รายงานวิจัยฉบับสมบูรณ์

โครงการ Chlorophyll fluorescence and antioxidant systems as a potential biomarker suite for the assessment of heavy metal exposure in the seedlings of the common mangrove, *Rhizophora mucronata*

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เมษายน พ.ศ. 2562

สัญญาเลขที่ MRG6080076

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและต้นสังกัด

Project Code : MRG6080076

Project Title : Chlorophyll fluorescence and antioxidant systems as a potential biomarker suite for the assessment of heavy metal exposure in the seedlings of the common mangrove, *Rhizophora mucronata*

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Project Period : 2 Years

บทคัดย่อ

ต้นโกงกางใบใหญ่ (*Rhizophora micronata* Lam.) เป็นพืชที่พบได้ทั่วไปในพื้นที่ป่าชายเลนซึ่งเป็นพื้นที่เสี่ยง ต่อการเผชิญกับปัญหาการปนเปื้อนโลหะหนัก การศึกษาการตอบสนองทางสรีรวิทยา และกลไกการทนต่อสภาพปนเปื้อนโลหะหนักโดยเฉพาะอย่างยิ่งโลหะหนักที่พบได้ทั่วไป และเป็นธาตุอาหารที่จำเป็นต่อการเจริญเติบโตของพืช เช่นทองแดงและสังกะสีจึงมีความสำคัญต่อการทำความเข้าใจกลไกที่ทำให้พืชชนิดนี้ทนต่อสภาพที่มีโลหะหนักมากเกินไป รวมถึงการประเมินผลกระทบของการปนเปื้อนโลหะหนักในพืชป่าชายเลน การทดลองที่1 ถูกแบ่งเป็น 5 ชุด การทดลอง ได้แก่ ชุดควบคุม, ชุดทองแดง 50 มิลลิกรัม ($CuCl_2$), ชุดทองแดง 100 มิลลิกรัม, ชุดสังกะสี 50 มิลลิกรัม ($ZnCl_2$) และ ชุดสังกะสี 100 มิลลิกรัม หลังจากการทดลอง 3 และ 7 วัน รากและใบถูกนำมาวัดการสะสมของทองแดงและสังกะสี, ประสิทธิภาพการสังเคราะห์ด้วยแสง, กิจกรรมของเอนไซม์ superoxide dismutase และ peroxidase, ปริมาณ non-protein thiols, การสะสมอนุมูลอิสระ (reactive oxygen species) และ lipid peroxidation พบว่า R. mucronata มีการจำกัดการขนส่งทองแดงและสังกะสีที่จะถูกส่งไปที่ใบ และสะสมไว้ที่รากทำให้ใบไม่ได้รับความเสียหายอย่างไรก็ตามปริมาณโลหะหนักที่สูงในรากไม่เหนี่ยวนำให้เกิดความเครียดออกซิเดชั่นหรือการกระตุ้นระบบต้านอนุมูลอิสระเนื่องจากโลหะหนักส่วนใหญ่ถูกเก็บไว้ที่ผนังเซลล์จึงสรุปได้ว่าต้นกล้า *R. mucronata* สามารถสะสมและจำกัดการขนส่งโลหะหนักเพื่อป้องกันไม่ให้เกิดความเสียหายแก่ส่วนที่ใช้ในกระบวนการการสังเคราะห์ด้วยแสง การทดลองที่ 2 แบ่งเป็น 3 ชุด การทดลอง ได้แก่ ชุดควบคุม, ชุดทองแดง 100 มิลลิกรัม และ ชุดสังกะสี 100 มิลลิกรัม หลังจากการทดลอง 1 และ 5 วัน วัดกิจกรรมการสังเคราะห์ด้วยแสง การสะสมอนุมูลอิสระ (reactive oxygen species) และ lipid peroxidation และติดตามการแสดงออกของยีน phytochelatin synthase พบว่าการแสดงออกของยีนนี้ถูกยับยั้งเมื่อ ต้นกล้า *R. mucronata* ได้รับโลหะหนักทองแดง ส่งผลให้เกิดการยับยั้งการสังเคราะห์ด้วยแสงและมีการแสดงออกของอาการเกิดพิษจากโลหะหนัก เช่น ลำต้นโค้งงอและใบเหี่ยว ส่วนต้นกล้า *R. mucronata* ได้รับโลหะหนักสังกะสีถึงแม้จะมีการแสดงออกของยีนนี้ลดลงในวันแรกแต่สามารถเพิ่มการแสดงออกของยีนนี้ได้ในวันที่ 5 และไม่มีการตอบสนองทางสรีรวิทยา แสดงให้เห็นว่า phytochelatin มีบทบาทสำคัญต่อการทนพิษจากโลหะหนักใน ต้นกล้า *R. mucronata*

คำสำคัญ : *Rhizophora mucronata*, copper, zinc, phytotoxicity, trace metal tolerance, phytochelatin

Abstract

Rhizophora mucronata is a common mangrove growing in habitats subjected to heavy metal (HM) contamination. Understanding their physiological responses to copper (Cu) and zinc (Zn) excess and underlying tolerance mechanisms is crucial to assess impacts of metal pollution on mangrove community. In the first experiment, seedlings were treated with Cu or Zn (0, 50 or 100 mg per plant) by means of a single addition. At day 3 and 7, Cu and Zn accumulation, photosynthetic efficiency, superoxide dismutase and peroxidase activity, non-protein thiols, reactive oxygen species and lipid peroxidation in roots and leaves were measured. *R. mucronata* restricted Cu and Zn translocation, thus accumulated HM mainly in roots while kept the leaves unaffected. However, high root HM did not induce oxidative stress nor anti-oxidative defense as HM were largely deposited in cell wall. We concluded that HM tolerance strategies of *R. mucronata* seedlings are exclusion and restriction of translocation to the vital photosynthetic tissue. The second experiment consists of three treatments: controls, 100 mg Cu and 100 mg Zn. After 1 and 5 day, photosynthetic activity, total reactive oxygen species and lipid peroxidation were measured and the expression of phytochelatin synthase (*pcs*) was assessed. Cu-treated plants showed a significant decrease in transcripts encoding phytochelatin synthase, a reduction in photosynthetic efficiency and phytotoxicity symptoms such as bent stem and wilted leaves. On the other hand, Zn-treated *R. mucronata* showed a down-regulation of pcs on day 1 but the expression was recovered on day 5 and there was no alteration in physiological features in this treatment. The results suggest that phytochelatin plays a critical role in heavy metal tolerance in *R. mucronata.*

Keywords: *Rhizophora mucronata*, copper, zinc, phytotoxicity, trace metal tolerance, phytochelatin

Executive Summary

The project is divided into two parts

Part 1

Objectives

This part aims to investigate the physiological responses of *Rhizophora mucronata* seedlings to copper (Cu) and zinc (Zn) contamination in order to better understand the mechanisms underlying tolerance to these two metals of this common mangrove species.

Materials and Methods

One-month-old seedlings of *Rhizophora mucronata* were obtained from the Mangrove Resources Development Station 38 (Songkhla, Thailand) and acclimated under ambient light and temperature at the greenhouse facility of the Department of Biology, Prince of Songkla University for 5 weeks before starting the experiment. Seedlings were subsequently divided into five groups of treatments (20 seedlings/treatment). Each seedling was planted in each pot containing the soil collected from the station (sandy clay loam soil containing 60% sand 28% clay and 12% silt, organic matter=2.0±0.3%, pH=5.20±0.03) and each plant was considered a replicate. Heavy metal (HM)-treated plants were watered with 100 mL of ¼ Hoagland solution with the salinity of 8 ppt containing 50 or 100 mg of Cu ($CuCl_2$) or Zn ($ZnCl_2$). The same solution without Cu and Zn addition was used as a control. All pots of plants were watered daily, and nutrients were re-supplied with ¼ Hoagland solution on day 3. On day 3 and 7 after treatment, photosynthetic parameters were recorded, and soil, plant roots, and leaves were collected. Each individual seedling was collected and stored separately. After harvested, plant leaves and roots were immediately washed with de-ionized water and dried using paper towel. Materials used for analysis of Cu and Zn content were oven-dried or immediately analyzed whereas materials used for biochemical assays were frozen in liquid N_2 and stored at -80˚C until use.

Roots, leaves and soil were dried at 80 ˚C (n=4). Approximately 0.1 g of dried tissues was ground and analyzed for Cu and Zn content, using an inductively coupled plasma optical emission spectrometry (ICP-OES Optima 5300 DV ICO/OES, Perkin Elmer, USA). Translocation factor (TF) was calculated as the ratio of HM in the leaves to its roots the following the equation: HM (leaf)/HM ($root$). Bioconcentration factor (BCF in roots) was calculated as the ratio of heavy metal concentration (HM) in the tissue to the concentration of same metal in soil, following equation: $BCF=HM$ ($root$)/HM ($soil$).

Fractionation of root tissue was modified from Li et al. (2016). First 0.35 g of fresh root samples (n=4) were homogenized with 14 mL of extraction buffer (50 mM Tris–HCl pH 7.5 containing 250 mM sucrose, and 1.0 mM dithioerythritol). The mixture was centrifuged at 300×g for 10 min at 4 °C. The supernatant represented the cytoplasmic fraction whereas the pellet represented the cell wall fraction. Both fractions were analyzed for Cu and Zn content as previously described and normalized to fresh weight.

Superoxide dismutase (SOD) activity was measured following Elavarthi and Martin (2010). Guaiacol Peroxidase (POX) activity was measured following Macfarlane and Burchett (2001). Non-protein thiols (NPT) content was measured following Devi and Prasad (1998). Total reactive oxygen species (ROS) was estimated according to

Phandee and Buapet (2018). Lipid peroxidation (LPO) was estimated following Jambunathan (2010). Fresh weight was used for normalization. All the analyses of SOD, POX, NPT, ROS and LPO were done with 3 technical replicates and 6 biological replicates.

Photosynthetic efficiency was assessed as the maximum quantum efficiency of photosystem II (Fv/Fm) and the effective quantum efficiency of photosystem II (ΦPSII) by using a pulse-amplitude modulated chlorophyll fluorometer (Mini-Pam, Walz, Germany) on the leaf of the second leaf pair (n=6). The Fv/Fm was calculated as $(F_m-F_0)/F_m$, where F_0 is the fluorescence yield of the dark-adapted sample and Fm is the maximum fluorescence after saturating light pulse is applied. The ΦPSII was calculated as $(F_m'-F)/F_m'$, where F is fluorescence yield of the light-adapted sample and Fm′ is the maximum fluorescence after saturating light pulse is applied.

Heavy metal content, superoxide dismutase activity, peroxidase activity, non-protein thiols content, reactive oxygen species and lipid peroxidation were tested using three-way ANOVA (Treatment, day after treatment and plant parts as categorical factors). The photosynthetic parameters were analyzed using repeated measures ANOVA (Treatment as categorical factor and day after treatment as a within-group factor). Fisher's least significant difference (LSD) test was used to compare the effect of heavy metal exposure across time of measurements and between plant parts (leaf and root).

Results and Discussion

There was no change in heavy metal (HM) content in controls. HM-enriched plants showed high accumulation of both copper (Cu) and zinc (Zn) in the roots whereas there was no change in accumulation in the leaves (Fig. 1a-d). We observed an increase in Cu in root tissues compared to control but not between the two Cu treatments (Fig. 1c) whereas accumulation of Zn in the roots showed dose and time-dependent response (Fig. 1d). A predominant accumulation of Cu and Zn in the roots and a translocation factor (TF) below 1 (Table 1) indicate that *Rhizophora mucronata* seedlings are excluder (Kaewtubtim et al. 2016), in line with previous studies in various mangrove species such as *R. mucronata* (TF=0.2 for Cu and Zn, Kaewtubtim et al. 2016), the congeneric species, *R. mangle* (TF=0.02 for Cu; 0.36 for Zn, Silva et al. 1990) and *R. stylosa* (TF=0.57 for Cu; 0.43 for Zn, Alongi et al. 2005). It has been suggested that under toxic concentration, translocation of Cu and Zn to the leaves is limited due to restriction at the endodermis by casparian strip in *Avicennia marina* (MacFarlane and Burchett 2000) and at the exodermis by lignification thickening in *Bruguiera gymnorrhiza* (Cheng et al., 2012). Nevertheless, *R. mucronata* seedlings showed large accumulation of Cu and Zn in the roots relative to the concentration present in the soil described as the bioaccumulation factors (BCF). However, BCF of HM-treated roots (Table 1) are lower than what was observed in *R. mucronata* in a previous study (2.6 for Cu and 6.6 for Zn, Kaewtubtim et al. 2016) but higher than in other congeneric species such as *R. mangle* (Silva et al. 1990) and *R. stylosa* (Alongi et al. 2003). The content of both HM in the root and leaf tissue was found to be higher than what was detected in *R. mucronata* in Pattani Bay, Thailand (Kaewtubtim et al. 2016). These previous investigations were conducted in adult plants in the natural settings with lower HM concentration in the sediment. The results suggest that BCF and tissue heavy metal content may vary depending on the plant developmental stage and heavy metal availability.

