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4 **ACCUMULATOR WEEDS UNDER MERCURIC AND LEAD EXPOSURE**

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GROWTH, PHOTOSYNTHESIS AND PROLINE ACCUMULATION OF FIVE METAL-ACCUMULATOR WEEDS UNDER MERCURIC AND LEAD EXPOSURE

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Running title: Growth, photosynthesis and proline accumulation of weeds

ABSTRACT

Heavy metals, especially lead and mercury contaminant, have spread widely because of their intensive utilization in industry or extraction in mining area which threatens our environment. The experiment aimed to examine the growth and some physiological parameters of five metal-accumulator weed species in response to mercury (Hg) and lead (Pb) treatment. Five weed species (*Branchiaria mutica, Cyperus kyllingia, Ipomea 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 growth, photosynthesis, lipid peroxidation and proline content were observed during the treatments. The result showed that both Hg and Pb decreased growth significantly, but the decrease was far higher in Hg than in Pb treatments. Hg treatment reduced photosynthetic rate dramatically under different photosynthetic photon flux density suggesting that heavy metal Hg until 0.5 mM caused the damage of photosynthetic apparatus in almost all species except in *I. aquatica*. Hg and Pb treatment caused dramatic increase in leaf MDA content, which was associated with the decrease of chlorophyll content significantly. Almost all 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. Only Hg treatment and not Pb that induced higher proline content in the leaves of threated plants without clear pattern of the increment among the species suggesting that proline may have a role as alarm stress rather than tolerant indicator.

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

INTRODUCTION

Heavy metal pollution is among the anthropogenic environmental problem that has been increasing during the last decades due to the increase of industrial development. Lead (Pb) and mercury (Hg) are two kinds of heavy metals that spread widely because of their intensive utilization or extraction in mining area. These metals are classified as heavy metals which have dangerous toxic effects on environment (McLusky and Elliot 2004) and can adversely affect the morphology, physiology, and biochemistry processes in plants and animals (Wuana and Okieimen 2011). For plants, photosynthesis 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 and 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 damage 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). Therefore, serious efforts are needed to tackle the problem of heavy metal pollution from our environment.

Phytoremediation is an alternative technology that has been believed to be able to overcome the problem of heavy metal pollution in soil and water. Phytoremediation is the use of plants to reduce or eliminate metal contaminant 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 the detoxification capacity of metals from intracellular antioxidant enzymes (Tangahu *et al*. 2011). Another important additional 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, then will be 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 than-ground biomass, (iii) widely distributed and highly branched root system, (iv) more accumulation of the target of heavy metals from soil, (v), translated from the targets of heavy metals, (vi) good adaptation to 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 discovered in single species, and therefore utilization of several species may important to support the success of phytoremediation process.

Some weed plants have great potential as a source of plants for phytoremediation programs, because, in addition to their rapid growth, these plants have extensive adaptability and wide spread in many ecosystems. The previous research showed that there are some hyper-accumulator plants, such as *Ipomea* 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 to grow

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97 well in the mining area in Indonesia that allegedly can act as accumulator plants (Juhaeti *et al*. 2005).
98 Five potential weed species from grasses and broadleaf weeds, namely *Branchiaria mutica, Cyperus*
99 *kyllingia, Ipomea aquatica, Mikania micrantha*, and *Paspalum conjugatum* were tested for their
100 ability to grow in water cultures treated with Hg and Pb. These species have been suggested to have
101 ability to accumulate Pb or Hg from environment (Sugiono *et al*. 2014; Bedabati and Gupta 2016;
102 Khan *et al*. 2018; Paz-Alberto *et al*. 2007).

103 The purpose of this study was to examine photosynthetic and physiological responses as well
104 as growth of the five weed species exposed to Hg and Pb treatments in water culture. This paper
105 presents the response of photosynthesis, some physiological properties, and the growth of those
106 species under different Hg and Pb toxicity.

107

108 **MATERIALS AND METHODS**

109 **Plant materials and water culture preparation**

110 In this experiment, some species of weeds (*Paspalum conjugatum, Cyperus kyllingia*, *Ipomea*
111 *aquatica*, *Mikania micrantha*, and *Branchiaria mutica*) were used and cultivated in water culture
112 using half strength Hoagland's solution. Hoagland solution was prepared in a plastic box contained 6
113 L of solution. One-month old plants were removed carefully from the polybag and the roots were
114 cleaned with water to remove soil and other solid media and then were planted in the box contained
115 Hoagland's solution. To stand properly, the plants were equipped by perforated stereo foam and
116 supported by fine sponge. To ensure air supply, each box was equipped by aerator. At the beginning,
117 all the plants were grown under half strength Hoagland's solution for 2 weeks to establish the initial
118 growth before heavy metal treatment.

119

120 **The treatment of mercury and lead**

121 The experiment was conducted using a completely randomized design with two factors, the first
122 factor was plant species of weeds (*P. conjugatum, C. kyllingia, I. aquatica, M. micrantha and B.*
123 *mutica*). The second factor was Hg and Pb treatments which comprised (0 [without Pb and Hg
124 treatment], Hg1 (0.25 mM of $Hg(NO_3)_2$), and Hg2 (0.5 mM of $Hg(NO_3)_2$), Pb1 (0.25 mM of
125 $Pb(NO_3)_2$), and Pb2 (0.5 mM of $(Pb(NO_3)_2)$. Each experiment unit had 3 replications with 6 plants
126 per box (unit experiment).

127 The treatment of Pb and Hg was given to the plants after 2 weeks establishment in the water
128 culture by adding lead nitrate $(Pb(NO_3)_2)$ and mercuric nitrate $(Hg(NO_3)_2)$ to the solution with
129 different concentrations. To keep the volume of the solution inside the box similar, distilled water
130 was added to each box so that the total volumes of all media were similar. The treatment of heavy
131 metals was given for 3 weeks to see the response of the treated plants.

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Observations were made by measuring the growth and development of the shoot and roots during the treatment. Many changes such as wilting, necrosis, discoloration of the leaves and roots were recorded along the treatment. Physiological analysis including photosynthesis, MDA, proline and chlorophyll content was carried out after 10 days of the treatment when the treated plants showed toxic symptoms. After 3 weeks of the treatment, the plants were harvested for the observation of growth parameters.

Photosynthesis measurement

Measurements of photosynthesis w*ere* carried out using Photosynthetic Gas Exchange Analyzer LiCOR LI-6400. Observations *were* made on the third leaf (fully expanded leaf) of each treatment with 3 replications. Observations were made for net photosynthetic rate (Pn), stomatal conductance (Gs) and transpiration rate (E) at a saturation level of 1500 µmol m^{-2} per second. Photosynthetic measurement was also carried out at different light intensity (100, 200, 400, 750, 1000 and 1500 µmol cm^{-2} per second) to analyze photosynthetic light curve. The average of photosynthetic light curve was calculated in response to Hg and Pb treatment using Microsoft Excel 2013.

Malondialdehyde (MDA) analysis

Lipid peroxidation was estimated by measuring MDA content as described by Ono *et al*. (1995). Fresh leaves (0.2 g) were ground in 0.5 ml of 0.1% (w/v) trichloracetic acid (TCA) at 4 °C. The leaf extract then *was* 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 then *was* incubated in the oven at 100°C for 30 minutes. After being cooled at the room temperature, 4 ml n-butanol *was* added to the solution, and then followed by centrifugation at 4200 rpm at 28°C for 20 minutes. The absorbance of the supernatant then *was* measured using a UV-VIS spectrophotometer (Shimadzu, UV-1700, Kyoto, Japan) at 532 nm and corrected for nonspecific turbidity by subtracting the absorbance at 520 nm. The concentration of MDA *was* calculated from its extinction coefficient (ε=155 L mmol^{-1} cm^{-1}).

