PHYTOREMEDIATION CAPABILITY OF WATER CLOVER (*MARSILEA CRENATA* (L). PRESL.) IN SYNTHETIC PB SOLUTION

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Abstract. The effect of lead (Pb) pollution on water clover resistance was determined by the Pb distribution and the percentage of free amino acids in plant tissues. Water clover plant from Surabaya, Indonesia was collected and plants of equal size were grown hydroponically. They were exposed into 0, 1, 5, 10 mgL^{-1} of Pb concentration for 10, 20, and 30 days. The experiment was a randomized completely block design with 3 replications. The Pb concentration was measured by inductively coupled plasma (ICP) and the free amino acid was analyzed by HPLC (High-Pressure Liquid Chromatography). The Pb concentration in plant organs and its free amino acids content indicated that water clover plant was resistance on Pb accumulation and affected their growth which potential for phytoremediation. The highest concentration of Pb was found in root tissue, followed by stem and leaf tissues. These findings indicated the respond of water clover plants resist on Pb that determined by the increased percentage of free amino acids and phytochelatins such as glutamic acid, cysteine, glycine, and proline.
Keywords: *free amino acid, phytoremediation, Pb, water clover*

Introduction

Contamination of heavy metals is increasingly widespread as a result of different activities such as lead (Pb) pollution. Pb is a heavy metal that causes poisoning in humans (Jaishankar et al., 2014). High Pb concentration can be found in industrial waste, in household detergents, other laundry products, and in cigarettes (Nakata et al., 2017). Pb is known as teratogenic and toxicity (Duruibe et al., 2007), such as inhibits the haemoglobin synthesis, causes kidney malfunction, affects reproductive systems and cardiovascular system, causes acute and chronic damage to the central nervous system and peripheral nervous system (Ghosh and Singh, 2005).

In plants, Pb causes a decrease of chlorophyll synthesis, inhibits photosynthesis, inhibits mineral collection, water imbalance and changes in the structure and permeability of the cell membrane, as well as other effects (Seregin and Ivanove, 1998).

In addition, Pb triggers the establishment of reactive oxygen species (ROS) and induces oxidative stress that causes the protein trafficking in plants (Yun et al., 2001; John et al., 2008; Stoyanova and Doncheva, 2002).

Nitrogen metabolism is central to plants response against heavy metals. It due to plants synthesize with a variety of metabolites when exposed to heavy metals and accumulate to the concentrations in the millimolar range, especially specific amino acids (Sharma and Dietz, 2006). Glycine and glutamic acid are involved in glutathione synthesis and phytochelatins, which plays a role in the binding of heavy metals. Arginine acts as a molecular marker and antioxidants in the polyamine synthesis (Sharma and Dietz, 2006; Khawas, 2011). Proteinogenic prolific amino acid as a radical

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scavenger, electron sink, macromolecules stabilizer, cell wall components (Matysik et al., 2002), osmoregulation and in metal binding (Sharma and Dietz, 2006). These amino acids are considered as important plant mechanisms against heavy metal stress.

Reduction of heavy metals concentration is urgently needed in the environment. Many methods have been developed to clean up the pollutant from water, but it seems that the plant is the cheapest and easiest method. “Phytoremediation” has been used widely, to remediate either contaminated soils or polluted water (Göthberg et al., 2004). The success of phytoremediation depends on growth rate and the plant ability to uptake the metals from the growth medium. Plants must produce sufficient biomass and able to accumulate a high concentration of heavy metals in their tissue.

The water clover is a hydrophyte plant with thin cuticle, constantly open stomata, has air bladder, and thin roots. A previous study of clover plants (*Marsilea crenata* Presl.) at wetlands of Wiyung, Ketintang, and Kendung in Surabaya city, Indonesia showed the plant ability to absorb Pb. The clover plant is commonly used as a vegetable by Surabaya city communities and the surrounding area. It indicates that the plant contains a concentration > 0.05 mg Pb kg^{-1}. This exceeds the quality standards provided by WHO (1996). Surprisingly, the plant growth is not affected by the heavy metal, as the plant was still growing with the average plant height of 13.9–19.4 cm. Wiadnya (2004) also observed Pb absorption by water clover in the limited volume media experiment. Previous research conducted by Nurhayati et al. (2015) which observe the stunted growth of water clover after treated in Pb media; the higher the Pb concentration, the slower the growth. Novi and Abdillah (2017) observed the ability of water clover and hydrilla to decrease the Pb concentration in paper liquid waste after 5, 10, and 15 days. Another plant in the same division, *Salvine minima*, also has the ability to accumulate Pb up to 0.1243 mgm^2 per day (Iha and Bianchini, 2015). These findings indicated the tolerance of water clover to heavy metal and the possibility to be categorized as an “accumulator or hyperaccumulator” plant.

