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Phytoremediation Potential of *Ceratophyllum* sp. on Arsenic-Contaminated Conditions

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ABSTRACT

Purpose: *Aquatic contamination with arsenic is a serious problem as people will be at risk of arsenic toxicity when using and drinking contaminated water. Phytoremediation is a possible way to remove arsenic from contaminated water; however, arsenic is usually toxic to aquatic plants and decreases the phytoremediation efficiency. Alleviating the toxicity of arsenic to plant growth may be possible. Thus, increasing the phytoremediation capacity to decontaminate arsenic-contaminated water, aided by plant growth regulators, is a challenge. Thus, this study aimed to increase the potential of Ceratophyllum sp. to remove arsenic from contaminated water when plant growth regulators were added.*

Research Method: *Ceratophyllum sp. was cultured in 2.50mg/L of arsenic-contaminated water for five days. The experiment was performed in a completely randomized design with one factor and five treatments (no plant growth regulator, salicylic acid, indole butyric acid, salicylic acid + calcium chloride and indole butyric acid + calcium chloride). Then the growth of Ceratophyllum sp., arsenic concentration in the plant biomass and the arsenic remaining in the water was determined at the end of the experiment.*

Findings: *The use of salicylic acid and salicylic acid + calcium chloride tended to increase the fresh and dry weight of Ceratophyllum sp. grown in arsenic-contaminated conditions, but both formulas of plant growth regulator did not increase the arsenic accumulation in the biomass of Ceratophyllum sp. Application of indole butyric acid in combination with calcium chloride tended to increase the accumulation of arsenic in the biomass of Ceratophyllum sp. so it was 121.9mg/kg, but the bioconcentration factor was only 39.2. The application of salicylic or indole butyric acid tended to increase the removal of arsenic from contaminated water; however, the amount of arsenic remaining under applications of both plant growth regulators was not significantly different from that without plant growth regulator application.*

Research Limitation: *There was no research limitation in this study.*

Originality/ Value: *Based on the results in this study, Ceratophyllum sp. had no potential to remove arsenic from contaminated water and the plant growth regulator used in this study was not necessary to be used in the stimulation of arsenic removal.*

Keywords: *Arsenic, Ceratophyllum sp., Phytoremediation, Plant growth regulator*

INTRODUCTION

Arsenic-contaminated water is a serious problem in some countries in the world. When the water used in agriculture is contaminated with arsenic, it increases human exposure in several ways. Plants, fish and other animals could uptake arsenic dissolved in water and increase the

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opportunity for humans to intake arsenic from water and contaminated food. There are several sources of arsenic contamination in water, such as mining, volcanic rock and volcanic emissions, and pesticide use in agriculture. Many countries in Latin America have arsenic contamination in groundwater (Bundschuh *et al*., 2021). This problem is also found in southeast Asia and Thailand. For example, arsenic contamination in groundwater at Beranang, Selangor, Malaysia was 23.70 - 54.40µg/L (Wahil *et al*., 2020). Arsenic in water at Ron Phibun District in Nakhon Si Thammarat, in the south of Thailand has been reported to be as high as 9,000 pg/L (Jones *et al*., 2008). Arsenic contamination in water has been reported at Mae Moh Valley, Lampang Province, Thailand as 325µg/L in mine sump water and 8µg/L in Mae Moh Reservoir water (Bashkin and Wongyai, 2002). A recent survey in Thailand reported that the arsenic concentration in a stream in the vicinity of the Mae Moh coal mine and power station in Lampang Province, Thailand was 10.54µg/L (Woon *et al*., 2021), which is slightly above 10µg/L (the limit of concentration for arsenic found in drinking water denoted by WHO (WHO, 2018)). In Thailand, the regulation of arsenic found in drinking groundwater should not exceed 0.05mg/l according to guidelines from the Department of Groundwater Resources, Thailand (Groundwater Information Technology Center, 2020).