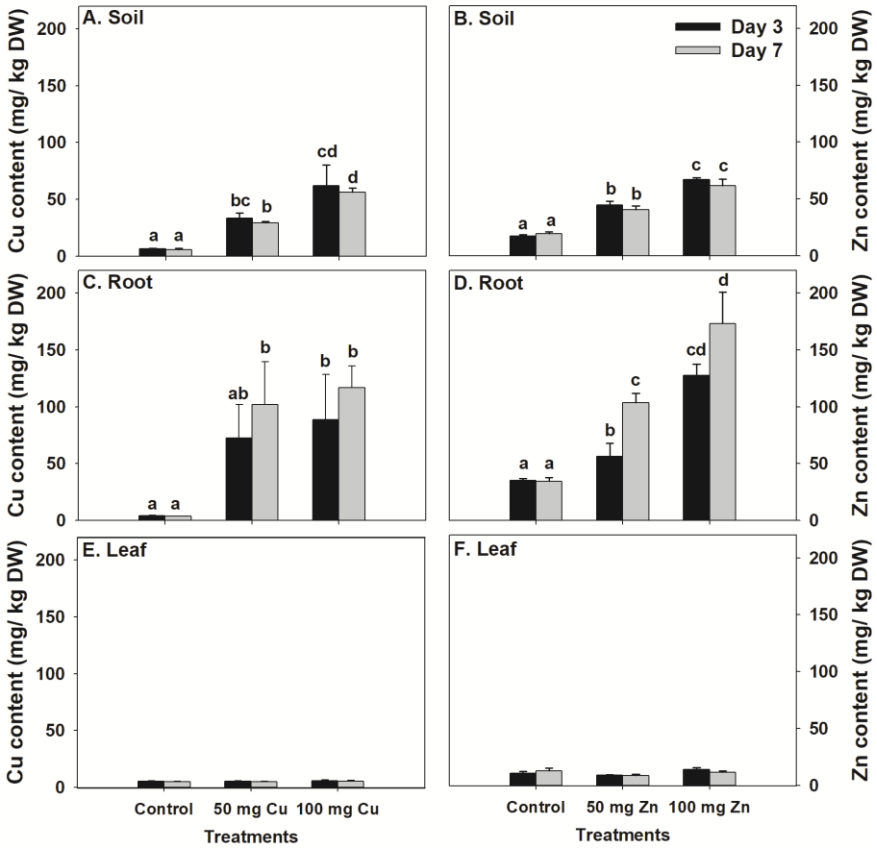

Fig.1 The accumulation of Cu and Zn in the soil (a,b), the leaves (c,d) and the roots (e,f) of *Rhizophora mucronata* seedlings exposed to control, Cu and Zn on day 3 and 7. Error bars show standard error of mean, n = 4. Bars without shared letters are statistically different (Fisher's LSD post-hoc test, $p<0.05$

The distributions of Cu and Zn in the two fractions of the root tissue of *R. mucronata* are shown in Fig. 2. Up to 94% of Cu and 80% of Zn were retained in the cell wall. Previous investigations have provided evidence of the role of cell wall in HM toxicity regulation (see review in Krzesłowska 2011; Printz et al. 2016). It has been suggested that cell wall has a strong capacity for binding HM, including Cu and Zn, and may act as a barrier preventing their entry into the
plant cytoplasm. This high binding capacity is attributed to sorption by cell wall components such as lignin, pectin, certain polysaccharides and proteins (Colzi et al. 2011; Krzesłowska 2011; Lang and Wernitznig 2011). In addition, high HM in the cell wall fraction may be due to HM efflux at the cell membrane. Various families of transporters have been identified and it has been proposed that they play a role in maintaining HM homoeostasis (see review in Singh et al. 2016).

Table 1 Accumulation and translocation of Cu and Zn in *Rhizophora mucronata* seedlings after 3 and 7 days of heavy metals treatment.

Parameter/ Treatment	Cu treatment		Zn treatment	
	Day 3	Day 7	Day 3	Day 7
Translocation Factor				
Control	1.27 ± 0.17	1.33 ± 0.08	0.30 ± 0.03	0.04 ± 0.05
50 mg	0.09 ± 0.05	0.09 ± 0.05	0.16 ± 0.03	0.09 ± 0.01
100 mg	0.10 ± 0.06	0.04 ± 0.00	0.12 ± 0.03	0.07 ± 0.01
Root Bioconcentration Factor				
Control	0.68 ± 0.09	0.80 ± 0.12	2.18 ± 0.13	2.15 ± 0.44
50 mg	3.02 ± 0.25	4.81 ± 1.45	1.37 ± 0.29	2.72 ± 0.14
100 mg	1.45 ± 0.01	2.00 ± 0.30	2.36 ± 0.54	2.82 ± 0.14

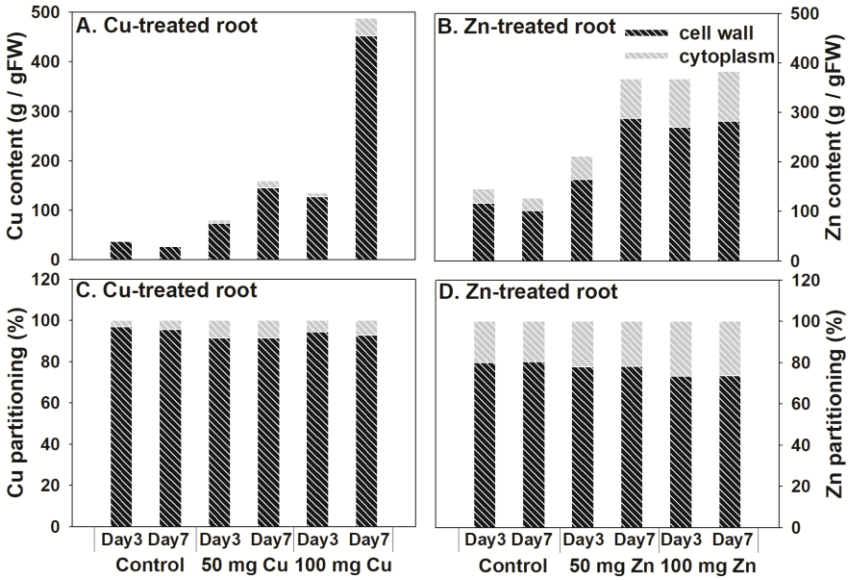

Fig.2 Distribution of Cu and Zn in the root cells of *Rhizophora mucronata* seedlings exposed to control, Cu and Zn on day 3 and 7. The content of Cu and Zn in cell wall and cytoplasmic fractions is shown in a,b whereas the percentage partitioning between the two fractions is shown in c,d.

Sequestration of HM in cell wall likely explains why only minor changes in physiological status were detected in the roots despite high levels of Cu and Zn in the tissue. Although HM content in cytoplasmic fraction still exhibited dose-dependent response, the concentrations detected within the root cell did not impose substantial toxicity. Root tissue did not show an over-production of reactive oxygen species (ROS; Fig. 3b) nor an increase in lipid peroxidation (Fig. 3d). In addition, there was no change in the superoxide dismutase (SOD) activity nor in the non-protein thiols (NPT) content in the roots (Fig. 4b,f). This lack of response implies that high Cu and Zn in the root tissue did not induce oxidative stress which is likely a result of HM retention in the cell wall which limits cellular uptake and consequently prevents HM toxicity. The activity of POX, however, was affected by HM addition. There was a reduction in POX activity on day 3 in treatments with 50 mg of Cu, 100 mg of Cu and 100 mg of Zn (Fig. 4d). On day 7, a partial recovery was observed in Cu-treated plants while a full recovery was observed in Zn-treated plants (Fig. 4d). This may be due to a structural modification of POX induced by Cu and Zn, thus affecting the enzyme functionality (Yruela 2009). Toxicity of Cu was stronger than Zn as it was detected at lower concentration and it imposed more chronic impacts than Zn. A number of studies have demonstrated that toxicity of Cu is more pronounced than Zn

which may be due to its redox-active nature (See review in Küpper and Andresen 2016; Nanda and Agrawal 2016). However, the toxicity mechanisms of HM on the activity of enzymes, particularly antioxidant enzymes, remain to be elucidated.

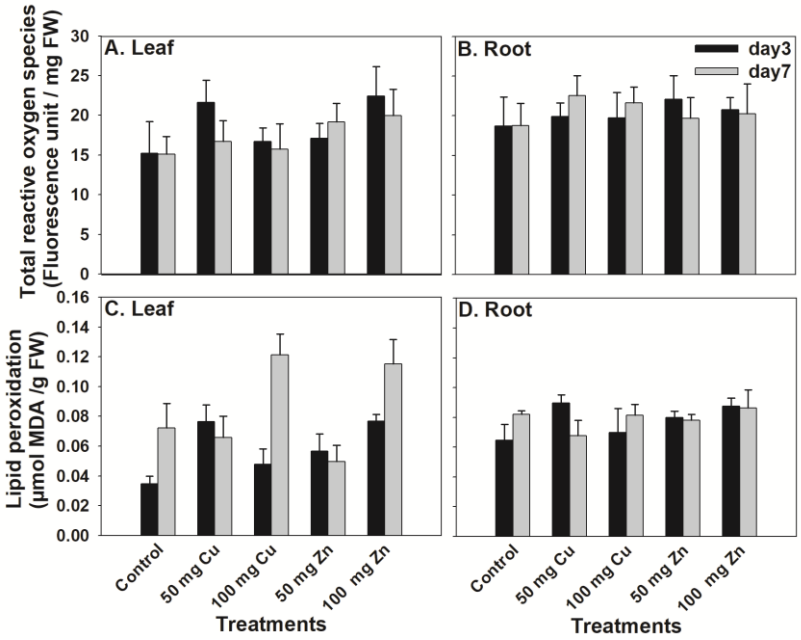

Fig.3 Reactive oxygen species (ROS) accumulation (a,b) and lipid peroxidation measured as MDA content (c,d) in the leaves and roots of *Rhizophora mucronata* seedlings exposed to control, Cu and Zn on day 3 and 7. Error bars show standard error of mean, n=6

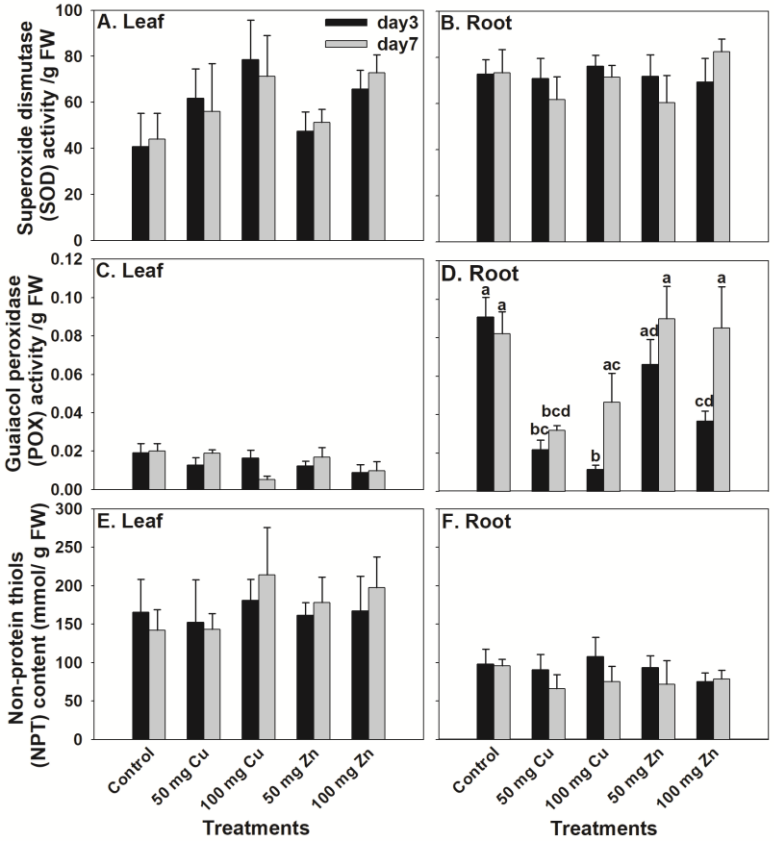

Fig.4 Superoxide dismutase (SOD) activity (a, b), guaiacol peroxidase (POX) activity (c,d) and non-protein thiols (NPT) content (e,f) in the leaves and roots of *Rhizophora*

mucronata seedlings exposed to control, Cu and Zn on day 3 and 7. Error bars show standard error of mean, n = 6. For Fig. 4d, bars without shared letters are statistically different (Fisher's LSD post-hoc test, $p<0.05$