Pb pollution does not seem to be declining in the future because Pb pollution sources are an unavoidable aspect in modern human life (Yang et al., 2000). Therefore, it is necessary to investigate the different of Pb concentration and exposure duration on plant tissue growth, and the percentage of free amino acids in clover plants that grow on Hogland solution. This research aim is to evaluate the capability of water clover as an agent for phytoremediation to heavy metal particularly the lead (Pb) based on its growth and the accumulation of Pb capacity.

Materials and methods

The water clover plant was grown in medium containing Pb solution to investigate the resistance and free amino acid. The medium was added with Pb solution (depend on the experimental treatments), i.e: (1) 0 Pb as the control; (2) 1.0 mgL^{-1} of $Pb(NO_3)_2$ solution; (3) 5.0 mgL^{-1} of $Pb(NO_3)_2$ solution; and (4) 10.0 mgL^{-1} of $Pb(NO_3)_2$ solution. The Pb concentration was based on the previous study (Herawati, 2008; Rachmadiati et al., 2012). These treatments were conducted in a split-plot design with 3 replications.

Water clover was collected from wetlands at Kendung (Surabaya, East Java, Indonesia). Initially, the plants were grown in a plastic chamber containing 20 L of Hoagland’s media in a glass house over 5 days. This was done to reduce environmental contamination and adapt the plants to glasshouse conditions. Subsequently, plants of water clover were selected with 90 g biomass. Each plant was grown for 10 days in a

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plastic chamber containing 20 L distillation water and Hoagland's solution (Göthberg et al., 2004).

These acclimated plants were used for further study. Water clover plants were selected with stems length of 20-25 cm and roots length of 8-10 cm. The plants were cleaned with distilled water and placed in glass aquarium of 40 cm length, 30 cm width, and 35 cm depth which consist of 5 L distilled water and Hoagland's solution. Every aquarium was filled with 100 g of the acclimated water clover. The variety pH of the medium was applied from 5.4 – 6.9 at the beginning of the experiment, and 6.5 – 7.0 at the end of the experiment. The plants were illuminated sunlight 12:12 h at light-dark cycle with photon flux density at 389 candles. All of the plant samples (destructive sampling) from each glass aquarium were harvested at 10, 20, and 30 days. Then, the biomass yield and metal content were measured (*Fig. 1*).

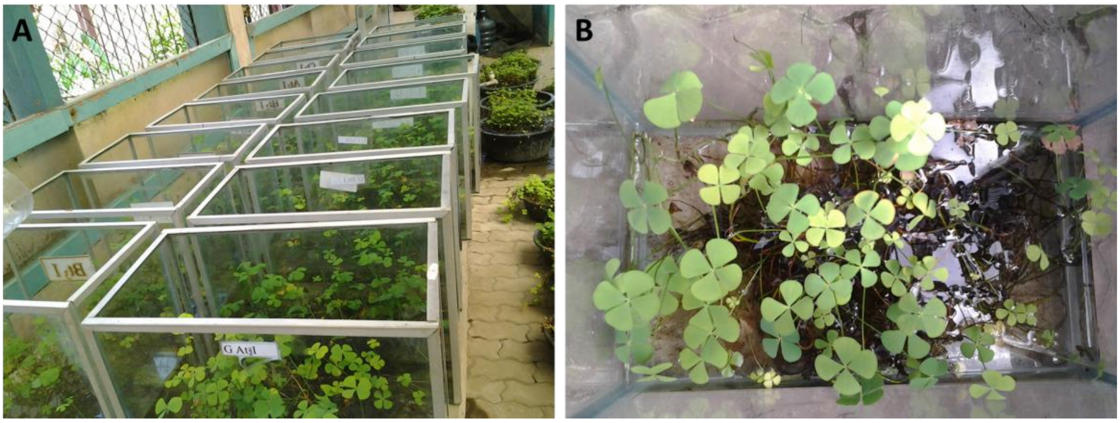

Figure 1. *Water clover (Marsillea crenata) plant in the glass aquarium experiment. A. General view. B. Overhead view*

