Analysis of metal accumulation and Phytochelatin Synthase (PCS) gene expression in *Saccharum spontaneum* L. as regards its function for remediation of lead (Pb) contamination

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Abstract. Pollution caused by heavy metals, has become a serious problem. Adverse effects arising from the increased use of heavy metals in a variety of human activities lead to any environmental degradation. Lead (Pb) is one of most common contaminants in the environment and highly toxic. Pb is less mobile, so its compound tends to accumulate in soil and sediments. Definitely, efforts are needed to remove this contaminant in the environment. *Saccharum spontaneum* L. is a perennial grass which has potential to be used as an accumulator plant to clean up pollutants. The ability of this plant as metal accumulator was tested in this study. *S.pontaneum* plants were treated using Pb in the concentrations of 0 ppm (control), 100 ppm, 200 ppm, and 300 ppm for 8 weeks. The results showed that there was an increase in the percentage of relative accumulation of Pb in the treated plants. This also indicated that plant roots accumulated more Pb than shoots. Meanwhile, expression of Phytochelatin synthase (PCS) gene increased 1.3-to-3.5-fold inductions in roots by increasing concentration of Pb treatments for 24 h. PCS gene expression showed the higher induction in the roots than in the shoots of *S.spontaneum* plant under Pb treatments.

1. Introduction

Heavy metal pollution is currently a serious problem worldwide. These inorganic pollutants generally come from anthropogenic activities in various sectors, such as industry, domestic, agriculture, and medical which then generate disposal waste into waters, soil, and atmosphere. This pollution spreads widely in the environment which in turn will also affect organisms [1,2]. Although some heavy metals are essential elements for living things, most of them can be toxic at high concentrations due to the formation of complex compounds in the cells. One of the important things regarding heavy metals as inorganic pollutants is that once they enter the environment, they cannot be decomposed. They persist and accumulate in the environment, affect, and pollute the food chain, which is then due to the toxicity of these heavy metals, various health problems arise in humans [3].

Among heavy metals, lead (Pb) is one of the most common hazardous contaminants. Naturally, it presents as a microelement in trace amounts in the soil and water, and in organisms even though it has no biological function [4]. Pb mainly persists in the environment as its stable ion Pb$^{2+}$[5]. Pb will not dissolve into the soil if the soil is not acidic, which means that under acidic conditions (pH < 5.5) Pb is
more mobile. Hence, it is very difficult to remove lead from the soil once it has strongly bounded in the soil matrix [6].

Phytoremediation or green remediation by using hyperaccumulator plants is an in-situ remediation technique to remove harmful contaminants from the environment. This technique emphasizes the ability of plants to absorb contaminants optimally without disturbing their growth. Certainly, the ability of plants to absorb and accumulate contaminants depends on the plant species used for remediation [7.8]. Accumulator plants for phytoremediation techniques must at least have a high resistance ability to survive in a polluted environment. The plant must be able to absorb maximum contaminants, translocate from the root, and then accumulate in above-ground organs [9]. Several plant species have shown their potential as accumulators of Pb through the ability to accumulate heavy metals in plant organs at high concentrations. It has been reported that *Tagetes minuta* L. and *Bidens pilosa* L. have a considerable potential for phytoremediation of soil polluted by Pb by accumulating the high values of Pb concentration in leaves [10], whereas *Athyrium wardii* showed higher lead concentration deposited in the roots than in the shoots [11].

Phytochelatin synthase (PCS) is an enzyme found in plants, fungi, nematodes, and all groups of algae including cyanobacteria. The specific γ-glutamylcysteine dipeptidyl transpeptidase (Phytochelatin synthase, PCS) catalyzes the formation process of phytochelatins synthesized from an oligomer of glutathione. Phytochelatin synthase (PCS) plays a crucial part in heavy metal tolerance in plants via the synthesis of phytochelatins (PCs), which can chelate heavy metals (HMs) in the vacuole and decrease cell damage [12]. Phytochelatins have a function as chelators, therefore it is very important for heavy metal detoxification [13]. In recent studies, the PCS gene has been used in the transgenic plants for the purpose of phytoremediation. The expression of *Ceratophyllum demersum* phytochelatin synthase, CdPCS1, enhances the heavy metal accumulation in *Escherichia coli* and Arabidopsis [14].

*Saccharum spontaneum* which belongs to the Poaceae family, is an erect perennial grass. This plant has a height of up to three meters and spreads massively using rhizomatous roots. This grass species can also thrive in marginal soils where no other crop can be cultivated and can locate itself in a variety of environments. Moreover, *S. spontaneum* can also be found as the most abundant grass species on the polluted area [15–17]. Therefore, this plant has the potential to be used as an accumulator plant to clean up metal pollutants in the environment. Due to this function, our study aimed to analyze the metal accumulation and Phytochelatin synthase (PCS) gene expression in *Saccharum spontaneum* under Pb contamination.

