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HEAVY METAL ACCUMULATOR PLANTS FOR GOLD MINING PHYTOREMEDIATION PROGRAM: MORPHO-PHYSIOLOGICAL AND HISTOCHEMICAL ANALYSIS

(Dr. HAMIM)

PROGRAM THRUST: RESTORATION OF DEGRADED ECOSYSTEM

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1. Research Title	: **Heavy metal accumulator plants for gold mining phytoremediation program: Morpho-physiological and histochemical analysis**
2. Research Coordinator	:
a. Name	: Dr. Ir. Hamim, M.Si.
b. Sex	: Male
c. Occupation	: Lecturer
3. Institution	:
a. Name of Institution	: Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University
b. Address	: Jl Agathis, Kampus IPB Darmaga, Bogor, Indonesia 16680
c. Telephone/Fax	: +62(251)8622833
d. Email address	: hamimhar@gmail.com ; hamim@ipb.ac.id
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Endorsed by
Deputy Director for Programme
SEAMEO BIOTROP

Research Coordinator

Dr. Jesus C. Fernandez

Dr. Ir. Hamim, M.Si.
NIP.: 19650322 199002 1 001

Approved by
Director of SEAMEO BIOTROP

Dr. Ir. Irdika Mansur, M.For.Sc
NIP 19660523 199002 1 001

1. INTRODUCTION

1.1. Background

According to the Directorate General of Plantation (2012), Indonesia has a very wide critical land, reaching 59.2 million ha, some of which are post mining lands that need to be remediated and regreened. Data from the Ministry of Forestry (2014) indicates that for the year 2013 alone the Ministry of Forestry has issued forest land use permit for mining exploration and exploitation of 214 business units with total area + 807,070 ha. The total with previous years the area is very large, and therefore the remediation program of mining land in Indonesia is quite large. Many problems arise related to gold mining, especially environmental problems (Krisnayanti & Anderson 2014), because in additional to soil structure damage and cyanide accumulation, it also produces metal contaminants. For example, the remaining mud of mining land or tailings contain heavy metals such as Pb by about 55-63.2 ppm (Syarif and Juhaeti 2003). Tailings from gold mining also contain some other heavy metals, such as arsenic, cadmium, and mercury at high levels (Arets et al., 2006; Purnomo et al., 2015; Setyaningsih, 2017). Existing tailings in mining areas are usually unfavourable substrates for plant growth due to extreme pH, low organic and nutrients, trace elements in high concentrations, reduced soil thickness, defective soil structures, and low water availability (Bench et al. 2015).

Heavy metals pollution has become a serious threat to the environment and food security. Unlike organic pollutants, biodegradation of heavy metals is just out of question and hence are continuously accumulating in the environment (Sarwar et al., 2010). Heavy metals are known to induce various toxic effects on plants, which influence morphological, physiological and biochemical processes in the plant (Kumar et al., 2014). Furthermore, accumulation of these heavy metals in agricultural soils and water resources poses a great threat to human health due to potential risk of their entry into food chain (Sarwar et al., 2010).

To improve the function of land contaminated by heavy metals requires physical or chemical treatments which needs considerable cost. Phytoremediation is a technology that can reduce heavy metal contaminants from the environment with relatively low cost and more effective. The basic principle of phytoremediation is utilizing the plants to clean up contaminated soil or water, and therefore, phytoremediation is friendlier to the environment (Kumar et al. 2013) and known as cost-effective technology (Cherian and Oliveira 2005). The basic principle of phytoremediation is utilizing the plants to clean contaminated soil or water. Therefore, phytoremediation is more friendly to the environment (Kumar et al. 2013), which the process can be completed *in situ* as well as *ex situ*, and easy to implement (Moenir and Misbachul 2010).

Since several essential elements come from metal groups, all plants have ability to absorb metals, but in varying amounts. Some plants have hyper-accumulator properties, which are able to accumulate metals with high concentrations in the root and shoot tissues, and can be used for the purpose of phyto-remediation. In one of the process known as phyto-extraction heavy metals are absorbed by the plant roots and translocated into the shoot to be recycled or disposed when the plants are harvested (Chaney

et al. 1995). The process of pollutant remediation from the soil occurs because certain types of plants can release substances carriers that bind to certain pollutant substances, then accumulate in plant tissues (Suhendrayatna et al. 2009). The accumulation of heavy metals in plant tissue can be detected by histochemical methods. The histochemical method is essential for the analysis, localization, and distribution of molecules in cells and tissues (Kiernan 2008).

For this purpose, some authors suggested to utilize the plants that have ability to absorb and accumulate more metal into the cell that is known as hyper-accumulator. Based on previous research, there are some plants that are hyper-accumulator, such as *Ipomea* sp. (Juhaeti et al., 2005), *Imperata cylindrica* (Howard et al., 2003), and *Paspalum conjugatum* (Mudarisna et al., 2014). Quimando et al. (2015) also suggested that, two *Phyllanthus* species *Phyllanthus erithrotycus* and *Phyllanthus securinegiodes* were known as hyper-accumulator plants. There are many weed species such as *Ischaemum Timorense*, *Cynodon dactylon*, *Cyperus kyllingia*, *Mikania cordata* (Burm.f.) B.L.Robinson, *Calopogonium mucunoides* that were also found to grow well in the mining area in Indonesia that allegedly can act as accumulator plants (Syarif and Juhaeti, 2003). Meanwhile, systematic efforts need to be carried out to determine the types of plants from many groups of weeds, shrubs, and trees that are potential for phytoremediation or even phytomining purposes.

There are many weed species in Indonesia such as *Ischaemum Timorense*, *Cynodon dactylon*, *Cyperus kyllingia*, *Mikania cordata* (Burm.f.) B.L.Robinson, *Calopogonium mucunoides* that are found to grow well in the mining area that allegedly can act as accumulator plants (Syarif and Juhaeti, 2003). Several types of shrub and trees such as *Jatropha curcas,* and *candlenuts* (*Reutealis trisperma* [Blanco]) have been tested for adaptability on marginal land including tin mining area, but the plant has not yet been tested for adaptation to gold mine lands (Hamim *et al*., 2017). Therefore, systematic efforts need to be carried out to determine the types of plants from many groups of weeds, shrubs, and trees that are potential for phytoremediation purpose.

This research is part of an umbrella research in the Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University to find out *plant consortium* that have good capacity for phytoremediation in gold mining area, by utilization several plants including shrubs and trees and the combination with microbes such as mycorrhizas and plant growth-promoting rhizobacteria.

1.2. Objectives

The objectives of the study are: (1) to analyse morphological, anatomical and physiological characters of several plants in response to heavy metal contaminant, (2) to identify the location and entry path of metal accumulation in these plants, and (3) to analyse further prospect of these plants in phytoremediation of heavy metal contaminated land in gold mining area.

1.3. Expected output

1. The data of morphological, anatomical and physiological characters of plants in response to heavy metal contaminant exposed by water culture.
2. The data of bio-accumulator capacity of the treated plants.
3. The potency of selected plants as phytoremediator of gold mining lands.
4. Two papers published in International Journal (Biotropia or other journal)

2. BENEFIT AND IMPORTANCE OF RESEARCH

This is a basic research that has benefit and importance to support and establish the implementation of phytoremediation concept by utilizing potential plants that have ability to grow well under contaminated land especially gold mine tailings and to constantly reduce contaminant from the site by physiological mechanism as well as to improve soil fertility as presented in the graph.