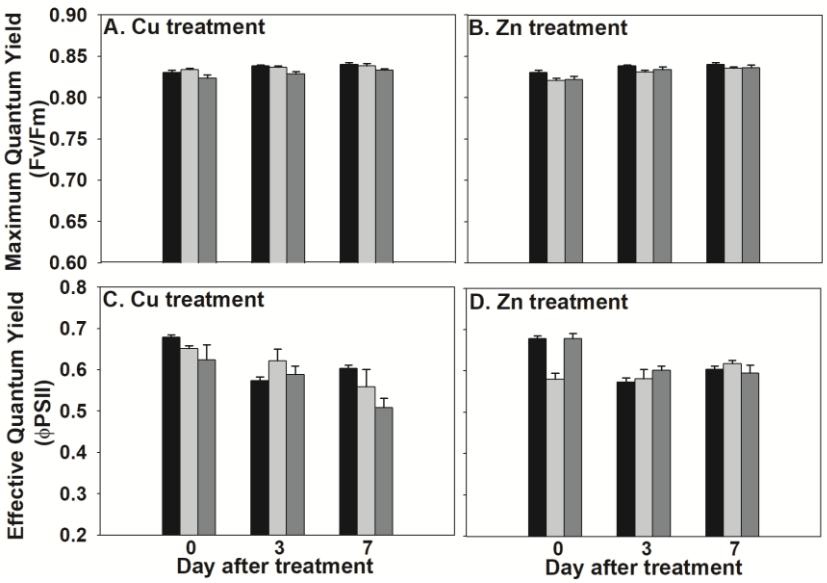

Fig.5 The maximum quantum yield (Fv/Fm a,b) and the effective quantum yield (ϕPSII c,d) of *Rhizophora mucronata* seedlings exposed to control, Cu and Zn on day 3 and 7. Error bars show standard error of mean, n = 6

Limited cellular uptake in the root as well as restricted translocation of HM to the leaves are likely explanations for the absence of change in the physiological status of the leaves. These results correspond with no change in HM content in leaf tissue. There were variations in ROS and lipid peroxidation in leaf tissue (Fig.3 a,c). However, such variations cannot be accounted for by HM toxicity as there was no change in HM accumulation in the leaves. Our results indicate that root sequestration in *R. mucronata* seedlings successfully prevent harmful HM-induced oxidative stress on photosynthetic tissue. This is supported by no change in detoxification capacity measured as the activity of SOD and POX and NPT content (Fig. 4 a,c,e). Similarly, both the maximum quantum efficiency of photosystem II (Fv/Fm) and the effective quantum efficiency of photosystem II (ϕPSII) remained unaffected in both Cu and Zn treatments (Fig. 5). These results once again suggest that exclusion mechanisms in *R. mucronata* seedlings successfully allow the plants to carry on their photosynthetic processes, as well as other physiological processes. Tolerance mechanisms to copper and zinc excess in *R. mucronata* seedlings in this study is summarized in supplementary Fig. 1. We conducted the experiment using the salinity of 8 ppt following the results of the previous studies which showed that low salinity (3-8 ppt) provided the optimal condition for initial growth of *R. mucronata* seedlings (Hoppe-Speer et al. 2011; Kodikara et al. 2018). However, Songkhla lake, our system of interest, is brackish with large seasonal variation in salinity (0–30 ppt, Rattanama et al. 2016). Since HM bioavailability and plant HM uptake vary with salinity (Stevens et al. 2003; Fritioff et al. 2005), the phytotoxic effects under different salinity regime may consequently differ from the results of the present study.

For application purposes, Cu and Zn in the roots of *R. mucronata* seedlings may be used as a biomarker for Cu and Zn contamination, due to a dose-dependent response evidenced in the present study. Since uptake and translocation of HM in this plant

were lower than the level reported in other hyperaccumulator plants, phytoremediation potential of *R. mucronata* may instead be associated with phytostabilization (Lorestani et al. 2013). Our findings highlight the ecological significance of mangroves which not only contribute as primary producer and important habitat but also function as long-term sequestration sites for HM pollutants, thus protecting adjacent ecosystems such as seagrass meadows and coral reefs.

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Part 2

Objectives

This part focuses on the role of sequestration in tolerance to Cu and Zn exposure in *Z. mucronata seedlings* by assessing the physiological responses in parallel with the expression of phytochelatin synthase (*pcs*) in their roots.

2. Materials and Methods

2.1 Experimental Design

The experimental design was adapted from Zhang et al. (2007). Two months-old seedlings of *Rhizophora mucronata* were obtained from the Mangrove Resources Development Station 38, Songkla Province, Thailand. The plants were allowed to acclimate in the greenhouse facility at the Department of Biology Prince of Songkla University for one month. Healthy seedlings with similar size were sorted and each seedling was subsequently planted in a glass container painted black (pre-washed overnight with 1% HCl to prevent metal adsorption, Ritter et al. 2014) filled with 1L of 1/4 Hoagland's solution (containing 8‰ NaCl). The experiment consists of 3 treatments (with 10 containers per treatment); control, 100 mg Cu (prepared from the stock solution of $CuCl_2$) and 100 mg Zn (prepared from the stock solution of $ZnCl_2$). The concentration was pre-determined from the previous study (Torasa et al. 2019). The experiment lasted for 5 days and measurements and samplings were done on day 0, day 1 and day 5.

2.2 Heavy metal content in plant tissue

To determine Cu and Zn content in plant tissue, roots and leaves were dried at 80 ˚C, using a hot air oven, for one week. Approximately 0.1 g of dried tissues was ground and analyzed for Cu and Zn content, using an inductively coupled plasma optical emission spectrometry (ICP-OES Optima 5300DV ICO/OES, Perkin Elmer, USA), at Central Equipment Division, Faculty of Science, Prince of Songkla University.

2.3 Measurements of photosynthetic activity

Pulsed Amplitude Modulated Fluorometry (Mini-PAM, Walz Germany) was adopted to investigate photosynthetic efficiency of the second leave pairs on day 0, day 1 and day 5. This method allows the examination of the processes related to photosynthesis in a non-destructive manner. The maximum quantum yield (Fv/Fm), a commonly-used indicator for stress and photoinhibitory damage, was determined after 20 minutes dark adaptation using dark leaf clips (Walz, Germany). In addition, rapid light curves (RLCs) were constructed on the same day and parameters derived from RLCs e.g. the maximum electron transport rates (ETRmax), the initial slope of the light response curve (α), and the minimum saturating irradiance (Ek), were calculated based on curve-fitting equation of Jassby and Platt (1982).

2.4 Estimation of total Reactive oxygen species (ROS) and Lipid peroxidation (LPO)

On day 1 and day 5, the plant tissue was harvested for analysis of total reactive oxygen species and lipid peroxidation. Since *R. mucronata* seedlings showed accumulation of Cu and Zn in the roots and restricted translocation, the analysis of total ROS and LPO was performed only in root tissue.

Total reactive oxygen species was estimated based on the fluorescence of 2',7'-dichlorofluorescein (DCF) (DCF) (adapted from Phandee and Buapet 2018). Highly fluorescent DCF is generated when a non-fluorescent 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) reacts with oxygen radicals, allowing an estimation of total ROS. In brief, 100 mg of root tissue was ground in liquid N_2 and mixed with 1 ml of 10 mM Tris-HCl pH 7.2. After centrifugation at 12,000 ×*g* at 4°C for 20 minutes, the supernatant of 100 µl was collected and diluted 10 times with 10 mM Tris–HCl pH 7.2. Finally, 10 µl of 1 mM DCFDA was added to the diluted extract followed by 10 minutes of dark incubation. Detection of fluorescence with the excitation at 490 nm and the emission at 530 nm was done using a spectrofluorometer (Jasco FP-8200, Jasco, Tokyo, Japan). Total ROS is expressed as relative fluorescence units /mg fresh weight of plant tissue.

Malondialdehyde (MDA), the end product of oxidative degradation of lipids by ROS, was determined using TBARS assay (adapted from Torasa et al 2019). MDA reacts with thiobarbituric acid (TBA) yielding TBARS, a product which can be measured spectrophotometrically. Root tissue of 200 mg was ground in liquid N_2, homogenized with 4 ml of 0.1 % trichloroacetic acid (TCA) and centrifuged at 10000 *xg* for 15 minutes. Subsequently, 1 ml of the supernatant was mixed with 2 ml of 20% TCA and 2 ml of 0.5% TBA and heated at 95 °C for 30 minutes before immediately placed on ice. The mixture was subsequently centrifuged at 10,000 xg for 10 minutes to collect the supernatant. The absorbance of the supernatant was read at 532 and 600 nm using a spectrophotometer (UV1720, Yoke instrument, China). The nonspecific absorbance at 600 nm was subtracted from the values at 532 nm and then divided by an extinction coefficient of 155 $mM^{-1}cm^{-1}$. Fresh weight of tissue was used for normalization.

2.5 Determination of expression of phytochelatin synthase

2.5.1 Primer design

The primers for six candidate reference genes; 18S Ribosomal RNA (*18s*), Actin (*actin*), Elongation factors 1α (*elofα*), Ubiquitin (*ubi*), Ribulose-1,5-biphosphate carboxylase large subunit (*rbcl*) and β-tubulin (*tub*) were designed following Saddhe et al (2018). These genes were annotated in NCBI sequence database of *Rhizophora apiculata* (MH277331, MH279969, MH310424, MH310425, KP697362 and MH310423). The gene specific primers of Phytochelatin synthase (*pcs*) were designed based on coding open reading frame regions of *Kandelia candel* PCs genes (GU556628.1). All primers were synthesized by the Macrogen Co., Ltd., (South Korea) and the primer sequences are presented in Table 1.

2.5.2 RNA extraction and synthesis of cDNA

The root tissue samples were taken from Rhizophora mucronata seedlings exposed to Cu and Zn on day 1 and 5. The tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until further use. Plant material was homogenized with mortar and pestle in liquid nitrogen. The total RNA was extracted following the manufacturer's protocol of RNAplant Plus Reagent (Tiangen, Chaina) until RNA precipitation step. After that, the purification step was using QIAshredder Mini Spin Column following the manufacturer's instructions of RNeasy Plant Mini kit (QAIGEN, Netherlands). The quality and concentration of RNA samples were determined by agarose gel electrophoresis and Nanodrop 2000 machine (Thermo scientific, United States). For

cDNA synthesis, all the reagents and protocols used were purchased from the RevertAid First Strand cDNA Synthesis kit (Thermo scientific, United States).

2.5.3 Selection of reference genes for semi-quantitative PCR

Six reference genes genes were amplified using Platinum taq dna polymerase (Invitrogen, United States). The PCR amplification was performed in 50 µl reactions containing 5 µl reaction buffer , 5 µl MgCl2 (25mM), 1 µl dNTP (10 µM), 0.25 µl Taq-polymerase (Invitrogen, United States) (5 U/µl), 1 µl of each primer (10 mM) and 1 µl of template cDNA. The thermal cycle was an initial 3 min of denaturation at 95℃, followed by 9 cycle of denaturation/annealing/extension with 30 sec at 95°C for denaturation, 30 sec at the annealing temperature, and 1 min at 72°C for extension; the initial annealing temperature was set at 48°C and increased 0.5 degree every cycle, to reach the temperature at 52°C, followed by 35 cycles of 30 sec of denaturation at 95℃, 30 sec of annealing at 50℃ and 1 min of elongation at 72℃, and a final elongation of 10 min at 72℃. The quality of the reference genes were checked by electrophoresis on 1.5% agarose gel and stained with ethidium bromide (EtBr).

2.5.4 Differential gene expression analysis

18S Ribosomal RNA was used as areference gene to standardize the results. The sequences of the specific primers of Phytochelatin synthase (PCs) used for RT-PCR reactions are defined in Table 1. PCR was performed using 5 µl reaction buffer , 5 µl MgCl2 (25mM), 1 µl dNTP (10 µM), 0.25 µl Taq-polymerase (Invitrogen, United States) (5 U/µl), 1 µl of each primer (10 mM) and 1 µl of template cDNA. The reaction conditions used for semi-quantitative RT -PCR was an initial 3 min of denaturation at 95℃, followed by 9 cycles of denaturation/annealing/extension with 30 sec at 95°C for denaturation, 30 sec at the annealing temperature, and 1 min at 72°C for extension; the initial annealing temperature was set at 48°C and increased 0.5 degree every cycle, to reach the temperature at 52°C, followed by 35 cycles of 30 sec of denaturation at 95℃, 30 sec of annealing at 50℃ and 1 min of elongation at 72℃, and a final elongation of 10 min at 72℃. The samples which were analyzed and compared to each other (8S Ribosomal RNA and PCs treated samples) were loaded on the same gel following the same settings of the image analyses. Densitometric analysis of EtBr-stained gel bands was performed using ImageJ software.

