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GROWTH, PHOTOSYNTHESIS AND PROLINE ACCUMULATION OF METAL-ACCUMULATOR WEEDS

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ABSTRACT

Intensive utilization of heavy metals, particularly lead and mercury in industry or extraction in mining areas, have caused the wide spread of these contatminants, thereby further threatening the environment. Consequently, the growth and some physiological responses of five metal-accumulator weed species were examined in response to mercury (Hg) and lead (Pb) treatments. These weed species (*Branchiaria mutica, Cyperus kyllingia, Ipomoea aquatica, Mikania micrantha,* and *Paspalum conjugatum)* were grown in water culture using half strength Hoagland's solution and subjected to $Hg(NO_3)_2$ and $PB(NO_3)_2$ at 0, 0.25 and 0.5 mM for 3 weeks. The plant growth, photosynthesis, lipid peroxidation and proline content were observed. Both Hg and Pb significantly decreased the growth, but the response was more remarkable in Hg than in Pb treatments. Hg treatment reduced photosynthetic rate dramatically under different photosynthetic photon flux density suggesting that heavy metal Hg up to 0.5 mM had damaged the photosynthetic apparatus in almost all species except in *I. aquatica.* Hg and Pb treatments caused dramatic increase in leaf MDA content, which was associated with the significant decrease of chlorophyll content. Most of the species were tolerant to Pb treatment up to 0.5 mM except *M. micrantha,* while only *C. kyllingia* and *I. aquatica* were tolerant to Hg treatment up to 0.5 mM. The Hg treatment also induced a higher proline content in the leaves of treated plants. Even without a clear increment pattern among the species, this suggested that proline may have a role as alarm stress rather than as tolerant indicator.

Keywords: heavy metals, metal toxicity, phytoremediation, stress physiology, weeds

INTRODUCTION

Heavy metal pollution is among the recently increasing anthropogenic-related environmental problems resulting from massive industrial development. Lead (Pb) and mercury (Hg) are two heavy metals that spread widely because of their intensive utilization or extraction from mining areas. These are classified as heavy metals which have dangerous toxic effects on the environment (McLusky & Elliot 2004) and can adversely affect the morphology, physiology and biochemistry processes in plants and animals (Wuana & Okieimen 2011). Photosynthesis in plants is a physiological process that is very sensitive to heavy metal toxicity both *in vitro* and *in vivo*, because they can hamper the work of Photosystem 2 (PSII) (Sheoran & Singh 1993). In addition, the accumulation of heavy metals, such as Pb and Hg in plants, has also been observed to cause the formation of ROS (Reactive Oxygen Species) which can react with macromolecules, such as DNA, pigments, proteins, lipids and other cellular molecules that cause a series of damaging processes known as oxidative stress (Ali *et al.* 2013; Singh *et al.* 2016). Heavy metals have also been reported to cause plasma membrane leakage, changes in antioxidant enzyme activity in plants, and induce the expression of genes that encode superoxide dismutase, peroxidase and catalase (Zhou *et al.* 2007). As such, heavy metal pollution is a serious problem of our environment.

Phytoremediation is an alternative technology believed to overcome the problem of heavy

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metal pollution in soil and water. It is the use of plants to reduce or eliminate metal contaminants present in the growing media (Tangahu *et al.* 2011). Plants have a variety of defense mechanisms in detoxifying heavy metals including the process of metal crushing in the cytosol by high affinity of ligands, such as amino acids and organic acids, and two classes of peptides, namely phytochelatins (PCs) and metallothioneins (MTs) at the intra and intercellular level (Hall 2002). Non-enzymatic synthesized compounds, such as proline (Pro) are also known to increase plant capacity to detoxify metal (Tangahu *et al.* 2011). Another important component of the plant defense system is the symbiotic association with arbuscular mycorrhiza (Leung *et al.* 2013; Setyaningsih *at al.* 2018). Arbuscular mycorrhizae can effectively detoxify heavy metals, increase antioxidant defense activities of plants and reduce metal absorption by host plants. Metal ions will be bound to the hyphae cell wall and then emitted as some extracellular biomolecules (Emamverdian *et al.* 2015; Leung *et al.* 2013).

To support the success of the phytoremediation program, the selection of plants that have superior properties for phytoremediation is very important, such as: (i) high growth rate; (ii) production of more above-ground biomass; (iii) widely distributed and highly branched root system; (iv) high accumulation of the targeted heavy metals from the soil; (v) tolerance to the toxic effects of the targeted heavy metals; (vi) better adaptation to the prevailing environmental and climatic conditions; (vii) resistance to pathogens and pests; (viii) easy cultivation and harvest, and (ix) repulsion to herbivores to avoid food chain contamination (Ali *et al.* 2013). Those preferable characters may not be possessed by one single species, and therefore, utilization of several species is important to support the success of phytoremediation process.

Some weed plants have great potential as plant sources for phytoremediation programs, because in addition to their rapid growth, these plants have extensive adaptability and wide distribution in many ecosystems. Some hyper-accumulator plants include *Ipomoea* sp. (Juhaeti *et al.* 2005), *Imperata cylindrica* (Howard *et al.* 2003) and *Paspalum conjugatum* (Mudarisna *et al.* 2014). Many weed species such as *Ischaemum timorense*, *Cynodon dactylon*, *Cyperus kyllingia*, *Mikania cordata*, *Calopogonium mucunoides* were also found growing well in the mining areas in Indonesia and were allegedly acting as accumulator plants (Juhaeti *et al.* 2005). Five potential weed species from grasses and broadleaf weeds, namely *Branchiaria mutica*, *Cyperus kyllingia*, *Ipomoea aquatica*, *Mikania micrantha* and *Paspalum conjugatum* were tested for their ability to grow in water cultures treated with Hg and Pb and have shown their ability to accumulate Pb or Hg from the environment (Sugiono *et al.* 2014; Bedabati & Gupta 2016; Khan *et al.* 2018; Paz-Alberto *et al.* 2007).

The purpose of this study was to examine the photosynthetic and physiological responses, as well as growth of the five weed species exposed to different Hg and Pb toxicity in water culture.

MATERIALS AND METHODS

Plant Materials and Water Culture Preparation

Species of weeds (*Paspalum conjugatum*, *Cyperus kyllingia*, *Ipomoea aquatica*, *Mikania micrantha* and *Branchiaria mutica*) were used and cultivated in water culture using half strength Hoagland's solution prepared in a plastic box containing 6 L of solution. One-month old plants were removed carefully from the polybag and the roots were washed with water to remove the soil and other solid media and were then planted in the box containing the Hoagland's solution. To stay upright, the plants were supported by perforated styrofoams and fine sponge. For continuous air supply, each box was equipped with aerator. All plants were grown under half strength Hoagland's solution for 2 weeks to establish the initial growth before the heavy metal treatment.