2. Materials and methods

2.1. Analysis metal accumulation

This study was conducted by using *Saccharum spontaneum* L. plant as the planting material collected from Citarum river bank, Mekar Mulya Village, District of Bekasi, West Java Indonesia. The plant was treated in an *ex-situ* research experiment performed by a completely randomized design in the greenhouse. The treatments consisted of various concentrations of Pb, namely 0 ppm (control), 100 ppm, 200 ppm, and 300 ppm, with 4 replications, in the form of watering plants using Pb treatments. Plants were planted in the pot-based growth medium containing 8 kg of lead contaminated soil collected from Cikarang river bank, Sukamulya Village, District of Bekasi, West Java Indonesia.

The plant was subjected under the treatments in order to ascertain the absorption capability of Pb in the shoot and the root. The growth and performance of the plants were assessed during the growing period. After harvesting on 8-week plants, Pb level was analyzed in the soil medium, in the root and the shoot of plants by using AAS (Atomic Absorption Spectro-photometry).

2.2. Analysis PCS gene expression

Template used for quantitative real time PCR was RNA samples isolated from roots and shoots of young *S. spontaneum* plants after subjected to Pb treatments (0 ppm, 100 ppm, 200 ppm, 300 ppm, and 400 ppm) for 24 h. Plants were induced by Pb treatments using the hydroponics method where only the plant
roots direct contacted with the pollutant solution. Total RNA extraction in the samples was performed by RNA Simple Total RNA Kit from Tiangen Biotech in which this step was initiated by crushing the samples using liquid nitrogen. RNA produced then was quantified by using nanodrop and read in the ratio of absorbance at 260 nm and 280 nm. A ratio of A260/A280 which the value is in between 1.9 – 2.1, is generally accepted as pure RNA.

Primers used in this study were designed by using primer-design software PrimerQuest Tool program (www.itddna.com). Primers used for PCS gene were designed form PCS of Phragmites australis with sequences of 5’-CGTGGATCTGAGGGAATGAA-3’ and 5’-GAACAGCAGCAGAACAGTTAGA-3’ for forward and reverse primers, respectively. In regard to normalize the target gene expression, a reference gene, ACT, was used as endogenous control, with the sequences of 5’-GCCATTCTTCTGTTGACCT-3’ for forward primer and 5’-GCTTCTCTTGTGATGCCTCCCT-3’ for reverse primer.

The expression of PCS gene in S. spontaneum plants was analysed by quantitative real time PCR (qRT-PCR) method and performed by using one-step real time PCR with SYBR®Green as fluorescence dyes. The real time PCR machine used in this study was Bio-rad CFX Connec Real Time System. The one-step real time PCR kit applied to the samples was SensiFAST™ SYBR® No-ROX One-Step Kit from BIOLINE which consisted of 2x SensiFAST™ SYBR® One-Step mix, as well as separate reverse transcriptase enzyme and Ribonuclease Inhibitor. The cycling conditions were as recommended by the manufacturer for 10 minutes at 45°C for reverse transcription process and followed by 2 minutes at 95°C for polymerase activation. The further process was denaturation in 95°C for 5 second, annealing in 60°C at 10 seconds, and extension in 72°C for 5 seconds, all for 40 cycles. At the end of the run, melting curve was generated by heating the amplicon at 600 to 90°C in order to confirm the specificity of the amplicon in each primer pair. The amount of transcript accumulation was then analyzed by using 2^∆∆Ct method [18].

3. Results dan discussion
An ex-situ research experiment was designed to identify the ability of S. spontaneum plants as lead (Pb) accumulators. This study was conducted according to research results that S. spontaneum has the ability to survive and invade naturally the polluted areas.

Figure 1 shows that after 8 weeks, there was an increase in Pb levels deposited in the soil which was in line with the increase in Pb level of the treatments. The same result was also found in the study related to the use of wastewater toward the environment. It is reported that the heavy metals will be accumulated in the environment by constant use of treated wastewater (TWW) for irrigation for a long time [19]. About this study, Pb level in the soil describes the level of its toxicity. This means that the higher the level of Pb in the soil, the higher the level of pollution and toxicity. The evaluation of Pb toxicity depends on the quantification of the metal content in the material.

The presence of metal pollutants in the soil greatly influences the metal content of the plants that grow on it, unless there is a mechanism that inhibits the absorption of the metals by plants. The potential for metal uptake by plants will also be influenced by the higher concentration of heavy metals in the soil. Pb treatments applied in the growth medium affected the metal accumulation in the roots and the shoot of S. spontaneum plant (Figure 2). This result indicated that S. spontaneum had the ability to absorb and accumulate Pb on the plant organs. The increase of Pb content in plants correlated with an increase of Pb concentration in the treatments. However, in the same of Pb treatments performed in this study, plant roots showed higher Pb levels than the shoots.

