Adaptation pattern of trembesi (Samanea saman Jacq. Merr.) seedling to lead exposure at nutrient culture based on the characteristics of organic acid

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Abstract. Lead (Pb) is known as one of the dangerous heavy metals which may spread in the environment due to industrial, domestic, agricultural and other human activities. The use of plants for cleaning up contamination which is known as phytoremediation requires information on the ability of plants to tolerate heavy metal exposure. The aim of this study was to determine the adaptation pattern of trembesi (Samanea saman Jacq. Merr) seedlings to lead (Pb) exposure based on the characteristics of organic acids content. Trembesi seedlings were grown on nutrient culture with Pb treatment of 0.5, 1.5 and 2.5 mM for 7 days. The percentage of seedling life was counted and the organic acids content was observed using High Performance Liquid Chromatography (HPLC). The results showed that the life percentage of trembesi seedling exposed to lead up to 1.5mM reached 100%, but it decreased to 60% when it was exposed to 2.5 mM of lead. Trembesi seedlings produced all three organic acids investigated as oxalic, malic and citric acid. The increase of lead exposure from 0.5 to 1.5 and 2.5 mM, caused a significantly increase of oxalic acid exudation from 0.346 to 0.545 and 0.668 µg/mL respectively. At the same time, they also induced the accumulation of citric acid from 46.4481 to 63,668 and 83,516 µg/g respectively. This shows that trembesi has the adaptability to lead by a combination of external and internal mechanisms involving organic acids accumulation.

1. Introduction

Lead is a dangerous heavy metal that can affect the human body function disorder, such as the kidneys, liver, central nervous system and others [1, 2]. Its existence in the environment can be caused by various human activities, such as industry, mining, agriculture as well as other domestic activities [2, 3]. It has been reported that the concentration of Pb reached 275 mg Kg⁻¹ on
agricultural land near the mining industry in Yunan province [4]. Pb content in the tailings resulted from a gold mining industry can reach 71 - 98 ppm [5, 6, 7, 8], while the safe threshold for the presence of Pb in the environment is 50 - 300 mg Kg\textsuperscript{-1} [9, 10].

The activity of revegetation on ex-mining land with heavy metal content needs to consider the types of plants that are able to grow on land with low fertility, tolerant to heavy metals exposure, and able to carry out remediation of heavy metals that contaminate the land [7]. Remediation activity using plants which is also known as phytoremediation is the use of plants to clean contaminated areas by removing, degrading, isolating toxic and hazardous materials from the environment [11, 12, 13]. Plants that are able to remediate heavy metals are normally adaptable to heavy metal exposure through some physiological properties. The secretion and accumulation of organic acids as chelator is one of the tolerant mechanism of plants to metal stress [14, 15]. Organic acids such as citrate, oxalate, malate, malonate, aconitate and tartrate are able to produce chelating agent for heavy metals with oxygen donor function in metal ligands [16]. It has been reported earlier that *Paraserianthes falcataria* exposed to lead was able to secrete oxalic, malic and citric acids [17].

Trembesi (*Samanea saman* Jacq. Merr.) is known as one of the legume trees that is often used for revegetating marginal lands and able to adapt widely to various types of soil and acidity as well as able to tolerate drought water at certain periods [18]. The preliminary research results also showed that trembesi seedlings were able to grow on tailings media contained heavy metals. Therefore, trembesi has been suggested as a candidate plant for heavy metal remediator. The investigation of the physiological adaptations of this plants to heavy metal exposure will help to predict the mechanism patterns of the plants in the phytoremediation process. The purpose of the experiment was to investigate the adaptation pattern of trembesi to heavy metal lead in a nutrient culture based on live percentage and organic acid secretion and accumulation.