Mercury and Lead Treatments

The experiment was conducted using a completely randomized design with two factors: firstly, the weed species (*P. conjugatum*, *C. kyllingia*, *I. aquatica*, *M. micrantha* and *B. mutica*) and secondly, the Hg and Pb treatments, namely control or "0" treatment (without Pb and Hg treatments, Hg1 {0.25 mM of $Hg(NO_3)_2$} and Hg2 {0.5 mM of $Hg(NO_3)_2$}, Pb1 {0.25 mM of $Pb(NO_3)_2$} and Pb2 {0.5 mM

of ($Pb(NO_3)_2$). Each experiment unit had 3 replications with 6 plants per box as the experimental unit.

Pb and Hg treatments were applied to the plants after 2 weeks of establishment in the water culture added with lead nitrate ($Pb(NO_3)_2$) and mercuric nitrate ($Hg(NO_3)_2$) at different concentrations. To maintain the same volume of the solution inside the box, distilled water was added to each box, so that the total volumes of all media were equal. The heavy metals treatments were administered for 3 weeks to observe the responses of the treated plants.

The shoot and root growth and development were measured during the 3-week treatment. Morphological changes, such as wilting, necrosis, discoloration of the leaves and roots were recorded along with the treatment. Physiological analyses including photosynthesis, MDA, proline and chlorophyll content were carried out after the 10-day period when the treated plants started showing toxic symptoms. After 3 weeks of treatment, the plants were harvested for measuring other growth parameters.

Photosynthesis Measurement

Measurements of photosynthesis were carried out using the Photosynthetic Gas Exchange Analyzer LiCOR LI-6400. Observations were made on the fully expanded third leaf with 3 replications for each treatment. Measurements were made for net photosynthetic rate (Pn), stomatal conductance (Gs) and transpiration rate (E) at a saturation level of 1,500 $\mu mol/m^2/s$. To analyze the photosynthetic light curve, photosynthesis measurement was also carried out at different light intensity (100, 200, 400, 750, 1000 and 1500 $\mu mol/m^2/s$). The average of photosynthetic light curve was calculated in response to Hg and Pb treatments using Microsoft Excel 2013.

Malondialdehyde (MDA) Analysis

Lipid peroxidation was measured using the MDA content based on method developed by Ono *et al.* (1995). Fresh 0.2 g-leaves were ground and mixed with 0.5 mL of 0.1% (w/v) trichloracetic acid (TCA) at 4 °C. The leaf extract was then added to 3 mL of 1% H_3PO_4 and 1 mL of 0.6% of TBA that was dissolved in 20% of TCA. The solution was then oven-incubated at 100 °C for 30 min. After cooling down at room temperature (27 °C), the solution was added with 4 mL n-butanol, and then centrifuged at 4,200 rpm at 28 °C for 20 min. The supernatant absorbance was then measured at 532 nm using a UV-VIS spectrophotometer (Shimadzu, UV-1700, Kyoto, Japan) and corrected for nonspecific turbidity by subtracting the absorbance at 520 nm. The concentration of MDA was calculated using its extinction coefficient ($\varepsilon = 155$ L/mmol/cm).

Chlorophyll Content Analysis

Chlorophyll content was analyzed using a method developed by Yoshida *et al.* (1976). Two grams of fresh leaves were ground and applied with 80% of acetone (p.a. Merck KGaA, Darmstadt, Germany) and then was filtered using Whatman paper no. 1 into 100 mL volumetric flask until all the chlorophyll were dissolved in the acetone solution, before the solution in the volumetric flask finally reached exactly 100 mL. A 5 mL chlorophyll solution was taken from the 100 mL volumetric flask, then it was poured into a 50 mL volumetric flask and was diluted using 80% of acetone until 50 mL. The absorbance of chlorophyll solution was measured using spectrophotometer (Shimadzu, UV-1700, Kyoto, Japan) at 645 nm and 663 nm wavelength (λ). Chlorophyll content was measured using the following formula:

$$\text{Chl a} = 0.0127.\ A663 - 0.00269.\ A645$$
$$\text{Chl b} = 0.0229.\ A645 - 0.00468.\ A663$$
$$\text{Total Chl} = \text{Chl a} + \text{Chl b} = 0.0202.\ A645 + 0.00802.\ A663$$

Chl a = Chlorophyll a; Chl b = Chlorophyll b
A645 = the absorbance at the λ of 645 nm
A663 = the absorbance at the λ of 663 nm

The regression curve between chlorophyll and MDA contents in response to heavy metal treatments was calculated using Microsoft Excel 2013.

Proline Analysis

Proline content of leaves was analyzed following Bates *et al.* (1973). Homogenized tissues (150 mg) from leaves were mixed with 3 mL of 3% sulfosalicylic acid and centrifuged at

10,000 rpm for 15 min. One mL of supernatant was mixed with 1 mL of glacial acetic acid and 1 mL of acid-ninhydrin (1.25 g ninhydrin dissolved in 30 mL glacial acetic acid and 20 mL 6 M phosphoric acid), incubated for 1 h at 100 °C and then cooled in an ice bath. The reaction mixture was extracted with 2 mL of toluene and mixed vigorously for 20 s. The chromophore containing toluene was aspirated from the aqueous phase and the absorbance was measured at 520 nm. Reference standards of proline from 5 to 60 µM were prepared and analyzed in the same way to obtain a calibration curve.

RESULTS AND DISCUSSION

Plant Growth Response

The growth responses of *B. mutica, C. kyllingia, I. aquatica, M. micrantha* and *P. conjugatum* differed among the five species, including shoot and root length, leaf number and the plant biomass. The mercury (Hg) and lead (Pb) treatments dramatically influenced plant growth, even though this varied among the species. All species showed a similar response pattern toward Hg treatment; the plant growth significantly ($p < 0.05$) reduced, except for the root growth of *I. aquatica* which did not decrease (Figs. 1 & 2). All plants subjected to 0.5 mM of Hg showed the most negative effects (Figs. 1 & 2). *C. kyllingia* and *M. micrantha* even died within 10 days after the treatment. On the other hand, the plant morphological response to Pb treatment was not as remarkable as to Hg, even though the 0.5 mM Pb treatment significantly decreased some morphological parameters particularly for *I. aquatica* and *M. micrania* (Figs. 1 & 2).

Responses of shoots were more prominent than the roots' responses to both Hg and Pb treatments (Figs. 1 & 2). The shoot length reduction ranged from 54% to 87% due to 0.5 mM of Hg, while it only caused 12 - 56% root length reduction. Only the root length of *I. aquatica* was not affected by Hg treatment (Fig. 1). Even though Pb treatment did not cause prominent damage, it significantly reduced the shoot length of *I. aquatica* and *M. micrantha* and the root length of *M. micrantha* (Fig. 1). Meanwhile, only *I. aquatica* and *C. kyllingia* remained alive until the end of the 3 week-treatment of 0.5 mM Hg.

