BIODIVERSITAS
Volume 20, Number 12, December 2019
Pages: 3690-3697

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d201231

Lead (Pb) toxicity effect on physio-anatomy of bead-tree, jatropha, castor bean and Philippine-tung grown in water culture

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Manuscript received: 7 November 2019. Revision accepted: 23 November 2019.

Abstract. *Hamim, Hanifatunisa, Hadisunarso, Setyaningsih L, Saprudin D. 2019. Lead (Pb) toxicity effect on physio-anatomy of bead-tree, jatropha, castor bean and Philippine-tung grown in water culture. Biodiversitas 20: 3690-3697.* Heavy metal contamination in both land and water has been intensively studied because of their broad impact for the environment. Bead-tree (*Melia azedarach*), Jatropha (*Jatropha curcas*), castor bean (*Ricinus communis*) and Philippine tung (*Reutealis trisperma*) are kinds of non-edible oil-producing species, that are able to grow well on degraded lands and may have potential use for phytoremediation of heavy metals contaminated areas. This study aimed to analyze the response of bead-tree (*Melia azedarach*), jatropha (*Jatropha curcas*), castor bean (*Ricinus communis*) and Philippine-tung (*Reutealis trisperma*) to lead (Pb) contaminant in water culture experiment based on morphological, physiological and anatomical parameters. Two months old plants were transferred to the media containing Hoagland solution. After three weeks of planting, $Pb(NO_3)_2$ treatments with five concentrations, i.e. control (0 mM), 0.5 mM, 1 mM, 2 mM, and 3 mM were added together with Hoagland replacement and the plants were treated for three weeks. The growth, anatomical and physiological parameters were observed during the treatment until three weeks. The results showed that Pb treatment, especially with higher concentration, dramatically decreased plant growth, such as plant height, number of leaves, as well as shoot and root dry weight of all the species. Lead treatment triggered the emergence of free radicals and oxidative stress as indicated by a significant increase of malondialdehyde (MDA) content, while it decreased chlorophyll content of all the species. The higher concentration of $Pb(NO_3)_2$ caused the thickness of upper epidermis, lower epidermis, and spongy mesophyll tissue to decrease significantly which contributed to the decrease of leaves thickness. Among the species, Philippine-tung was the most tolerant of Pb toxicity. This species is potential to be used in phytoremediation program of lead-contaminated land such as gold mine area as well as heavy industrial areas.

Keywords: Lead, Pb, metal toxicity, MDA, non-edible oil-producing plant, leaf anatomy

INTRODUCTION

Industrial and agricultural developments for urban as well as rural communities have been progressing which no doubt can cause environmental pollution. Among the pollutants, heavy metal contamination in both land and water has been intensively studied because of their broad impact on the environment. Heavy metals especially from non-essential elements such as Pb, Hg, and Cd (Ashraf 2006), may even become more dangerous due to the process of bioaccumulation along the food chains (David et al. 2012). Lead (Pb) is an example of heavy metal pollutant produced from industrial wastes such as gold mine tailings (Hilmi et al. 2018) which cause a decrease in soil quality and adversely affects plants, animals, humans, and ecosystems. The toxic effects of Pb on plants result in a decrease of plant ability to absorb mineral nutrition, lower water balance, inhibition of root growth, blackening of root system and leaves chlorosis (Nas and Ali 2018). Lead (Pb) in the environment is not biodegradable and due to continuous use, its accumulation in the environment is constantly rising with increasing hazards (Nas and Ali 2018). Therefore, fundamental efforts are needed to reduce such environmental pollution that occurs.

Environmental pollution due to heavy metals such as lead (Pb) contaminant can be minimized by plants that have hypertensive properties, meaning that they are able to accumulate metals with high concentrations in their roots and shoot tissues, so that these hyperaccumulator plant can be used for phytoremediation purposes. Phytoremediation is a waste cleaning technology considered as innovative, economical, and relatively safe to reduce pollutants, including heavy metals, from the environment (Salt et al. 1995). The basic principle in phytoremediation is using green plants, particularly that have ability to absorb higher amount of pollutant materials to reduce contaminants from soil or water. The advantage of phytoremediation techniques is its ability to produce secondary discharge materials that are less toxic, and more environmentally friendly (Kumar et al. 2013).