2. Materials and Methods

2.1. Research materials and location
Trembesi seed, Aquadest, nutrient stock (1.5 mM Ca(NO\textsubscript{3})\textsubscript{2}, 4H\textsubscript{2}O; 1.0 mM NH\textsubscript{4}NO\textsubscript{3}; 1.0 mM KCl; 0.4 mM MgSO\textsubscript{4}; 1.0 mM KH\textsubscript{2}PO\textsubscript{4}; 0.5 mg/L MnSO\textsubscript{4}; 0.02 mg/L CuSO\textsubscript{4}.5H\textsubscript{2}O; 0.05 mg/L ZnSO\textsubscript{4}.7H\textsubscript{2}O; 0.05 mg/L H\textsubscript{2}BO\textsubscript{3}; 0.01 mg/L (NH\textsubscript{4})6 MoO\textsubscript{24}.4H\textsubscript{2}O; 0.068mM Fe-EDTA). NaOH and HCl as the solution for pH adjustment, liquid nitrogen, the standar of oxalic acid, citric acid, and malic acid for HPLC, KH\textsubscript{2}PO\textsubscript{4}, H\textsubscript{3}PO\textsubscript{4} 10% and ethanol 95%. The equipment for organic acid isolation such as Solid Phase Extraction (SPE) waters Sep-Pak C18 Classic Cartridge, KCKT Waters ser E2695 with detektor UV-VIS 2489 series with the condition of XTerraC18150mmx4.6mm. The experiment was carried out in the Chemistry Laboratory and Glasshouse of the University of Nusa Bangsa, Bogor, Indonesia.

2.2. Preparation of water culture
Liquid culture media used in the experiment was based on the Sopandie solution (Sopandie, 1990) then added with metal lead Pb(NO\textsubscript{3})\textsubscript{2}. The composition of the adaptation medium was half strength of the nutrient dose of Sopandie (1990) without Pb. The treatment medium was 1/3 dose of sterile Sopandie nutrient solution which was combined with Pb(NO\textsubscript{3})\textsubscript{2} treatment in 4 different concentrations i.e.: 0 (Pb0), 0.5 (Pb0.5), 1.5 (Pb 1.5) and 2.5 (Pb2.5) mM. The solution pH was always controlled to be held in the range of 4.5 to 5 using NaOH or HCl.

2.3. Water culture planting and treatment
The trembesi seeds were obtained from the collection of the Citarum-Ciliwung Watershed Management Center Bogor, West Java. Trembesi seeds that have been sterilized with 10% of NaClO\textsubscript{3} and alcohol were then germinated for 30 days on previously sterilized zeolite media. Furthermore, the seeds were transferred to the adaptation medium for 2 days and then the plants were exposed to lead treatment for 7 days. After 7 days of stress, the trembesi seedlings were
harvested and cleaned for growth and morphological measurement and further organic acid analysis

2.4. Growth of seedling analysis
After the treatment, the seedlings were observed to analyse the changes in leaf color and leaf morphological conditions. The number of seedlings mortality was also calculated every day during Pb exposure for 7 days.

2.5. Organic acid analysis
Organic acid secretion was measured by analysis of the organic acids in the culture medium, while the accumulation was determined by the analysis of organic acids’ content in the plant tissues. Samples were filtered and cleaned up into a Solid Phase Extraction (SPE), eluted using 0.45 µm millipore filter and then injected into the KCKT Waters E2695 series system with UV-VIS detector 2489 series, XTerraC18150mmx4.6mm. Organic acids were detected by a UV detector at a wavelength of 210 nm. Identification and quantification of organic acids were carried out by comparing standard chromatograms with samples based on retention time and area. Calculation of organic acid levels was carried out using the Empower 2 software standard to calculate the organic acid content in liquid media and plant tissue (Table 1).

| No | Organic acids | Retention time | Area (AU) |
|----|---------------|----------------|-----------|
| 1  | Oxalic acid   | 3.577          | 655263    |
| 2  | Malic acid    | 7.475          | 95114     |
| 3  | Citric acid   | 8.653          | 39842     |

2.6. Data analysis
The percent of seedling life was calculated as follow:

\[
\text{Percent of life} = \frac{X}{Y} \times 100\% \tag{1}
\]