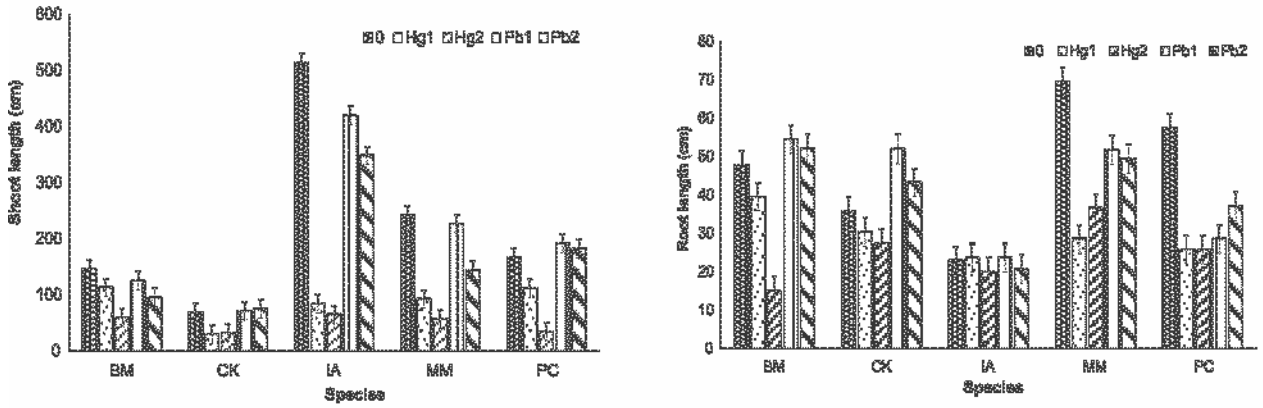

Figure 1 Shoot and root length of the species after 3 weeks exposure to Hg and Pb at different concentrations
Notes: 0 = control (without heavy metal treatments); Hg1 = 0.25 mM of Hg; Hg2 = 0.5 mM of Hg; Pb1 = 0.25 mM of Pb; Pb2 = 0.5 mM of Pb; BM = *Branchiaria mutica*; CK = *Cyperus kyllingia*; IA = *Ipomoea aquatica*; MM = *Mikania micrantha* and PC = *Paspalum conjugatum*.

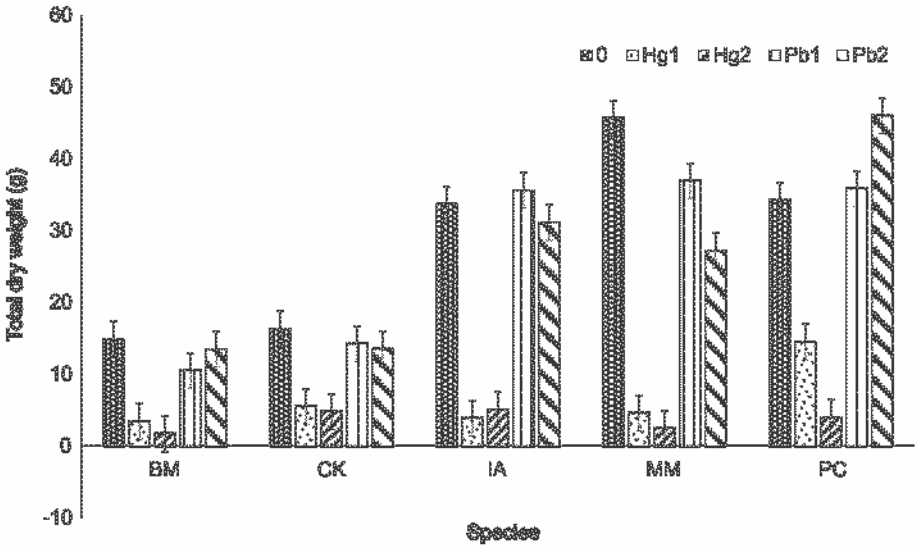

Figure 2 Total dry weight of the species after 3 weeks exposure to Hg and Pb at different concentrations
Notes: 0 = control (without heavy metal treatments); Hg1 = 0.25 mM of Hg; Hg2 = 0.5 mM of Hg; Pb1 = 0.25 mM of Pb; Pb2 = 0.5 mM of Pb; BM = *Branchiaria mutica*; CK = *Cyperus kyllingia*; IA = *Ipomoea aquatica*; MM = *Mikania micrantha* and PC = *Paspalum conjugatum*.

Heavy metals have been known to cause inhibition of root and canopy growth, and plant production (Peralta *et al.* 2001; Kibra 2008). Toxic metal, particularly lead and mercury, have affected several plants, including *Triticum aestivum* (Patra & Sharma 2000), *Phaseolus vulgaris* L. (Zengin & Munzuroglu 2005), tomatoes (Cho & Park 1999), and many others. Hg at high concentrations is very toxic that it damages the cells and causes physiological changes (Ortega-Villasante *et al.* 2005). The accumulation of Hg can also inhibit plant growth, causing plant productivity to decline. In this study, the lengths of shoots and roots and total dry weight of the five plant species decreased dramatically due to Hg stress even at only 0.25 mM concentration (Fig. 1), while Pb treatment of up to 0.5 mM only caused a relatively small decrease in growth except for *M. micrantha* (Figs. 1 & 2).

Shoot and root length as well as dry weight are the most readily observed indicators of plant growth in response to environmental stresses. Heavy metals inhibit cell division and elongation, absorption of water and nutrients, and slow down enzymatic activities thereby eventually inhibiting the plant growth (Shahid *et al.* 2015). The accumulation of Hg inhibited root and canopy growth, decreased the root-canopy ratio, and dry weight and dissolved protein content in the canopy of the *Triticum aesticum* plant (Patra & Sharma (2000). In this current study, the greatest decrease in dry weight was found in *M. micratha* plants both at 0.25 mM and 0.5 mM Hg concentrations as well as at 0.5 M Pb treatment (Fig. 2). The lower dry weight of plants showed that the physiological processes in plants were disrupted due to heavy metal toxicity, so that the growth was far from optimal.

Analysis of Photosynthesis

The species had almost similar responses to the heavy metal treatments, exhibiting an average net photosynthetic rate (Pn) of 13.5 μmol/m^2/s, while the control plants showed a stomatal conductance (*Gs*) values approximately 211 mmol/m^2/s. The effect of lead (Pb) treatment up to 0.5 mM did not significantly reduce Pn of all species (Fig. 3). However, the treatment of mercury (Hg) particularly at 0.5 mM caused dramatic decrease of Pn of almost all species except *I. aquatica* (Fig. 3). The *C. kyllingia* and *M. micrantha*, which had the lowest photosynthetic rate, died after 10 days of the 0.5 mM Hg treatment (Fig. 3). *P. conjugatum* and *B. mutica* also had decreased Pn at 0.5 mM Hg, up to 33% and 69%, respectively (Fig. 3). However, the 0.25 mM Hg treatment did not cause significant photosynthetic reduction after 10 days. The effect of Hg treatment on Gs values was almost similar on the Pn of the species.