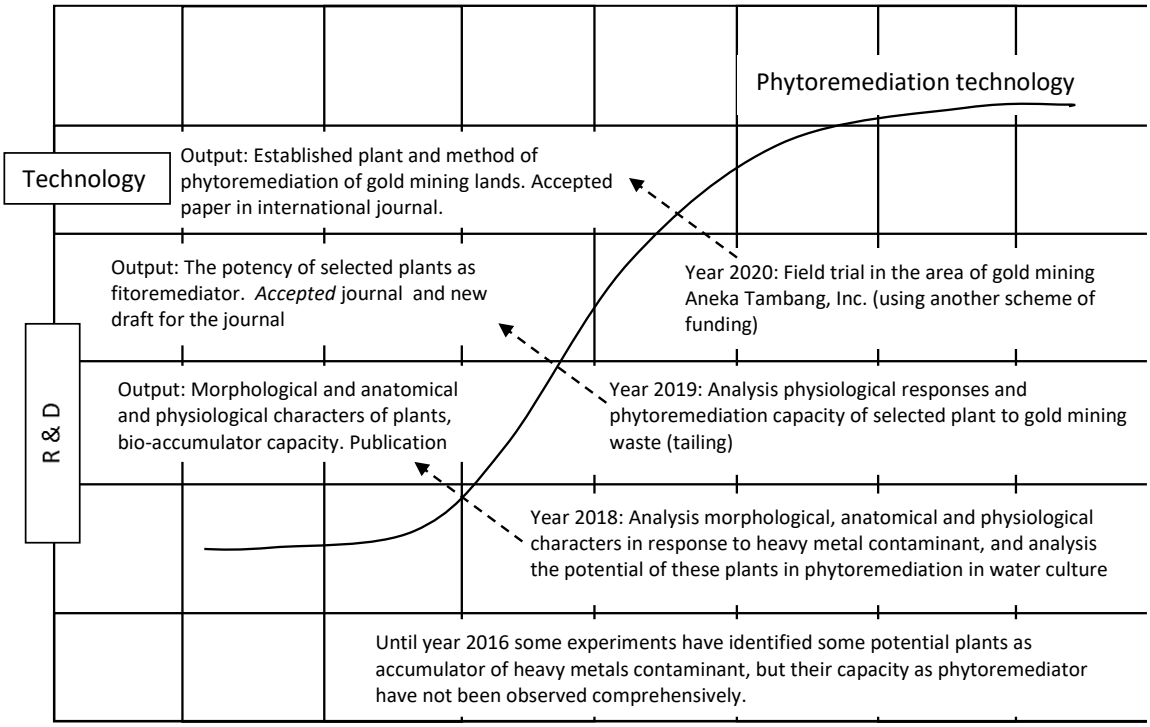

Figure 1. Benefit and the importance of research "***Heavy metal accumulator plants for gold mining phytoremediation program: Morpho-physiological and histochemical analysis***"

3. METHODS

3.1. Plant materials and water culture preparation

In this experiment, some species of plants (*Paspalum conjugatum, Cyperus kyllingia, Ipomea aquatica, Mikania micrantha,* and *Branchiaria mutica*) *were* used. Before experiment, the Hoagland's stock solution was prepared which consisted of macro nutrients (KNO_3, $Ca(NO_3)_2.4H_2O$, $NH_4H_2PO_4$, $MgSO_4.7H_2O$) and micronutrients (KCl, H_3BO_3, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, H_2MoO_4, and NaFeEDTA). Media solution then is prepared in a big container before distributed to experiment box contained 6 L of solution. One month old plants were removed carefully from the polybag and the roots were cleaned with water to remove soil and other solid media and then were planted in the box contained Hoagland's solution. To stand properly, the plants were equipped by perforated stereo foam and supported by fine sponge. To ensure air supply, each box is equipped by aerator. At the beginning, all the plants were grown under half strength Hoagland's solution for 3 weeks.

3.2. Treatment with Pb and Hg

The treatment of Pb and Hg was given to the plants after 2 weeks establishment in the culture by adding lead nitrate ($Pb(NO_3)_2$) and mercuric nitrate ($Hg(NO_3)_2$) to the solution with different concentrations (0 ml [control], 0.5, and 1 mM ($Pb(NO_3)_2$) or ($Hg(NO_3)_2$). To keep the volume of the solution inside the box similar, distilled water was added to each box so that the total volume of all media are similar. The treatment of heavy metals was given for 20 days to see the response of the treated plants.

The experiment was conducted using a completely randomized design with two factors, the first factor was plant species of weeds (*Paspalum conjugatum, Cyperus kyllingia, Ipomoea aquatica, Mikania cordata and Branchiaria mutica*). The second factor was Pb and Hg treatments which comprised (P0 [without Pb and Hg treatment], P1, 0.25 mM, and P2 0.5 mM of ($Pb(NO_3)_2$, H1 0.25 mM, and H2 0.5 mM of ($Hg(NO_3)_2$). Each experiment unit *was* repeated 3 times with 6 plants per box (unit experiment).

Observations was 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. After 20 days of the treatment, the plants were harvested for the observation of morphological, and anatomical parameters. For morphological analysis, root length, stem length, number and leaf area were measured. Physiological analysis was carried out after 10 days of the treatment, which including photosynthesis, proline and chlorophyll content. The accumulation of lipid peroxidation which indicative of the content of ROS was calculated by measuring malondialdehyde content. Anatomical analysis using light microscope and histochemical analysis was carried out to observe different structure of leaves and roots all the plant and accumulation pattern of

the heavy metal. At the end of the treatment, Pb and Hg content of the roots and leaves *was* measured to analyze the accumulation capacity of the plants to Pb and Hg.

3.3. Analysis of leaves and roots anatomy

For anatomical analysis, roots and leaf samples were dipped into fixative solution using 70% of ethanol (p.a. Merck KGaA, Darmstadt, Germany) until measurement. Leaves and roots are cut using microtome and then were analyzed to observe epidermis, corticle and vascular tissues. After cutting, leaves are dipped for 10 minutes in sodium hypochlorite to remove debris and chlorophyll, and then are rinsed for 10 minutes using distilled water. Finally the leaves are dipped into safranin for 15 minutes for coloring. After coloring the tissues are placed in the object glass which had been dripped by glycerin 10% for observation.

3.4. Histochemical analysis for Pb and Hg accumulation

Samples of leaves and roots of the plants are cut across 30 μm thick using frozen microtome (Yamato type RV-240). The observed Pb accumulation *was* performed using sodium rodizonate reagent. The sieved sample *was* immersed in 0.4% sodium rodizonate for 60 minutes, washed with distilled water and immersed in 0.1% light green dye in 1% acetic acid for 2 minutes. The incision *was* then placed on top of the object glass and drops glycerin, then covered with a glass cover. Observations are made using a light microscope (Olympus BX51) equipped with an indomicro camera. The presence of lead (Pb) in plant cells or tissues *was* characterized by a red color (Harahap 2004).

The accumulation of mercury (Hg) in plant tissue can be seen using dithizone reagents. Sample of plant organ that has been slashed, soaked in dithizone solution, then let stand for 15 minutes at room temperature. The incision *was* placed on top of the object glass and spilled with 30% glycerin, then covered with a cover glass. Observations are made using a light microscope (Olympus BX51) equipped with an indomicro camera. The presence of mercury in cells or tissues *was* characterized by the presence of brown color in the observed tissue (Hussain et al. 2010).

3.5. Chlorophyll content analysis

Chlorophyll content *was* analyzed using method developed by Yoshida et al. (1976). Two grams of fresh leaves are ground using 80% of acetone (p.a. Merck KGaA, Darmstadt, Germany) and then are filtered using Whatman paper no: 1 into 100 ml of volumetric flask until all the chlorophyll are dissolved into the acetone solution, before finally the solution in the volumetric flask reach 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, Kyoto, Japan) at the 645 nm and 663 nm wavelength (λ). Chlorophyll content was measured using formula as follow[28]:

$$\text{Chl a} = 0.0127.\ A663 - 0.00269.\ A645$$

$$\text{Chl b} = 0.0229.\ A645 - 0.00468.\ A663$$

Total Chl = Chl a + Chl b = 0.0202. A645 + 0.00802. A663

Chl a = Chlorophyll a; Chl b = Chlorophyll b

A663 = the absorbance at the λ of 663 nm

3.6. Photosynthesis measurement

Measurements of photosynthesis *was* carried out using Photosynthetic Gas Exchange Analyzer LiCOR LI-6400. Observations *was* 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 1000 mmol/cm^2 per second. The measurement was also carried out at different light intensity (100, 200, 400, 750, 100 and 1500 mmol/cm^2 per second) to analyzed photosynthetic light curve.

3.7. Lipid peroxidation analysis

Lipid peroxidation *was* estimated as described in Mihara *et al*. (1985) with some modifications based on Ono *et al*. (1990). Fresh roots (0.2 g) are ground in 0.5 ml of 0.1% (w/v) trichloracetic acid (TCA) at 4 °C. The root 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 100ºC for 30 minutes. After cooling at the room temperature, 4 ml n-butanol *was* added to the solution, and then followed by centrifugation at 4200 rpm at 28ºC 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).

3.8. Proline Analysis

Proline content of leaves *was* analyzed following Bates et al. (1973). Homogenized tissues (150 mg) from leaves are 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 1h 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 are prepared and analyzed in the same way to obtain a calibration curve.

3.9. Analysis of Pb and Hg in the roots and shoot content

After 20 days (at the end of treatment) the plants *were* harvested by separating roots and shoot part of the plant for Pb and Hg analysis. The roots and shoots are dried using oven at 80ºC for 3 days until

the weight was constant. The dried matters then are analysed using Atomic Absorbance Spectroscopy (AAS) to measure Pb and Hg content from the roots and shoot of the plants.

4. RESULTS AND DISCUSSIONS

4.1. Plant Morphology

In this experiment five species that was suggested as metal accumulator were used i.e. *Branchiaria mutica*, *Cyperus kyllingia*, *Ipomea aquatica*, *Mikania micrantha*, and *Paspalum conjugatum*. After grown for 3 weeks in the polybags, and then moved to water culture with Hoagland solution for 2 weeks, the plants were treated with Hg(NO3)2 and Pb(NO3)2 for three weeks. Morphological and physiological analysis were carried out among the plants to distinguish the different response of the plant to heavy metal treatment.