Phytoremediation is an alternative method to remove arsenic contamination in water. Many aquatic plants have been reported to remove arsenic via phyto-accumulation or phyto-filtration, using hyper-accumulating plants to adsorb and absorb contaminants in water (Rahman and Hasegawa, 2011). There are many aquatic plants that have been reported to accumulate arsenic. *Cyperus papyrus* growing in 175mg/kg wetland soil accumulated 130-172mg As/kg plant (Jomjun *et al*., 2010). *Lemna minor* was reported to remove 70% of arsenic (initial concentration = 0.5mg/L arsenic) within 15 days (Goswami *et al*., 2014). *Vallisneria natans*, a submerged aquatic plant species, could accumulate arsenic with a bioconcentration factor of 1,300 within 21 days. The plant uptake of As (V) was via a phosphate transporter and then reduction to As (III) within the plant tissue (Chen *et al*., 2015). *Ceratophyllum*, a genus of submerged and rootless aquatic plants used as a model plant in this study has also been reported to accumulate arsenic (Xue *et al*., 2012). For example, the maximum accumulations of arsenate and arsenite in *Ceratophyllum demersum* were 862 and 963µg As/g dry weight after exposure to arsenate and arsenite at concentrations of 10µM for four days, respectively (Xue *et al*., 2012). Khang *et al*. (2012) also reported that *C. demersum* could accumulate 227.5g As/g dry weight with no sign of toxicity appearance after exposure to 40µM of arsenite for 24 hours. The other advantages of *Ceratophyllum* sp. for use in phytoremediation are that it is also usually found in natural water habitats in Thailand (Rayan *et al*., 2021), has ecological tolerance and fast growth with high biomass accumulation (Dawson, 2008).

However, high concentrations of arsenic and a long exposure time to arsenic have also been reported to be toxic to aquatic plants, including *Ceratophyllum* sp. (Khang *et al.,* 2012; Xue *et al.,* 2012). Several toxic effects of arsenic on plants include stunted growth, reduce biomass and inhibition of root growth (Abbas *et al*., 2018). Exposure to arsenic also inhibited chlorophyll synthesis in *C. demersum* (Mishra *et al*., 2016). Using a plant growth regulator is an established method to alleviate metal toxicity in plants (Chouychai and Somtrakoon, 2022), and several plant growth regulators have been used to alleviate metal toxicity in plants. For example, salicylic acid has been reported to decrease cadmium toxicity in rice via enhanced antioxidant defense activities and decreasing hydrogen peroxide concentration (Guo *et al*., 2007). Indole-3-acetic acid could increase the cell number and protein content of green alga *Acutodesmus obliquus* that was exposed to lead-contaminated water. In addition, indole-3-acetic acid exposure decreased the malondialdehyde and hydrogen peroxide content in alga cells (Piotrowska-Niczyporuk *et al*., 2018). It is possible that the use of salicylic acid or auxin, with and without calcium ions that are involved

in the plant signaling system (Medvedev, 2005), will alleviate arsenic toxicity to *Ceratophyllum* sp. To the best of our knowledge, studies on using plant growth regulators to alleviate arsenic toxicity in *Ceratophyllum* sp. have rarely been found; however, plant growth regulators have been reported to be used in alleviating the toxicity of metal to other plants as described above. Thus, salicylic acid and indole butyric acid alone or in combination with calcium chloride were applied to *Ceratophyllum* sp. to alleviate arsenic toxicity and increase the arsenic accumulation in this study. This knowledge will be beneficial for improving the phytoremediation efficiency of arsenic-contaminated water.