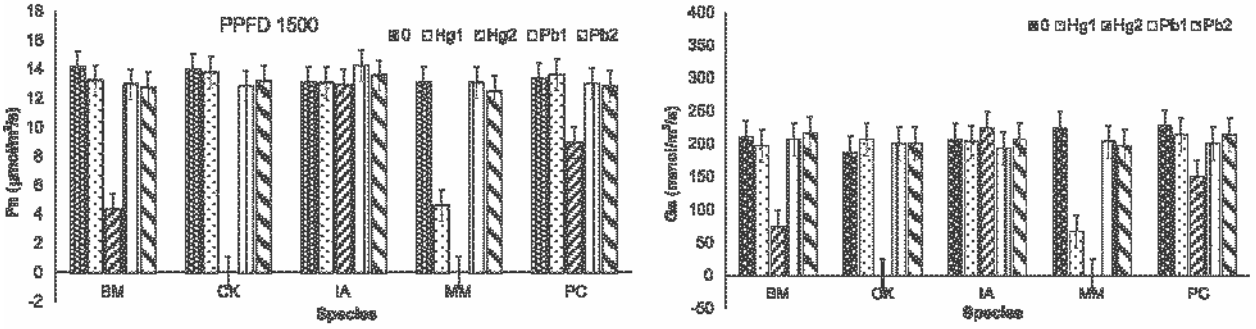

Figure 3 The average of net photosynthetic rate (Pn) and stomatal conductance (Gs) of five species (BM, CK, IA, MM and PC) in response to Hg and Pb treatments (0, 0.25 and 0.5 mM) 10 days after Hg and Pb exposure
Notes: BM = *Branchiaria mutica*; CK = *Cyperus kyllingia*; IA = *Ipomoea aquatica*; MM = *Mikania micrantha* and PC = *Paspalum conjugatum*.

To further understand the photosynthetic responses of each species to Hg and Pb treatments, the analysis of photosynthetic light curve was carried out using different photosynthetic photon flux density (PPFD), starting from 100 to 1,500 μmol/m²/s. This light curve was also important to understand the consistency of the data and to determine the maximum photosynthesis under environmental stress. Results showed that every species had different curve with unique photosynthetic rates indicating the responses of the species to the given treatments (Fig. 4). In general, photosynthetic characteristics recorded even at lower PPFD (100 μmol/m²/s) were almost similar among the treatments. The maximum photosynthesis was reached under the PPFD of approximately 750 μmol/m²/s (Fig. 4). The photosynthesis graphs showed that the treatment with 0.5 mM of Hg (Hg2) caused dramatic decrease of photosynthesis in all light intensities, except in *I. aquatica* and *P. conjugatum*, while Pb treatment did not have this effect, except in some points of PPFD. For *M. micratha* the effect of Hg was even larger because at 0.25 mM, Hg had also decreased the photosynthetic rate (Fig. 4).

To construct the light curve of photosynthesis for all the species responses to the treatments at different PPFD, the average single treatment of Hg and Pb was calculated and the light curve was plotted using logarithmic equation (Fig. 5). The graph showed three different groups of curves with the lowest curve representing the plants treated by 0.5 mM of Hg. The second group of curves was the highest photosynthesis light curve representing the control plants and Pb-treated plants which had almost similar curve (Fig. 5). The third group of curves represented the plants treated with 0.25 mM of Hg. This photosynthetic curve indicated high photosynthetic rate, but it was still lower than the second curve (Fig. 5). This curve was created especially because of the response of *M. micrantha* which had lower photosynthetic rate under 0.25 mM of Pb treatment (Fig. 4). The second and the third curves showed that at PPFD of 1,500 μmol/m²/s, the photosynthesis was still not saturated so that the photosynthetic rate was still possible to increase when the PPFD increased (Fig. 5). The distinction of the curve was also reflected in the stomatal conductance (Gs) curve in response to Hg and Pb treatments at different PPFD (Fig. 6). The Gs values decreased in response to higher PPFD with different pattern depending on the heavy metal treatment. The plants treated with Hg of 0.5 mM had the lowest Gs at all PPFD (Fig. 6).