Harvested plants were divided into three parts: roots, stem, and leaves. Each part of the plant was placed at the dried oven at 80 °C for 48 h and weighed to determine its dry weight. To further analysis, 5 g of each plant was taken and dried plant tissues with a mill. Then, 0.5 g of powdered sample was diluted with 5 ml of HNO_3 and 50 ml of deionized double distilled water. Pb analysis was performed by using the extraction method (Gothberg et al., 2004). Then, the digested samples and 50 ml of media samples were analyzed by inductively coupled plasma (Teledyne Leeman Labs) to determine the Pb concentration. The total accumulation and partitioning of the metals by the plants were calculated. Plant biomass yield was measured on dry weight basis.

Several different steps were performed in order to identify free amino acids. Initially, the reagent kit was prepared, followed by solvent preparation and sample preparation of HPLC (High-Pressure liquid Chromatography) with hydrolysis processes and derivatization (Waters, 2017).

Preparation of reagent kit

Reagent kit was prepared by heating at 55 °C. The vial 24 (AcrQ fluo reagent powder) was heated 2-3 min. Then, 1 ml of AccQ fluorine reagent diluent was inserted into vial 2A followed by heating and mixing until the powder spread evenly.

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Solvent preparation

Solvent solutions were prepared by mixing 19 g Sodium Acetate and 2.27 g TEA into 1 L of water. Then, 40% of phosphoric acid (+ 6 mL or more) was added until pH 5.1 followed by adding 5 mL ACN (ACETONITRILE) and water (Waters, 2017).

Hydrolysis

Hydrolysis was performed with 100 mg of the sample that accurately weighed. The sample was placed into tube and mixed with 5 ml 6N HCL. Drying sample was performed by using Nitrogen or Argon; each tube was closed and placed into an oven at 112 °C for 22 h and the sample was filtered by using 0.45 pm of filter paper. Then, 100 ml of filtered sample was diluted with 5 ml of MiliQ water (Waters, 2017).

Derivatization

The 50 pL of diluted sample was mixed with 350 pL AccQ derivatization buffer and 100 pL AccQ fluor reagent to derivatize. The mixed solution was shaken briefly and placed into the water that has been heated at 55 °C for 10 min. Then, the sample can be injected into the HPLC (Waters, 2017).

Data analysis

Experimental design

The research was a completely randomized design with three replicates for each treatment. The first treatment was the heavy metal concentration containing K1: 0 mgL^{-1}; K2: 1 mgL^{-1}; K3: 5 mgL^{-1}; and K4: 10 mgL^{-1} of Pb. The second treatment was the variety time of observation, 10^{th}, 20^{th}, and 30^{th} day respectively.

Variables

The observed variables were: (1) the plant’s biomass; (2) Pb content in roots, stems, and leaves; (3) the Pb removal percentage; and (4) the produced free amino acid percentage in the root.

Statistical analysis

The observation data was analyzed by ANOVA and followed by Tukey *ad hoc* test with a 95% confidence level using Genstat 15 edition resources statistical program. Then, the free amino acid was analyzed descriptively based on the percentage and total protein.

Results

The growth of water clover at various medium conditions is presented in *Table 1*. There was a positive growth of water clover at 10, 20, and 30 days observation. It seems that water clover was easily adapted to the new environment. Therefore, water clover was not to require adaptation periods. The growth of water clover at various Pb concentrations was significantly different ($p < 0.05$). It indicated that the higher Pb concentration, the slower the growth of the water clover (*Table 1*). This is due to the more Pb is absorbed water clover plants and affects the metabolism. Although the

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growth tended to slow, the ability of plants to absorb Pb remains “high”, which can be seen in *Table 2*. Interestingly, there were no visible poisoning symptoms beside the slowed growth after morphological observation (data not shown).

Table 1. *The growth of water clover at different Pb concentrations*

Pb concentration (mg L^{-1})	Dried biomass at (g/plant)		
	10 days	**20 days**	**30 days**
0	15.470^{dA}	16.555^{dB}	17.384^{dC}
1	14.525^{cA}	15.547^{cB}	16.366^{cC}
5	13.510^{bA}	14.308^{bB}	14.770^{bC}
10	12.810^{aA}	13.475^{aB}	3.895^{aC}

Means followed by the same letters, in the same column are not significantly different (p = 0.05), small letter for concentration and capital letter for detention time

As shown in *Table 1*, it demonstrated that the water clover grows well in lead-polluted solution. However, compared to early observation, the growth of water clover was much slower. The capability of water clover to remove Pb from solution was shown in *Table 2*. There was significantly different from the removal by water clover.