Chlorophyll content analysis

Chlorophyll content *was* analyzed using method developed by Yoshida *et al*. (1976). Two grams of fresh leaves were ground using 80% of acetone (p.a. Merck KGaA, Darmstadt, Germany) and then were filtered using Whatman paper no. 1 into 100 ml of volumetric flask until all the chlorophyll were dissolved into the acetone solution, before finally the solution in the volumetric flask reaches exactly 100 ml. A 5 ml of chlorophyll solution *was* taken from 100 ml volumetric flask, then it *was* put into 50 ml of volumetric flask and *was* diluted using 80% of acetone until 50 ml. The absorbance of chlorophyll solution *was* measured using spectrophotometer (Shimadzu, UV-1700,

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167 Kyoto, Japan) at the 645 nm and 663 nm wavelength (λ). Chlorophyll content was measured using

168 formula as follow[28]:

169 $\qquad$ Chl a = 0.0127. A663 – 0.00269. A645

170 $\qquad$ Chl b = 0.0229. A645 – 0.00468. A663

171 $\qquad$ Total Chl = Chl a + Chl b = 0.0202. A645 + 0.00802. A663

172 $\qquad$ Chl a = Chlorophyll a; Chl b = Chlorophyll b

173 $\qquad$ A645 = the absorbance at the λ of 645 nm

174 $\qquad$ A663 = the absorbance at the λ of 663 nm

175 The regression curve between chlorophyll and MDA contents in response to heavy metal treatments

176 was calculated using Microsoft Excel 2013.

177

178 Proline Analysis

179 Proline content of leaves *was* analyzed following Bates *et al.* (1973). Homogenized tissues

180 (150 mg) from leaves were mixed with 3 mL of 3% sulfosalicylic acid and centrifuged at 10,000 rpm

181 for 15 min. One mL of supernatant *was* mixed with 1 mL of glacial acetic acid and 1 mL of acid-

182 ninhydrin (1.25 g ninhydrin dissolved in 30 mL glacial acetic acid and 20 mL 6 M phosphoric acid),

183 incubated for 1h at 100 °C and then cooled in an ice bath. The reaction mixture *was* extracted with 2

184 mL of toluene and mixed vigorously for 20 s. The chromophore containing toluene *was* aspirated

185 from the aqueous phase and the absorbance *was* measured at 520 nm. Reference standards of proline

186 from 5 to 60 μM are prepared and analyzed in the same way to obtain a calibration curve.

187

188 RESULTS AND DISCUSSION

189 Plant Growth response

190 Among five species, the plants have different growth characteristics including shoot and root

191 length, leaf number as well as plant biomass. The treatment using mercury (Hg) and lead (Pb)

192 dramatically influenced plant growth, even though there was variation among the species. For all the

193 species (*B. mutica, C. kyllingia, I. aquatica, M. micrantha,* and *P. conjugatum*), there was a similar

194 pattern of Hg treatments which significantly (P<0.05) reduced plant growth, except for root growth

195 of *I. aquatica* which did not decrease in response to Hg treatments (Figures 1-2). The most negative

196 effect was shown by all the plants subjected to 0.5 mM of Hg (Figures 1-2), which even caused *C.*

197 *Kyllingia* and *M. micrantha* dead 10 days after the treatment. On the other hand, response of plant

198 morphology to Pb treatment was not as big as to Hg, even though at 0.5 mM of Pb, the treatment

199 significantly decreased some morphological parameters especially for *I. aquatica* and *M. micranta*

200 (Figures 1-2).

Response of shoot was more prominent than roots in response to both Hg and Pb treatment (Figures 1 and 2). For shoot length, the reduction was in the range of 54 to 87% due to 0.5 mM of Hg, while it only caused 12 – 56% reduction of root length. For root length parameter, only *I. aquatica* that was not affected by Hg treatment (Figure 1). Even though Pb treatment did not cause prominent damage, it reduced significantly (P<0.05) shoot length of *I. aquatica* and *M. micrantha* and root length of *M. micrantha* (Figure 1). Meanwhile, only *I. aquatica* and *C. kyllingia* that still stood until the end of the treatment at 0.5 mM of Hg (3 weeks).
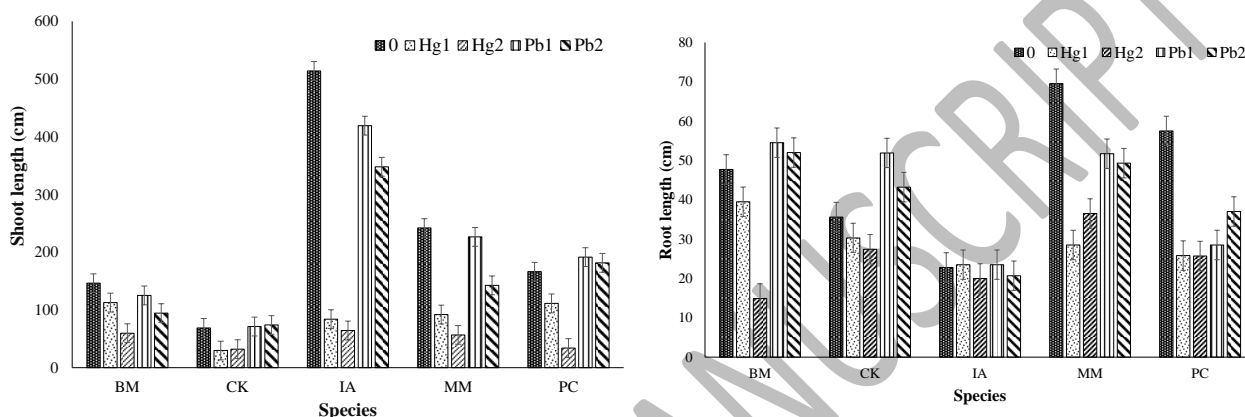
Figure 1. Shoot and root length of the species after 3 week exposure to Hg and Pb with different concentrations. 0: control (without heavy metal treatment); Hg1: 0.25 mM of Hg; Hg2: 0.5 mM of Hg; Pb1: 0.25 mM of Pb and Pb2: 0.5 mM of Pb. BM: *Branchiaria mutica,* CK: *Cyperus kyllingia,* IA: *Ipomea aquatica,* MM: *Mikania micrantha,* and PC*: Paspalum conjugatum.*
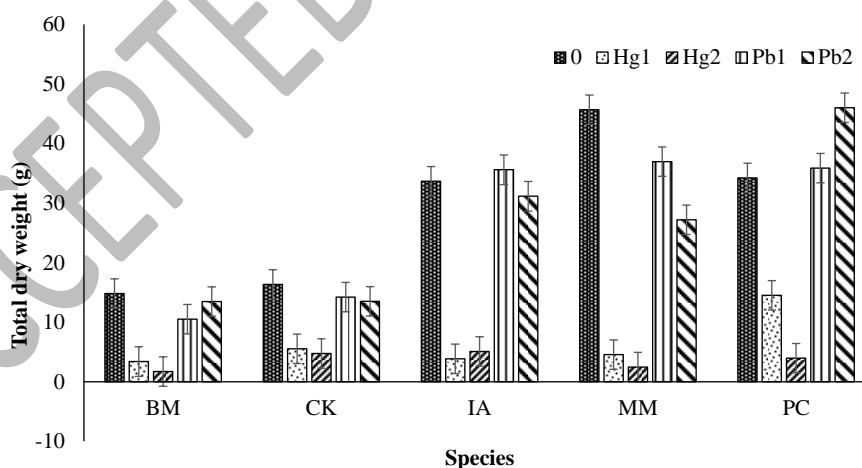
Figure 2. Total dry weight of the species after 3 week exposure to Hg and Pb with different concentrations. 0: control (without heavy metal treatment); Hg1: 0.25 mM of Hg; Hg2: 0.5 mM of Hg; Pb1: 0.25 mM of Pb and Pb2: 0.5 mM of Pb. BM: *Branchiaria mutica,* CK: *Cyperus kyllingia,* IA: *Ipomea aquatica,* MM: *Mikania micrantha,* and PC*: Paspalum conjugatum.*