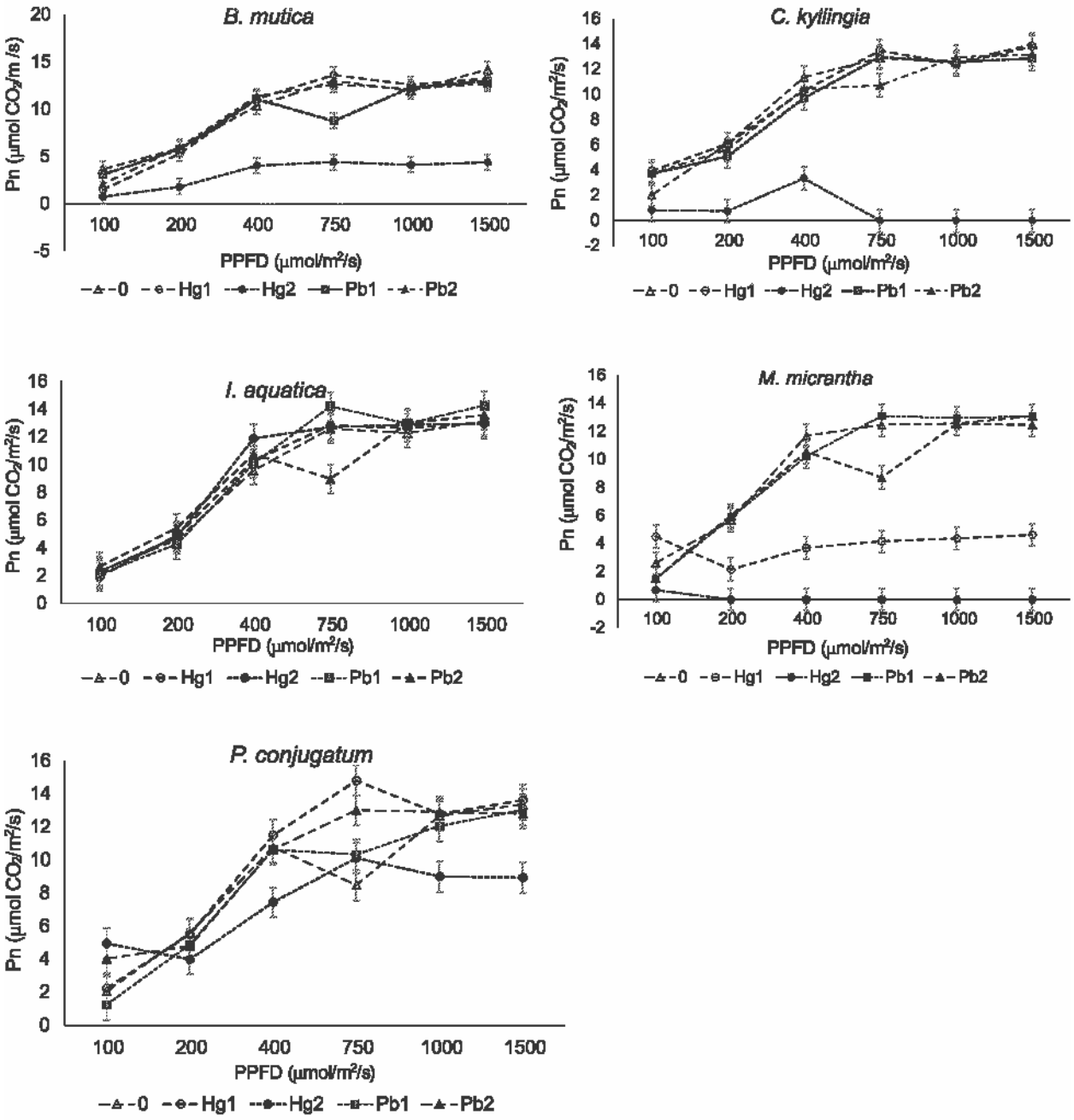

Figure 4 Net photosynthetic rate (Pn) of five species (BM, CK, IA, MM and PC) in response to Hg and Pb treatments (0, 0.25 and 0.5 mM) under different PPFD (from 100 until 1,500 µmol/m^2/s)
Notes: BM = *Branchiaria mutica*; CK = *Cyperus kyllingia*; IA = *Ipomoea aquatica*; MM = *Mikania micrantha* and PC = *Paspalum conjugatum*.

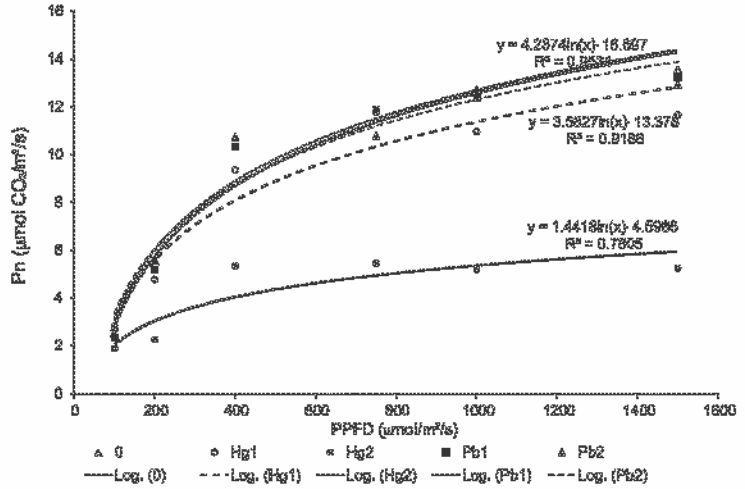

Figure 5 Photosynthetic light curve of five species (BM, CK, IA, MM and PC) in response to heavy metal treatments at different PPFD (from 100 until 1,500 μmol/m²/s)
Notes: 0 = control (without heavy metal treatments); Hg1 = 0.25 mM of Hg; Hg2 = 0.5 mM of Hg; Pb1 = 0.25 mM of Pb; Pb2 = 0.5 mM of Pb; BM = *Branchiaria mutica*; CK = *Cyperus kyllingia*; IA = *Ipomoea aquatica*; MM = *Mikania micrantha* and PC = *Paspalum conjugatum*.

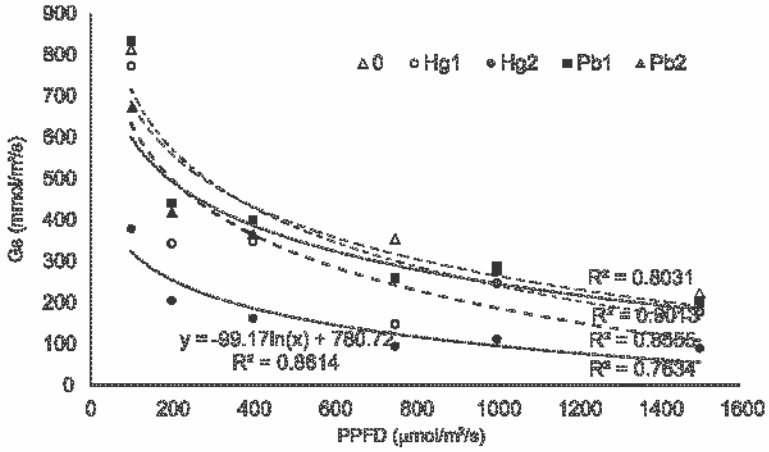

Figure 6 Stomatal conductance (Gs) of five species (BM, CK, IA, MM and PC) in response to heavy metal treatments at different PPFD (from 100 until 1500 μmol/m²/s).
Notes: 0 = control (without heavy metal treatments); Hg1 = 0.25 mM of Hg; Hg2 = 0.5 mM of Hg; Pb1 = 0.25 mM of Pb; Pb2 = 0.5 mM of Pb; BM = *Branchiaria mutica*; CK = *Cyperus kyllingia*; IA = *Ipomoea aquatica*; MM = *Mikania micrantha* and PC = *Paspalum conjugatum*.

Photosynthesis is a physiological process that is very sensitive to heavy metal toxicity both *in vitro* and *in vivo*, especially the Photosystem 2 (PSII) (Sheoran & Singh 1993). The effects of heavy metal toxicity on photosynthesis can occur either directly or indirectly (Aggarwal *et al.* 2011) i.e., directly, through inhibition of light reactions and oxygen formation, NADP reduction and photophosphorylation, while indirectly, through the inhibition of chlorophyll synthesis or the increase of chlorophyll damage.

The similarly decreasing patterns of Pn and Gs due to heavy metal stress (Figs. 1 - 4) suggested that metal toxicity may affect water absorption which was indicated by the decrease of relative water content (Fig. 7), resulted in the decrease of stomatal conductance. The decrease of stomatal conductance is a general response of plants under water deficit (Hamim 2005), but in many cases dehydration was also shown by plants under heavy metal toxicity by Pb in *Helianthus annuus* and barley (Kastori *et al.* 1992; Vassilev *et al.* 1998) or *Beta vulgaris* under Zn toxicity (Sagardoy *et al.* 2010). Among the five study species, *I. aquatica* and *P. conjugatum* had the best performance with a relatively steady or not fluctuating photosynthesis under Hg and Pb treatments which may be considered an indicator of their adaptability to these heavy metals.