Table 2. *Percentage removal Pb by water clover*

Pb concentration (mg L^{-1})	Percentage removal Pb by plants		
	10 days	**20 days**	**30 days**
1	100 ± 0.04^{aA}	98.00 ± 0.04^{aA}	100 ± 0.04^{aA}
5	100 ± 0.04^{aA}	99.90 ± 0.04^{aA}	99.40 ± 0.04^{aA}
10	100 ± 0.04^{aA}	99.90 ± 0.04^{aA}	99.65 ± 0.04^{aA}

Means followed by the same letters, in the same column are not significantly different (p = 0.05)

The water clover plants were able to absorb Pb concentration in the organs, although their growth was considerably slower as shown in *Table 1*. It is important noted that water clover plants are able to remove Pb from the solution. Then, the highest Pb concentration of water clover was found in root tissues (*Table 3*).

The presence of Pb in root tissues of water clover was relatively constant with the plant of age, except for 10 days after treatment. Pb concentration in roots tissues of water clover was not differences. As shown in *Table 4*, Pb concentration in stem tissues indicated significant differences compared to leave tissues.

Table 3. *Pb concentration in various plant tissues*

Day	1 mg L^{-1} Pb (mg kg^{-1})			5 mg L^{-1} Pb (mg kg^{-1})			10 mg L^{-1} Pb (mg kg^{-1})		
	Roots	**Stems**	**Leaves**	**Roots**	**Stems**	**Leaves**	**Roots**	**Stems**	**Leaves**
10	0.8 ± 0.15^{bA}	0.40 ± 0.03^{bA}	0.18 ± 0.00^{aA}	1.93 ± 0.05^{bA}	2.29 ± 0.05^{bA}	0.58 ± 0.05^{bA}	3.80 ± 0.05^{bA}	2.65 ± 0.05^{bA}	0.82 ± 0.05^{bA}
20	1.2 ± 0.05^{bB}	0.09 ± 0.05^{bB}	0.13 ± 0.01^{aB}	7.88 ± 0.05^{bB}	5.01 ± 0.05^{bB}	0.01 ± 0.05^{bB}	9.74 ± 0.05^{bB}	6.79 ± 0.05^{bB}	0.01 ± 0.05^{bB}
30	1.3 ± 0.05^{bB}	0.20 ± 0.01^{bB}	0.12 ± 0.01^{aB}	9.10 ± 0.05^{bB}	0.58 ± 0.05^{bB}	0.02 ± 0.05^{bB}	13.12 ± 0.05^{bB}	0.72 ± 0.05^{bB}	0.02 ± 0.05^{bB}

Means followed by the same letters for the same time of measurements, in the same column (small letters) and rows (capital letters) are not significantly different (p = 0.05)

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The amino acid content was found to see the plants response against heavy metal stress. A significant difference in the percentage of each amino acid produced by the clover water plants were recognized (*Table 4*).

As presented in *Table 4*, there were 17 types of amino acids. The percentage of amino acids decreased and had low value (0.000) except for cysteine after 20 days of Pb exposed. In addition, lysine and isoleucine also indicated a low value (0.000) on the concentration of 5 mg L^{-1} after 20 days of Pb exposed.

Table 4. *Free amino acids (%) of water clover exposed to lead at 20 days (n = 3)*

Amino acid	**Concentration of Pb (mg L^{-1}) at … days**		
	1	**5**	**10**
	20	**20**	**20**
Aspartic acid	0.086±0.006[a]	0.269±0.030[a]	0.126±0.104[a]
Serine	0.290±0.014[a]	0.286±0.006[b]	0.107±0.042[c]
Glutamic acid	0.137±0.006[a]	0.136±0.001[a]	0.126±0.015[a]
Glycine	0.155±0.001[a]	0.135±0.006[a]	0.100±0.050[a]
Histidine	0.182±0.045[a]	0.133±0.039[a]	0.051±0.023[a]
Agrinine	0.266±0.051[a]	0.199±0.129[a]	0.076±0.035[a]
Threonine	0.318±0.053[a]	0.205±0.121[a]	0.086±0.042[a]
Alanin	0.353±0.033[a]	0.269±0.086[ab]	0.094±0.050[b]
Proline	0.372±0.201[a]	0.116±0.164[a]	0.095±0.057[a]
Cystine	0.230±0.014[a]	0.014±0.020[b]	0.000±0.000[b]
Tyrosine	0.159±0.153[a]	0.050±0.013[a]	0.041±0.021[a]
Valine	0.246±0.105[a]	0.247±0.132[a]	0.090±0.042[a]
Metheonine	0.036±0.036[a]	0.013±0.018[a]	0.012±0.007[a]
Lysine	0.135±0.191[a]	0.440±0.269[a]	0.425±0.057[a]
Isoleucine	0.110±0.156[a]	0.188±0.103[a]	0.072±0.028[a]
Leucine	0.231±0.141[a]	0.281±0.140[a]	0.730±0.823[a]
Phenylalanine	0.339±0.013[a]	0.163±0.066[ab]	0.059±0.028[b]