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223   Heavy metals have been known to cause inhibition of root and canopy growth and plant
224   production (Peralta *et al*. 2001; Kibra 2008). Metal toxic effects, especially lead and mercury, have
225   been reported in several plants, including *Triticum aestivum* (Patra and Sharma 2000), *Phaseolus*
226   *vulgaris* L. (Zengin and Munzuroglu 2005), tomatoes (Cho and Park 1999), and several other plants.
227   According to Ortega-Villasante *et al*. (2005) Hg at high concentrations is very toxic to cells which
228   induces damage to cells and causes physiological changes. The accumulation of Hg can also inhibit
229   plant growth, causing plant productivity to decline. In this study, the value of shoot and root length,
230   and total dry weight in the five plant species decreased dramatically due to Hg stress which was given
231   even only at 0.25 mM concentration (Figure 10), while Pb treatment treated up to 0.5 mM only caused
232   a relatively small decrease except for *M. micrantha* (Figures 1-2).

233   Shoot and root length as well as dry weight are the indicators of the most commonly observed
234   plant growth to see plant responses to the environmental stress. This happens because heavy metals
235   caused inhibition of cell division and elongation, absorption of water and nutrients, and the decrease
236   of enzymatic activity so that the growth rate was inhibited (Shahid *et al*. 2015). Based on the research
237   of Patra and Sharma (2000) the accumulation of Hg inhibited root and canopy growth, decreased the
238   root-canopy ratio, and dry weight and dissolved protein content in the canopy of the *Triticum*
239   *aesticum* plant. In this experiment, the greatest decrease in dry weight was found in *M. micratha*
240   plants both at 0.25 mM and 0.5 mM Hg concentrations as well as at 0.5 M Pb treatment (Figure 2).
241   The lower dry weight of plants showed that the physiological processes in plants were disrupted due
242   to heavy metal toxicity so that the growth was less optimal. To investigate further, some physiological
243   analyses were presented below.

244

Analysis of Photosynthesis

246   The analysis of net photosynthetic rate (*Pn*) of five species in response to heavy metal treatment
247   showed that all the species had almost similar *Pn* by the average of 13.5 μmol m^{-2} s^{-1} for control
248   plants, with stomatal conductance (*Gs*) values approximately 211 mmol m^{-2} s^{-1}. The effect of lead
249   (Pb) treatments up to 0.5 mM did not significantly reduce *Pn* of all species (Figure 3). However, the
250   treatment of mercury (Hg) especially at 0.5 mM caused dramatic decrease of *Pn* almost all species
251   except *I. aquatica* (Figure 3). The *C. Kyllingia* and *M. micrantha* were dead after 10 days of the
252   treatment with 0.5 mM of Hg, and therefore they had the lowest photosynthetic rate (Figure 3). The
253   0.5 mM of Hg also decreased *Pn* of *P. conjugatum* and *B. mutica* up to 33% and 69% respectively
254   (Figure 3). However, the treatment with 0.25 mM of Hg did not cause photosynthesis reduction
255   significantly after 10 days of the treatment. The effect of mercury treatment on *Gs* values was also
256   almost similar with the *Pn* among the species that were used in the experiment.
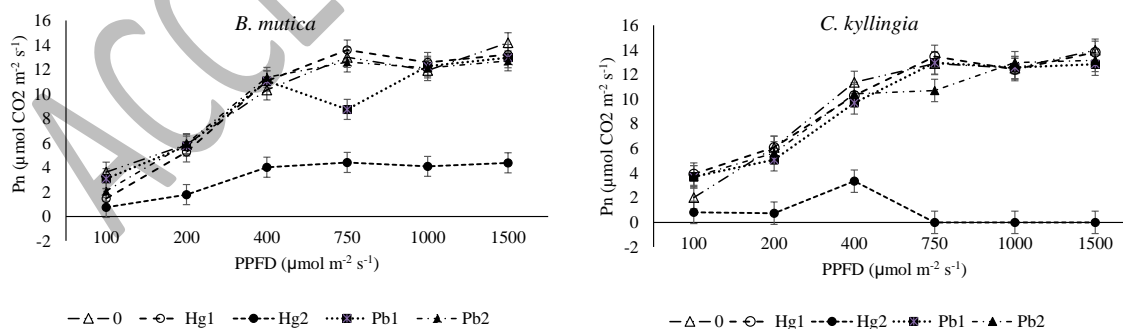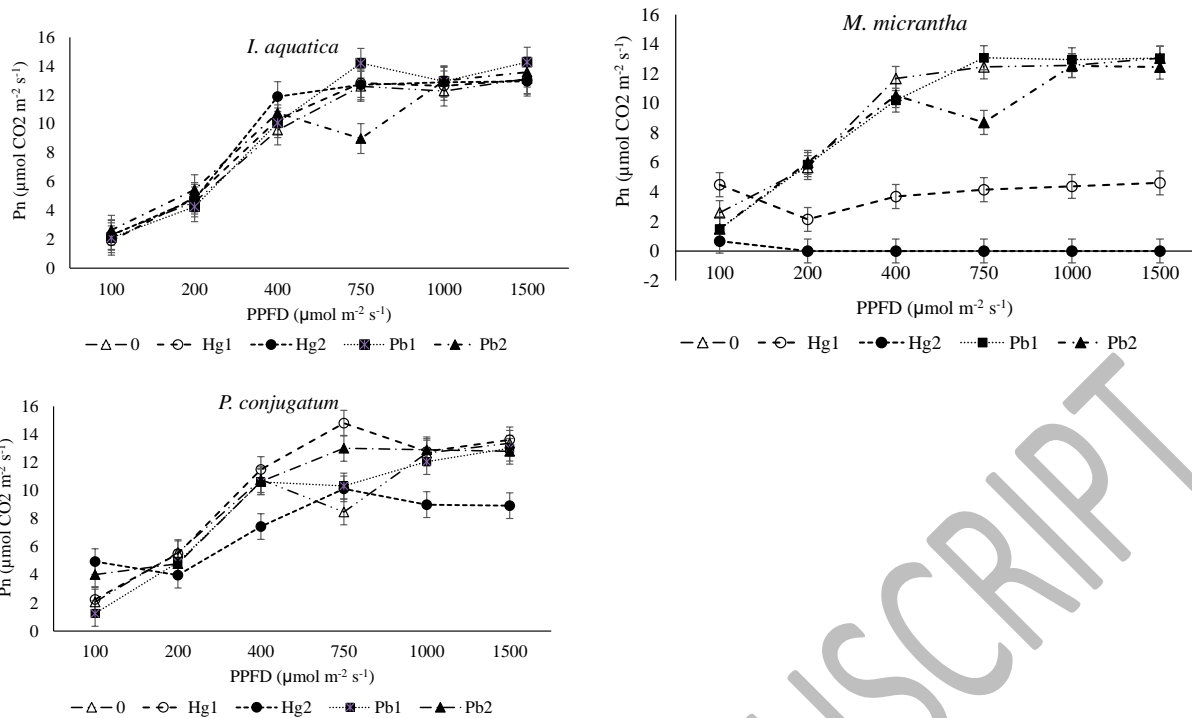
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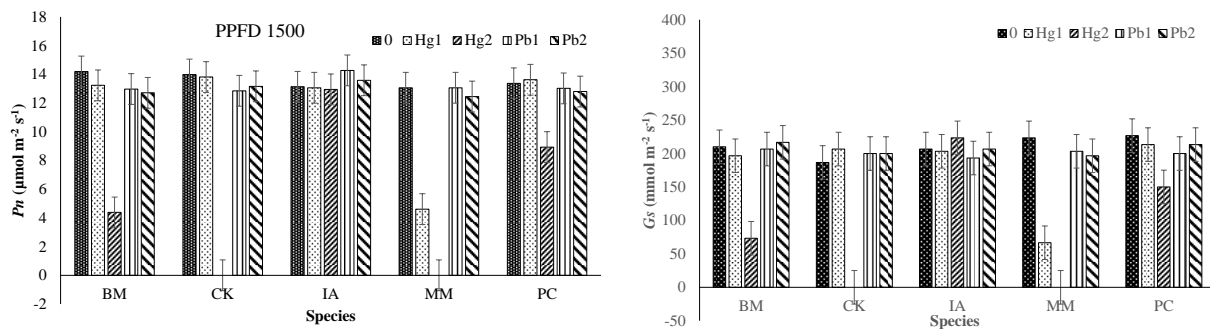
Figure 3. The average of net photosynthetic rate (*Pn*) and stomatal conductance (*Gs*) of five species (BM: *Branchiaria mutica,* CK: *Cyperus Kyllingia,* IA: *Ipomea aquatica,* MM: *Mikania micrantha,* and PC*: Paspalum conjugatum*) in response to Hg and Pb treatments (0, 0.25 and 0.5 mM) 10 days after Hg and Pb exposure.