2.6 Statistical analysis

Statistica Academic (StatSoft, Tulsa, USA) was the platform used for all the statistical analyses. The effects of Cu and Zn exposure on Fv/Fm and RLC-derived parameters were tested using repeated-measures ANOVA (treatments as the categorical factor and days after treatment as the within-group factor). The differences in tissue trace metals, total ROS and LPO among the treatments were analyzed using two-way ANOVA. One-way ANOVA was used to assess the difference between the *pcs* transcript level of control and treated samples. Fisher's least significant difference (LSD) test or unequal N HSD post-hoc test was used to compare the physiological and gene expression data among treatments and days after treatments. A $p\text{-value}<0.05$ was considered to be significantly different. Cochran's test was used to test the assumption of homogeneity of variances before ANOVA was performed.

Table 1 Reference genes and target genes investigated in *Rhizophora mucronata*. Accession numbers and primers sequences are indicated.

No.	Gene label	Accession number	Gene description	Primer sequence (5'-3')
1	18SrRNA_F 18SrRNA_R	MH277331	18S rRNA	F-CCGTCCTAGTCTCAACCATAAAC R-GCTCTCAGTCTGTCAATCCTTG
2	Actin_F Actin_R	MH279969	Actin	F-ATCACACCTTCTACAACGAGC R-CAGAGTCCAACACGATACCAG
3	EF1α_F EF1α_R	MH310424	Elongation Factor 1	F-AGCGTGTGATTGAGAGGTTC R-AGATACCAGCCTCAAAACCAC
4	UBQ_F UBQ_R	MH310425	Ubiquitin	F-CACTTCGACCGCCACTAC R-AGGGCATCACAATCTTCACAG
5	RbcL_F RbcL_R	KP697362	Rubisco Large Subunit	F-ATGTCACCACAAACAGAGACTAAAGC R-GTAAAATCAAGTCCACCRCG
6	β-TUB_F β-TUB_R	MH310423	β-tubulin	F-ACCTCCATCCAGGAGATGTT R-GTGAACTCCATCTCGTCCATTC
7	GSP_PCSF GSP_PCSR	GU556628.1	Phytochelatin synthase (PCs)	F-GCCTTGGAGATGGTTTGATG R-GCCACCAATAGGTGAAAAATG

3. Results

3.1. Physiological responses

Content of copper and zinc in plant tissue

Cu and Zn content in leaves and roots of control remained similar throughout the experiment (Fig. 1). Treated plants showed significantly higher Cu and Zn accumulation in the roots than control (approximately 500 times and 300 times higher than controls for Cu and Zn, respectively, Fisher's LSD post-hoc test, $p < 0.05$). The root bioaccumulation of Cu was not time-dependent, that is, root Cu content on day 5 remained at the same level as on day 1(Fig. 1A) whereas Zn accumulation in root decreased slightly on day 5 (Fig. 1B, Fisher's LSD post-hoc test, $p < 0.05$). On the contrary, Cu and Zn content in leaves of treated-plants was not affected by trace metal addition (Fig. 1 C,D).

Changes in photosynthetic parameters

There was no significant difference in the maximum quantum yield of PSII (Fv/Fm) among treatments and days and values ranged from 0.80 to 0.82 (Fig 2A). However, Cu and Zn exerted different effects on the light use efficiency measured as the rapid light curves (RLCs) of *R. mucronata* (Fig. 2B, Table 2). On day 0, plants in all treatments exhibited comparable RLCs. On day 1, Cu exposure resulted in a significant decrease in the maximum electron transport rates (ETR_{max}) and the initial slope of the light response curve (α) compared to control (Unequal N HSD post-hoc test, $p < 0.05$) whereas Zn exposure did not affect any RLCs-derived parameter. Similar response was observed on day 5 (Fig. 2B, Table 2) but percentage reduction in ETRmax induced by Cu exposure compared to control was higher than on day 1 (from 27% decrease on day 1 to 47% decrease on day 5) and a reduction in Ek in Cu-treated plants was also detected (Unequal N HSD post-hoc test, $p < 0.05$).

Oxidative-related parameters and observed phytotoxicity symptoms

Total reactive oxygen species and lipid peroxidation in the roots showed slight variations among treatments, but there was no statistical difference (Fig. 3A,B). While there was no clear sign of damage nor stress in the roots (Fig 4 A,B), the plants exhibited evident toxicity symptoms in the treatment with Cu excess such as slight wilting of leaves and bent stems on day 5 (Fig 4 C,D).

3.2 Differential expression of phytochelatin synthase (pcs)

The transcript levels of *pcs* in the roots of *R. mucronata* was evaluated via semi-quantitative RT-PCR analysis (Fig. 6). Cu exposure resulted in suppressed *pcs* expression. The transcript levels of *pcs* in Cu-treated roots on both day 1 and day 5 was lower than those of controls (Fisher's LSD post-hoc test, $p < 0.05$) and there was no significant difference between days after treatment. On the other hand, Zn exposure resulted in suppressed pcs expression on day 1 (Fisher's LSD post-hoc test, $p < 0.05$) but the transcript levels of *pcs* in Zn-treated roots was restored to the same level as control on day 5.

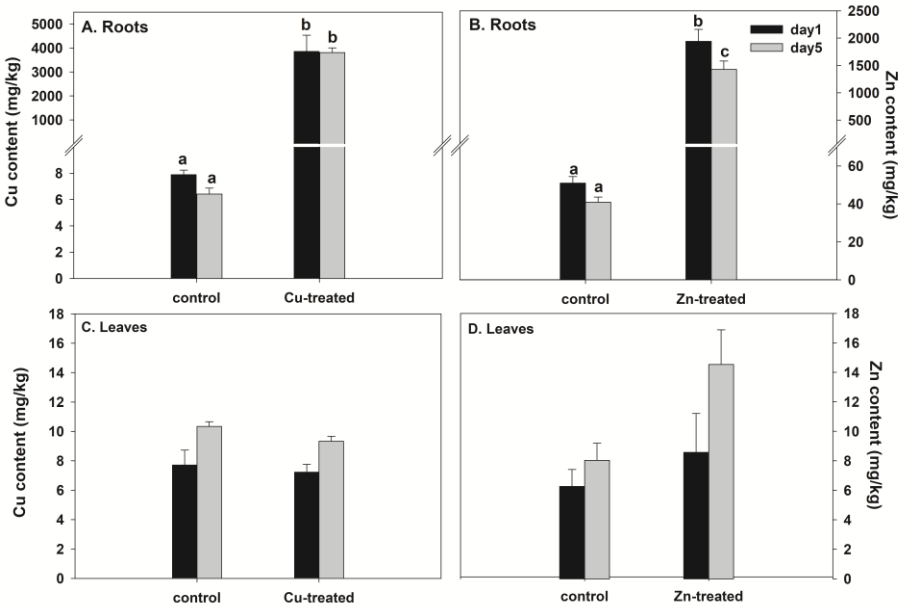

Fig. 1 The accumulation of trace metals in the (A,B) roots and (C,D) leaves of *Rhizophora mucronata* exposed to 200 mg Cu (A,C) and 200 mg Zn (B,D) compared to controls on day 1 (black bars) and 5 (gray bars). Error bars show standard error of mean, n=4. Bars without shared letters are statistically different (Fisher's LSD post-hoc test, $p < 0.05$).

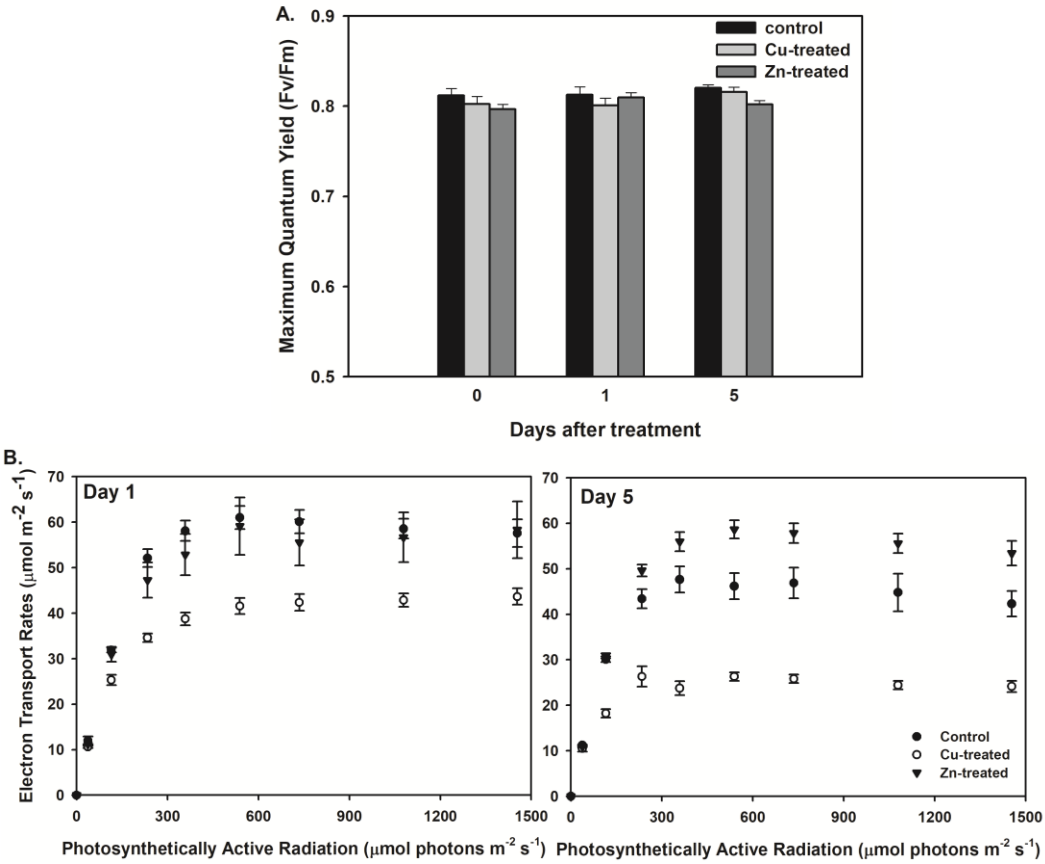

Fig. 2 Photosynthetic response of *Rhizophora mucronata* seedlings exposed to controls, Cu and Zn. (A) Time course of the maximum quantum yield (Fv/Fm) of *Rhizophora mucronata* after up to 5 days exposed to control, Cu and Zn: control (black bars), Cu (light grey bars) and Zn (dark grey bars). (B) Electron transport rates (ETRs) as a function of irradiance (PAR) measured on the second leaf pair of *R. mucronata* exposed to control, Cu and Zn on day 1 and 5. There was no difference among treatments in day 0 (not shown in graphs).

Error bars show standard error, n=5.

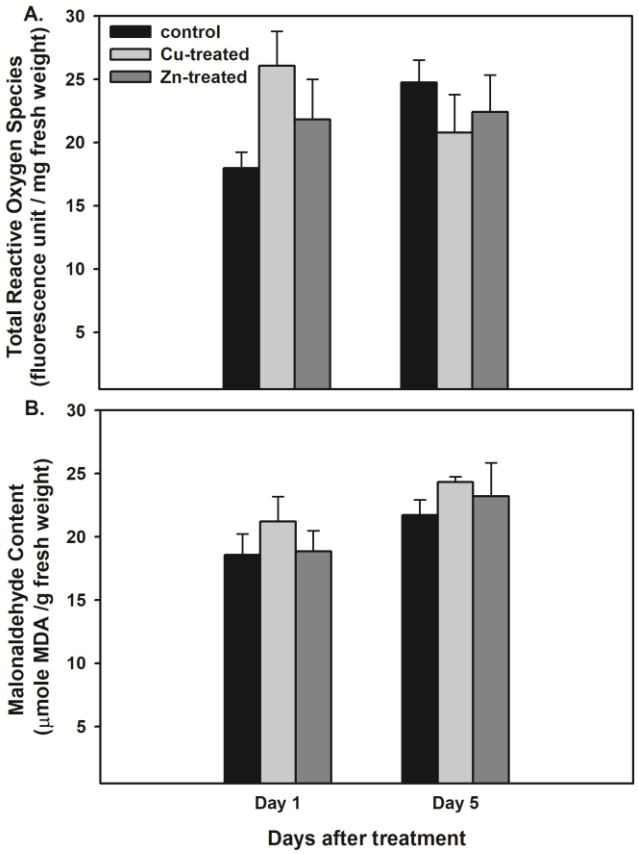

Fig. 3 Oxidative stress-related parameters measured in the roots of *Rhizophora mucronata* seedlings exposed to controls, Cu and Zn. on day 1 and 5. (A) Total reactive Oxygen Species and (B) Lipid peroxidation. Error bars show standard error of mean, n=5.