The presence of metal toxicity in the soil is one of the stress factors for the plants. These stress factors can affect plant growth and even productivity. However, plant species, known as hyperaccumulators, have the ability to absorb even large amounts of toxic elements, resulting in an increase in their concentration in aboveground biomass over 100-times compared to conventional plants. These plants have developed tolerance mechanism to toxic elements in the polluted environment. Some weeds, especially grass species, are known to have the ability to accumulate a large amount of metal elements in their roots and shoots [20,21]. In addition, this type of plant also has several other superior characters,
such as having a fast growth rate, well-developed root system, large biomass, and long-term growth cycle.

Figure 1. Pb levels on the growth medium. The concentration of Pb was analyzed in the growth medium containing lead (Pb) contaminated soil. Pb treatments with various concentrations (0 ppm, 100 ppm, 200 ppm, and 300 ppm) were applied in the growth medium for a period of 8 weeks.

Figure 2. Pb levels on Saccharum spontaneum plants. Pb was measured in the roots and the shoots after the plants were subjected under Pb treatments (0 ppm, 100 ppm, 200 ppm, and 300 ppm) on the growth medium containing lead (Pb) contaminated soil for 8 weeks.

The mechanism of heavy metal tolerance in plants is regulated by a complex network of genes involved in the process of extraction, chelation, uptake, detoxification, accumulation in the roots, and translocation to the shoots. One of the mechanisms by plants to detoxify HM is the production of short-chain thiol-rich repeats having low molecular weight peptides which known as phytochelatins (PCs). This peptide is synthesized from sulfur-rich glutathione (GSH) by the enzyme phytochelatin synthase (PCS) with the general structure (γ-glutamyl-cysteine) - glycine (up to 11) which presents a high affinity for binding to heavy metal [22].

Figure 3 shows the results of PCS gene expression analysis in Saccharum spontaneum plants under Pb treatments of 0 ppm - 400 ppm for 24 hours. The results indicated that the expression of PCS gene was higher in the roots than in the shoots with an enhanced range of 1.3 to 3.5-fold induction as compared to the controls. This result corresponds with the result in Figure 2 where the higher of Pb accumulation was detected in the root than that of in the shoots.
Recently, genes encoding PCS have been cloned and characterized in various plant species, such as *Arabidopsis thaliana* [23], *Oryza sativa* [24], *Brassica juncea* [25], *Triticum aestivum* [26], and *Thlaspi caerulescens* [27]. PCS is constitutively expressed and induced when exposed to heavy metals which act as the main inducers for its activity. When plants are exposed to heavy metals, phytochelatins for metal detoxification are immediately synthesized de novo from GSH. It is related to the condition that exposure to heavy metals can trigger a dramatic depletion of GSH reserves in the cells. The phytochelatins loaded with heavy metals are pumped, like the glutathione conjugates, into the vacuoles. This transport is ATP dependent. Because of the acidic environment in the vacuole, the heavy metals are liberated from the phytochelatins and finally deposited there as sulfides, often as microcrystalline sulfide complexes [28]. In relation to this study, phytochelatin binds to Pb ions leading to sequestration of Pb ions in plants and thus serves as an important component of the detoxification mechanism in plants. The capacity of plants to sequester heavy metal ions by binding them to phytochelatins has been utilized to detoxify soils which are polluted with heavy metals. This procedure then is as called phytoremediation.

**Figure 3.** Expression analysis of Phytochelatin synthase (PCS) gene in *Saccharum spontaneum*. The PCS gene expression was measured in the roots (black bar) and the shoot (white bar) of the plant. The plants were subjected in Pb treatments for 24 h before extracted the RNA form the roots and the shoots.

Understanding the molecular mechanisms of metal resistance in plants will be the key points especially in the engineering of transgenic plants to the purpose of phytoremediation. Certainly, the isolation and overexpression of phytochelatin synthase genes has prompted some understanding of their role in metal stress tolerance. Transgenic tobacco plants expressing CdPCS1, PCS gene from *Ceratophyllum demersum* cv. L, showed several-fold increased PC content and precursor non-protein thiols with an enhanced of metal accumulation without significant decrease in plant growth [29]. Furthermore, overexpression of MnPCS1 and MnPCS2, PCS gene isolated from *Morus notabilis*, in Arabidopsis and tobacco enhanced Zn\(^{2+}/Cd^{2+}\) tolerance in most transgenic individual [30]. Therefore, the PCS gene as a source to develop the transgenic plants is the right choice in increasing plant tolerance to heavy metals.

**4. Conclusion**

The results from this study presented that *Saccharum spontaneum* had the ability to accumulate heavy metal Pb in the plant organs. Pb treatments subjected to the growth medium was able to induce Pb accumulation in the roots and shoots of plants. Pb accumulation in the roots was higher than in the shoots. PCS gene expression in the roots showed 1.3-to-3.5-fold inductions which were higher than that
of to the shoots under Pb treatments. Pb accumulation in the roots correlated with an increased phytochelatin synthase (PCS) expression in *S.spontaneum* plant.

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