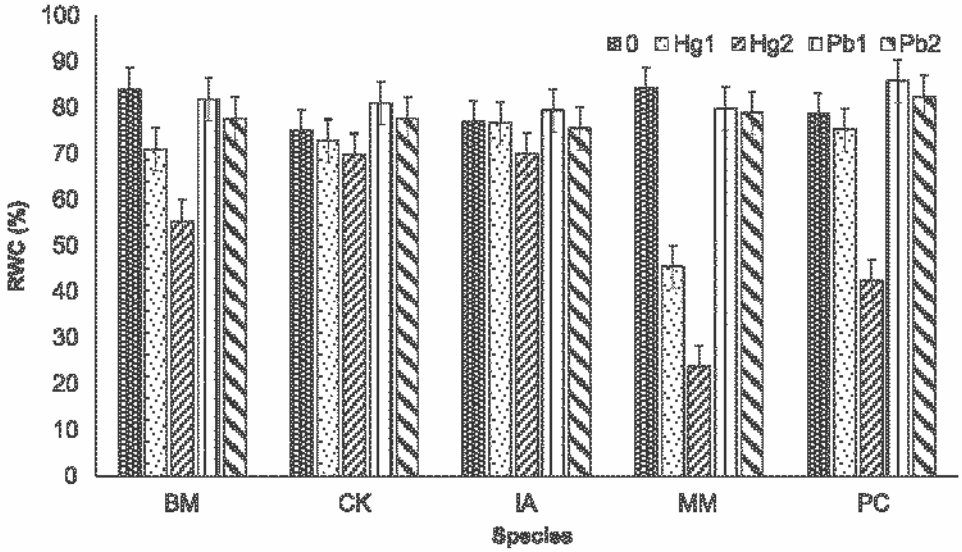

Figure 7 Relative water content (RWC) of five species (BM, CK, IA, MM and PC) after 10 days exposure to Hg and Pb at different concentrations

Notes: 0 = control (without heavy metal treatments); Hg1 = 0.25 mM of Hg; Hg2 = 0.5 mM of Hg; Pb1 = 0.25 mM of Pb; Pb2 = 0.5 mM of Pb; BM = *Brachiaria mutica*; CK = *Cyperus kyllingia*; IA = *Ipomoea aquatica*; MM = *Mikania micrantha* and PC = *Paspalum conjugatum*.

Analysis of Total Chlorophyll and Leaf MDA

The heavy metals dramatically decreased the chlorophyll content of all species (Fig. 8) particularly for plant treated with Hg at 0.5 mM. *B. mutica* had the most dramatic decrease by Hg treatment, while *I. aquatica* was the least affected (Fig. 8). On the contrary, the treatment using Pb until 0.5 mM only significantly decreased chlorophyll content of *C. kyllingia*, *I. aquatica* and *M. micrantha*, but not of *B. mutica* and *P. conjugatum* (Fig. 8).

Decrease in chlorophyll content is a general symptom of heavy metal toxicity in plant. The decrease of chlorophyll content happened in all of the heavy metal treatments of *Phaseolus vulgaris* seedlings, with the most remarkable decrease in mercuric (Hg) treatment followed by Cd and Cu, while Pb had the least effect (Zengin & Munzuroglu 2005). The dramatic decrease of chlorophyll and photosynthesis due to heavy metal stress was also observed in poplar plants (Chandra & Kang 2016), as well as in perennial grass *Phragmites australis* (Ayeni *et al.* 2012). In this study the similar pattern was observed in the chlorophyll content of the five weeds with the most affected species, *B. mutica* and *M. micrantha* by Hg treatments and *I. aquatica* by Pb treatments.

Membrane systems, including the chloroplast, are considered the main target of oxidative stress due to heavy metals. This happens because polyunsaturated fatty acids as the main component of lipid membranes are very sensitive to heavy metals. The Hg treatment given in high concentrations reduced the total chlorophyll content of the five plant species (Fig. 8). Hg stress induces photoreduction inhibition of protochlorophyllide, so the total chlorophyll value of leaves decreases with increasing Hg concentration as observed in wheat leaves (Solymosi *et al.* 2004). This decrease occurs because heavy metals can cause chlorophyll biosynthesis inhibition through the inhibitive action of two highly sensitive enzymes, i.e., α-aminolaevulinic acid (ALA) dehydratase and protochlorophyllide reductase which play an important role in the early and final stages of chlorophyll biosynthesis (de Filippis *et al.* 1981). Mercury was also reported to cause magnesium ions replacement in photosynthetic pigments (Kupper *et al.* 1998).

Malondialdehyde (MDA) content also varied among the species with the highest content found in *I. aquatica* followed by *M. micrantha*, while the lowest was found in *C. Kyllingia* (Fig. 9). Heavy metal Hg and Pb treatments caused the significant increase of MDA content in leaves of almost all species. However, the

treatments did not induce the significant increase in roots (data not shown). Only in *P. conjugatum* roots treated with 0.5 mM, did the MDA content increased significantly. Treatment with Hg significantly increased the leaf MDA of all species with the range of 2-fold increase in *C. kyllingia* until 13-fold increase in *P. conjugatum* as compared to the control, even though the highest leaf MDA was observed in *I. aquatica* exposed to 0.5 mM Hg (Fig. 9). Contrary to Hg, the Pb treatment caused the low increase of leaf MDA content in *B. mutica* and *C. kyllingia* (approximately 2 fold) but very high (7 until 33 fold) in *I. aquatica*, *M. micrantha* and *P. conjugatum* with the highest MDA content observed in *I. aquatica* (Fig. 9).

The MDA content is an index of the level of cellular damage after stress treatment, which is the main cytotoxic product of lipid peroxidation and indicators of free radical production (Fu & Huang 2001; Hamim *et al.* 2017). Higher increase of MDA content is an indication of oxidative stress which shows the main destructive factor in plants due to environmental stress, including heavy metals (Wu *et al.* 2003; Shanker *et al.* 2004). This study showed that Hg and Pb had significantly affected lipid peroxidation as indicated by the higher MDA values (Fig. 9). The increase of MDA content has also been observed in several plants subjected to heavy metal stress, such as in sorghum treated with Cd (Kumar & Pathak 2018), tree species *Reutealis trisperma* grown in goldmine tailing (Hilmi *et al.* 2018) and water hyacinth (*Eichhornia crassipes*) treated with high Pb (Malar *et al.* 2014).