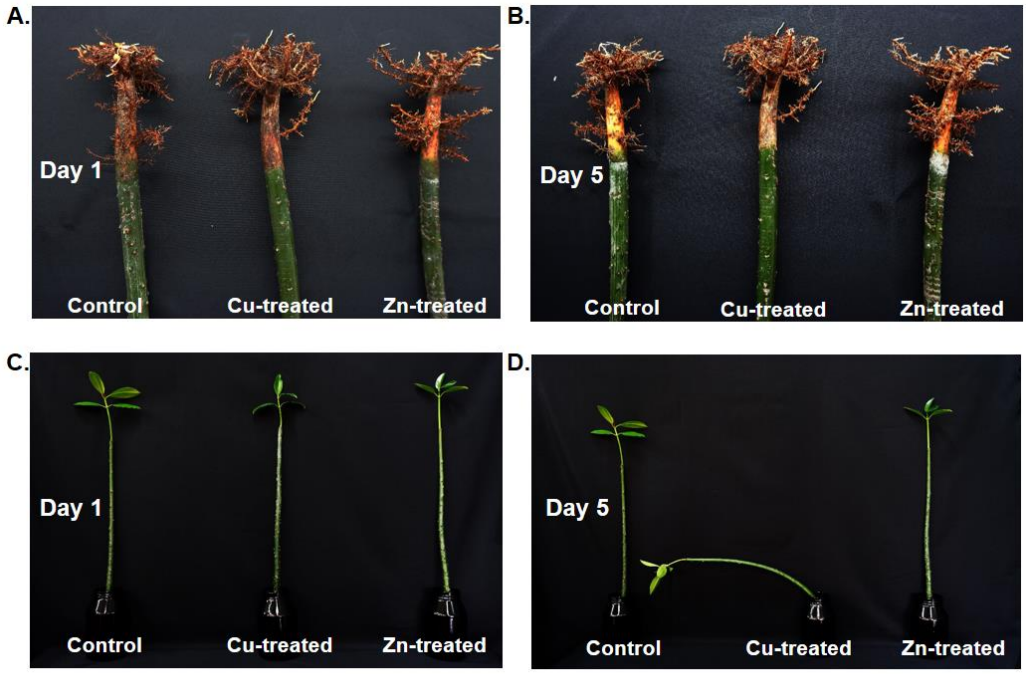

Fig. 4 Photograph of the roots (A,B) and whole plants (C,D) of *Rhizophora mucronata* seedlings exposed to Cu and Zn on day 1 and 5.

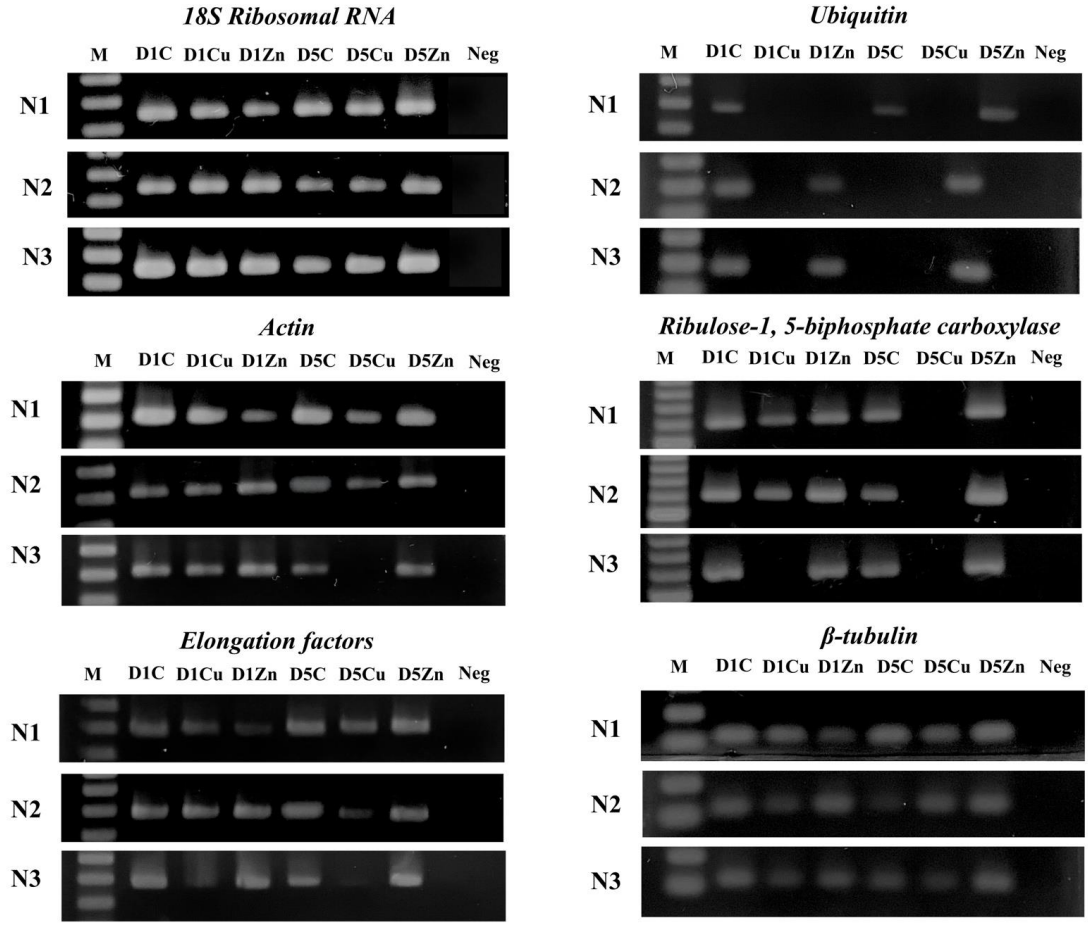

Fig. 5 Amplification product of candidate reference genes of R. mucronata exposed to control, Cu and Zn on day 1 and 5. PCR products on 1.5% agarose gel stained with ethidium bromide. Amplification products of six candidate reference genes (18S Ribosomal RNA, Actin, Elongation factors 1α, Ubiquitin, Ribulose-1,5-biphosphate carboxylase and β-tubulin) selected for gene validation samples. M: 100 bp DNA ladder. Lanes 1, 2, 3, 4, 5, 6 and 7 were the gene products of D1C (control day 1), D1Cu (Cu-treated day1), D1Zn (Zn-treated day1), D5C (control day 5), D5Cu (Cu-treated day5), D5Zn (Zn -treated day5) and negative control.

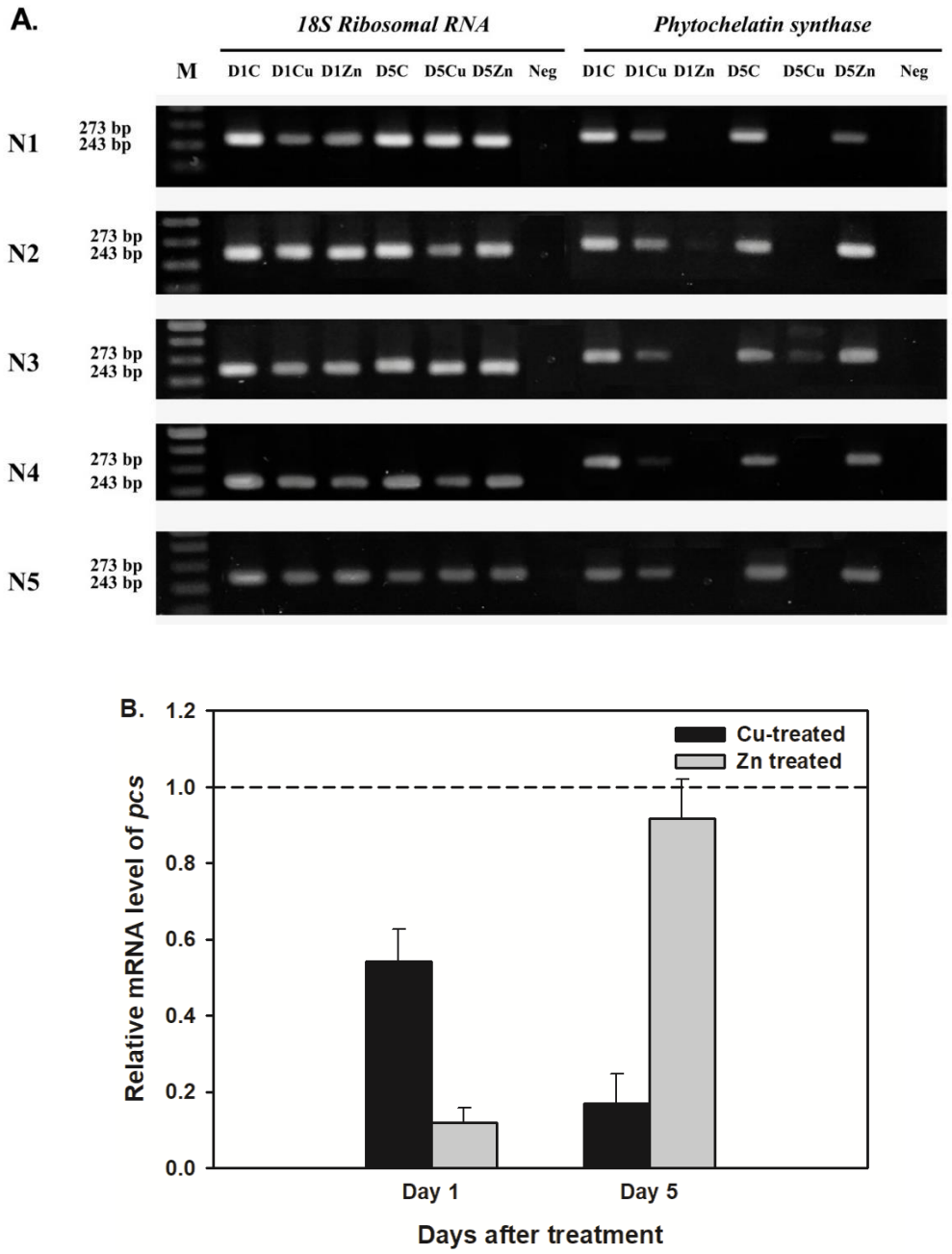

Fig. 6 Expression patterns of *pcs* in the roots of *R. mucronata* treated with Cu or Zn sampled on day 1 and day 5 of the treatment. *18s* was used as an internal control. Evaluations of expression patterns of *pcs* via semi-quantitative RT-PCR analysis (A). Expression of *pcs* relative to control derived from semi-quantitative RT-PCR analysis (B)

Table 2 Photosynthetic parameters derived from rapid light curves. (mean ± SE, n = 5). Values within the same day without shared letters are statistically different (Fisher's LSD post-hoc test, $p < 0.05$).

	ETRmax	Alpha	Ek
Day0			
Control	53.01 ± 3.60	0.30 ± 0.00	171.20 ± 10.48
Cu-treated	52.28 ± 4.80	0.30 ± 0.01	174.40 ± 10.94
Zn-treated	50.20 ± 2.10	0.28 ± 0.01	179.06 ± 10.24
Day1			
Control	57.74 ± 1.68^{a}	0.33 ± 0.01^{ac}	177.81 ± 9.58
Cu-treated	42.09 ± 1.61^{b}	0.24 ± 0.01^{b}	177.43 ± 8.04
Zn-treated	57.23 ± 5.59^{a}	0.30 ± 0.01^{bc}	190.78 ± 14.47
Day 5			
Control	48.19 ± 2.23^{ac}	0.31 ± 0.01^{a}	153.11 ± 5.86^{bc}
Cu-treated	25.40 ± 0.92^{b}	0.25 ± 0.02^{b}	107.15 ± 11.49^{b}
Zn-treated	55.38 ± 1.97^{c}	0.32 ± 0.01^{a}	174.74 ± 5.73^{c}

4. Discussion

An addition of Cu and Zn did not change the bioaccumulation in the leaves but on the contrary large accumulation was detected in the roots, suggesting that *R. mucronata* seedlings restricted their translocation of Cu and Zn from the roots to the photosynthetic tissues. This is in line with our previous study on *R. mucronata* which reported a translocation factor of 0.04-0.10 for Cu and 0.07-0.12 for Zn (Torasa et al. 2019) and the study conducted by Kaewtubtim et al. (2016) on the same species. It has been suggested that under excessive concentration of trace metals, cell wall plays a significant role in trace metals immobilization, thus reduce translocation as evidenced in our earlier work (Torasa et al. 2019). In addition to cell wall sequestration, casparian strip (MacFarlane and Burchett 2000) and the lignification thickening of exodermis (Cheng et al. 2010, 2012) have been proposed to play a role in preventing trace metals from entering the vascular tissue of *Avicennia marina* and *Bruguiera gymnorrhiza*, respectively.