Means followed by the same letters in the same rows are not significantly different (p = 0.05)

Discussion

The water clover showed stunted growth after exposed with Pb even though there was no morphological alteration. It indicated that water clover plants are able to accumulate with Pb in the long period based on the growth of biomass after observed in 20^{th} and 30^{th} day. These findings suggested the potency of water clover as a biological agent for phytoremediation due to the ability to accumulate heavy metal in

its tissues. Similar research was conducted using the *Salvinea culcullata* water plant by Phetshombat et al. (2006), which can accumulate Pb until 16.36 $mgKg^{-1}$ under 40 mgL^{-1} but showed chlorosis in its leaf after long exposure.

The Pb exposure eventually affects the metabolism of the plant (Gomez et al., 2009). Similar to Cd exposure, Pb was found to slow the growth in several aquatic plants, such as *Lymnochasris flava* species and *Ipomoea aquatica* (Rachmadiarti et al., 2012). Pb interferes the tissue permeability (Fahr, 2013), increases the bound pectin to middle lamella of cell walls which inhibit the cell expansion (Krzeslowska, 2011), and affects the growth hormones, auxin metabolism, and its carrier. Pb also trigger the disorder in

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some plant metabolism such as photosynthetic pathway and peroxidase induction that plays a role in indole acetic acid degradation for growth and cell multiplication (Hoffman et al., 1985; McComb et al., 2012).

The mechanism behind the higher Pb concentration in the roots may include the binding of the positively charged toxic metals ion to negative charges in the cell walls (Matysik et al., 2002). In most plants, a larger proportion of the metal was retained in the roots and thereby prevented from interfering with the sensitive metabolic reaction in the shoot. It due to an internal mechanism to avoid toxic metal concentration in the shoot (Fahr et al., 2013).

The root has the tendency to accumulate a high concentration of Pb compared to in shoot (Gothberg et al., 2004). About 95% roots of Pb accumulated in a number species of brassica family other plants were in the roots (Kumar et al., 1995). However, concentration Pb in leaves are lower than in the stems because the leaves of water clover are very thin and Pb can lose through leaves transpiration. The stem accumulated high concentration of Pb compared to in leaves. It due to the possibility of the stem in the water (growing media). In addition, water clover as ferns (*Pteridophyta*) that has a vascular cylinder on the rod shape of concentric systems amphichiral in the xylem. The starch content and the veins size in the xylem have a larger size than the phloem. In addition, Mitel Lamela connected between the xylem cells.

In contrast, there was no significant difference in Pb content in leaves of clover water. It may occur due to the concentration of treatment. At low concentration of Pb until it reaches 0 in the leaves. It can be caused by water clover has a small, thin, and wide structure of leaves. Therefore, the absorption of the nutrients including Pb trigger small translocate into the leaves. In addition, Pb has been concentrated in the roots and accumulated in the stem. It due to the epidermal tissue on water clover leaves consists of a layer. Thus, the accumulation of Pb is less than the stem. The Cu metal content presented on other types of clover plants (*Marsilea quadrifolia*) is lower than *Echornia crassipes* and *Vallisneria spiralis.* Although the content of Pb on clover leaves is lower than on the stem, but the content of Pb in the roots of the clover is higher than in shoot (stem + leaves).

The content of Pb in the leaves indicated that the dosage of Pb concentration at the bellow limit allowed for foodstuffs (0.5 mg/kg^{-1}) unless the treatment of 10 mgL^{-1} at 10 days of observation (WHO, 1996). It suggested that clover water have been used to remediate Pb synthetic solution at a concentration of up to 10 mgL^{-1} with 20 days and it can be consumed based on the food security terms.