Figure 1. Morphology of five species that were used in the experiment: (1) *Branchiaria mutica*, (2) *Cyperus kyllingia*, (3) *Ipomea aquatica*, (4) *Mikania micrantha*, and (5) *Paspalum conjugatum*.

Among the five species, the plants have different growth characteristics including shoot and root length, leaf number as well as plant biomass. The treatment using Hg and Pb also influenced plant growth, even though there was variation among the species. There was similar pattern of Hg treatments

which significantly reduced plant growth of all the species, except for root growth of *Ipomea aquatica* which did not decrease in response to Hg treatments (Figures 2-5). The most negative effect was shown by all the plants subjected to 0.5 mM of Hg (Figures 2-5). On the other hand, response of plant morphology to Pb treatment was not as big as Hg, even though at 0.5 mM of Pb significantly decreased some morphological parameters especially for *M micranta* (Figures 2, 4 and 5).

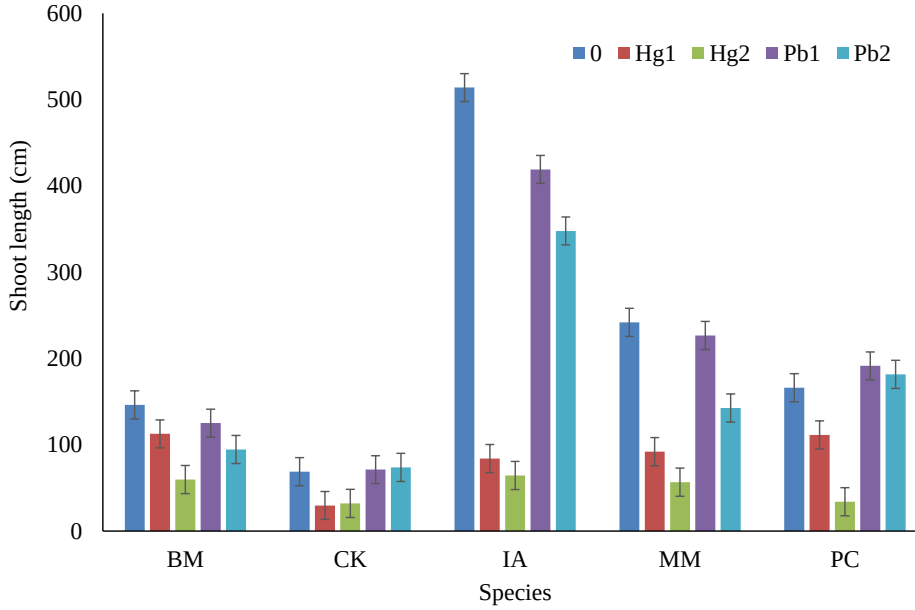

Figure 2. Shoot length of the species after 3 weeks exposure to Hg and Pb with different concentrations. 0: control (without heavy metal treatment); Hg1: 0.25 mM of Hg; Hg2: 0.5 mM of Hg; Pb1: 0.25 mM of Pb and Pb2: 0.5 mM of Pb. BM: *Branchiaria mutica*, CK: *Cyperus kyllingia*, IA: *Ipomea aquatica*, MM: *Mikania micrantha*, and PC*: Paspalum conjugatum.*

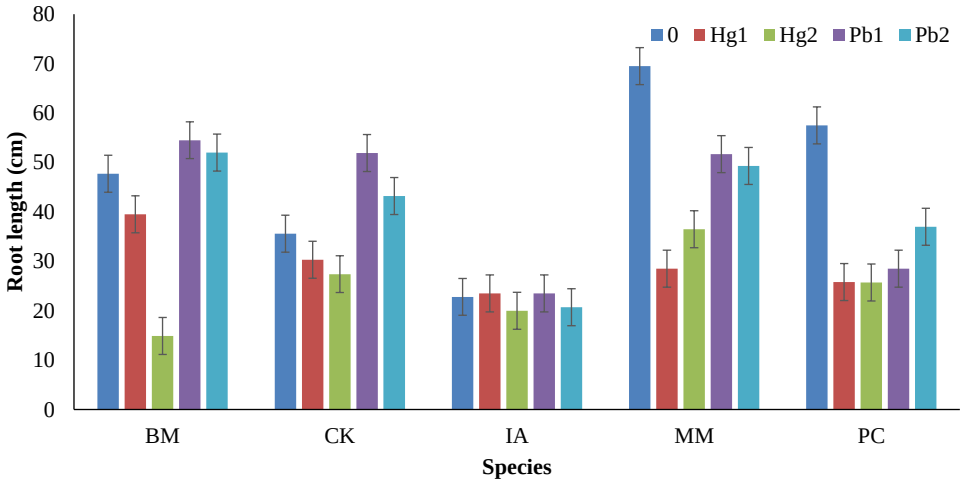

Figure 3. Root length of the species after 3 weeks exposure to Hg and Pb with different concentrations. 0: control (without heavy metal treatment); Hg1: 0.25 mM of Hg; Hg2: 0.5 mM of Hg; Pb1: 0.25 mM of Pb and Pb2: 0.5 mM of Pb. BM: *Branchiaria mutica*, CK: *Cyperus kyllingia*, IA: *Ipomea aquatica*, MM: *Mikania micrantha*, and PC*: Paspalum conjugatum.*

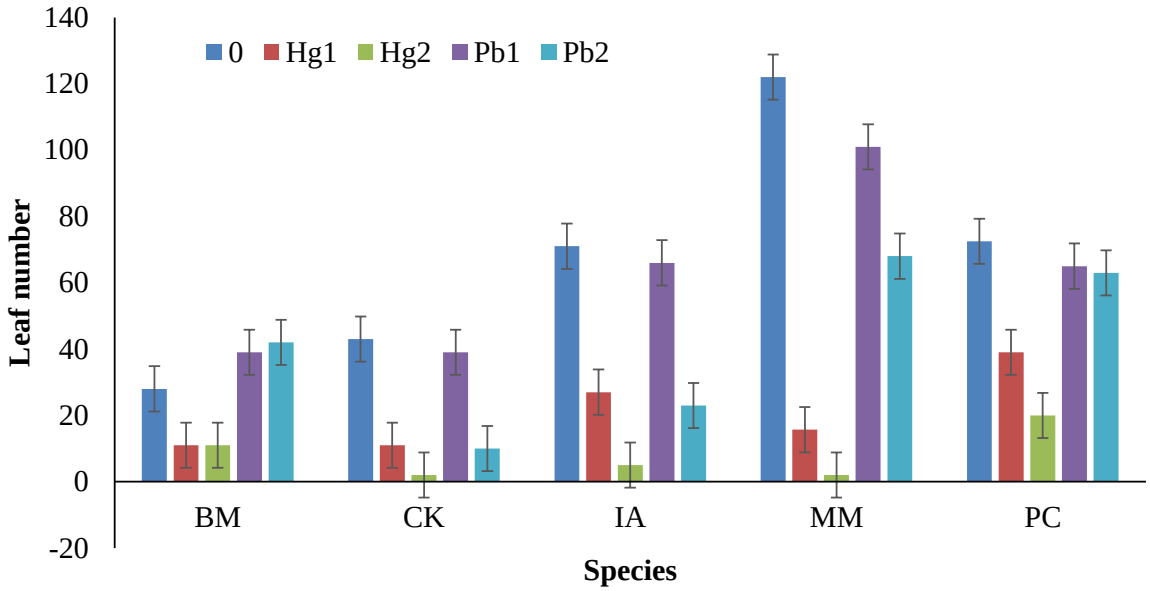

Figure 4. Leaf number of the species after 3 weeks exposure to Hg and Pb with different concentrations. 0: control (without heavy metal treatment); Hg1: 0.25 mM of Hg; Hg2: 0.5 mM of Hg; Pb1: 0.25 mM of Pb and Pb2: 0.5 mM of Pb. BM: *Branchiaria mutica*, CK: *Cyperus kyllingia*, IA: *Ipomea aquatica*, MM: *Mikania micrantha*, and PC*: Paspalum conjugatum.*