Nevertheless, restricted translocation did not prevent a down-regulation of photosynthetic efficiency as observed in rapid light curves (RLCs) of Cu-treated plants. Although the maximum quantum yield of PSII was unaffected, suggesting a lack of damage to the photosynthetic apparatus, Cu exposure resulted in a significant alteration in light use efficiency particularly the maximum electron transport rates (ETR_{max}). Copper toxicity has multiple inhibiting sites in photosynthetic light reactions (Dewez et al., 2005; Barón et al., 2018) including water-splitting systems

(Rijstenbil et al., 1994), quinone and pheophytin (Mohanty et al., 1989; Yruela et al., 1993, 1996) which may result in a decrease in electron transport rate. A reduction in light use efficiency observed in this study may serve as photoprotection to limit absorbed light energy thus prevent over-excitation of electron transport chain and ROS generation under metal stress (Takahashi and Badger, 2011; Burdett et al., 2014; Buapet et al., 2017; Phandee and Buapet, 2018; Buapet et al. 2019). However, photoinhibition due to stress should not be excluded since slight wilting of leaves and bent stem were also observed in Cu-treated plants. The other plausible explanation is the disturbance of water balance induced by excess Cu. Studies have shown that Cu exposure reduced plant water uptake and stomatal conductance (Kastori et al 1992; Burzyński and Klobus 2004; Rucińska-Sobkowiak 2016), which may consequently reduce photosynthetic electron transport rates. Parameters derived from RLCs, particularly ETR_{max}, were found to be very responsive to Cu toxicity as the effects were detected from day 1 after exposure. This impact on photosynthesis was not observed in our previous study (Torasa et al. 2019), suggesting the effects of different experimental set-ups. In our previous study, we planted the seedlings in soil whereas in the present study, the seedlings were grown in hydroponic experiment. Soil particles are known to contribute to metal immobilization thus lessen the bioavailability of trace metals, and hence lower the toxicity (Liu et al 2014; Natesan et al 2014; Marchand et al 2016).

Neither sign of oxidative stress measured as total reactive oxygen species (ROS) and lipid peroxidation in the roots nor visual difference of *R. mucronata* roots among treatments was detected despite large accumulation of Cu and Zn. These results highlight that *R. mucronata* was, even at seedlings stage, very effective at preventing oxidative damage induced by excessive trace metals. Studies have elucidated the roles of various defensive mechanisms associated in ROS detoxification in mangrove plants including antioxidant enzymes such as superoxide dismutase and glutahthione peroxidase and non-enzymatic antioxidants such as glutathione, ascorbate and polyphenols (Zhang et al. 2007; Caregnato et al. 2008; Huang and Wang 2010; Wang et al. 2014).

Distinct differential expression of phytochelatin synthase (*pcs*) in the roots was observed among treatments. Cu exposure markedly reduced transcripts encoding phytochelatin synthase from day 1 onwards, suggesting that this Cu concentration may be too excessive and led to disturbed transcription processes and reduced mRNA levels of *pcs*. Previous studies have demonstrated that PC accumulation in plants is regulated dependent on dosage of trace metals. Under toxic concentrations of Cu, PC content in metallophytes increased until a certain concentration where it showed a marked reduction (Schat and Kalff 1996; Schat et al 2002). Although being up-regulated by Cu exposure, *pcs* expression in a mangrove plant, *Avicennia germinans*, decreased with increasing Cu concentration (Gonzalez-Mendoza et al 2007). In addition, our recent work has demonstrated that high Cu level is able to inhibit transcription processes of various genes associated with antioxidant and detoxification systems in a seagrass, *Zostera muelleri* (Buapet et al. 2019). In contrast, there was a substantial inhibition of *pcs* expression in the roots of Zn-treated plants on day 1 but the transcript level of *pcs* recovered to the level similar to control on day 5. This recovery corresponds with Zn level in the roots which decreased on day 5, allowing the transcription process to restore. While this study could not attribute the role in defense against oxidative stress to phytochelatin since a substantial down-regulation in *pcs* did not promote any increase in ROS or lipid peroxidation, a constitutive expression of *pcs* appeared to be essential to prevent a phytotoxicity symptoms.

Exposure to Cu clearly affected the plant health on day 5, probably in part, because of a failure to uphold phytochelatin level in the root cells. However, it should not be excluded that other sequestration mechanisms are involved in plant tolerance to trace metals, such as metallothioneins and ABC transporters which may cooperatively contribute in neutralization and compartmentalization of trace metals in the roots of *R. mucronata* (Zhou and Goldsbrough, 1994; Giordani et al., 2000; Guo et al., 2003; Contreras-Porcia et al., 2011; Laporte et al., 2016; Sharma et al., 2016).

Mangrove degradation emphasize the pressing need to understand the mechanisms underlying the key stressors that threaten the survival of the mangrove ecosystem and to establish an effective suite of biomarkers as preventive tools. Based on combined photosynthetic responses and molecular investigation, we suggest that Cu imposed more severe toxicity in *R. mucronata* seedlings compared to Zn, leading to a fast response at the cellular level. The exact mechanisms of Cu toxicity in *R. mucronata* remain to be elucidated but it may partly involve an inhibition of phytochelatin biosynthesis. Due to their responsiveness, we suggest that Cu content in the roots and RLC parameters should be integrated into a biomarker suite for the detection of Cu contamination in *R. mucronata*. Note that trace metal exposure in the present study was by means of a single addition, which resulted in a plateau in Cu bioaccumulation after the first day or even a slight decrease in Zn bioaccumulation at the end of the experiment. The response may differ should plants are exposed with multiple-pulse exposure or slow-released source. Further studies using systemic approaches such as transcriptome and proteome analysis will provide valuable dataset for identifying other candidate genes and mechanisms which have high specificity to exposure to Cu and Zn excess.

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Appendix

Project outputs

Publication

Torasa S, Boonyarat P, Phongdara A, Buapet P. 2019. Tolerance mechanisms to copper and zinc excess in Rhizophora mucronata Lam. seedlings involve cell wall sequestration and limited translocation. Bulletin of Environmental Contamination and Toxicology. https://doi.org/10.1007/s00128-019-02589-y

Conference presentation

The 5 th international Mangrove Macrobenthos and Management meeting (MMM5) 1-5 July 2019 Singapore.

Bulletin of Environmental Contamination and Toxicology
https://doi.org/10.1007/s00128-019-02589-y

Tolerance Mechanisms to Copper and Zinc Excess in *Rhizophora mucronata* Lam. Seedlings Involve Cell Wall Sequestration and Limited Translocation

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Received: 11 November 2018 / Accepted: 6 March 2019

Abstract

Rhizophora mucronata is a common mangrove growing in habitats subjected to heavy metal (HM) contamination. Understanding their physiological responses to copper (Cu) and zinc (Zn) excess and underlying tolerance mechanisms is crucial to assess impacts of metal pollution on mangrove community. Seedlings were treated with Cu or Zn (0, 50 or 100 mg per plant) by means of a single addition. At day 3 and 7, Cu and Zn accumulation, photosynthetic efficiency, superoxide dismutase and peroxidase activity, non-protein thiols, reactive oxygen species and lipid peroxidation in roots and leaves were measured. *R. mucronata* restricted Cu and Zn translocation, thus accumulated HM mainly in roots while kept the leaves unaffected. However, high root HM did not induce oxidative stress nor anti-oxidative defense as HM were largely deposited in cell wall. We concluded that HM tolerance strategies of *R. mucronata* seedlings are exclusion and restriction of translocation to the vital photosynthetic tissue.

Keywords *Rhizophora mucronata* · Copper · Zinc · Phytotoxicity · Physiology

Mangroves are heavy metal (HM) accumulation sites, because of anthropogenic activities such as agricultural run-off, domestic and industrial wastewater discharges, port activities, and mining (Kaewtubtim et al. 2016; Pumijumnong and Danpradit 2016). Although copper (Cu) and zinc (Zn) play an important role as plant micronutrients, they can be harmful at excessive concentration. Several studies have shown that mangrove habitats, particularly of *Rhizophora* sp., deposit Cu and Zn (Kaewtubtim et al. 2016; Pumijumnong and Danpradit 2016). A positive correlation between the concentration of HM in sediment and in plant biomass under field conditions was reported (Kamaruzzaman et al. 2011; Souza et al. 2015). Similar trends were observed in Cu and Zn contaminated treatments under experimental conditions (Macfarlane and Burchett 2002; Macfarlane et al. 2007).

Plants which are able to grow in contaminated areas have efficient defensive mechanisms to prevent toxicity effects. One of the strategies is to limit Cu and Zn uptake into the cell. HMs can be sequestered in the cell wall (Colzi et al. 2011; Krzesłowska 2011; Lang and Wernitznig 2011) whereas control of HMs uptake by various families of transporters upon exposure to excessive HMs have been reported (Singh et al. 2016). Overabundance of HMs in the cell, including Cu and Zn ions, triggers the formation of reactive oxygen species (ROS) and finally lead to oxidative stress (Shahid et al. 2014). ROS damage biological molecules, particularly lipids, through a chain of oxidation reactions (Shahid et al. 2014). Moreover, toxicity mechanisms of Cu and Zn are not limited to oxidative damage but also include disruption of photosynthesis via replacement of Mg in the light-harvesting complex II (LHC II) (Thomas et al. 2016) and inhibition of photosynthetic electron transport (Yruela

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00128-019-02589-y) contains supplementary material, which is available to authorized users.

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Published online: 13 March 2019

et al. 1996) as well as carbon fixation (Monnet et al. 2001). To prevent disturbance of physiological processes once HMs are present in the cell, plants rely on other defensive mechanisms such as detoxification of ROS by antioxidant systems (Shahid et al. 2014; Singh et al. 2016), neutralization of free metal ions by forming complexes with phytochelatin, and metallothionein (Gonzalez-Mendoza et al. 2007) and excretion of HMs (Bothe and Słomka 2017). Under the HM-contaminated condition, the antioxidant systems consisting of enzymatic and non-enzymatic substances are up-regulated (Singh et al. 2016). The antioxidant enzymes, such as superoxide dismutase (SOD) and peroxidase (POX), are responsible for ROS elimination (Shahid et al. 2014; Singh et al. 2016). Glutathione, one of the major constituents of non-protein thiols (NPT), functions as a non-enzymatic antioxidant. It directly regulates the redox balance in the cell and is also used as a substrate for ROS elimination via enzymatic reaction such as glutathione peroxidase (GPX) (Macfarlane and Burchett 2001; Rahman et al. 2012).

HM pollution is among the major threats to mangrove health and may contribute to mangrove degradation (Pumijumnong 2014; Sandilyan and Kathiresan 2014). *Rhizophora mucronata* L. is one of the most common mangrove species, growing in estuaries, tidal creeks, and mudflat areas in Thailand and is often exposed to HM contamination (Kaewtubtim et al. 2016; Pumijumnong 2014; Pumijumnong and Danpradit 2016). Understanding their physiological responses under Cu and Zn contamination and their underlying tolerance mechanisms is crucial for predicting the plant response to HM pollution at the organismal and community level. However, little is known about such physiological effects or about the mechanisms that help mangroves cope with HMs, particularly Cu and Zn. Previous works have been conducted mainly on *Avicennia marina, Avicennia germinans, Kandelia candel* and *Bruguiera gymnorrhiza* (Macfarlane and Burchett 2002; Gonzalez-Mendoza et al. 2007; Rahman et al. 2012). Cu and Zn contamination was found to reduce plant growth (Macfarlane and Burchett 2002), photosynthetic activity and pigment contents (Macfarlane and Burchett 2001) while increase the activity of antioxidant enzymes such as SOD, POX, catalase, and GPX (Macfarlane and Burchett 2001; Rahman et al. 2012). These results suggest that exposure to excessive Cu and Zn affects photosynthetic activity, induces oxidative stress and increases ROS scavenging activities in both enzymatic and non-enzymatic systems in mangroves. Nevertheless, the effects of HMs in the genus *Rhizophora* remain unexplored. This study focuses on seedlings as they are in the most critical stage and their survival could determine the success of the establishment and colonization of the mangrove forest. In addition, *R. mucronata* seedlings have not yet fully developed their secretion systems, thus there is low tendency of glandular excretion of metal chloride complexes from their leaves and barks. This may render them more vulnerable to HM toxicity but at the same time making them a potential bioindicator of HM contamination.

Materials and Methods

One-month-old seedlings of *R. mucronata* were obtained from the Mangrove Resources Development Station 38 (Songkhla, Thailand) and acclimated under ambient light and temperature at the greenhouse facility of the Department of Biology, Prince of Songkla University for 5 weeks before starting the experiment. Seedlings were subsequently divided into five groups of treatments (20 seedlings/treatment). Each seedling was planted in each pot containing the soil collected from the station (sandy clay loam soil containing 60% sand 28% clay and 12% silt, organic matter = 2.0 % ± 0.3%, pH = 5.20 ± 0.03) and each plant was considered a replicate. HM-treated plants were watered with 100 mL of ¼ Hoagland solution with the salinity of 8 ppt containing 50 or 100 mg of Cu ($CuCl_2$) or Zn ($ZnCl_2$). The same solution without Cu and Zn addition was used as a control. All pots of plants were watered daily, and nutrients were re-supplied with ¼ Hoagland solution on day 3. On day 3 and 7 after treatment, photosynthetic parameters were recorded, and soil, plant roots, and leaves were collected. Each individual seedling was collected and stored separately. After harvested, plant leaves and roots were immediately washed with de-ionized water and dried using paper towel. Materials used for analysis of Cu and Zn content were oven-dried or immediately analyzed whereas materials used for biochemical assays were frozen in liquid N_2 and stored at − 80°C until use.