To understand further about the characteristic of photosynthesis of each species in response to Hg and Pb treatments, the analysis of light curve of photosynthesis was carried out using different photosynthetic photon flux density (PPFD), starting from 100 to 1500 µmol m^{-2} s^{-1}. This light curve was also important to understand the consistency of the data and to determine the maximum photosynthesis under environmental stress. The data showed that every species had different curve with the uniqueness of photosynthetic rate values which determined the response of the species to the given treatments (Figure 4). In general photosynthesis was recorded even at lower PPFD (100 µmol m^{-2} s^{-1}) with almost similar values among the treatments. The maximum photosynthesis was reached under the PPFD of approximately 750 µmol m^{-2} s^{-1} (Figure 4). The photosynthesis graphs showed that the treatment with 0.5 mM of Hg (Hg2) caused dramatic decrease of photosynthesis in all light intensity except in *I. aquatica* and *P. conjugatum*, while Pb treatment did not have this effect, except in some point of PPFD. For *M. micratha* the effect of Hg was even larger because at 0.25 mM, Hg also decreased photosynthesis (Figure 4).

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Figure 4. Net photosynthetic rate (*Pn*) of five species (BM: *Branchiaria mutica,* CK: *Cyperus kyllingia,* IA: *Ipomea aquatica,* MM: *Mikania micrantha,* and PC*: Paspalum conjugatum*) in response to Hg and Pb treatments (0, 0.25 and 0.5 mM) under different PPFD (from 100 until 1500 μmol m^{-2} s^{-1}).

To construct the light curve of photosynthesis for all the species in response to the treatments and different PPFD, the average of single treatment of Hg and Pb was calculated and the light curve was plot using logarithmic equation as presented in Figure 5. The graph showed that there were 3 different groups of curves with the lowest curve represented the curve of the plants treated by 0.5 mM of Hg. The second group of curves was the highest photosynthesis light curve represented by some curves including control plant and Pb-treated plants which had almost similar curve (Figure 5). The third curve was the curve of the plants treated by 0.25 mM of Hg. This photosynthetic curve indicated high photosynthetic rate, but it was still lower than the second curve (Figure 5). This curve was created especially because the response of *M. micrantha* which had lower photosynthesis under 0.25 mM of Pb treatment (Figure 4). The second and the third curves showed that at the PPFD of 1500 μmol m^{-2} s^{-1} the photosynthesis was still not saturated so that the photosynthetic rate was still possible to increase when the PPFD increased (Figure 5). The distinction of the curve was also reflected in the stomatal conductance (*Gs*) curve in response to Hg and Pb treatments and different PPFD (Figure 6). The *Gs* values decreased in response to higher PPFD with different pattern depended on the heavy metal treatment. The plants treated with Hg of 0.5 mM had the lowest *Gs* at all PPFD (Figure 6).

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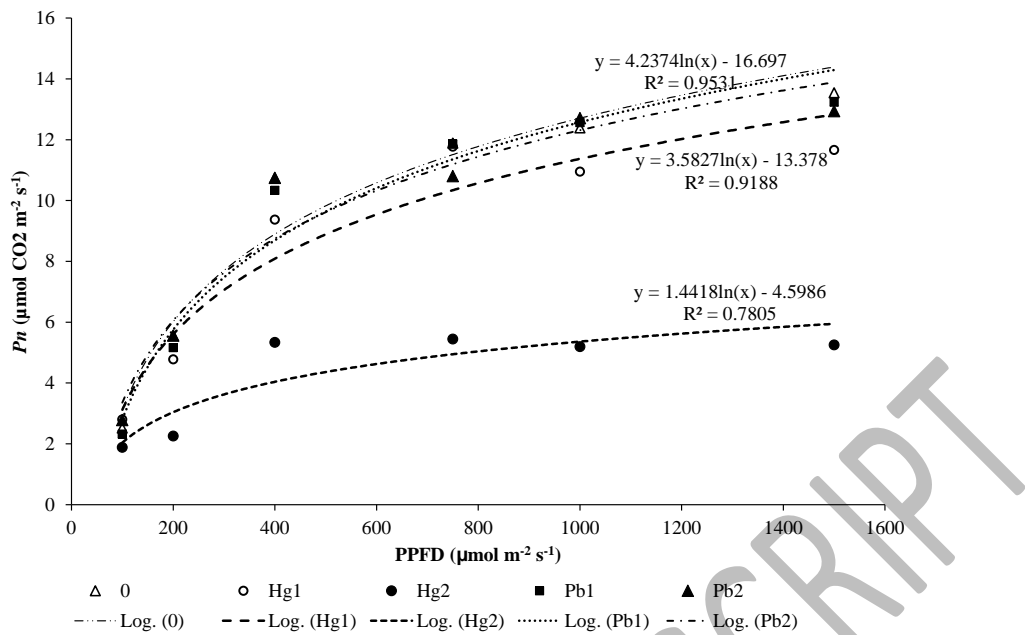
Figure 5. Photosynthetic light curve of all the species (*Branchiaria mutica, Cyperus kyllingia, Ipomea aquatica, Mikania micrantha,* and *Paspalum conjugatum*) in response to heavy metal treatments (0: control (without heavy metal treatment); Hg1: 0.25 mM of Hg; Hg2: 0.5 mM of Hg; Pb1: 0.25 mM of Pb and Pb2: 0.5 mM of Pb) with different PPFD (from 100 until 1500 μmol m^{-2} s^{-1}).
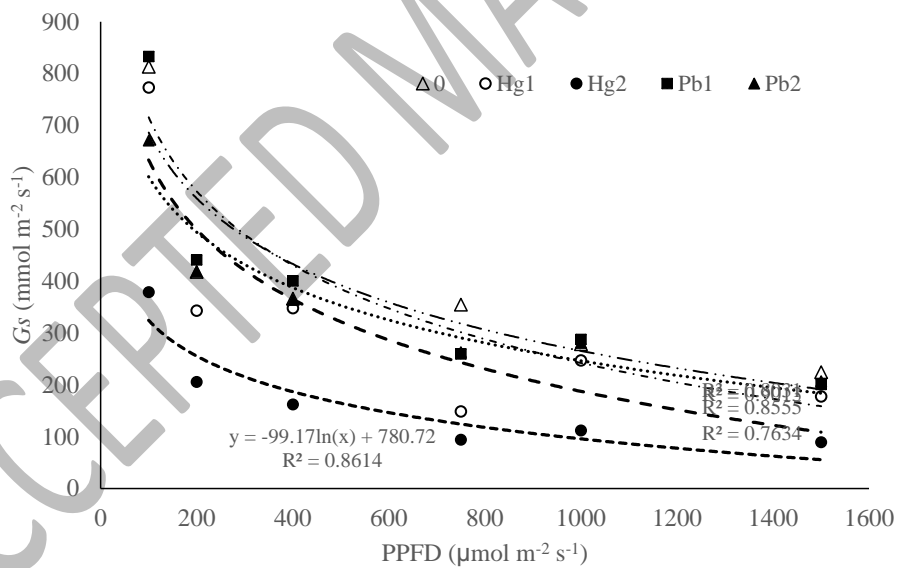