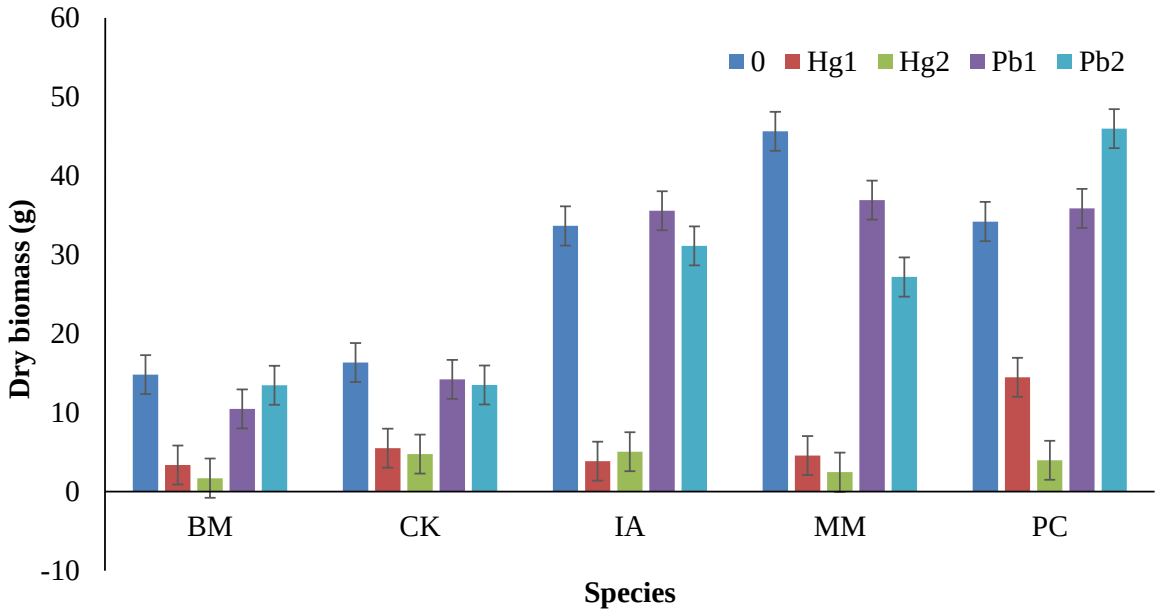

Figure 5. Dry biomass of the species after 3 weeks exposure to Hg and Pb with different concentrations. 0: control (without heavy metal treatment); Hg1: 0.25 mM of Hg; Hg2: 0.5 mM of Hg; Pb1: 0.25 mM of Pb and Pb2: 0.5 mM of Pb. BM: *Branchiaria mutica*, CK: *Cyperus kyllingia*, IA: *Ipomea aquatica*, MM: *Mikania micrantha*, and PC*: Paspalum conjugatum.*

Response of shoot was more prominent than roots in response to Hg and Pb treatment (Figures 2 and 3). For shoot length, the reduction was in the range from 54 to 87 % due to 0.5 mM of Hg, while it only caused 12 – 56 % reduction on root length. In response to Hg treatment, *I. aquatica*, *M. micrantha*, and *P. conjugatum* were the most repressed shoot growth, while *B. mutica* and *C. kyllingia* were the least affected. However, for root length parameter, only *I. aquatica* that was not affected by Hg treatment (Figure 4). To observe further about ability of those species to survive under higher heavy metal contaminant, physiological parameter were observed.

Heavy metals have been known to cause inhibition of root and canopy growth and plant production (Peralta et al. 2001; Kibra 2008). Metal toxic effects, especially lead and mercury, have been reported in several plants, including *Triticum aestivum* (Patra and Sharma 2000), *Phaseolus vulgaris* L. (Zengin and Munzuroglu 2005), tomatoes (Cho and Park 1999), and several other plants. According to (Ortega-Villasante et al. 2005) Hg at high concentrations is very toxic to cells which induces damage to cells and causes physiological changes. The accumulation of Hg can also inhibit plant growth, causing plant productivity to decline. In this study, the value of plant height, number of leaves, and total dry weight in the five plant species decreased dramatically due to Hg stress which was given even only at 0.25 mM concentration (Figure 2-5), while Pb treatment treated up to 0.5 mM only caused a relatively small decrease except for *M. micrantha* (Figure 5).

Plant height and number of leaves are indicators of the most commonly observed plant growth to see plant responses to the environmental stress. Decreasing the value of plant height and number of leaves may occur due to the accumulation of high Hg in plant tissues. This happens because heavy metals can cause inhibition of cell division and elongation, absorption of water and nutrients, and the decrease enzymatic activity so that the growth rate was inhibited (Shahid et al. 2015). Based on the research of Patra and Sharma (2000) 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. 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, while the smallest decrease was shown by *C. kyllingia* plants (Figure 5). The lower dry weight of plants shows that the physiological processes in plants were disrupted due to heavy metal toxicity, so that the growth was less optimal (Supriono 2010).

4.2. Physiological parameters

In this experiment, physiological parameters observed including malondialdehyde (MDA) content of shoot and roots, chlorophyll content, photosynthetic rate and proline content. MDA content was 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 6). 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 (Figure 7). Only in *P. conjugatum* roots treated with 0.5 mM Pb the MDA content increased significantly (Figure 7).

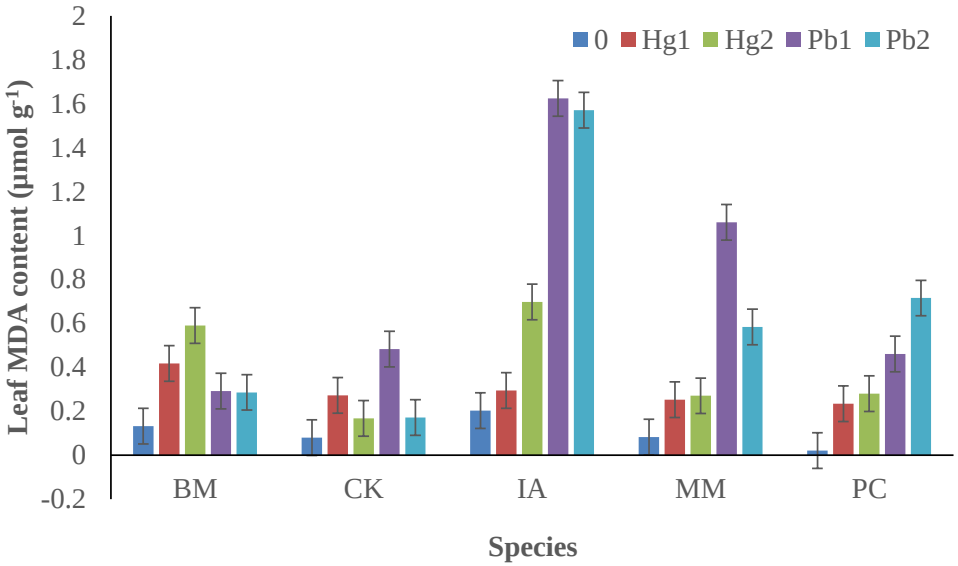

Figure 6. Leaf MDA content of the species after 10 days exposure to Hg and Pb with different concentrations. 0: control (without heavy metal treatment); Hg1: 0.25 mM of Hg; Hg2: 0.5 mM of Hg; Pb1: 0.25 mM of Pb and Pb2: 0.5 mM of Pb. BM: *Branchiaria mutica*, CK: *Cyperus kyllingia*, IA: *Ipomea aquatica*,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

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 control, even though the highest leaf MDA was shown by *I. aquatica* exposed to 0.5 mM Hg (Figure 6). 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 6).

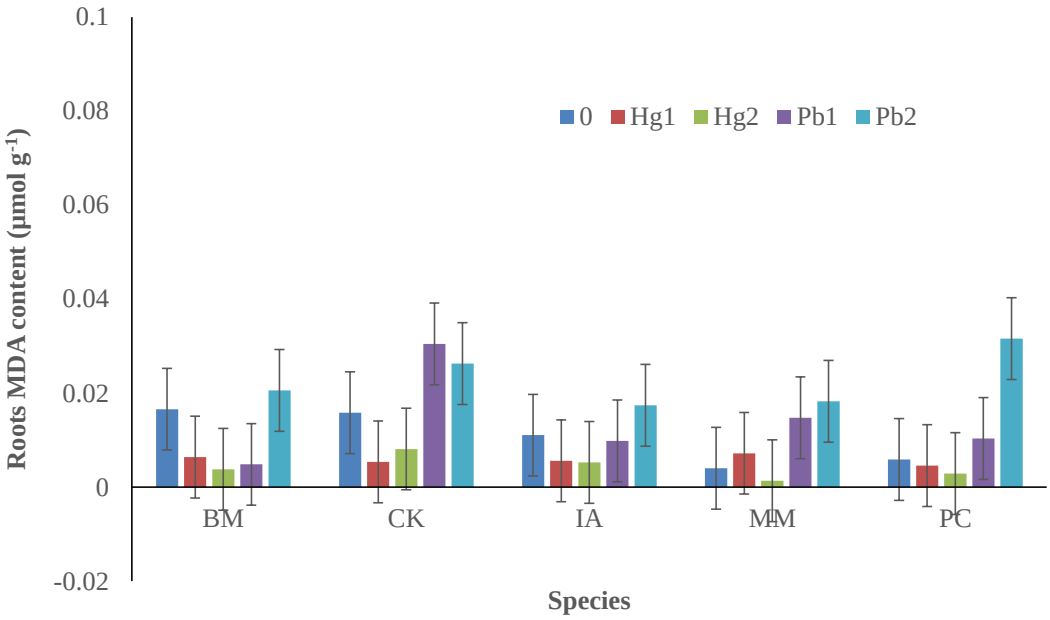

Figure 7. Root MDA content of the species after 10 days exposure to Hg and Pb with different concentrations. 0: control (without heavy metal treatment); Hg1: 0.25 mM of Hg; Hg2: 0.5 mM of Hg; Pb1: 0.25 mM of Pb and Pb2: 0.5 mM of Pb. BM: *Branchiaria mutica*, CK: *Cyperus kyllingia*, IA: *Ipomea aquatica*, MM: *Mikania micrantha*, and PC*: Paspalum conjugatum.*

From the analysis of chlorophyll content indicated that heavy metal treatment caused the decrease of chlorophyll content of all species dramatically (Figure 8). 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* had the least affected (Figure 8). Different from Hg, the treatment using Pb until 0.5 mM only decreased chlorophyll content of *C. kyllingia*, *I. aquatica* and *M. micrantha*, but not of *B. mutica* and *P. 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 different correlation between MDA and chlorophyll content in response to Hg and Pb treatment. Figure 9 showed that the increase of MDA content due to Hg treatment was closely related to the decrease of chlorophyll content which 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 Hg (Figure 9).