Some under-utilized biodiesel-producing plants such as jatropha (*Jatropha curcas*), castor bean (*Ricinus communis*), bead-tree (*Melia azedarach*) and Philippine-tung (*Reutealis trisperma*) may have potential function to be used in phytoremediation process, since these plants are able to grow well on dry and marginal lands as well as on gold mine tailings (Hamim et al. 2017; Hilmi et al. 2018), and even able to absorb heavy metal contaminant (Romeiro et al. 2006; Khamis et al. 2014). These plants can produce non-edible oil feedstock for biodiesel and can be developed in degraded or contaminated land, while at the same time they can also involve in phytoremediation process. In addition, another advantage of these plants is that they produce biodiesel from non-edible oil, so that the absorbed metals may not enter to the natural food chain that endangers other living things.

Previous experiments found that jatropha and bead-tree had ability to grow well in marginal land and can be used for phytoremediation of lead and cadmium polluted soils (Mangkoedihardjo and Surahmaida 2008; Romeiro et al. 2006), while bead-tree was also able to be utilized in phytoremediation of soils contaminated with cadmium up to 40 ppm and lead up to 800 ppm (Khamis et al. 2014). Philippine-tung had also wide adaptability and can grow well in a variety of agroecosystem conditions (Pranowo et al. 2015) including post-mining land that produces heavy metal-containing waste (Hilmi et al. 2018). However, the comparison of these species based on morphophysiological and anatomical properties in response to Pb treatment has not been elucidated. This data is important to determine the most appropriate species for phytoremediation program in Pb contaminated areas such as gold mine tailing.

The objective of this experiment was to analyze the response of bead-tree (*Melia azedarach*), jatropha (*Jatropha curcas*), castor bean (*Ricinus communis*) and Philippine-tung (*Reutealis trisperma*) to lead (Pb) contaminant in water culture experiment based on morphological, physiological and anatomical parameters.

MATERIALS AND METHODS

Plant preparation

The experiment was carried out from April to September 2018 in the Greenhouse, Laboratory of Plant Physiology and Genetics, as well as in the Laboratory of Plant Resources and Ecology, Department of Biology Faculty of Mathematics and Natural Sciences, Bogor Agricultural University (IPB University), Bogor, Indonesia. The seedlings of four non-edible oil producing-species, i.e. bead-tree (*Melia azedarach*), jatropha (*Jatropha curcas*), castor bean (*Ricinus communis*) and Philippine-tung (*Reutealis trisperma*) were prepared by growing the seeds in the small polybag for 2 months before the plants were treated with Pb in water culture. During the preparation, the plants were supplied with enough water and fertilized with commercial fertilizer N: P: K (12: 12: 12).

Before the treatment, water culture installation was prepared using 6 liters plastic containers with a dimension of 20x25x15 cm^3 equipped with stereo-foam to cover the top of the container and to support the plants. For every container, an aerator was installed to provide enough oxygen for experimental plants. The water culture media was also prepared before treatment using Hoagland solution which provided complete macro and micronutrient including Fe-EDTA (Taiz and Zeiger 2010). Two months old plants were then transferred to the container contained full strength of Hoagland solution, where for every container contained four plants of the same species. Before treatment, all the plants were grown for three weeks in water culture for acclimatization.

Experimental design and Pb treatments

The experiment was carried out using Completely Randomized Design with 2 factors. The first factor was four species of non-edible oil producing-species, i.e. bead-tree (*Melia azedarach*), jatropha (*Jatropha curcas*), castor bean (*Ricinus communis*) and Philippine-tung (*Reutealis trisperma*). The second factor was Pb treatment comprised five different Pb concentrations, i.e. 0 Mm (control), 0.5 mM, 1 mM, 2 mM and 3 mM. After three weeks of acclimatization, the plants were ready for the treatment with Pb $(NO_3)_2$. The application of Pb treatment was provided together with replacement of Hoagland solution. After Pb application, the plants were grown for three weeks for observation and analysis.

Morphological analysis

After the treatment, morphological parameters including plant height, the increase of leaves number, and leaf area were observed. Plant height and leaf area were measured twice a week after the treatment. After three weeks, leaf area and leaf morphology were analyzed using scanner and ImageJ application.

Lipid peroxidation analysis

Lipid peroxidation was analyzed by measuring the level of malondialdehyde (MDA) as the end product of lipid peroxidation using the method of Hodges et al. (1999). The leaves of the plants were weighed for 0.5 g each using an analytical balance, then each leaf sample was pulverized using a pestle mortal to be mixed in a 15 ml falcon tube contained 5 ml of Thiobarbituric acid (TBA), and 5 ml of Trichloroacetic acid (TCA). Falcon tubes contained the mixture was put into a water bath at 80ºC, for 5 minutes. Then the mixture was centrifuged for 15 minutes. Leaf MDA content was measured using a spectrophotometer at 450 nm, 532 nm, and 600 nm. The MDA content was calculated using the formula:

[MDA] (µmol/ g FW) = 6.45 x (D532-D600)-0.56 x D450;

FW= Fresh weight

Chlorophyll analysis

Chlorophyll analysis was carried out based on the method of Quinet et al. (2012) using 80% acetone solution as a solvent. The 0.5 g leaf samples from the plants were weighed using an analytical balance. Subsequent samples were ground using mortar with additional 80% of acetone solution and they were centrifuged for 10 minutes. Chlorophyll content of leaf extract was measured using a spectrophotometer at long wave of 470 nm, 646 nm, 663 nm. Chlorophyll analysis was carried out 17 days after treatment (DAT). Formula for calculation of chlorophyll content was as follow:

[Total Chlorophyll] (μg/ g FW) = 7.15 (A663) + 18.71 (A646); FW= Fresh weight.

Leaf anatomy analysis

Leaf anatomical analysis was carried out to observe the thickness of the leaf including upper epidermis, palisade tissue, spongy tissue, and lower epidermis. The leaf was sliced transversely and was observed using light microscope (Olympus CX23) assisted with Optilab Viewer 2.2. The measurement of the tissues was carried out using ImageJ application.

Histochemical analysis

Histochemical analysis was carried out based on method used by Seregin and Kozhevnikova (2011) with modification. Fresh leaf was sliced transversely using hand microtome, and then the sample was dyed with dithizone reagent. The existence of Pb was indicated by the appearance of red/orange color when the samples were observed using light microscope (Olympus CX23). To support the analysis, the observation was assisted by Optilab Viewer 2.2.

Data analysis

The data were analyzed using analysis of variance (One-Way ANOVA) followed by the Duncan Multiple Range Test (DMRT) using SPSS 16.0 program. To determine the degree of tolerance level, the Principal Component Analysis (PCA) was applied using Metabo-Analyst 4.0 (Xia and Wishart. 2016). Data standardization was carried out using Microsoft Excel 2013 before the data was calculated using PCA analysis.

RESULTS AND DISCUSSION

Plant height

Plant height is a general morphological parameter strongly influenced by environmental changes including heavy metal stress. The Pb treatment for 3 weeks significantly decreased plant height of almost all the species except Philippine tung (Figure 1). The biggest decrease in plant height occurred in bead-tree which decreased up to 31.03% due to Pb treatment, while the lowest decrease occurred in Philippine tung which was only 9.39% compared to control plants.

Increase of leaf number (ILN)

The leaf number was influenced by Pb treatment as well as different species. Statistical analysis showed significantly different responses of ILN due to Pb treatment. ILN value in Philippine-tung did not decrease in response to Pb concentration, while in jatropha and castor bean ILN decreased significantly due to higher Pb treatment (Table 1). The ILN of bead tree tended to decrease due to Pb treatment but statistically not significant. In average ILN decreased constantly from the concentrations of 0.5 mM Pb to 3 mM (Table 1).

Leaf area (LA)

The treatment with Pb for three weeks showed no significant effect on leaf area (LA) despite a slight decrease (Table 2). The decrease in LA in bead-tree plants was 39.30%, followed by Philippine-tung (28.47%), jatropha (21.51%), and the least was castor bean plants which were only 11.37%. The decrease of leaf area was associated with the increase of Pb concentration from 0.5 mM to 3 mM (Table 2).

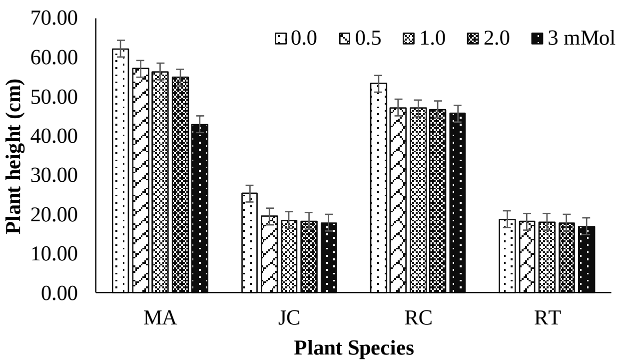

Figure 1. Plant height of bead-tree (MA), jatropha (JC), castor bean (RC) and Philippine-tung (RT) treated with different concentrations of lead (0 Mm, 0.5 mM, 1 mM, 2 mM, and 3 mM). The bar represents standard of error based on Duncan analysis at 0.05% of α.

Table 1. Increase of leaf number of bead-tree (MA), jatropha (JC), castor bean (RC) and Philippine-tung (RT) treated with different concentrations of lead (0 Mm, 0.5 mM, 1 mM, 2 mM, and 3 mM).

