel & Polle 2002; Stroinski & Kozlowska 1997). These observations were consistent with Lichtenthaler (1998), who described plant's stress responses as being bell-shaped curves and considered the decreasing part as an exhaustion phase in which the defense systems were overloaded, leading to chronic damage and cell death.

Conclusion

In a sum, the accumulation of Zn and its effect on antioxidant enzymes in roots and leaves were dependent on the concentration of Pb exposure within the range Zn: 2-6 ppm; Pb: 0.4-0.8 ppm. The treatment of Zn with Pb exposure had increased the accumulation of Zn in leaves but reduced its uptake in roots. Due to their sensitivities to Zn with Pb, changes in the levels of SOD and CAT can be used as a biomarker to monitor the toxicity of Pb and Zn exposure in *C. asiatica*.

Acknowledgements

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G.H. Ong & C.K. Yap*
Department of Biology, Faculty of Science
Universiti Putra Malaysia
43400 UPM, Serdang, Selangor
Malaysia

M. Maziah
Department of Biochemistry
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
43400 UPM, Serdang, Selangor
Malaysia

S.G. Tan
Department of Cell and Molecular Biology
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
43400 UPM, Serdang, Selangor
Malaysia

*Corresponding author; email: yapckong@hotmail.com