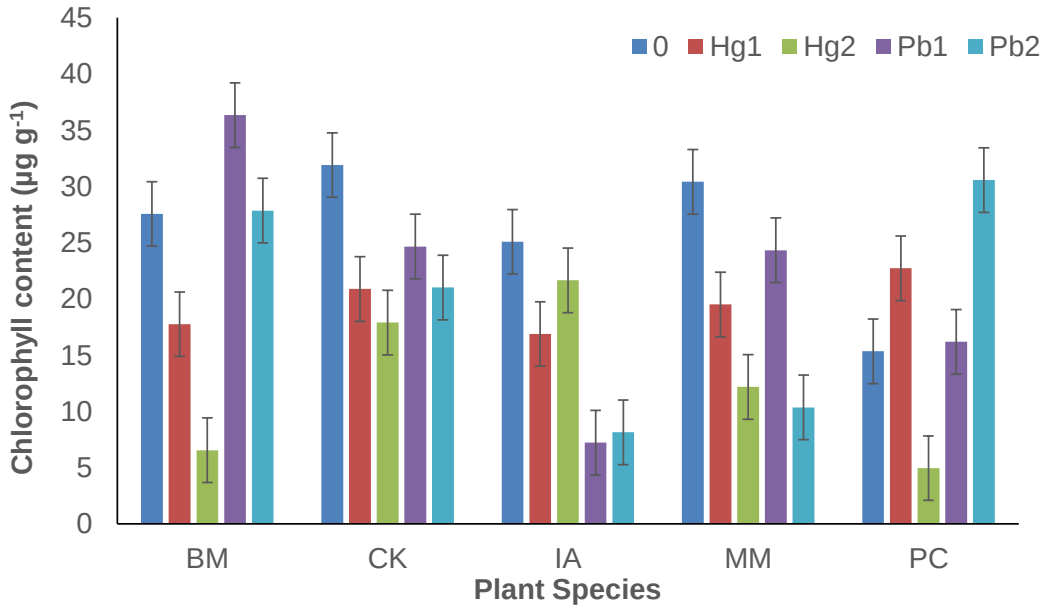

Figure 8. Chlorophyll content of the species after 10 days exposure to Hg and Pb with different concentrations. 0: control (without heavy metal treatment); Hg1: 0.25 mM of Hg; Hg2: 0.5 mM of Hg; Pb1: 0.25 mM of Pb and Pb2: 0.5 mM of Pb. BM: *Branchiaria mutica*, CK: *Cyperus kyllingia*, IA: *Ipomea aquatica*, MM: *Mikania micrantha*, and PC*: Paspalum conjugatum.*

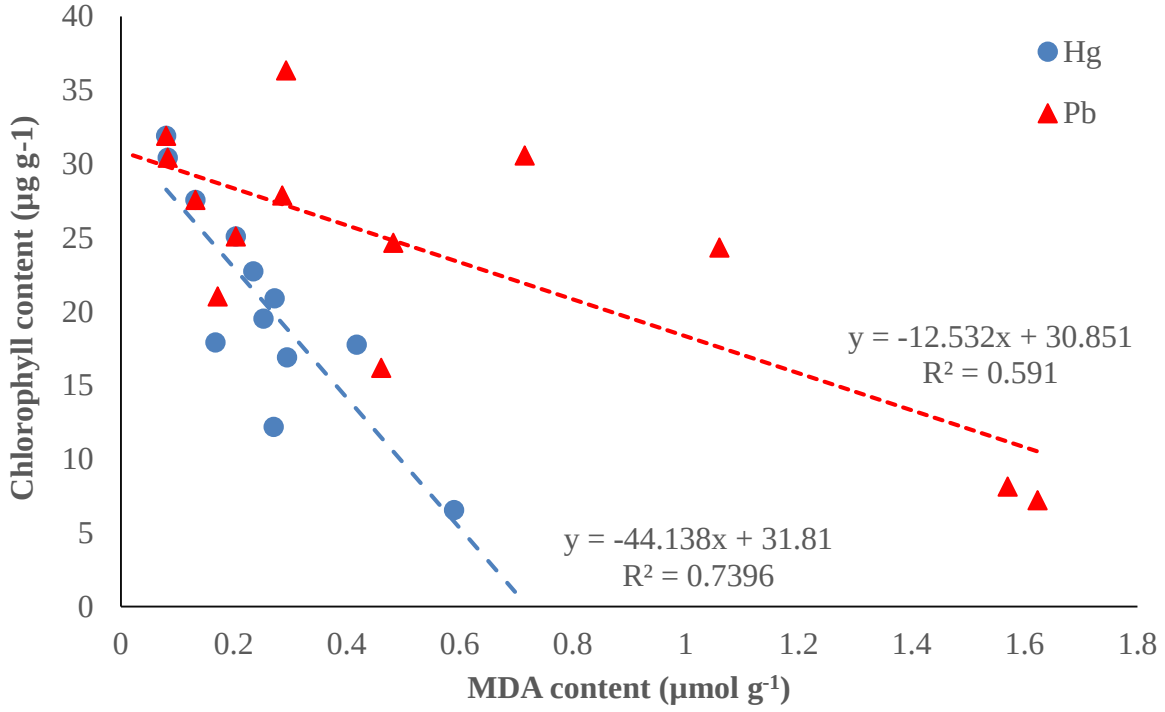

Figure 9. The regression graph between MDA and chlorophyll content of all species in response to Hg and Pb treatment. There was different slope among both treatment, where Hg treatment had steeper, while Ph had slightly sloping.

The content of malondialdehyde is an index to evaluate the level of damage to plants after stress treatment, which is the main cytotoxic product of lipid peroxidation and indicators of free radical production (Fu and Huang 2001). The higher MDA value indicates that the plant is under tress, on the contrary the lower MDA value shows the more tolerant of the plant. In general, the negative effects of heavy metals are based on two mechanisms. First, there is an inhibition of the enzyme's work when metal ions react with a functional group of proteins. Second, heavy metals can deactivate biomolecules through the transfer of essential metal ions from specific binding sites (Schutzendubel and Polle 2001). Inactivation of enzymes and biomolecules was initially considered the main cause of heavy metal toxicity. However, several studies have shown that oxidative stress is 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 haa a significant effect on lipid peroxidation as indicated by the higher MDA values due to treatment (Figure 6).

Membrane systems including chloroplast membranes 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. Data from the study showed that the Hg and Pb stresses given in high concentrations reduced the total chlorophyll content of the five plant species studied (Figure 8). Solymosi et al. (2004) reported that Hg stress induces photoreduction inhibition of protochlorophyllide in wheat leaves, so the total chlorophyll value of leaves decreases with increasing Hg concentration. This decrease occurs because heavy metals can cause chlorophyll biosynthesis to be inhibited through the work inhibition of two highly sensitive enzymes, namely enzymes (α-aminolaevulinic acid (ALA) dehydratase) and protochlorophyllide reductase which play a role in the early and final stages of chlorophyll biosynthesis (de Filippis et al. 1981). Mercury was also reported to cause magnesium ions to be replaced in photosynthetic pigments (Kupper et al. 1998).

4.3. Photosynthesis analysis

The analysis of photosynthetic rate of five species in response to heavy metal treatment showed that all the species had almost similar photosynthetic rate i.e. at the average was 13.5 μmol cm^{-2} $s^{-1}$for control plants (Figure 10). The effect of Pb treatments up to 0.5 mM did not significantly reduce photosynthetic rate of all species. However the treatment of Hg especially at 0.5 mM caused dramatic decrease of photosynthetic rate almost all species except *I. aquatica* (Figure 10). The *C. kyllingia* and *M. micrantha* had the lowest photosynthetic rate in response to 0.5 mM of Hg treatment.