Figure 6. Stomatal conductance (Gs) of all the species (*Branchiaria mutica, Cyperus kyllingia, Ipomea aquatica, Mikania micrantha,* and *Paspalum conjugatum*) in response to heavy metal treatments (0: control (without heavy metal treatment); Hg1: 0.25 mM of Hg; Hg2: 0.5 mM of Hg; Pb1: 0.25 mM of Pb and Pb2: 0.5 mM of Pb) with different PPFD (from 100 until 1500 μmol m^{-2} s^{-1}).

It has been well known that 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 and Singh, 1993). According to Aggarwal *et al*. (2011) the effects of heavy metal toxicity on

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photosynthesis can occur either directly or indirectly. Directly is through inhibition of light reactions and oxygen formation, NADP reduction and photophosphorylation, while indirectly is due to the inhibition of chlorophyll synthesis or the increase of chlorophyll damage.

The similar pattern of *Pn* and *Gs* decrease due to heavy metal stress (Figures 1-4) suggests that metal toxicity may affect water absorption indicated by the decrease of relative water content (Figure 6) which 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 such as *Helianthus annuus* and barley under Pb treatment (Kastori *et al*. 1992; Vassilev *et al*. 1998) or *Beta vulgaris* under Zn toxicity (Sagardoy *et al*. 2010). Among the five species, *I. aquatica* and *P. Conjugatum* had the best performance in photosynthesis which did not decrease under Hg and Pb treatments which may become an indicator of their adaptability to those heavy metal treatments.
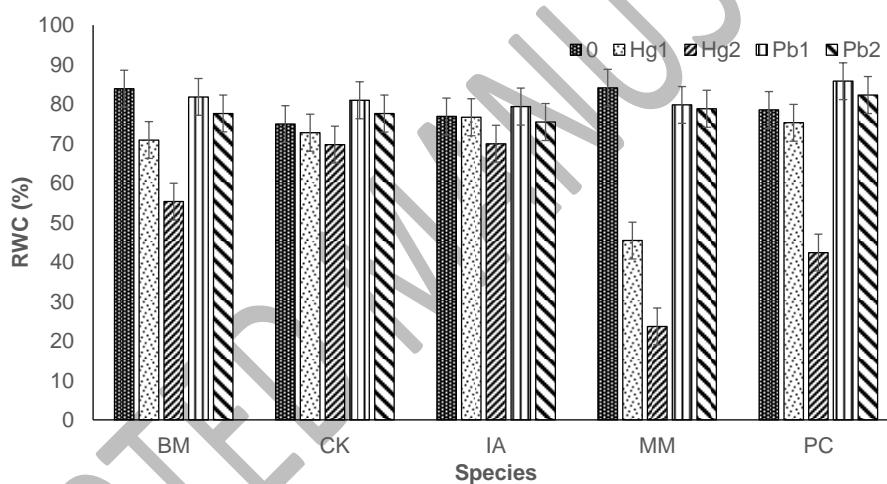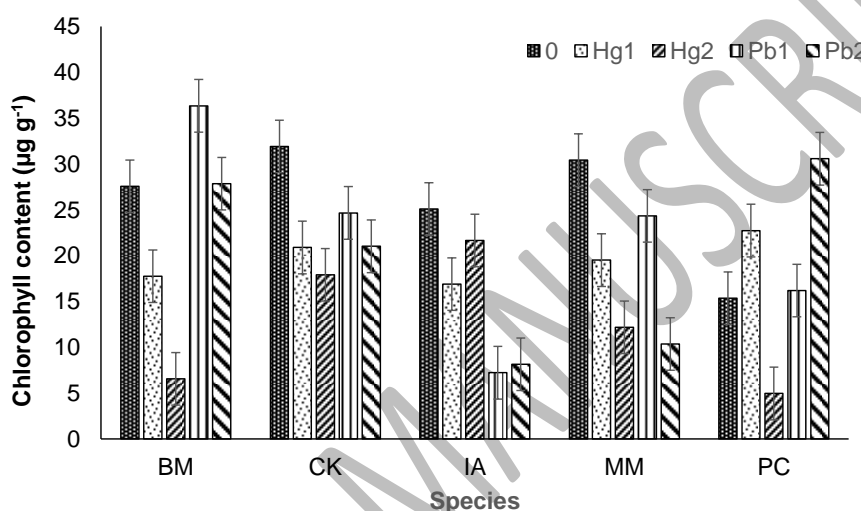

Figure 6. Relative water content (RWC) of five species after 10 days exposure to Hg and Pb with different concentrations. 0: control (without heavy metal treatment); Hg1: 0.25 mM of Hg; Hg2: 0.5 mM of Hg; Pb1: 0.25 mM of Pb and Pb2: 0.5 mM of Pb. BM: *Branchiaria mutica,* CK: *Cyperus Kyllingia,* IA: *Ipomea aquatica,* MM: *Mikania micrantha,* and PC: *Paspalum conjugatum.*

Analysis of total chlorophyll and leaf MDA

From the analysis of chlorophyll content indicated that heavy metal treatment caused the decrease of chlorophyll content of all species dramatically (Figure 7). The decrease of chlorophyll content was dramatic for plant treated with Hg at 0.5 mM especially for *B. mutica* and *M. micrantha*. Based on the decrease of chlorophyll content, *B. mutica* was the most affected by Hg treatment, while *I. aquatica* was the least affected (Figure 6). Different from Hg, 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* (Figure 7).