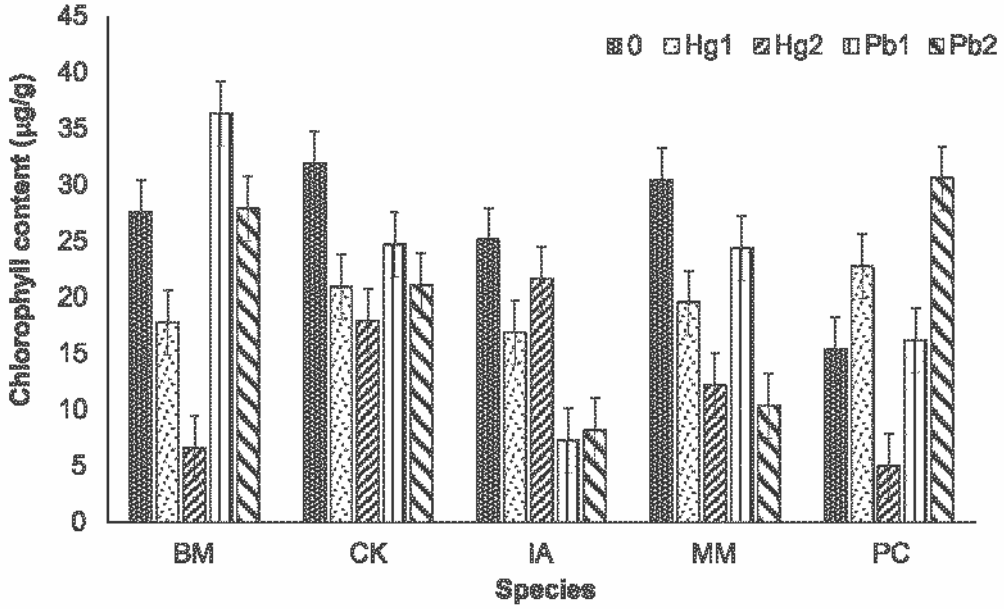

Figure 8 Chlorophyll content of five species (BM, CK, IA, MM and PC) after 10 days exposure to Hg and Pb at different concentrations

Notes: 0 = control (without heavy metal treatments); Hg1 = 0.25 mM of Hg; Hg2 = 0.5 mM of Hg; Pb1 = 0.25 mM of Pb; Pb2 = 0.5 mM of Pb; BM = *Branchiaria mutica*; CK = *Cyperus kyllingia*; IA = *Ipomoea aquatica*; MM = *Mikania micrantha* and PC = *Paspalum conjugatum*.

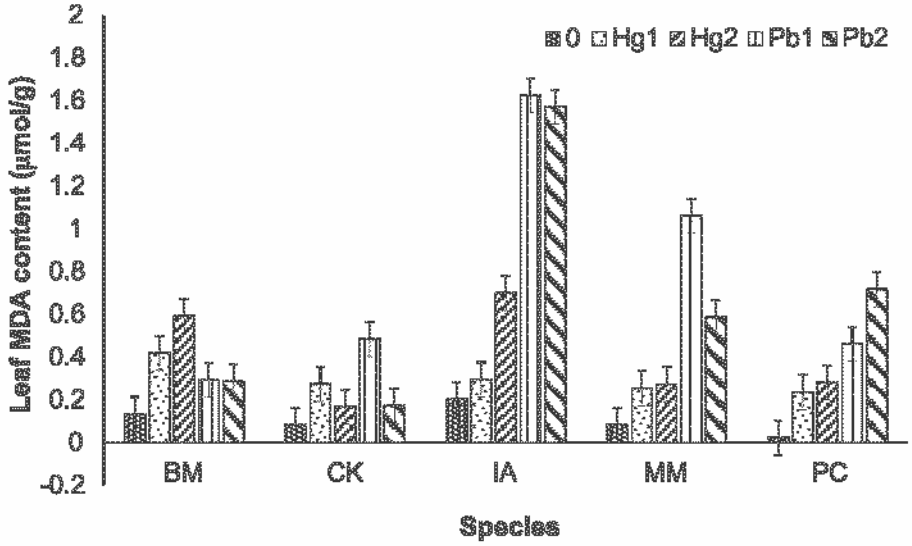

Figure 9 Leaf MDA content of five species (BM, CK, IA, MM and PC) after 10 days exposure to Hg and Pb at different concentrations

Notes: 0 = control (without heavy metal treatments); Hg1 = 0.25 mM of Hg; Hg2 = 0.5 mM of Hg; Pb1 = 0.25 mM of Pb; Pb2 = 0.5 mM of Pb; BM = *Branchiaria mutica*; CK = *Cyperus kyllingia*; IA = *Ipomoea aquatica*; MM = *Mikania micrantha* and PC = *Paspalum conjugatum*.

A close negative correlation existed between the increase of MDA content in response to Hg and Pb treatments and the decrease of chlorophyll content as indicated by the steep graph (Fig. 10). In contrast to Hg, even though it still caused the increase of MDA content and the decrease of chlorophyll content, the correlation was lower with less steep slope than that of Hg (Fig. 10), suggesting that the effect of Hg treatment on the decrease of chlorophyll was higher than that of Pb. The same result was observed in Zengin and Munzuroglu (2005) wherein the effect of Hg was far higher than Pb on the chlorophyll reduction of *P. vulgaris* seedlings.

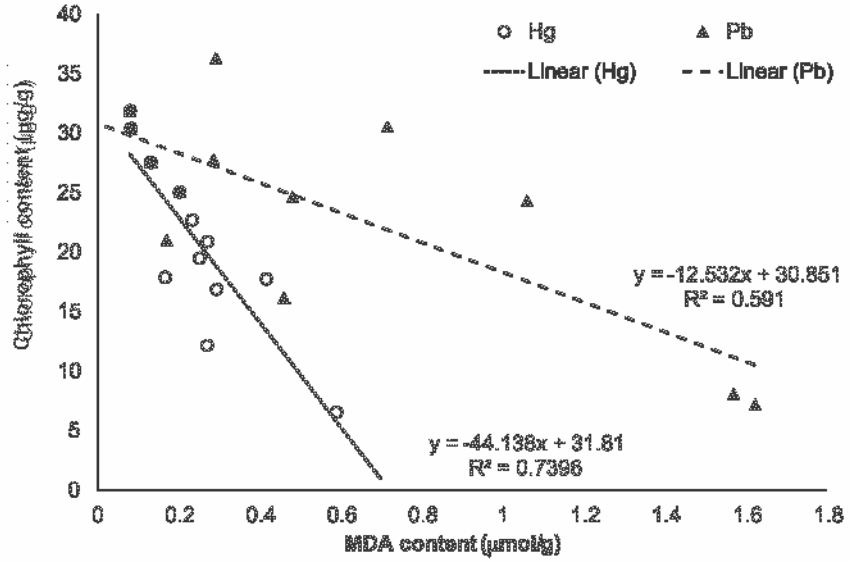

Figure 10 The regression graph between MDA and chlorophyll content of all species in response to Hg and Pb treatments

Notes: There was a different slope pattern between both treatments, where Hg treatment had a steeper slope, while Pb had a slight slope.