Roots, leaves and soil were dried at 80°C (n = 4). Approximately 0.1 g of dried tissues was ground and analyzed for Cu and Zn content, using an inductively coupled plasma optical emission spectrometry (ICP-OES Avio® 500, Perkin Elmer, USA) at the Central Equipment Division, Faculty of Science, Prince of Songkla University. The limit of detection was 0.4 and 1 µg/L for Cu and Zn, respectively. For quality control, blanks and standards were digested and analyzed with each sample batch (R^2 of the standard curve > 0.995) and all samples and standards were analyzed in duplicates (%RSD ≤ 10%). Translocation factor (TF) was calculated as the ratio of HM in the leaves to its roots the following the equation: *HM* (leaf)/*HM* (root). Bioconcentration factor (BCF in roots) was calculated as the ratio of HM concentration in the tissue to the concentration of same metal in soil, following equation: *BCF* = *HM* (root)/*HM* (soil).

Fractionation of root tissue was modified from Li et al. (2016). First 0.35 g of fresh root samples (n = 4) were homogenized with 14 mL of extraction buffer (50 mM Tris–HCl pH 7.5 containing 250 mM sucrose, and 1.0 mM

dithioerythritol). The mixture was centrifuged at 300×*g* for 10 min at 4°C. The supernatant represented the cytoplasmic fraction whereas the pellet represented the cell wall fraction. Both fractions were analyzed for Cu and Zn content as previously described and normalized to fresh weight.

SOD activity was measured following Elavarthi and Martin (2010). Guaiacol POX activity was measured following Macfarlane and Burchett (2001). NPT content was measured following Devi and Prasad (1998). Total ROS was estimated according to Phandee and Buapet (2018). Lipid peroxidation (LPO) was estimated following Jambunathan (2010). Fresh weight was used for normalization. All the analyses of SOD, POX, NPT, ROS and LPO were done with three technical replicates and six biological replicates.

Photosynthetic efficiency was assessed as the maximum quantum efficiency of photosystem II (Fv/Fm) and the effective quantum efficiency of photosystem II (ΦPSII) by using a pulse-amplitude modulated chlorophyll fluorometer (Mini-Pam, Walz, Germany) on the leaf of the second leaf pair (n = 6). The Fv/Fm was calculated as $(F_m - F_0)/F_m$, where F_0 is the fluorescence yield of the dark-adapted sample and Fm is the maximum fluorescence after saturating light pulse is applied. The ΦPSII was calculated as $(F_m' - F)/F_m'$, where F is fluorescence yield of the light-adapted sample and Fm′ is the maximum fluorescence after saturating light pulse is applied.

HM content, SOD activity, POX activity, NPT content, ROS and LPO were tested using three-way ANOVA (Treatment, day after treatment and plant parts as categorical factors). The photosynthetic parameters were analyzed using repeated measures ANOVA (Treatment as categorical factor and day after treatment as a within-group factor). Fisher's least significant difference (LSD) test was used to compare the effect of HM exposure across time of measurements and between plant parts (leaf and root).

Results and Discussion

There was no change in HM content in controls. HM-enriched plants showed high accumulation of both Cu and Zn in the roots whereas there was no change in accumulation in the leaves (Fig. 1a–d). We observed an increase in Cu in root tissues compared to control but not between the two Cu treatments (Fig. 1c) whereas accumulation of Zn in the roots showed dose and time-dependent response (Fig. 1d).

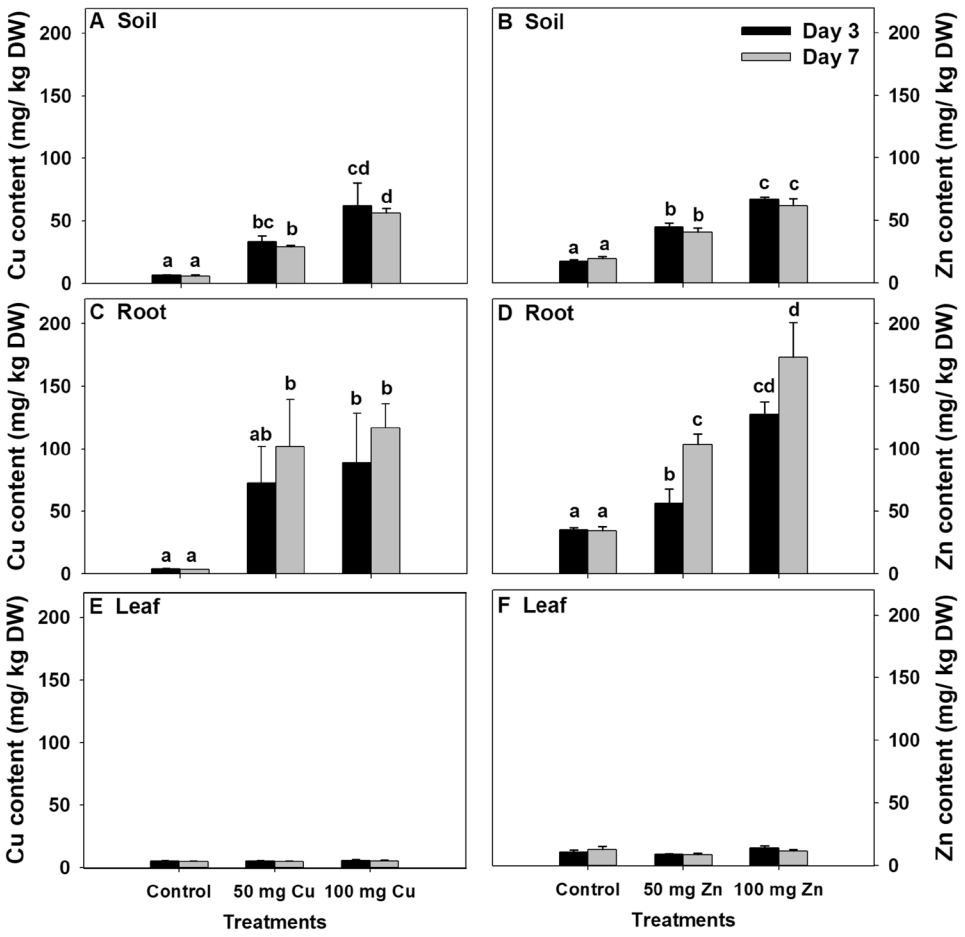

Fig. 1 The accumulation of Cu and Zn in the soil (**a, b**), the roots (**c, d**) and the leaves (**e, f**) of *R. mucronata* seedlings exposed to control, Cu and Zn on day 3 and 7. Error bars show standard error of mean, n = 4. Bars without shared letters are statistically different (Fisher's LSD post-hoc test, $p < 0.05$)

A predominant accumulation of Cu and Zn in the roots and a TF below 1 (Table 1) indicate that *R. mucronata* seedlings are excluder (Kaewtubtim et al. 2016), in line with previous studies in various mangrove species such as *R. mucronata* (TF = 0.2 for Cu and Zn, Kaewtubtim et al. 2016), the congeneric species, *Rhizophora mangle* (TF = 0.02 for Cu; 0.36 for Zn, Silva et al. 1990) and *Rhizophora stylosa* (TF = 0.57 for Cu; 0.43 for Zn, Alongi et al. 2005). It has been suggested that under toxic concentration, translocation of Cu and Zn to the leaves is limited due to restriction at the endodermis by casparian strip in *A. marina* (MacFarlane and Burchett 2000) and at the exodermis by lignification thickening in *B. gymnorrhiza* (Cheng et al. 2012). Nevertheless, *R. mucronata* seedlings showed large accumulation of Cu and Zn in the roots relative to the concentration present in the soil described as the bioaccumulation factors (BCF). However, BCF of HM-treated roots (Table 1) are lower than what was observed in *R. mucronata* in a previous study (2.6 for Cu and 6.6 for Zn, Kaewtubtim et al. 2016) but higher than in other congeneric species such as *R. mangle* (Silva et al. 1990) and *R. stylosa* (Alongi et al. 2003). The content of both HM in the root and leaf tissue was found to be higher than what was detected in *R. mucronata* in Pattani Bay, Thailand (Kaewtubtim et al. 2016). These previous investigations were conducted in adult plants in the natural settings with lower HM concentration in the sediment. The results suggest that BCF and tissue HM content may vary depending on the plant developmental stage and HM availability.

Table 1 Accumulation and translocation of Cu and Zn in *R. mucronata* seedlings after 3 and 7 days of HMs treatment

Parameter/treatment	Cu treatment		Zn treatment	
	Day 3	Day 7	Day 3	Day 7
TF				
Control	1.27 ± 0.17	1.33 ± 0.08	0.30 ± 0.03	0.04 ± 0.05
50 mg	0.09 ± 0.05	0.09 ± 0.05	0.16 ± 0.03	0.09 ± 0.01
100 mg	0.10 ± 0.06	0.04 ± 0.00	0.12 ± 0.03	0.07 ± 0.01
Root BCF				
Control	0.68 ± 0.09	0.80 ± 0.12	2.18 ± 0.13	2.15 ± 0.44
50 mg	3.02 ± 0.25	4.81 ± 1.45	1.37 ± 0.29	2.72 ± 0.14
100 mg	1.45 ± 0.01	2.00 ± 0.30	2.36 ± 0.54	2.82 ± 0.14

The distributions of Cu and Zn in the two fractions of the root tissue of *R. mucronata* are shown in Fig. 2. Up to 94% of Cu and 80% of Zn were retained in the cell wall. Previous investigations have provided evidence of the role of cell wall in HM toxicity regulation (see review in Krzesłowska 2011; Printz et al. 2016). It has been suggested that cell wall has a strong capacity for binding HM, including Cu and Zn, and may act as a barrier preventing their entry into the plant cytoplasm. This high binding capacity is attributed to sorption by cell wall components such as lignin, pectin, certain polysaccharides and proteins (Colzi et al. 2011; Krzesłowska 2011; Lang and Wernitznig 2011). In addition, high HM in the cell wall fraction may be due to HM efflux at the cell membrane. Various families of transporters have been identified and it has been proposed that they play a role in maintaining HM homoeostasis (see review in Singh et al. 2016).

Sequestration of HM in cell wall likely explains why only minor changes in physiological status were detected in the roots despite high levels of Cu and Zn in the tissue.

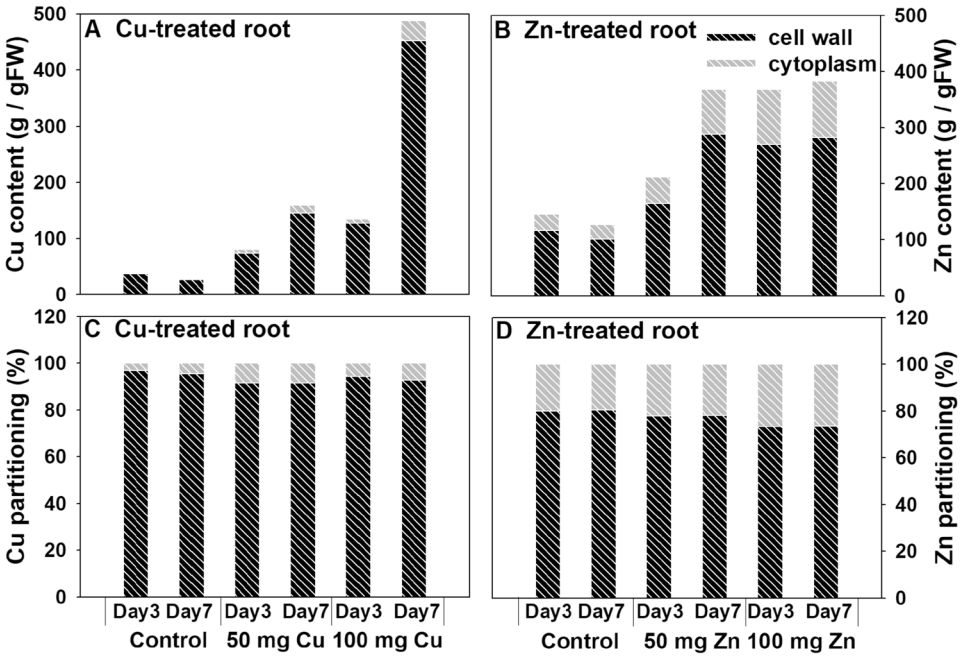

Fig. 2 Distribution of Cu and Zn in the root cells of *R. mucronata* seedlings exposed to control, Cu and Zn on day 3 and 7. The content of Cu and Zn in cell wall and cytoplasmic fractions is shown in **a, b** whereas the percentage partitioning between the two fractions is shown in **c, d**

Although HM content in cytoplasmic fraction still exhibited dose-dependent response, the concentrations detected within the root cell did not impose substantial toxicity. Root tissue did not show an over-production of reactive oxygen species (ROS; Fig. 3b) nor an increase in LPO (Fig. 3d). In addition, there was no change in the SOD activity nor in the NPT content in the roots (Fig. 4b, f). This lack of response implies that high Cu and Zn in the root tissue did not induce oxidative stress which is likely a result of HM retention in the cell wall which limits cellular uptake and consequently prevents HM toxicity. The activity of POX, however, was affected by HM addition. There was a reduction in POX activity on day 3 in treatments with 50 mg of Cu, 100 mg of Cu and 100 mg of Zn (Fig. 4d). On day 7, a partial recovery was observed in Cu-treated plants while a full recovery was observed in Zn-treated plants (Fig. 4d). This may be due to a structural modification of POX induced by Cu and Zn, thus affecting the enzyme functionality (Yruela 2009). Toxicity of Cu was stronger than Zn as it was detected at lower concentration and it imposed more chronic impacts than Zn. A number of studies have demonstrated that toxicity of Cu is more pronounced than Zn which may be due to its redox-active nature (See review in Küpper and Andresen 2016; Nanda and Agrawal 2016). However, the toxicity mechanisms of HM on the activity of enzymes, particularly antioxidant enzymes, remain to be elucidated.