Generally, only a small portion of metal is brought up by the roots and transported to the shoot. It has been reported that the exclusion of metal from networks above the ground is a tolerance strategy against metals (Taylor and Crowder, 1983). The root has a higher tolerance than the shoot. In addition, it has a tendency to decrease translocation by increasing metal concentrations in the roots and included the general characteristics of various type of metals and plants. The binding of toxic metal through the positive ions to negative ions in the cell walls of the root, the formation of *metal phytate*, and khelasi on the phytochelatin followed by an accumulation in vacuoles are the mechanism by reducing transport and improving metal tolerance (Gothberg et al., 2004).

Kamel (2008) reported the adaptability of plants to extract metals from the water and the aptitude of plants to transfer metals from plant root to shoot. The concentration free amino acid in roots of water clover at some concentrations up to 30 days as shown in

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Table 4. The percentage of these amino acids was decreased. It due to the adaptation to heavy metal exposure which relatively high from 5 to10 ppm from amino acids. There are 6 amino acids (arginine, proline, leucine, valine, serine and glycine) and has acts an important role in the regulation of osmotic plant (Mansour, 2000). Arginine is involved in the synthesis of polyamines and acts as a molecular marker and antioxidant (Sharma and Dietz, 2006).

In the present study, the presence of proline showed its role moving between tissue and protect plants against stress. It due to its role as a major compound for carbon and nitrogen source, protect of cytoplasmic enzymes, and maintenance of cellular structures. High sensitivity in the metabolism of proline synthesis and its degradation has advantageous to control the adverse metabolic processes (Kamel, 2008). Proline serves as a radical scavenger, electron sink, stabilizer of macromolecules, cell wall components (Matysik et al., 2002), as well as osmoregulator and metal binding (Sharma and Dietz, 2006). Proline has been found at *Helianthus annuus* which accumulate with Pb, Cd, Cu, and Zn (Kastori et al., 1992). The existence of Ni in *Alyssum lesbiacum* plant was found to increase histidine in the xylem (Kramer et al., 1997). Hyperaccumulation of Ni triggers by histidine that translocates Ni from roots to shoot (Kerkeb and Kramer, 2003).

The percentage of amino acids in plants growing on Pb treatment has been investigated. The continued increase of amino acids occurred on plant exposed with Pb from 10 days up to 20 days. In contrast, reduced amino acid occurred on day 30, followed by the slow growth of plants. The increase of these amino acids in accordance with the Pb concentration which is absorbed by the roots did not a difference between 20 and 30 days (*Table 4*). Syam et al. (2010) reported that the biosynthetic pathway of *Salvinia minima* triggers Pb^{2+} detoxification. It has been indicated for other organisms, as part of the mechanism to cope with this metal in roots and leaves.

The root of water clover plant contains the amino acid, glutamic acid, and cysteine. They are dimeric amino acid formed by the oxidation of two cysteine residues that make a disulfide bond covalently. This organosulfur compound has the formula $(SCH_2CH(NH_2)CO_2H)_2$) and glycine. It is related to the multitude of amino acid protein from contaminated locations through the biosynthesis of phytochelatin and metallothioneins. The synthesis of phytochelatins and metallothioneins are the response of plants to trigger several metals or metalloid. Because of the similarity with metallothioneins, the phytochelatins have been known as class III of metallothioneins. Phytochelatins have a major function as detoxification. Meanwhile, metallothioneins has function in assisting the translocation of some metals. A gene family encodes metallothioneins, while phytochelatins produced enzymatically (Christopher and Peter, 2002). Plants also develop detoxification by inducing the protein synthesis due to the stress of phytochelatins and metallothioneins and high intensity in mango and apricot (Khawas, 2011).

Conclusion

The water clover growth, the concentration of Pb and free amino acid in plant organs indicated that water clover plants were survived and continued their growth. The highest concentration of Pb was found in root tissue followed by stem and leaf tissues. The phytochelatin production by clover plant as a resistance respond. In addition, it was followed by an increased percentage of free amino acids and the presence of

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phytochelatins such as glutamic acid, cysteine, glycine and proline. Based on this study result, it is imperative to investigate further the phytoremediation capability of water clover by increasing the Pb concentration and in the smaller time frame.

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Conflict of interests. The authors declare that there is no conflict of interests.

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