Proline Analysis

Proline content is among the physiological parameters which normally increase when the plant is subjected to environmental stresses, such as drought, salinity and even heavy metal stresses. The experiment also showed the similar tendency, especially when the plants were treated with Hg at 0.25 and 0.5 mM (Fig. 11). Proline content of all species significantly increased from 2-fold until 9-fold increase at 0.25 mM of Hg treatments and even until 15-fold increase at 0.5 mM of Hg treatment. The highest proline content was presented by *M. micrantha* at 0.5 mM of Hg followed by *P. conjugatum* and *I. aquatica* (Fig. 11). However, there was no clear pattern between proline content and plant adaptability to Hg stress, because *M. micrantha* (the most affected by Hg) and *I. aquatica* (the least affected by Hg) had high proline content. In contrast to Hg, the treatment using Pb at 0.25, as well as, 0.5 mM did not affect the increase of proline content of all species. The regression data presenting proline content in relation to Hg or Pb treatments indicated that these two parameters had different graph patterns and correlation coefficients (Fig. 11).

Proline is amino acid that in many cases increased dramatically in response to several environmental stresses, such as drought stress (Lum *et al.* 2014; Mwenye *et al.* 2016), salinity stress (Theriappan *et al.* 2011), as well as heavy metal stress (Zengin & Munzuroglu 2005; Theriappan *et al.* 2011). Previous study recorded that the induction of proline accumulation was also found in some crops such as *Cajanus cajan*, *Vigna mungo* and *Triticum aestivum* subjected to heavy metals (Alia & Saradhi 1991). This amino acid was found to have an important role as biochemical scavenger of ROS induced by abiotic stress. However, in this study, the increase of proline happened when the plant underwent severe stress due to metal toxicity (Fig. 12), and there was no correlation between proline accumulation and metal tolerance among the five species, suggesting that the increase of proline indicated an alarm stress rather than the role of reducing the damage of heavy metal stress in these species.

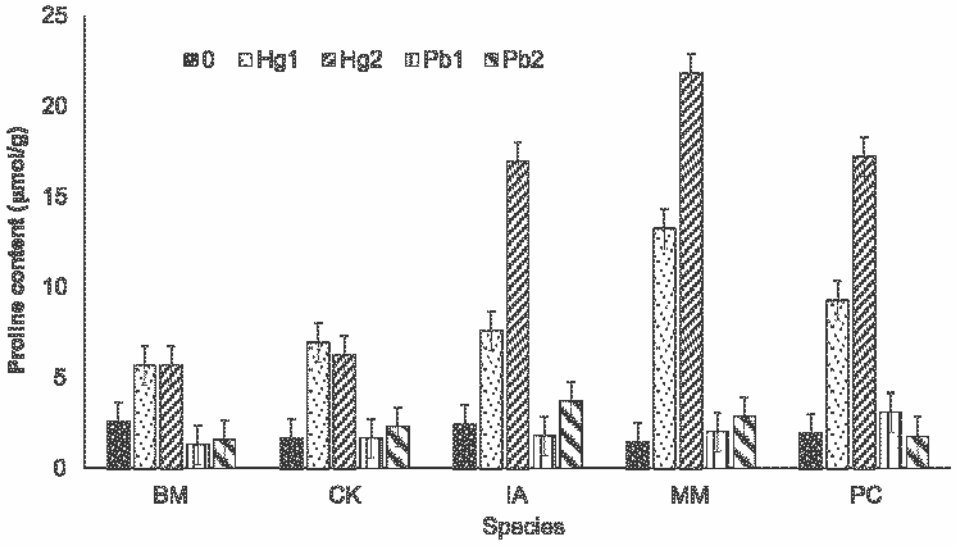

Figure 11 Proline content of five species (BM, CK, IA, MM and PC) subjected to different concentrations of Hg and Pb

Notes: 0 = control (without heavy metal treatment); Hg1 = 0.25 mM of Hg; Hg2 = 0.5 mM of Hg; Pb1 = 0.25 mM of Pb and Pb2 = 0.5 mM of Pb; BM = *Branchiaria mutica*; CK = *Cyperus kyllingia*; IA = *Ipomoea aquatica*; MM = *Mikania micrantha* and PC = *Paspalum conjugatum*.

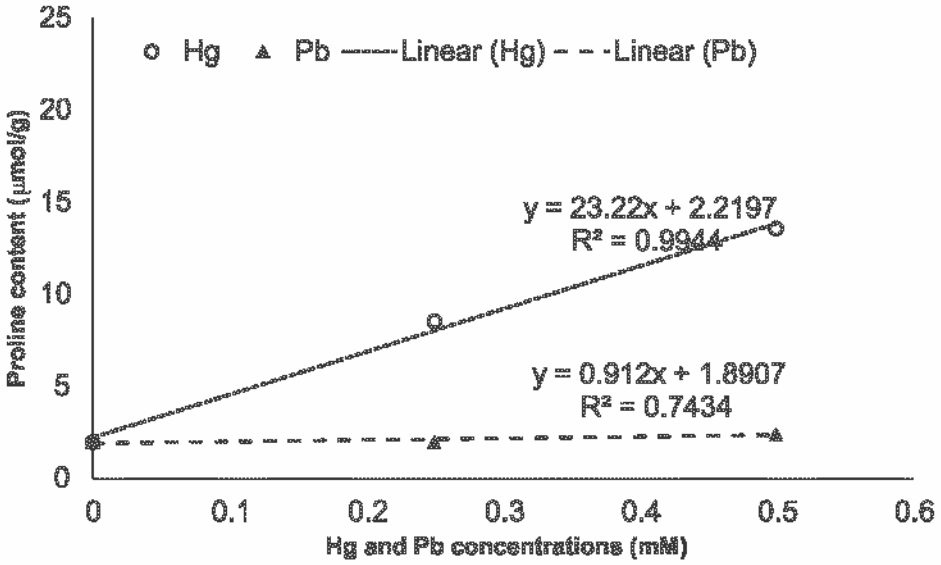

Figure 12 Regression of the average proline content from five species and the Hg and Pb treatments at different concentrations (0, 0.25 and 0.5 mM)
Note: The increase of Hg treatments induced proline content, but not with Pb treatments.

CONCLUSION

Heavy metal treatments using $Hg(NO_3)_2$ and $Pb(NO_3)_2$ at 0.25 and 0.5 mM had caused dramatic decrease in the growth of the studied five weeds (*B. mutica, C. kyllingia, I. aquatica, M. micrantha* and *P. conjugatum*) with Hg causing more prominent effects than Pb. Hg treatment also significantly reduced the net photosynthetic rate under different photosynthetic photon flux density, suggesting that heavy metal Hg until 0.5 mM had damage the photosynthetic apparatus of almost all the weed species. Most of the species were tolerant to Pb treatment up to 0.5 mM except *M. micrantha*, while only *C. kyllingia* and *I. aquatica* were tolerant to Hg treatment up to 0.5 mM. Hg and Pb caused dramatic increase in leaf MDA content, which was associated with the significant decrease of chlorophyll content. Only Hg treatment, not Pb, had induced higher proline content in the leaves of treated plant, but without a clear increment pattern among the species, suggesting that proline may have a role as alarm stress rather than as tolerant indicator. Among the five species, *C. kyllingia* and *I. aquatica* were the most tolerant to lead and mercury contaminant.

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