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347   The decrease of chlorophyll content is a general symptom of heavy metal toxicity in plant.
348   Zengin and Munzuroglu (2005) showed that the decrease of chlorophyll content happened to all heavy
349   metal treatment to *Phaseolus vulgaris* seedlings, with the most decrease happened in mercuric (Hg)
350   treatment followed by Cd and Cu, while Pb had the least effect. The dramatic decrease of chlorophyll
351   and photosynthesis due to heavy metal stress was also observed in poplar plants (Chandra and Kang
352   2016) as well as in perennial grass *Phragmites australis* (Ayeni *et al*. 2012). In this experiment the
353   similar pattern was observed for chlorophyll content in five weeds with the most affected species that
354   was observed in *B. mutica* and *M. micrantha* for Hg treatments and *I. aquatica* for Pb treatments.
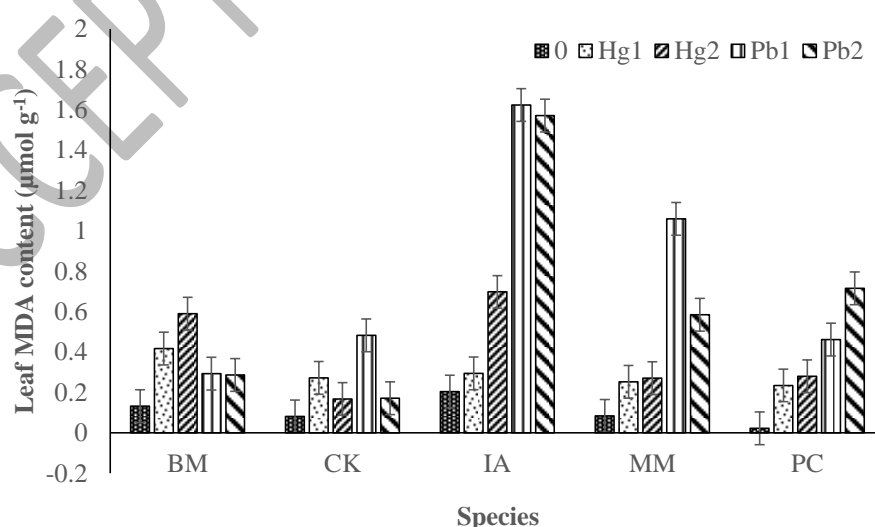
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356
357   Figure 7. Chlorophyll content of the species after 10 day exposure to Hg and Pb with different
358   concentrations. 0: control (without heavy metal treatment); Hg1: 0.25 mM of Hg; Hg2: 0.5
359   mM of Hg; Pb1: 0.25 mM of Pb and Pb2: 0.5 mM of Pb. BM: *Branchiaria mutica,* CK:
360   *Cyperus Kyllingia,* IA: *Ipomea aquatica,* MM: *Mikania micrantha,* and PC*: Paspalum
361   conjugatum.*
362

363   Membrane systems including chloroplast membranes are considered the main target of
364   oxidative stress due to heavy metals. This happens because polyunsaturated fatty acids as the main
365   component of lipid membranes are very sensitive to heavy metals. Data from the study showed that
366   the Hg treatment given in high concentrations reduced the total chlorophyll content of the five plant
367   species (Figure 7). Solymosi *et al*. (2004) reported that Hg stress induces photoreduction inhibition
368   of protochlorophyllide in wheat leaves, so the total chlorophyll value of leaves decreases with
369   increasing Hg concentration. This decrease occurs because heavy metals can cause chlorophyll
370   biosynthesis to be inhibited through the work inhibition of two highly sensitive enzymes, i.e. α-
371   aminolaevulinic acid (ALA) dehydratase and protochlorophyllide reductase which play an important
372   role in the early and final stages of chlorophyll biosynthesis (de Filippis *et al*. 1981). Mercury was
373   also reported to cause magnesium ions to be replaced in photosynthetic pigments (Kupper *et al*. 1998).

13

MDA content also varied among the species with the highest content was found in *I. aquatica* followed by *M. micrantha*, while the lowest was found in *C. Kyllingia* (Figure 8). Heavy metal treatment (Hg and Pb) caused the increase of MDA content significantly in leaves of almost all species. However, the treatment did not induce the significant increase in roots (data not shown). Only in *P. conjugatum* roots treated with 0.5 mM the MDA content increased significantly. Treatment with Hg increased leaf MDA of all species significantly with the range from 2 fold in *C. Kyllingia* until 13 fold in *P. conjugatum* compared to the control, even though the highest leaf MDA was shown by *I. aquatica* exposed to 0.5 mM Hg (Figure 8). Different from Hg, the treatment using Pb induced the increase of leaf MDA content only low 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 was shown by *I. aquatica* (Figure 8).

The content of malondialdehyde (MDA) is an index to evaluate the level of cellular damage after stress treatment, which is the main cytotoxic product of lipid peroxidation and indicators of free radical production (Fu and 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 the Hg and Pb treatment has a significant effect on lipid peroxidation as indicated by the higher MDA values due to the treatment (Figure 7). The increase of MDA content has also been observed in several plants subjected to abiotic stress including heavy metal stress such as in sorghum treated with Cd (Kumar and 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. Leaf MDA content of the species after 10 day exposure to Hg and Pb with different concentrations. 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:

14

Cyperus Kyllingia, IA: *Ipomea aquatica,* MM: *Mikania micrantha,* and PC: *Paspalum conjugatum.*

There was a close correlation between the increase of MDA content in response to Hg and Pb treatment and the decrease of chlorophyll content (Figure 9). There was a different correlation between MDA and chlorophyll content in response to Hg and Pb treatment. Figure 4 showed that the increase of MDA content due to Hg treatment was closely associated to the decrease of chlorophyll content indicated by the steep graph. Different from Hg, the treatment of Pb, even though it caused the increase of MDA content and the decrease of chlorophyll content, the correlation was lower with less steep than that of Hg (Figure 9), suggesting that the effect of Hg treatment on the decrease of chlorophyll was higher than Pb. This result is in line with Zengin and Munzuroglu (2005) who observed that the effect of Hg was far higher than Pb on chlorophyll reduction of *P. vulgaris* seedlings.
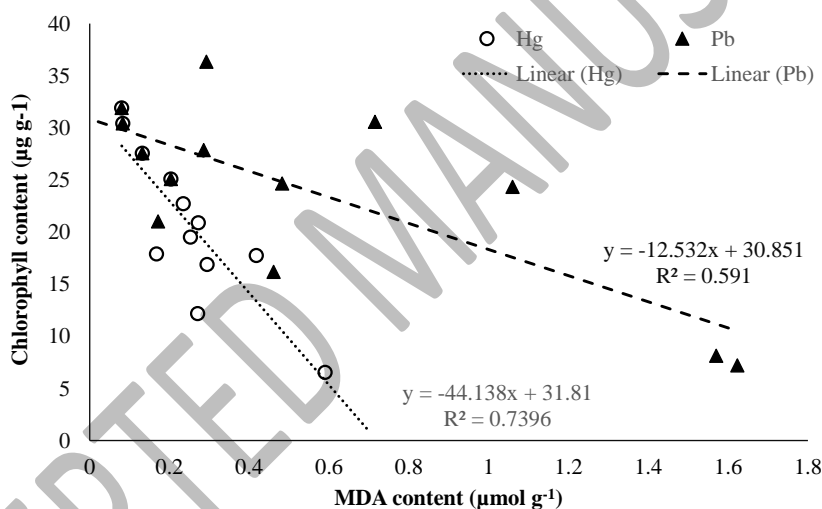
Figure 9. The regression graph between MDA and chlorophyll content of all species in response to Hg and Pb treatment. There was a different slope among both treatment, where Hg treatment had steeper, while Pb had slightly sloping.

Proline analysis

Proline content is among the physiological parameters which normally increase when the plant is subjected to environmental stress such as drought, salinity, and even heavy metal stress. The experiment also showed the similar tendency especially when the plants were treated with Hg at 0.25 and 0.5 mM (Figure 10). Proline content of all species increased significantly ($P<0.05$) from 2 until 9 fold for 0.25 mM of Hg treatments and even until 15 fold for 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* (Figure 10). 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

15

affected by Hg) had high proline content. Different from Hg, the treatment using Pb at 0.25 as well as 0.5 mM did not effect to 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 and coefficient correlation (Figure 11).