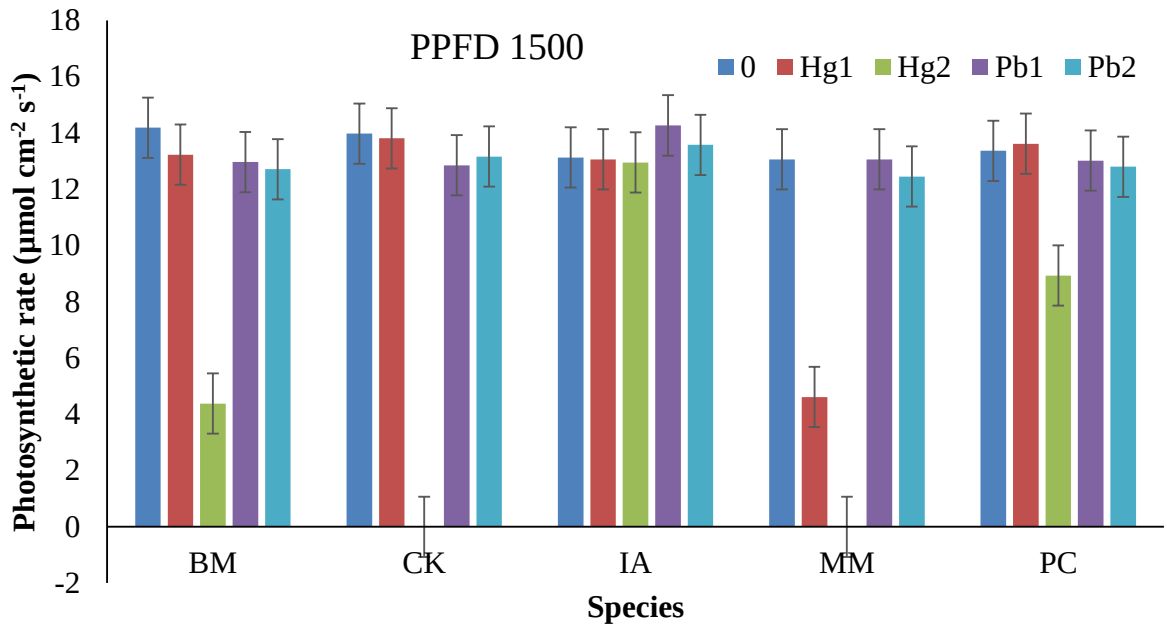

Figure 10. The average of photosynthetic rate of five species (BM: *Branchiaria mutica,* CK: *Cyperus kyllingia,* IA: *Ipomea aquatica,* MM: *Mikania micrantha,* and PC*: Paspalum conjugatum*) in response to Hg and Pb treatments (0, 0.25 and 0.5 mM).

To understand further about the characteristic of photosynthesis of each species in response to the treatment of Hg and Pb, the analysis of light curve of photosynthesis was carried out using different photosynthetic photon flux density (PPFD), starting from 100 to 1500 mmol cm^{-2} s^{-1}. This light curve 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 11). In general photosynthesis was recorded at event lower PPFD (100 mmol cm^{-2} s^{-1}) with almost similar values among the treatments. The maximum photosynthesis was reached under the PPFD of 750 mmol cm^{-2} s^{-1} (Figure 11). The photosynthesis graphs showed that the treatment with 0.5 mM of Hg 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 even larger because at 0.25 mM, Hg also decreased photosynthesis (Figure 11).

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 were calculated and the light curve were plot using logarithmic equation as presented in Figure 12. The graph showed that there were 3 different groups of light curve with the lowest curve represented the curve of the plants treated by 0.5 mM of Hg. The second curve was the highest photosynthesis light curve which represented by some curves including control plant and Pb-treated plants which had almost similar curve (Figure 12). 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 still lower than the second curve (Figure 12). This curve was created especially because the response of *M. micrantha* which had lower photosynthesis under 0.25 mM of Pb treatment (Figure 11). The second and the third curves showed that at the PPFD of 1500 mmol cm^{-2} s^{-1} the photosynthesis was still not saturated, so that the photosynthetic rate was still possible to increase when the PPFD increase (Figure 12).

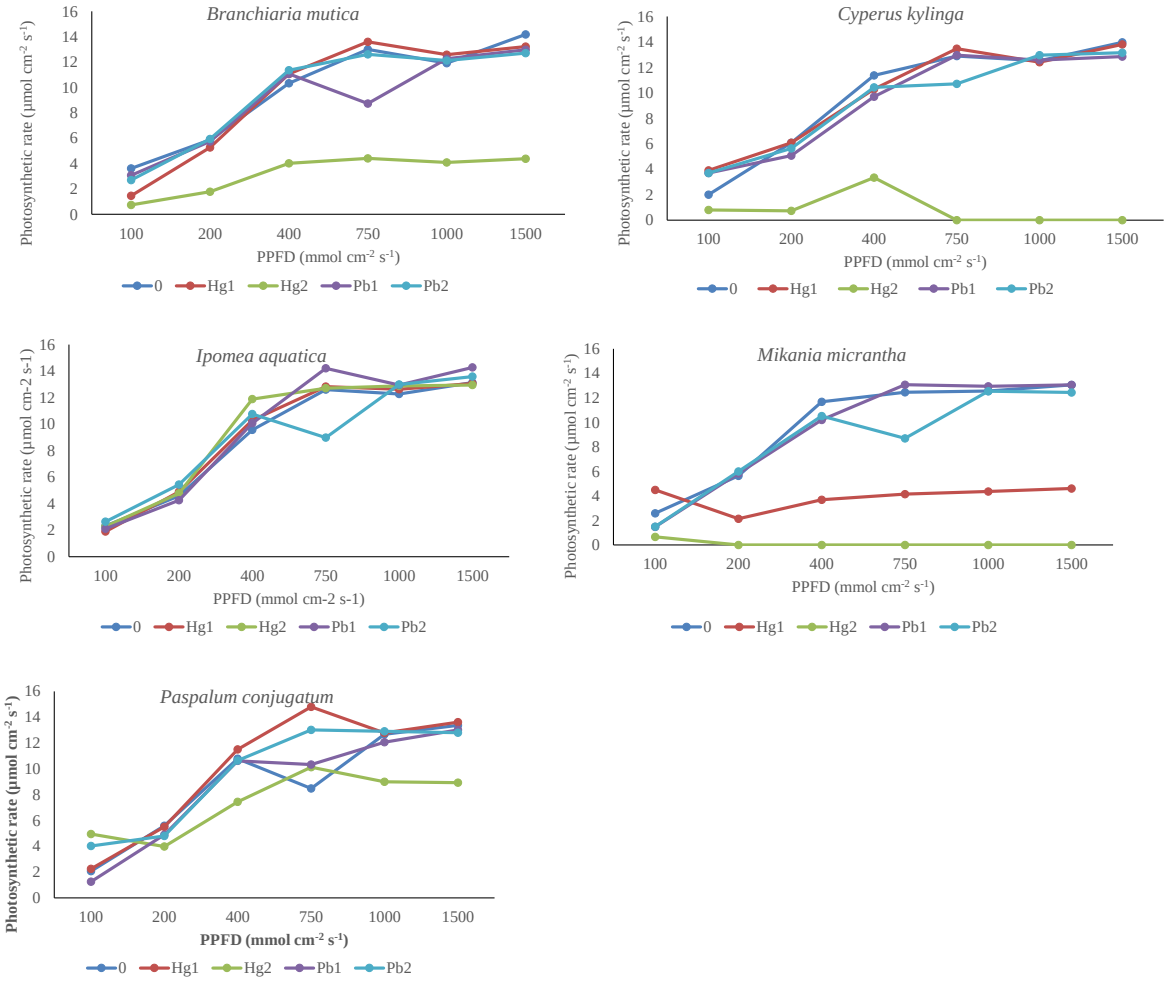

Figure 11. The photosynthetic rate of five species (BM: *Branchiaria mutica*, CK: *Cyperus kyllingia*, IA: *Ipomea aquatica*, MM: *Mikania micrantha*, and PC*: Paspalum conjugatum*) in response to Hg and Pb treatments (0, 0.25 and 0.5 mM) under different PPFD.