Species	Pb concentration (mM)				
	0	0.5	1	2	3
	Increase leaf number (n)				
Bead-tree	0.25c	0.17c	0.17c	0.17c	0.17c
Jatropha	2.17a	1.67a	1.08ab	0.67b	0.25c
Castor bean	1.83a	0.58b	0.92b	0.33c	0.33c
Philippine tung	0.36bc	0.40bc	0.40bc	0.40bc	0.40bc
Averages	1.15a	0.70b	0.64b	0.39bc	0.29c

Note: The number followed by similar alphabets is not significantly different at 5% of DMRT test.

Table 2. Leaves area of bead-tree (MA), jatropha (JC), castor bean (RC) and Philippine-tung (RT) treated with different concentrations of lead (0 Mm, 0.5 mM, 1 mM, 2 mM, and 3 mM).

Species	Pb Concentration (mM)				
	0	0.5	1	2	3
			Leaves area (cm)		
Bead tree	50.13 ab	48.53 ab	37.73 ab	35.17 ab	30.43 b
Jatropha	79.85a	71.34a	66.86a	62.67a	62.67a
Castor bean	51.00ab	48.44ab	47.13ab	46.87ab	45.52ab
Philippine tung	65.90a	57.75a	57.16a	47.98ab	47.14ab
Averages	61.72 a	56.51 a	52.21 ab	48.17ab	46.49ab

Note: The number followed by similar alphabets is not significantly different at 5% of DMRT test

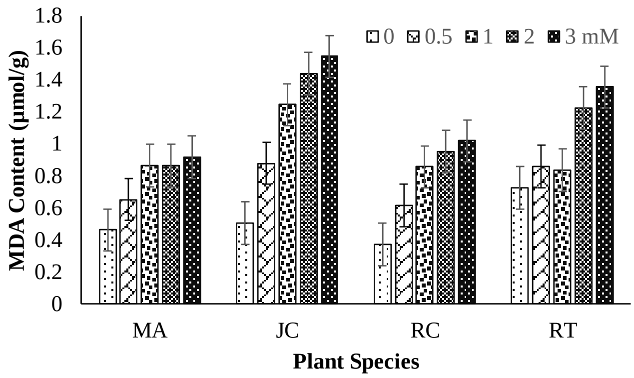

Figure 2. MDA content of bead-tree (MA), jatropha (JC), castor bean (RC) and Philippine-tung (RT) in response to different concentrations of lead (0 Mm, 0.5 mM, 1 mM, 2 mM, and 3 mM). The bar represents standard of error based on Duncan analysis at 0.05% of α.

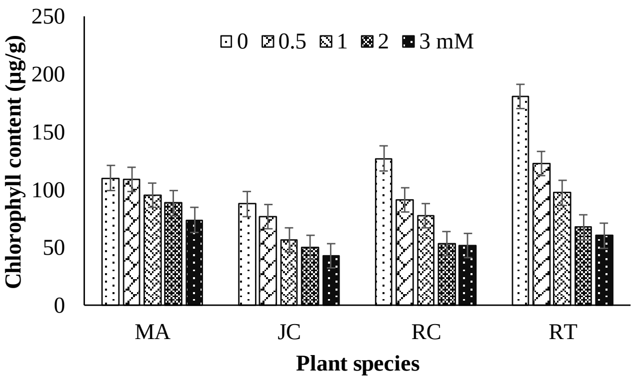

Figure 3. Chlorophyll content of bead-tree (MA), jatropha (JC), castor bean (RC) and Philippine-tung (RT) in response to different concentrations of lead (0 Mm, 0.5 mM, 1 mM, 2 mM, and 3 mM). The bar represents standard of error based on Duncan analysis at 0.05% of α.

Lipid peroxidation analysis

Pb stress strongly influenced the concentration of malondialdehyde (MDA) in the plants. The increased concentration of Pb treatment caused significant increase in leaf MDA content of the four species. Statistically, there was a significant interaction between plant species and Pb treatments. The MDA content increased dramatically in all the species due to Pb treatments, where MDA content increased up to 210% in jatropha, 175.67% in castor bean, 97.82% in bead-tree, and 84.93% in Philippine-tung (Figure 2). The increase of MDA was strongly associated with Pb concentration applied to the media with the coefficient correlation (R^2) values of more than 0.94 (data not shown). The higher MDA content is indicative that the plant was under the stress.