Proline is amino acid that in many cases increased dramatically in response to several environmental stress such as drought (Lum *et al*. 2014; Mwenye *et al*. 2016), salinity stress (Theriappan *et al.* 2011), as well as heavy metal stress (Zengin and 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 and Saradhi 1991). This amino acid has been suggested to have important role as biochemical scavenger of ROS induced by abiotic stress. However, the data showed that the increase of proline happened when the plant underwent severe stress due to metal toxicity (Figure 10), and there was no correlation between proline accumulation and metal tolerant among five species, suggesting that the increase of proline is an indicative of alarm stress rather than that of the role to reduce the damage of heavy metal stress in these species.
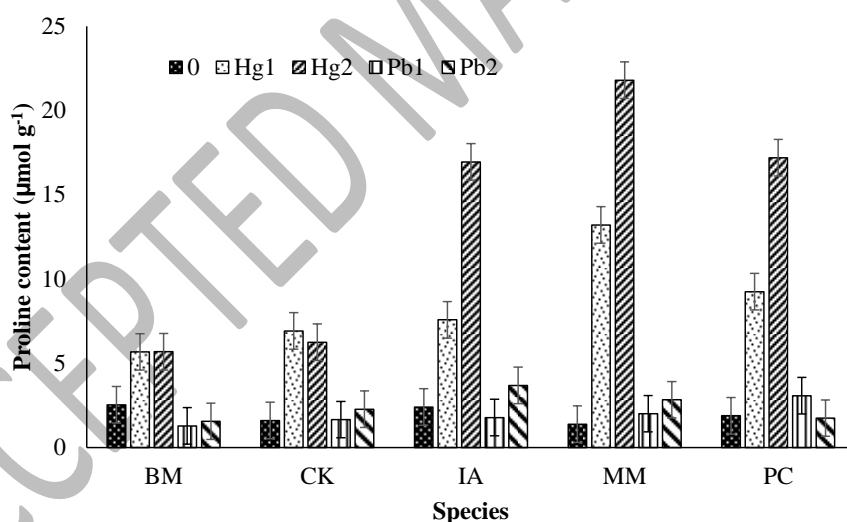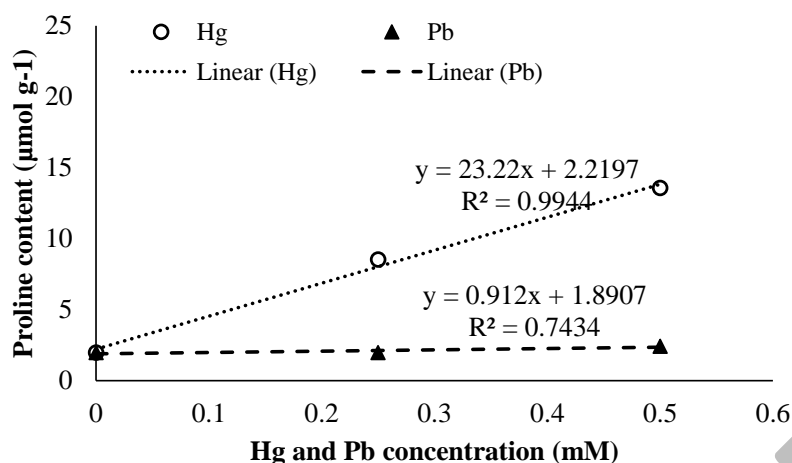

Figure 10. Proline content of five species subjected to different treatment of Hg and Pb. 0: control (without heavy metal treatment); Hg1: 0.25 mM of Hg; Hg2: 0.5 mM of Hg; Pb1: 0.25 mM of Pb and Pb2: 0.5 mM of Pb. BM: *Branchiaria mutica,* CK: *Cyperus kyllingia,* IA: *Ipomea aquatica,* MM: *Mikania micrantha,* and PC*: Paspalum conjugatum.*

Figure 11. The regression of the average of proline content from five species and the Hg and Pb treatments at different concentrations (0, 0.25 and 0.5 mM). The increase of Hg treatments induced proline content, but it did not happen to Pb treatments.

CONCLUSION

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

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REFERENCES

Aggarwal A, Sharma I, Tripathi BN, Munjal AK, Baunthiyal M, Sharma V: Metal Toxicity and Photosynthesis. In *Photosynthesis: Overviews on Recent Progress & Future Perspective Edition*. Edited by: Itoh S, Mohanty P, Guruprasad KN. New Delhi: IK International Publishing House. 2011:229-236.

479   Ali H, Khan E, Sajad MA. 2013. Phytoremediation of heavy metals—Concepts and applications.
480   Chemosphere. 91:689-881.

481   Alia P and P Saradhi. 1991. Proline accumulation under heavy metal stress. J Plant Physiol. 138: 554-
482   558.

483   Ayeni O, Ndakidemi P, Snyman R, Odendaal J. 2012.   Assessment of metal concentrations,
484   chlorophyll content and photosynthesis in *Phragmites australis* along the lower Diep river,
485   CapeTown, South Africa. Energy Environ. Res. 2(1): 128-139.

486   Bates LS, Waklren RP, and Teare ID. 1973. Rapid determination of free proline water stress studies.
487   Plant Soil. 39:205-207.

488   Bedabati C L, and Gupta A. 2016. Phytoremediation of lead using *Ipomoea aquatica* Forsk. in
489   hydroponic solution. Chemosphere. 156:407-411.

490   Chandra R and Kang H. 2016. Mixed heavy metal stress on photosynthesis, transpiration rate, and
491   chlorophyll content in poplar hybrids. Forest Sci. Tech. 12(2):55-61.

492   Cho U, Park J. 1999. Changes in hydrogen peroxide content and activities of antioxidant enzymes in
493   Tomato seedlings exposed to mercury. J Plant Biol. 42:41–48.

494   de Filippis LF, Hampp R, Ziegler H. 1981. The effect of sub-lethal concentration of zinc, cadmium
495   and mercury on Euglena II. Respiration, photosynthesis and photochemical activities. Arch
496   Microbiol. 128:407-411.

497   Emamverdian A, Ding Y, Mokhberdoran F, Xie Y. 2015. Heavy metal stress and some mechanisms
498   of plant defense response. Scient World J. 2015: 756120.

499   Fu J, Huang B. 2001. Involvement of antioxidants and lipid peroxidation in the adaptation of two cool-
500   season grasses to localized drought stress. Environ Exper Bot. 45:105-114.

501   Hall JL. 2002. Cellular mechanisms for heavy metal detoxification and tolerance. J Exp Bot.
502   53(366):1-11.

503   Hamim. 2005. Photosynthesis of C3 and C4 Species in response to increased CO_2 concentration and
504   drought stress. Hayati J Biosci. 12(4): 131-138.

505   Hamim, Hilmi M, Pranowo D, Saprudin D, Setyaningsih L. 2017. Morphophysiological changes of
506   biodiesel producer plants (Blanco) in response to gold-mining wastewater. Pak J Biol Sci.
507   20:423-435.

508   Hilmi M, Hamim, Sulistyaningsih YC, Taufikurahman. 2018. Growth, histochemical and
509   physiological responses of non-edible oil producing plant (*Reutealis trisperma*) to gold mine
510   tailings. Biodiversitas. 19(4):1294-1302.

511   Howard RL, Abotsi E, Van Rensburg ELJ and Howard S. 2003. Lignocellulose biotechnology:
512   issues of bioconversion and enzyme production. Afric J Biotech. 2(12):602-619.

513   Juhaeti T, Syarif F, Hidayati N. 2005. Inventory of potential plants for phytoremediation of degraded
514   land and water due to gold mining (*Inventarisasi tumbuhan potensial untuk fitoremediasi
515   lahan dan air terdegradasi penambangan emas*). Jurnal Biodiversitas 6(1):31-33.

516   Kastori R, Petrovic´ M, Petrovic´ N. 1992. Effect of excess lead, cadmium, copper, and zinc on water
517   relations in sunflower. J Plant Nutr 15:2427–2439.

518   Khan MM, Islam E, Irem S, Akhtar K, Ashraf MY, Iqbal J, Liu D. 2018. Pb-induced phytotoxicity in
519   para grass (*Brachiaria mutica*) and Castorbean (*Ricinus communis* L.): Antioxidant and
520   ultrastructural studies. Chemosphere. 200:257-265.