Note: X = Life number of seedling, Y = Number of all of seedling

Organic acid (OAC) secretion was calculated as follow:

\[
\text{OACs (} \mu g \text{ mL}) = \left( \frac{A_{\text{spl}}}{A_{\text{std}}} \right) \times C_{\text{std}} \times \frac{F_{\text{p}}}{\text{sample weight}} \tag{2}
\]

Organic acid accumulation was calculated as follow:

\[
\text{OACac (} \mu g \text{ g}) = \left( \frac{A_{\text{spl}}}{A_{\text{std}}} \right) \times C_{\text{std}} \times \frac{F_{\text{p}}}{\text{sample weight}} \times 1000 \tag{3}
\]

Note: A spl : area of sample; A std : Area of standard; C Std : standard concentration; Fp : factor of dilution

The pattern of organic acid content was calculated using the regression function.

Linear regression: \( Y=aX+b \); or Polynomial regression: \( Y=ax^2+bx+c \) \tag{4}

2.7. Statistical analysis
The experiment was carried out using a completely randomized design with four treatments of lead exposure levels, 0 mM, 0.5 mM, 1.5, mM and 2.5 mM. The effect of lead exposure treatment on seedling morphology as well as organic acid content secretion and exudation was analyzed statistically using Analysis of variance was followed by DMRT at a 95% level, using SPSS 16 software.
3. Results and Discussion

3.1. The live percentage of trembesi seedlings
After 7 days of lead exposure with the concentration of 0 mM until 2.5 mM, all trembesi seedlings were mostly still alive. However, on the third day of exposure to Pb 2.5 mM, some seedlings changed their leaves color to yellow and became dry. The following day, the stem of those plants gradually became dry and resulted in up to 40% mortality on day 7 (Figure 1). This showed that seedlings experienced chlorosis and necrosis as a result of Pb exposure due to the concentration had reached toxic levels [19]. This symptom was also reported in P. falcataria seedlings exposed to 5 mM Pb during 5 days [17], in Anthocephalus cadamba seedlings [7], and R. trisperma treated with gold mining wastewater contained heavy metal [20]. The presence of Pb in leaves to a certain concentration may be able to replace the role of Mg in the chlorophyll molecule, which in turn caused inhibition of chlorophyll formation and resulted in yellow leaves, and of course the inhibition of photosynthetic process [17, 19, 21]. It can even cause death if the toxic effect is occurred for a long time [17, 22].

![Figure 1](image-url) Figure 1. Live percentage of trembesi seedlings until 7 days of Pb exposure in water culture.

3.2. Organic acid content
Three kinds of organic acids including oxalic acid, malic acid and citric acid were found in trembesi seedlings exposed to lead, either in the form of secretion or accumulated substances. In the overall level of lead exposure, oxalic acid secretion was ranged from 0.197 - 0.668 µg/g which were 5-10 times higher than that of malic acid and citric acid. Oxalic acid secretion was produced at all levels of Pb stress treatment 0 - 2.5 mM, while for malic acid and citric acid, they were significantly (p<0.05) secreted when the seedlings were exposed to Pb 1.5 mM, but they decreased again when the exposure reached 2.5 mM Table 2.

Trembesi seedlings accumulated citric acid at each level of lead exposure and the accumulation was significantly increased after 1.5 and 2.5 mM, while malic acid and oxalic acid experienced an increase in the amount of new organic acids when they were exposed to Pb at 2.5 mM. This condition has also been reported to occur in sengon plants [17] that the citric acid produced is more than malic acid and oxalic acid when the plants were exposed to Pb. Citric acid was accumulated in the greatest concentration, up to 20 times greater than the accumulation of malic acid ranging from 44,765 - 83,516 µg/g compared to 2,384 - 2,730 µg/g respectively Table 3.

These three types of organic acids were also reported to be secreted and accumulated by several plants exposed to heavy metals, such as P. falcataria exposed to Pb [19] and Chinese garbage exposed to Zn [23]. However, these three organic acids accumulations were not found in other species, suggesting that each plant may have specific organic acids in response to heavy metal exposure. The use of large amounts of citric or oxalic acid to play a detoxification role of lead without
increasing biomass is thought to be one of the efforts of seedlings to save metabolic energy to produce organic acids [11, 16]. This suggests that trembesi seedlings have a defense mechanism against metal lead by secreting oxalic acid and detoxifying it in the tissue by citric acid accumulation.