Chlorophyll analysis

The increase in Pb concentration caused a significant decrease in leaf chlorophyll content of all the species (Figure 3). Among the four species, Philippine-tung had the highest total chlorophyll content at control level (without Pb treatment), while the lowest was occurred in jatropha plants (Figure 3). There was a significant interaction between the species and Pb concentration contributed to the decrease of chlorophyll content. The steepest decrease of chlorophyll due to Pb treatment was found in Philippine-tung which decreased up to 66.61% due to 3 mM of Pb treatment, while the modest (32.89%) was found in bead-tree (Figure 3). Chlorophyll content under the maximum Pb treatment (3 mM) was almost similar among the species except for bead-tree that still had higher chlorophyll content under the maximum Pb treatment.

Leaf anatomy and histochemical analysis

The treatment of Pb not only affected morphological and physiological characters of the plants but also affected anatomical structure. Application of Pb for three weeks significantly affected leaf anatomy. Higher concentration of Pb resulted in a decrease of leaf thickness, upper epidermal tissue, lower epidermal tissues, and spongy tissues. Conversely, palisade tissues of three species (i.e. bead-tree, castor bean and Philippine-tung) increased due to Pb application at 3 mM (Table 4). In contrast to the other three plants, the jatropha leaves decreased in thickness of palisade tissue (Table 4). The greatest decrease in leaf thickness occurred in castor bean that was 38.31%, while the lowest thickness decrease was found in Philippine-tung which was only 2.36%. The largest decrease in upper epidermal tissue occurred in castor bean (50.69%), and the lowest occurred in bead-tree (13.43%). The biggest decrease of the lower epidermis tissue occurred in jatropha (59.78%), while the lowest occurred in Philippine-tung (29.88%). The most decrease of spongy tissue occurred in jatropha (34.20%), and the lowest was Philippine-tung (10.37%).

Table 3. Leaves anatomical analysis of bead-tree, jatropha, castor bean and Philippine-tung including the width of leaves, sponge tissues, palisade tissues, upper epidermis, and lower epidermis

The width of (μm)	Treatments (mM)	Species			
		Bead-tree	Jatropha	Castor bean	Philippine-tung
Leaf	0	506.0^{a}	612.4^{a}	739.2^{a}	473.2^{a}
	3	412.4^{b}	586.4^{b}	456.0^{b}	462.0^{b}
Sponge tissues	0	188.8^{a}	321.6^{a}	284.8^{a}	186.4^{a}
	3	150.0^{b}	211.6^{b}	200.0^{b}	166.4^{b}
Palisade tissues	0	124.8^{a}	141.6^{a}	214.8^{a}	192.4^{a}
	3	179.6^{a}	215.2^{a}	145.2^{a}	200.8^{a}
Upper epidermis	0	53.6^{a}	118.8^{a}	115.2^{a}	81.2^{a}
	3	46.4^{a}	60.4^{b}	56.8^{b}	56.0^{a}
Lower epidermis	0	53.2^{a}	83.2^{a}	110.4^{a}	69.6^{a}
	3	36.4^{a}	50.0^{b}	44.4^{b}	48.8^{b}

Note: The number followed by a similar alphabet within the same column is not significantly different based on 5% of DMRT analysis