Limited cellular uptake in the root as well as restricted translocation of HM to the leaves are likely explanations for the absence of change in the physiological status of the leaves. These results correspond with no change in HM content in leaf tissue. There were variations in ROS and LPO in leaf tissue (Fig. 3a, c). However, such variations cannot be accounted for by HM toxicity as there was no change in HM accumulation in the leaves. Our results indicate that root sequestration in *R. mucronata* seedlings successfully prevent harmful HM-induced oxidative stress on photosynthetic tissue. This is supported by no change in detoxification capacity measured as the activity of SOD and POX and NPT content (Fig. 4a, c, e). Similarly, both the maximum quantum efficiency of photosystem II (Fv/Fm) and the effective quantum efficiency of photosystem II (ϕPSII) remained unaffected in both Cu and Zn treatments (Fig. 5). These results once again suggest that exclusion mechanisms in *R. mucronata* seedlings successfully allow the plants to carry on their photosynthetic processes, as well as other physiological processes. Tolerance mechanisms to Cu and Zn excess in *R. mucronata* seedlings in this study is summarized in supplementary material (Fig. S1). We conducted the experiment using the salinity of 8 ppt following the results of the previous studies which showed that low salinity (3–8 ppt) provided the optimal condition for initial growth of *R. mucronata* seedlings (Hoppe-Speer et al. 2011; Kodikara et al. 2018). However, Songkhla lake, our system of interest, is brackish with large seasonal variation in salinity (0–30 ppt, Rattanama et al. 2016). Since HM bioavailability and plant HM uptake vary with salinity (Stevens

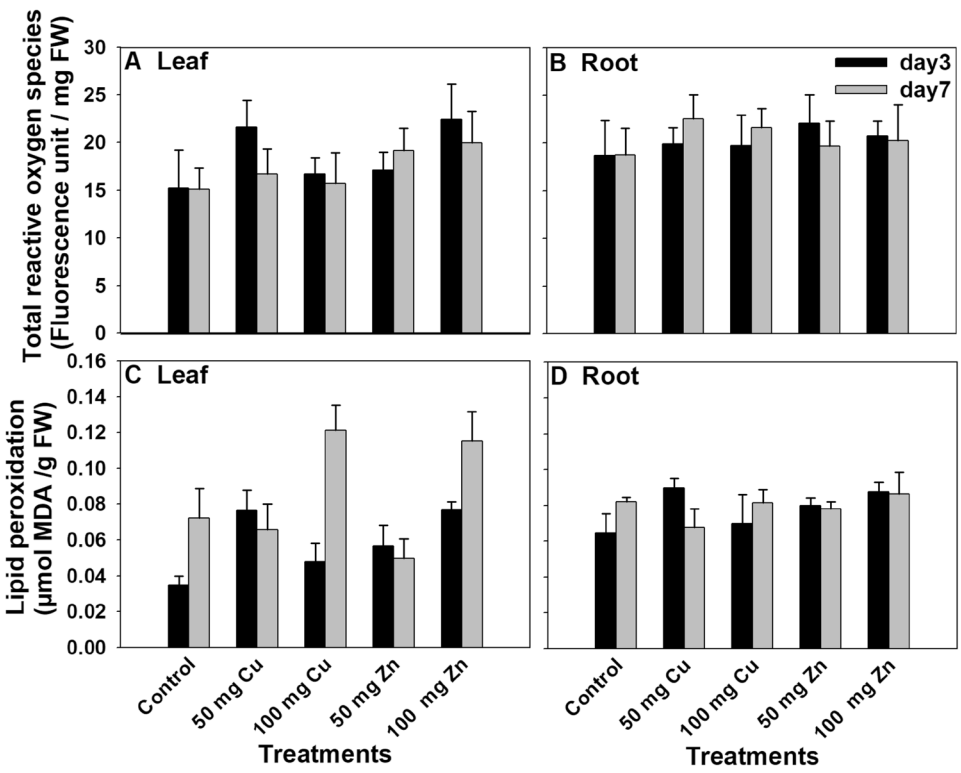

Fig. 3 ROS accumulation (**a, b**) and LPO measured as MDA content (**c, d**) in the leaves and roots of *R. mucronata* seedlings exposed to control, Cu and Zn on day 3 and 7. Error bars show standard error of mean, n = 6

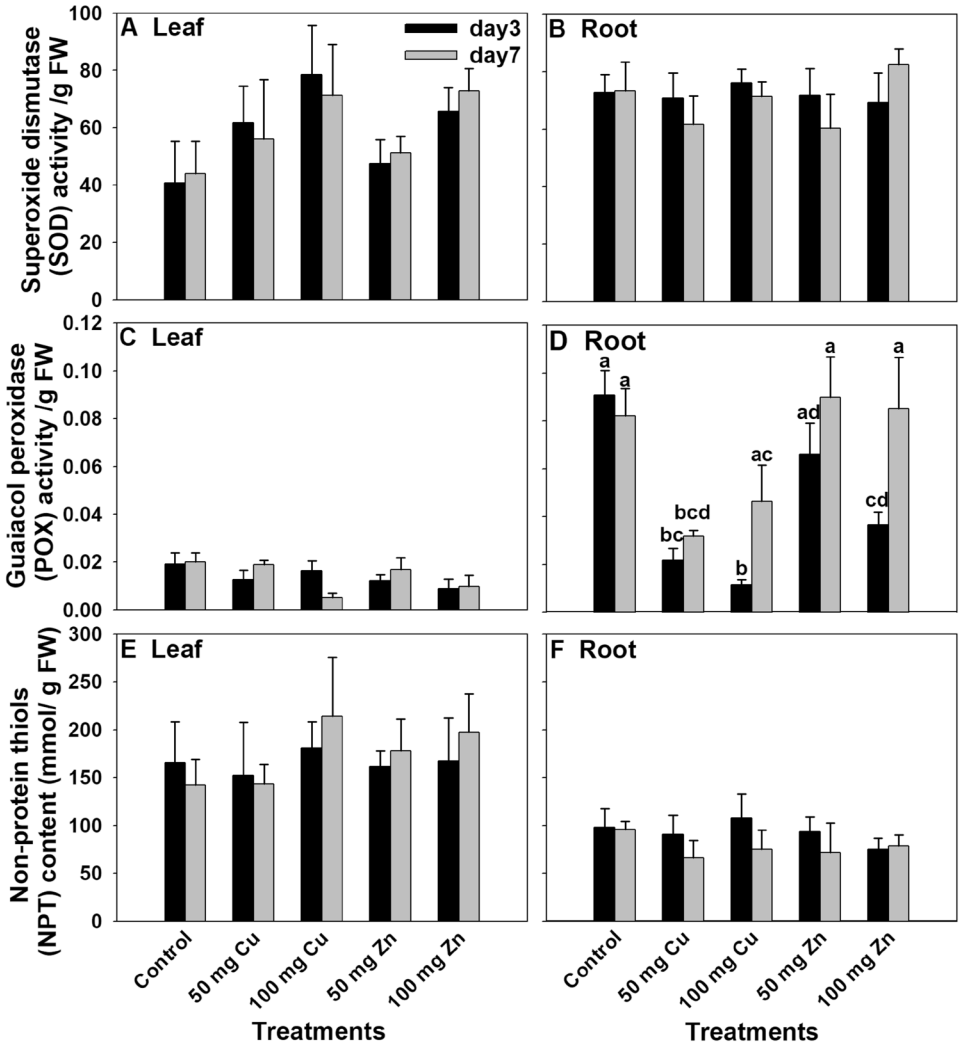

Fig. 4 SOD activity (**a, b**), guaiacol POX activity (**c, d**) and NPT content (**e, f**) in the leaves and roots of *R. mucronata* seedlings exposed to control, Cu and Zn on day 3 and 7. Error bars show standard error of mean, n = 6. For Fig. 4d, bars without shared letters are statistically different (Fisher's LSD post-hoc test, $p < 0.05$)

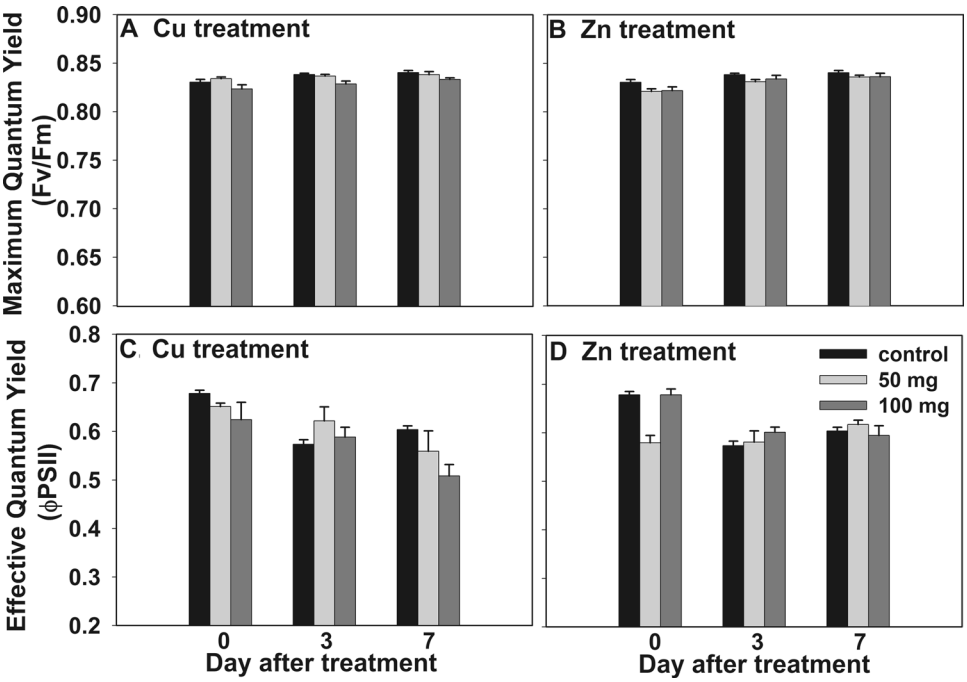

Fig. 5 The maximum quantum yield (Fv/Fm **a, b**) and the effective quantum yield (ϕPSII **c, d**) of *R. mucronata* seedlings exposed to control, Cu and Zn on day 0, 3 and 7. Error bars show standard error of mean, n = 6

et al. 2003; Fritioff et al. 2005), the phytotoxic effects under different salinity regime may consequently differ from the results of the present study.

For application purposes, Cu and Zn in the roots of *R. mucronata* seedlings may be used as a biomarker for Cu and Zn contamination, due to a dose-dependent response evidenced in the present study. Since uptake and translocation of HM in this plant were lower than the level reported in other hyperaccumulator plants, phytoremediation potential of *R. mucronata* may instead be associated with phytostabilization (Lorestani et al. 2013). Our findings highlight the ecological significance of mangroves which not only contribute as primary producer and important habitat but also function as long-term sequestration sites for HM pollutants, thus protecting adjacent ecosystems such as seagrass meadows and coral reefs.

Acknowledgements This work was supported by Thailand Research Fund (TRF Grant No. MRG6080076). We also thank Asst. Prof. Dr. Siriporn Pradit for technical advice.

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