Table 2. Organic acid secretion of trembesi seedling exposed to lead treatments for 7 days in water culture.

| Pb (mM) | Oxalate | Malate | Citrate |
|---------|---------|--------|---------|
| 0       | 0.197 ± 0.156 a | 0.000 ± 0.002 a | 0.000 ± 0.002 a |
| 0.5     | 0.346 ± 0.167 a | 0.040 ± 0.03 a | 0.028 ± 0.013 a |
| 1.5     | 0.545 ± 0.199 b | 0.106 ± 0.201 b | 0.081 ± 0.031 b |
| 2.5     | 0.668 ± 0.198 b | 0.062 ± 0.032 ab | 0.047 ± 0.023 ab |

Note: The number followed by different letter in the same column are significantly different in error rate 5% by Duncan test (DMRT).

Table 3. The organic acid accumulation of trembesi seedling exposed to lead treatment for 7 days in water culture.

| Pb (mM) | Oxalate | Malate | Citrate |
|---------|---------|--------|---------|
| 0       | 39.810 ± 6.782 b | 2.730 ± 0.325 a | 44.765 ± 2.98 a |
| 0.5     | 33.812 ± 5.678 b | 2.384 ± 0.321 a | 46.481 ± 3.898 a |
| 1.5     | 23.980 ± 4.032 a | 2.438 ± 0.354 a | 63.668 ± 11.543 b |
| 2.5     | 61.948 ± 8.761 c | 4.154 ± 0.335 b | 83.516 ± 10.674 c |

Note: The number followed by different letter in the same column are significantly different in error rate 5% by Duncan test (DMRT).

3.3. The pattern of organic acid content in response to Pb exposure

Among the three types of organic acids exudate produced by trembesi seedlings, oxalic acid levels continued to increase significantly (p ≤ 0.05) along with the increase of lead exposure (Table 2). The increase in oxalic acid secretion reached 20-70% with each increase in Pb exposure, with a linear upward trend of $y = 0.1612x + 0.0361$ and $R^2 = 0.9927$ (Figure 2). This condition indicates that trembesi seedlings have avoided being metabolized actively from Pb stress by an external adaptation strategy through the secretion of oxalic acid.
Figure 3. The pattern of organic acid content accumulated by trembesi in response to Pb up to 2.5 mM for 7 days.

Figure 2. The pattern of organic acid content secreted by trembesi in response to Pb up to 2.5 mM for 7 days.
The increase of Pb stress up to 2.5 mM in trembesi seedlings increased citric acid accumulation, reached 83.156 mg/L, or increased significantly (p<0.05) nearly 2 times for every 1 mM Pb exposure increase (Table 3), with a trend linear increase, $y = 13,344x + 26,248$; $R^2 = 0.9078$ (Figure 3). Meanwhile, the accumulation of Oxalate and malate did not show a consistent trend, it significantly decreased when exposed to Pb 1.5 mM, but increased again at 2.5 mM exposure, with a polynomial trend. This pattern shows that trembesi seedlings have ability to detoxify lead by accumulating citrate as a form of their internal adaptation strategy. Metals are bound by organic acids that are exuded by the roots, so that the metal is not transported across the cell membrane and remains outside the roots, or organic acid activation to detoxify heavy metal that enter the cytoplasm by chelating to be less toxic [16, 17, 24, 25].

4. Conclusions
Trembesi seedlings produce oxalic, malic and citric acids in response to Pb treatment. Oxalic acid secretion was 6 times greater than citric acid, and 5 times greater than malic acid, while trembesi seedlings accumulated citrate 20 times greater than malic acid and 1.5 times greater than oxalic acid. Trembesi seedlings increased oxalic acid secretion and citric acid accumulation when exposed to lead up to 2.5 mM with a linear trend which probably as part of external adaptation strategies to lead stress.

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