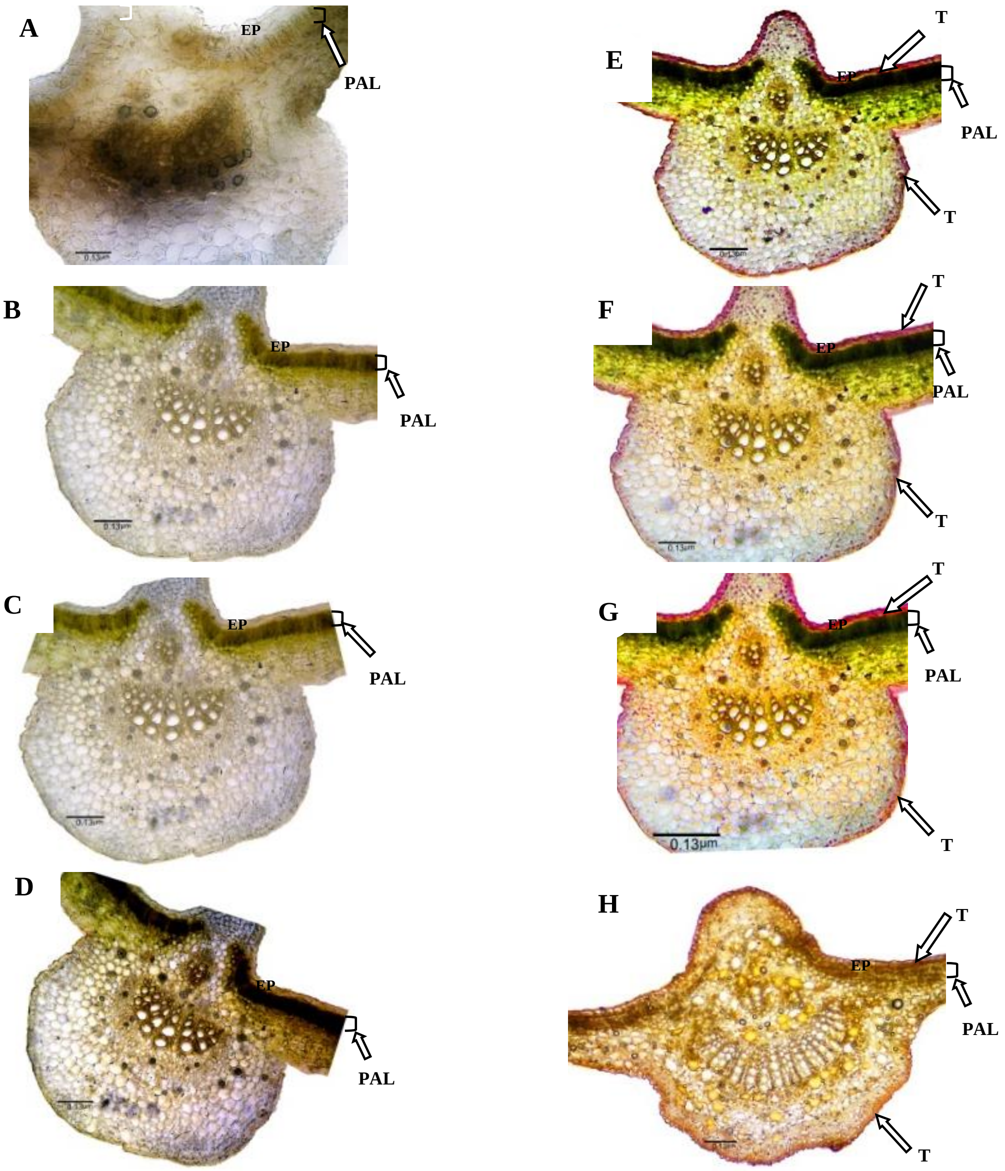

Figure 4. Histochemical analysis of lead heavy metal accumulation (Pb) in leaf tissues of bead-tree (A, E), jatropha (B, F), castor bean (c, g) and Philippine-tung (D, H). Note: A, B, C, D are the control plants (without Pb); E, F, G, H are the plants treated with 3 mM of Pb; EP: epidermis; PAL: palisade, T: lead (Pb)

Histochemical analysis was carried out using fresh incisions from leaves of bead-tree, jatropha, castor bean, and Philippine-tung by comparing control plants and the plants treated with 3 mM of Pb. The results showed that the accumulation of Pb in all species treated with Pb concentrations of 3 mM was positive, indicated by the presence of red/orange color in plant tissue (Figure 4). Most of Pb accumulation was found in epidermal tissues (Figure 4).

Principle component analysis (PCA)

To determine the most tolerant of Pb treatment among the species, Principle Component Analysis (PCA) was applied using parameters of plant height, leaf growth, leaf area, malondialdehyde, chlorophyll, and leaf thickness. Plant height rate and leaf growth were the largest components in component 1, whereas in component 2 malondialdehyde and leaf area were the largest components. Based on the analysis of the main components of the data, Philippine-tung is the most tolerant while the other three were grouped in a similar group (Figure 5).

Discussion

Lead (Pb) is a widely distributed heavy metal with negative biological implications. In this experiment, it was found that the increase of lead (Pb) concentration in the media significantly ($P<0.5$) decreased the growth of all four species based on plant height and the increase of leaves number, while leaves area despite decreasing but were not significantly different (Figure 1, Tables 1 and 2). There was a variation among the species, where bead-tree and Philippine-tung were not influenced by Pb treatment based on leave number, while jatropha and castor bean was strongly influenced. It has been understood that Pb accumulation in the plant body caused many negative impacts including inhibition of growth rate, chlorosis, necrosis, decreased water potential, changes in membrane function, inhibition of photosynthesis, respiration, altered metabolism, and the activity of several key enzymes (Ashfaque et al. 2016). Even though each species may have different responses depending on its tolerance capacity to heavy metal toxicity. Irma (2016), for example, found that Pb significantly decreased the growth of Amaranthus, while in maize Pb application did not affect plant growth (Figlioli et al. 2019). According to Liang et al. (2015), the symptoms of Pb toxicity can be seen from stunted growth and decreased leaf number. Excessive Pb absorption in plants caused toxic effect which disrupted plant metabolism, inhibited plant growth, and even caused plant death (Sharma and Dubey 2005).