521   Kibra MG. 2008. Effects of mercury on some growth parameters of rice (*Oryza sativa* L.). Soil and
522   Environ. 27(1):23–28.

523 Kumar P and Pathak S: Short-term response of plants grown under heavy metal toxicity. In *Heavy*
524   *Metals.* Edited by Saleh HEM and Aglan R. London:Intech Open; 2018:69-89.

525 Kupper H, Kupper F, Spiller M. 1998. In situ detection of heavy metal substituted chlorophylls in
526   water plants. Photosynth Res. 58:123–133.

527 Leung HM, Wang ZW, Ye ZH, Yung KL, Peng XL, Cheung KC. 2013. Interactions between
528   arbuscular mycorrhizae and plants in phytoremediation of metal-contaminated soils: A
529   review. Pedosphere. 23: 549-563.

530 Lum MS, Hanafi MM, Rafii YM, Akmar ASN. 2014. Effect of drought stress on growth, proline and
531   antioxidant enzyme activities of upland rice. J Anim Plant Sci. 24(5): 1487-1493.

532 Malar S, Vikram SS, Favas PJC, and Perumal V. 2014. Lead heavy metal toxicity induced changes
533   on growth and antioxidative enzymes level in water hyacinths [*Eichhornia crassipes* (Mart.)].
534   Bot Stud. 55: 54-65.

535 McLusky DS, Elliot M. *The Estuarine Ecosystem Ecology, Threats, and Management*. New York
536   (US): Oxford University Press Inc; 2004.

537 Muddarisna N, Krisnayanti BD, Handayanto E. 2014. Phytoextraction of mercury from polluted land
538   by small-scale gold mine tailing and their effect tp growth of maize. (*Fitoekstrasi merkuri*
539   *dari tanah tercemar limbah tambang emas skala kecil dan pengaruhnya pada pertumbuhan*
540   *tanaman jagung).* Jurnal Lahan Suboptimal. 4(1): 81-88.

541 Mwenye OJ, van Rensburg L, van Biljon A, van der Merwe R. 2016. The role of proline and root
542   traits on selection for drought-stress tolerance in soybeans: a review. South Afr J Plant Soil.
543   33:245-256.

544 Ono, K., Y. Yamamoto, A. Hachiya and H. Matsumoto, 1995. Synergistic inhibition of growth by
545   Aluminum and iron of tobacco (*Nicotiana tabacum* L.) cells in suspension culture. Plant Cell
546   Physiol., 36: 115–125.

547 Ortega-Villasante C, Rellan-Alvarez R, del Campo FF. 2005. Cellular damage induced by cadmium
548   and mercury in *Medicago sativa*. J Exp Bot. 56:2239–2251.

549 Patra M, Sharma A. 2000. Mercury toxicity in plant. Bot Rev. 66:379-409.

550 Paz-Alberto AM, Sigua GC, Baui BG, Prudente JA. 2007. Phytoextraction of lead-contaminated soil
551   using vetivergrass (*Vetiveria zizanioides* L.), cogongrass (*Imperata cylindrica* L.) and
552   carabaograss (*Paspalum conjugatum* L.). Environ Sci Pollut Res – Internat. 14: 498-504.

553 Peralta JR, Gardea TJL, Tiemann KJ, Gomez E, Arteaga S, Rascon E, Parsons JG. 2001. Uptake and
554   effects of five heavy metals on seed germination and plant growth in Alfalfa (*Medicago sativa*
555   L.). Bull Environ Contam Toxicol. 66:727–734.

556 Sagardoy R, Va´zquez S, Florez-Sarasa ID, Albacete A, Ribas-Carbo M, Flexas J, Abadı´a J, Morales
557   F. 2010. Stomatal and mesophyll conductance to CO_2 are the main limitations to
558   photosynthesis in sugar beet (Beta vulgaris) plants grown with excess zinc. New Phytol.
559   187:145–158.

560 Setyaningsih L, Wulandari AS, Hamim H. 2018. Growth of typha grass (*Typha angustifolia*) on gold-
561   mine tailings with application of arbuscular mycorrhiza fungi. Biodiversitas. 19(2): 454-459.

562 Shahid M, Khalid S, Abbas G, Shahid, N, Nadeem M, Sabir M, Aslam M, Dumat C: Heavy metal
563   stress and crop productivity. In *Crop Production and Global Environmental Issues*. Edited by
564   Hakeem KR. Switzerland (CH): Springer International Publishing; 2015:1-25.

565 Shanker AK, Djanaguiraman M, Sudhagar R, Chandrashekar CN, Pathmanabhan G. 2004.
566   Differential antioxidative response of ascorbate glutathione pathway enzymes and metabolites

567   to chromium speciation stress in green gram (*Vigna radiata* (L.) R.Wilczek. cv CO4) roots.
568   Plant Sci. 166:1035-1043.

569 Sheoran IS and Singh R: Effect of heavy metals on photosynthesis in higher plants. In *Photosynthesis:*
570   *Photoreactions to Plant Productivity*. Edited by Abrol YP, Mohanti P, Govinjee G. New
571   Delhi: Springer; 1993: 451-468.

572 Singh S, Parihar P, Singh R, Singh VP, Prasad SM. 2016. Heavy metal tolerance in plants: role of
573   transcriptomics, proteomics, metabolomics, and ionomics. Front Plant Sci. 6:11-43.

574 Solymosi K, Lenti K, Myśliwa-Kurdziel B, Fidy J, Strzałka K, Böddi B. (2004). Hg(2$^+$) reacts with
575   different components of the NADPH: protochlorophyllide oxidoreductase macrodomains.
576   Plant Biol. 6:358–368.

577 Sugiono CM, Nuraini Y, Handayanto E. The potency of *Cyperus kyllingia* Endl. for phytoremediation
578   of soil contaminated by gold mine mercuriy. J Tanah Sumberdaya Lahan. 1:1-8/

579 Tangahu BV, Abdullah SRS, Basri H, Idris M, Anuar N, Mukhlisin M. 2011. A review on heavy
580   metals (As, Pb, and Hg) uptake by plants through phytoremediation. Intern J Chem Engin.
581   2011: 939161.

582 Theriappan P. Gupta AK, Dhasarathan P. 2011. Accumulation of proline under salinity and heavy
583   metal stress in cauliflower seedlings. J. Appl. Sci. Environ. Manage. 15 (2) 251 – 255.

584 Vassilev A, Berova M, Zlatev Z. 1998. Influence of Cd^{2+} on growth, chlorophyll content, and water
585   relations in young barley plants. Biol Plant. 41:601–606.

586 Wu F, Zhang G, Dominy P. 2003. Four barley genotypes respond differently to cadmium: lipid
587   peroxidation and activities of antioxidant capacity. Environ Exp Bot. 50:67-78.

588 Wuana RA and Okieimen FE. 2011. Heavy metals in contaminated Soils: A review of sources,
589   chemistry, risks and best available strategies for remediation. Comm Soil Sci Plant Anal. 42:
590   111-122.

591 Yoshida S, Forna DA, Cock JH and Gomez KA. *Laboratory Manual for Physiological studies of rice*.
592   Los Banos. Philippines: IRRI; 1976.

593 Zengin FK and Munzuroglu O. 2005. Effect of some heavy metals on content of chlorophyll, proline
594   and some antioxidant chemicals in bean (*Phaseolus vulgaris* L.) seedlings. Acta Biol.
595   Cracovien. Ser. Bot. 47(2): 157-164.

596 Zhou ZS, Huang SQ, Guo K, Mehta SK, Zhang PC, Yang ZM. 2007. Metabolic adaptation to mercury-
597   induced oxidative stress in roots of *Medicago sativa*. JIB. 101:1-9.