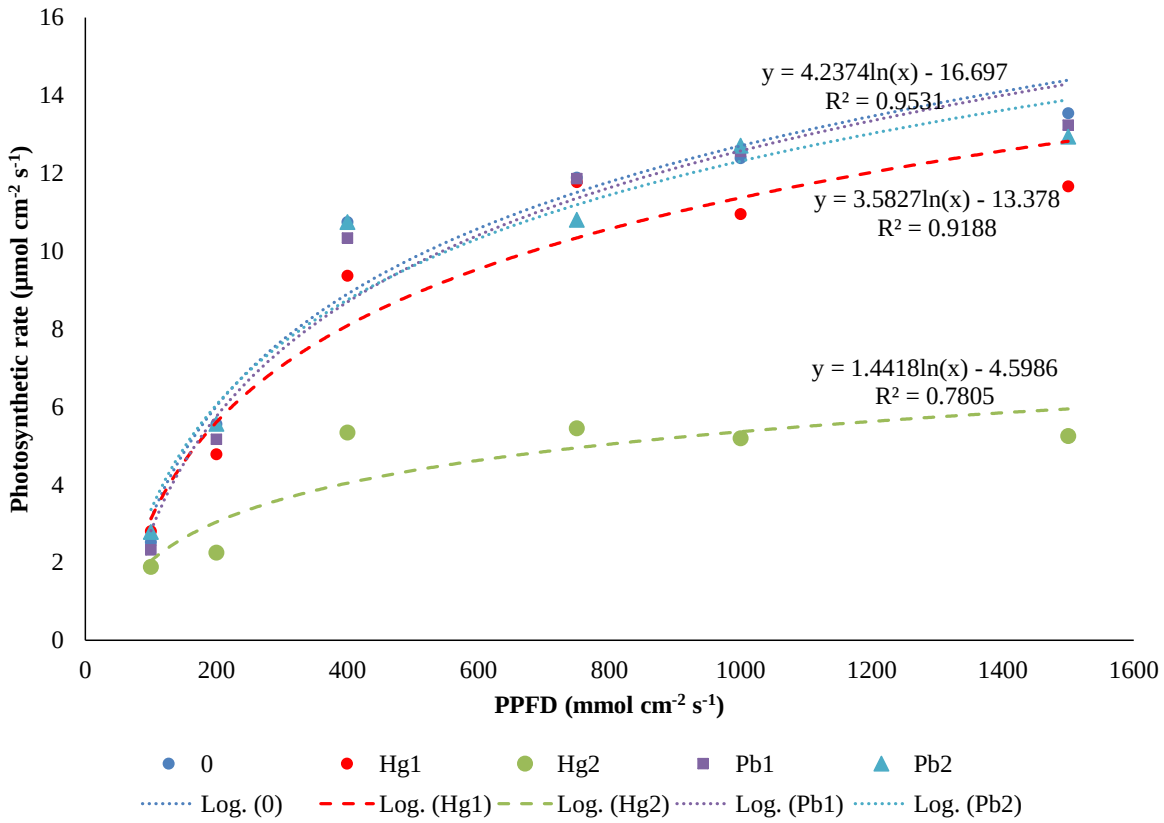

Figure 12. Photosynthetic light curve of all the species (*Branchiaria mutica, Cyperus kyllingia, Ipomea aquatica, Mikania micrantha,* and *Paspalum conjugatum*) in response to heavy metal treatments (0: control (without heavy metal treatment); Hg1: 0.25 mM of Hg; Hg2: 0.5 mM of Hg; Pb1: 0.25 mM of Pb and Pb2: 0.5 mM of Pb)

It has been known that photosynthesis is a physiological process that is very sensitive to heavy metal poisoning both in vitro and in vivo, especially the light system 2 (PSII) considering that heavy losses can hamper the work of PSII (Sheoran and Singh, 1993). According to Aggarwal et al. (2011) the effects of heavy metal toxicity on photosynthesis can occur either directly or indirectly. Directly is through inhibition of light reactions and oxygen formation, NADP reduction and photophosphorylation. While indirectly due to inhibition of chlorophyll synthesis or increased damage to chlorophyll. In Figure 6 it is also shown that the chlorophyll content decreases due to the treatment of Hg and Pb which indirectly affects the reduction of photosynthesis rate (Figure 10).

4.4. Proline analysis

Proline content is among the physiological parameter which normally increase when the plant was subjected to environmental stress such as drought, salinity, and even heavy metal stress. Proline content of all the species also indicated the similar result especially when the plants were treated with Hg at 0.25 and 0.5 mM (Figure 13). Proline content in all the species increased significantly 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

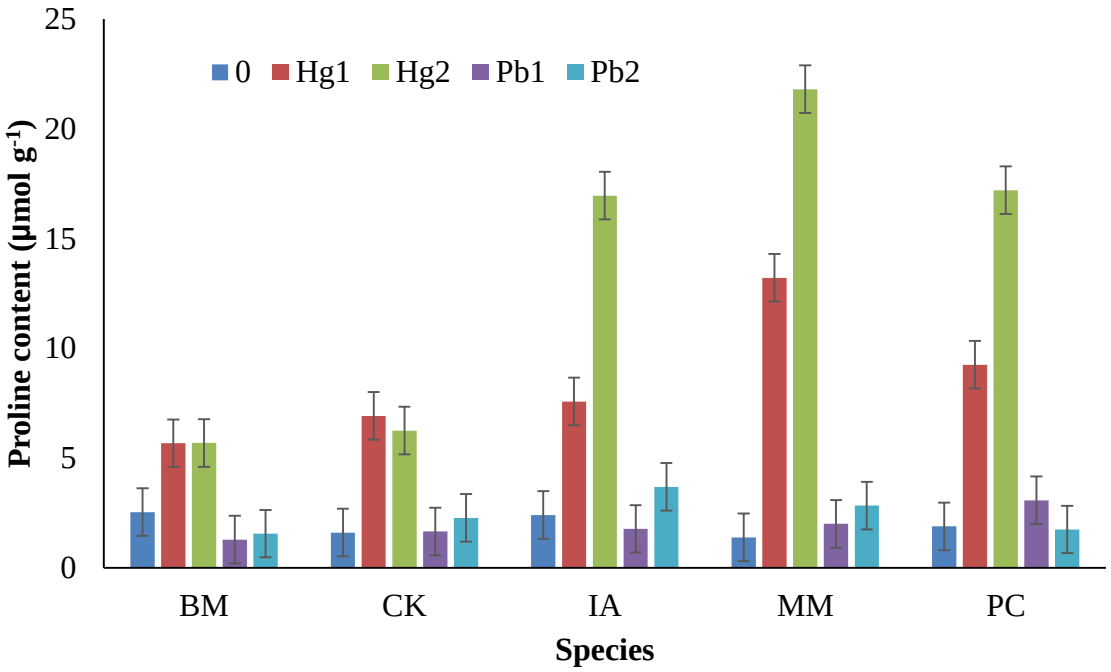

Figure 13. Proline content of five species subjected to different treatment of Hg and Pb. 0: control (without heavy metal treatment); Hg1: 0.25 mM of Hg; Hg2: 0.5 mM of Hg; Pb1: 0.25 mM of Pb and Pb2: 0.5 mM of Pb. BM: *Branchiaria mutica*, CK: *Cyperus kyllingia*, IA: *Ipomea aquatica*, MM: *Mikania micrantha*, and PC: *Paspalum conjugatum*.

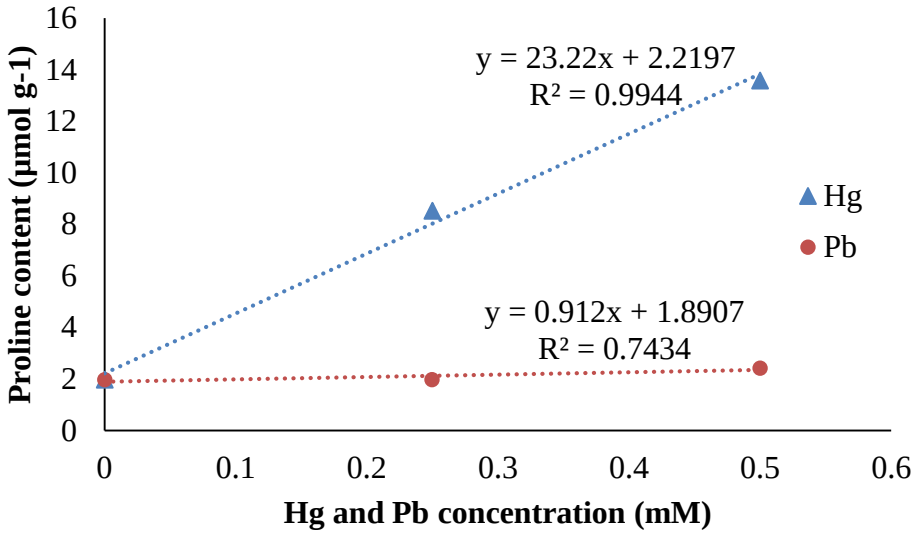

Figure 14. The regression of the average of proline content from five species and the Hg and Pb treatments at different concentrations (0, 0.25 and 0.5 mM). The increase of Hg treatments induced proline content, but it was not happen to Pb treatments.

highest proline content was presented by *M. micrantha* at 0.5 mM of Hg followed by *P. conjugatum* and *I. aquatica* (Figure 13). 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 and Hg or Pb treatments indicated that these two parameters had different graph and coefficient correlation (Figure 14). This data may support the assumption that the increase of proline content is indicative of alarm stress rather than the role of proline to reduce the damage of abiotic stress.