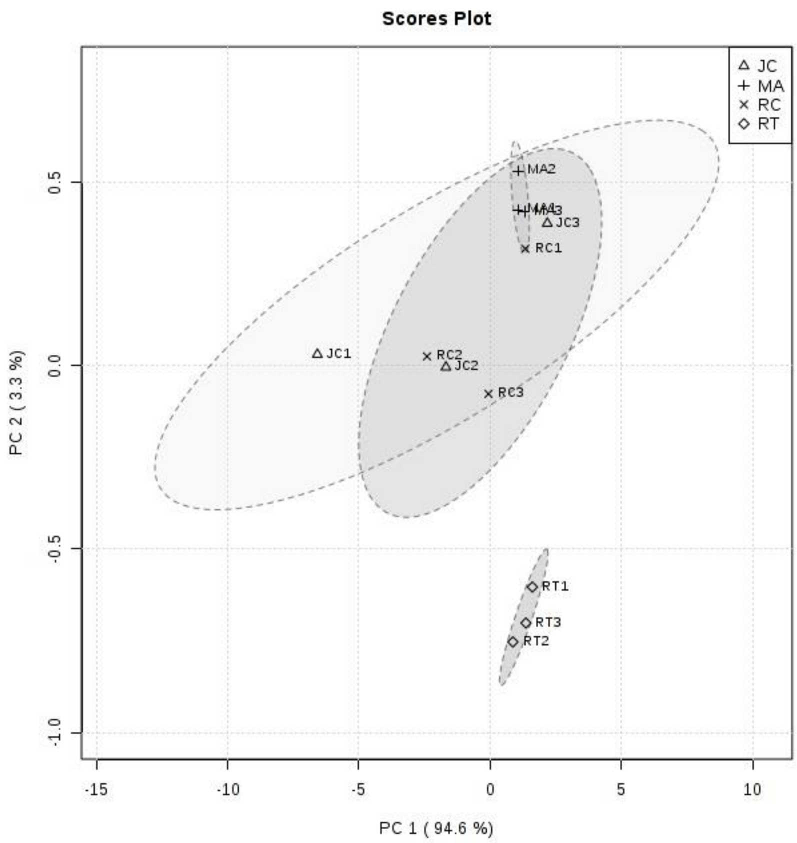

Figure 5. Two-dimensional score plot of the four species based on Principle Component Analisis (PCA) of plant height, increase leaf growth, leaf area, chlorophyll content, leaf width, and MAD content. Note: MA: *Melia azedarach*; JC: *Jatropha curcas;* RC: *Ricinus communis*; RT: *Reutealis trisperma.*

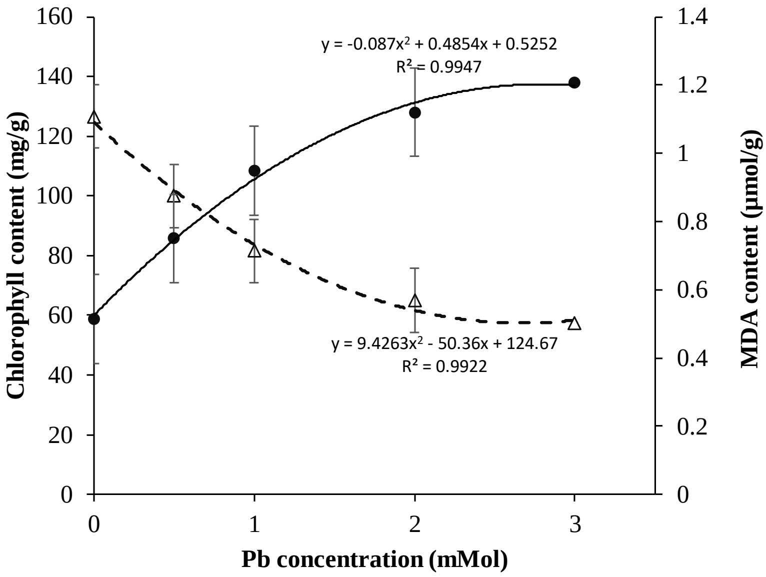

Figure 6. The regression of averages of MDA (●) and chlorophyll (Δ) content of all the species in response to different concentrations of lead (0 Mm, 0.5 mM, 1 mM, 2 mM, and 3 mM). The bar represents standard of error based on Duncan analysis at 0.05% of α.s.