4.5. Relative water content

Relative water content (RWC) is a physiological parameter as an indication of water of the plant under certain environment. This parameter sometimes was analysed to understand the effect of metal stress to the suppression of water channel in the cellular level which induce water stress. In this experiment, among the five species *B. mutica*, *M. micrantha* and *P. conjugatum* underwent RWC reduction significantly especially when treated with 0.5 mM of Hg (Figure 15). Even though not exactly fit, the reduction of RWC of the species was negatively associated to the proline increase (Figure 13). This data my support the idea that heavy metal toxicity may have association to the impair of water channel in the cellular level.

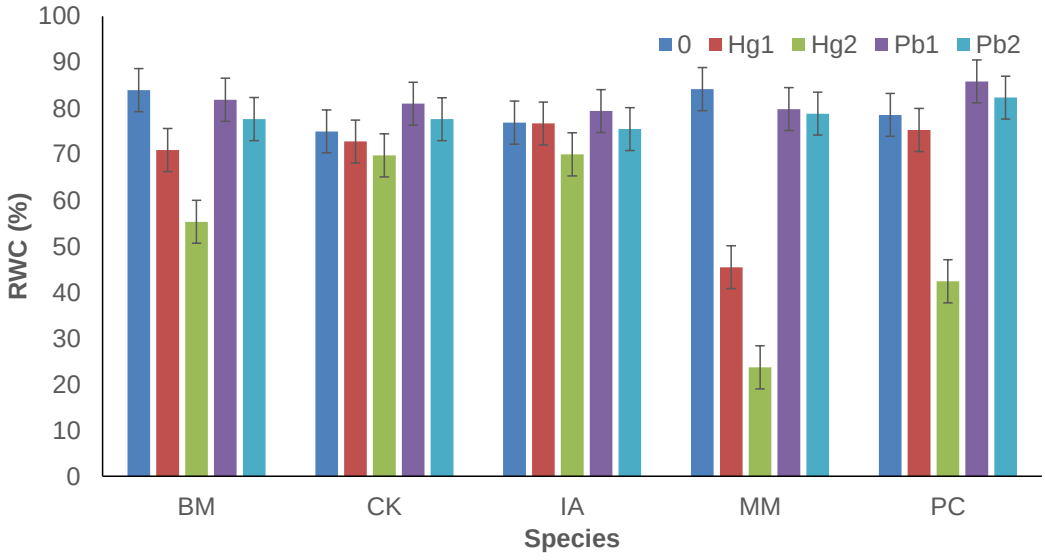

Figure 15. Relative water content (RWC) of five species after 3 weeks treated with different concentration of Hg and Pb. 0: control (without heavy metal treatment); Hg1: 0.25 mM of Hg; Hg2: 0.5 mM of Hg; Pb1: 0.25 mM of Pb and Pb2: 0.5 mM of Pb. BM: *Branchiaria mutica*, CK: *Cyperus kyllingia*, IA: *Ipomea aquatica*, MM: *Mikania micrantha*, and PC*: Paspalum conjugatum.*

4.6. Plant anatomy analysis

Anatomy analysis was carried out to observe the anatomical changes in response to heavy metal Hg and Pb treatments as part of adaptation mechanism to avoid the cellular damage. Histochemical analysis was also carried out to observe metal absorption into the cellular level. This analysis was needed to ensure the accumulation of the metal to above part of the plant, not only being conserved inside the root tissues. Figure 16 showed the graphs of leaf tissues of five species with different treatment using 0.5 mM of Hg and Pb. The application of dithizone reagent to detect Hg and Pb showed that for *B. mutica* 0.5 mM of Hg caused cellular damage, while Pb did not affect the cell neither Pb accumulation inside leave tissues. In *C, kyllingia* the cells were still alive, there was Hg accumulation found but only a view Pb was accumulated inside the cell. For *I. aquatica* there was Hg and Pb accumulation inside the leaf cell while the cell was still alive. In the *M. micranta* it was also found Pb accumulation, while in *P. conjugatum* Pb was only found in the epidermis cell wall but not the others.

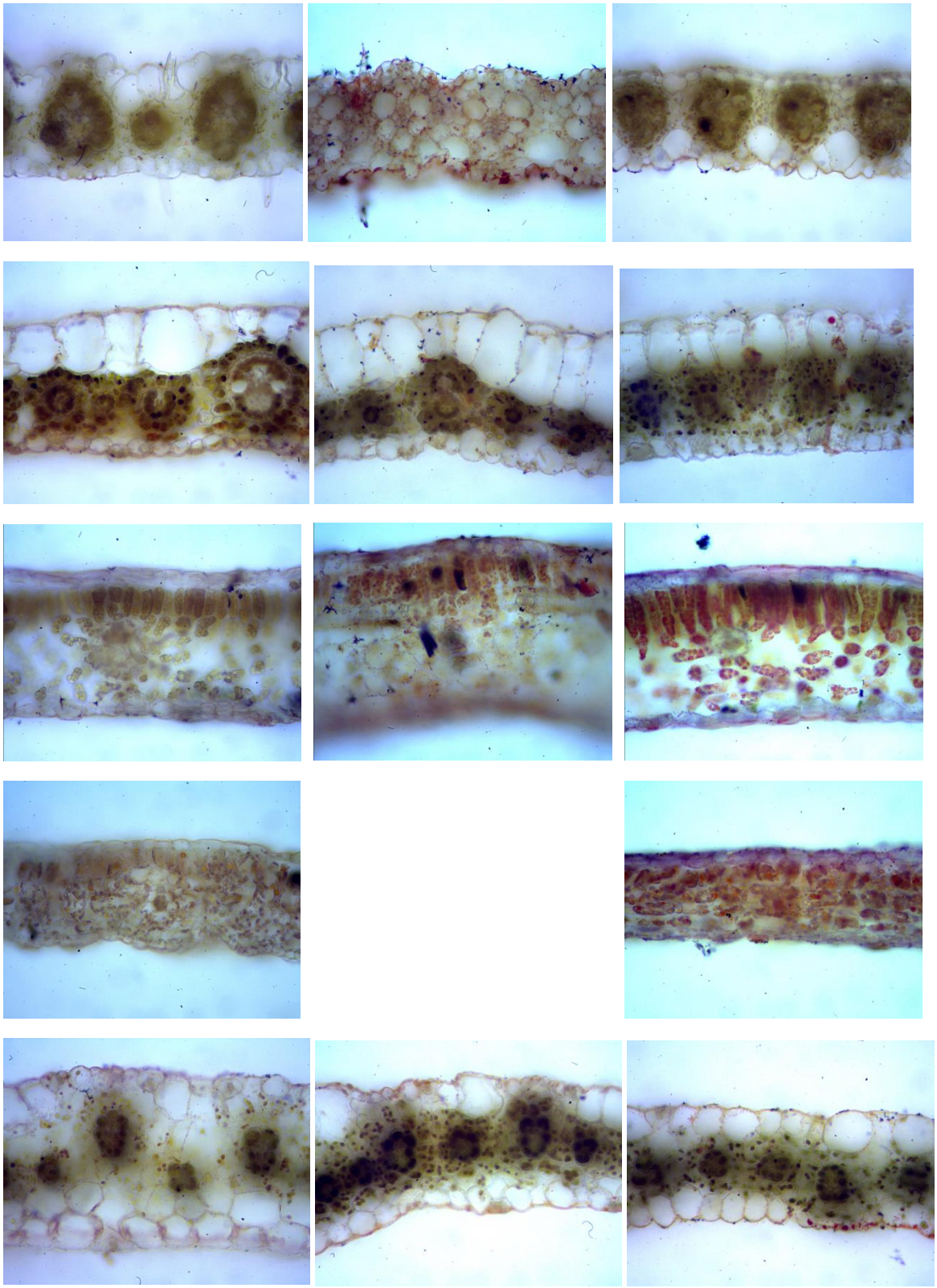

Figure 16. Leaf anatomy of five species (from the top to bottom: *B. mutica*, *C. kyllingia* , *I. aquatica*, *M. micrantha* and *P. conjugatum*) treated with 0 (left), 0.5 mM of Hg (middle) and 0.5 mM of Pb (right). For M. micrantha treated by 0.5 mM Hg, all the plant was dead before collecting samples for anatomy analysis.

5. CONCLUSION

Heavy metal treatments using Hg(NO3)2 and Pb(NO3)2 effectively induced growth decrease of five species that was used in this experiment (*Branchiaria mutica, Cyperus kyllingia, Ipomea aquatica, Mikania micrantha,* and *Paspalum conjugatum*), but the decrease was far higher in Hg than in Pb treatment. Hg and Pb treatment caused dramatic increase in leaf MDA content, which was associated with the decrease of chlorophyll content significantly. There was a close negative correlation between the increased of MDA and the decrease of chlorophyll content in all species. Only Hg treatment that induced higher proline content in the leaves of threated plants. Photosynthesis analysis showed that only Hg treatment that 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 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 *P. conjugatum* were tolerant to Hg treatment up to 0.5 mM.

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ANNEX