Interestingly, in this study, Pb stress had no significant effect on plant height, increased leave number as well as leaves area of Philippine-tung (*R. trisperma*), suggesting that this species is resistance to Pb. The adaptability of this species to Pb has also been confirmed in other experiments using soil media contained Pb, such as Hilmi et al. (2018) who observed various accessions of *R. trisperma* grown on gold mine tailings contained Pb, and Andriya et al. (2019) who compared this species with other non-edible oil-producing plants under gold mine tailings.

The effect of Pb on plant morphology was also strongly reflected in physiological characters. This can be seen from the increase in malondialdehyde (MDA) and the decrease in chlorophyll content (Figures 2 and 3). The increase in MDA content is associated to development of hydroxyl free radicals such as Reactive Oxygen Species (ROS) due to heavy metal such as Cu, Zn, Ni, Cr, Pb and Cd (Juknys et al. 2012). The reaction of ROS with the fatty acid composition of the cell membrane is known as lipid peroxidation which caused the breakdown of fatty acid chains into various toxic compounds and consequently caused the damage of the cell membrane. According to Reddy et al. (2005), Pb induced dramatic increase of MDA content in many species including horsegram (*Macrotyloma uniflorum*) and bengalgram (*Cicer arietinum*) plants. Our study showed that jatropha and castor bean had the most MDA upsurge, while Philippine-tung and bead-tree had the least (Figure 2). Our findings also highlighted the growth inhibition of Pb treatment to these species (Figure 1 and Table 2) which may be associated with the occurrent of oxidative stress due to Pb treatments.

In contrast, Pb treatment caused dramatic decrease in chlorophyll content of all the species (Figure 3). Philippine-tung underwent the largest chlorophyll reduction because the initial chlorophyll content was very high compared to that of other species, while under the highest Pb treatment, chlorophyll content of all the species was almost similar except bead-tree that had the highest chlorophyll content. The decrease of chlorophyll content as the response of heavy metal stress has been reported by many authors for several species such as *Phaseolus vulgaris* (Hamid et al. 2010), cotton (Bharwana et al. 2014), maize (Figlioli et al. 2016) and spring barley (Juknys et al. 2012). The decrease in chlorophyll content due to Pb toxicity also caused photosynthetic reduction (Hamid et al. 2010), which consequently produced stunted plant growth (Bharwana et al. 2014). There is a strong negative association between the increase of MDA and the decrease of chlorophyll content. The decrease of chlorophyll was mirroring the increase of MDA content (Figure 6). These two physiological aspects are closely related because the consequence of ROS development due to heavy metal resulted in chloroplast damage of membrane lipids and proteins including enzymes that actively involved in chlorophyll development. In addition, the damage of chlorophyll may be also happened directly because of oxidative stress in the chloroplast which increased chlorophyll degrading enzymes, chlorophyllase (Rao and Rao, 1981). Decreased chlorophyll content due to oxidative stress is also common in several abiotic stress conditions including drought (Wang et al. 2016), salt stress (Sevengor et al. 2011), as well as heavy metal stress (Bharwana et al. 2014; Juknys et al. 2012).

Histochemical analysis showed that Pb was absorbed and translocated into the leaves, indicated by red color in epidermal tissues (Figure 4). The existence of Pb in the leaves not only influenced morphophysiological properties but may also affect anatomical changes. In general, heavy metals can cause a decrease in leaf thickness (Tang et al. 2013). This experiment also showed that Pb stress reduced the thickness of the upper epidermal tissue, spongy tissue, lower epidermal tissue and leaf thickness (Table 4). All the species had similar patterns to reduce tissues and leaf thickness as response to Pb treatment, except for palisade and upper epidermal tissues. This may be associated with the important function of those tissues for the plants where photosynthetic pigments are mostly present in palisade tissues (Figure 4). In addition, the stomata where transpiration normally happens also exist in the lower part of the leaves (in lower epidermis). Consequently, the accumulation of Pb may happen more in the lower part of the leaves which including sponge and lower epidermal tissues. Among the four species, there was only Philippine-tung that had the lowest changes in leave thickness which may be associated with the tolerance capacity of this species to Pb treatment.

Finally, to distinguish the most tolerant among the four species, comprehensive analysis was conducted. Based on Principle Component Analysis (PCA) it showed that the four species were separated into 2 groups where Philippine-tung was separated from the other three (Figure 5). Among the four species, Philippine-tung is the most tolerant of Pb stress. The parameter supported to this was the small decrease in plant height, leaf area, leaf growth, leaf thickness and the increase MDA levels in plants that were not too large.

ACKNOWLEDGEMENTS

We would like to thank the Ministry of Research and Higher Education that supports this research through Program of *Penelitian Dasar Unggulan Perguruan Tinggi* (PDUPT) 2019. The authors have no potential conflicts of interest.

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