MATERIALS AND METHODS

Preparation of Arsenic-contaminated Water

One-eighth Hoagland modified basal salt mixture solution was prepared by weight with 1.63g of modified basal salt mixture (Phytotechnology Laboratories, USA) and dissolved in reverse osmosis water. Transfer an appropriate amount of the one-eighth Hoagland modified basal salt mixture solution into 1-liter polypropylene trays. Arsenic standard solution diluted in nitric acid (Chem Supply, Australia) was poured into the one-eighth Hoagland modified basal salt mixture solution to give a final concentration of arsenic as 2.50mg/L. Then, each plant growth regulator was prepared and poured into the one-eighth Hoagland modified basal salt mixture solution to give the final concentrations of each plant regulator to no plant growth regulator: 0.1mM of salicylic acid, 0.001mM of indole butyric acid, 0.1mM of salicylic acid + 20mM of calcium chloride and 0.001mM of indole butyric acid + 20mM of calcium chloride. The volume of the one-eighth Hoagland modified basal salt mixture solution in each tray was adjusted to 600mL. The pH of the water after preparation of the arsenic-contaminated water was acidic. Thus, the pH of the one-eighth Hoagland modified basal salt mixture solution in each treatment was adjusted to around 3.6-4.1, except trays with salicylic acid and calcium with a pH of around 3.3-3.4. The acidity of the medium was caused by using an arsenic standard solution diluted in nitric acid to prepare arsenic-contaminated water. Thus, every tray including the control treatment without arsenic standard solution addition had its pH adjusted at the beginning of the experiment to be the same.

Pot Experiment

The experiment was performed in a completely randomized design (CRD) with one factor and five treatments (no plant growth regulator, 0.1mM salicylic acid, 0.001mM indole butyric acid, 0.1mM salicylic acid + 20mM calcium chloride and 0.01mM indole butyric acid + 20mM calcium chloride), each treatment was performed in eight replicates. *Ceratophyllum* sp. was collected from a constructed pond at Mahasarakham University, rinsed with tap water and 15g of *Ceratophyllum* sp. was transferred to the experimental tray containing 2.50mg/L of arsenic. Trays containing 2.50mg/L of arsenic without *Ceratophyllum* sp. and trays without arsenic but cultured with *Ceratophyllum* sp. served as controls. The experiment was performed for five days. Then, the *Ceratophyllum* sp. was collected to determine the fresh weight and dry weight. Relative fresh weight was calculated by [(fresh weight on day five/fresh weight on day zero) x100] according to Ni *et al*. (2015). The arsenic in the biomass of *Ceratophyllum* sp. and water was sent for analysis at the Central Laboratory (Thailand) Co., Ltd., Khonkaen Branch. The arsenic in the biomass of *Ceratophyllum* sp. was analyzed using the methods described in the in house method based on AOAC (2016). Briefly, the plant biomass of all replicates in each treatment was combined before analysis because the biomass of the plant was not sufficient to separately analyze each replicate. After biomass combination in each treatment, the plant biomass was digested with nitric acid in a microwave (Mitestone, model Ethos up) at 180-200°C for 2 hr before analyzing because the biomass of the plant was not sufficient to be separately analyzed and the

analysis of arsenic with ICP-OES (Perkin Elmer, Model Optima 4300). The arsenic remaining in the water was analyzed by standard methods for the examination of water and wastewater APHA/AWWA/WFF (2017). Briefly, samples were digested with nitric acid and hydrochloric acid in a microwave at 180-200°C for 2h and analyzed with ICP-OES. The bioaccumulation factor was calculated from the arsenic concentrations (mg/kg) in *Ceratophyllum* sp. tissue divided by the arsenic concentration in the water.

Statistical Analysis

The data are shown as mean ± SE, and the statistical differences are shown as $P \leq 0.05$. Two-way ANOVA and were used for variance analysis among treatments of the arsenic toxicity to plant growth. One-way ANOVA was used for variance analysis among treatments of arsenic accumulation. The LSD method was used for pairwise comparisons of means.

RESULTS AND DISCUSSION

Growth of Ceratophyllum sp. in As-contaminated Condition

The biomass of *Ceratophyllum* sp. at the beginning of the experiment was approximately 15.28-15.45g for each treatment. The fresh weight of *Ceratophyllum* sp. grown in both non-contaminated and arsenic-contaminated water for five days was decreased when compared to the fresh weight of the plant at the beginning of the experiment. Using a plant growth regulator did not increase the fresh weight of *Ceratophyllum* sp. grown under non-contaminated water; however, using indole butyric acid and indole butyric acid + calcium chloride tended to decrease the fresh weight of *Ceratophyllum* sp. to only 13.12±0.61 and 12.86±0.36g, respectively. Meanwhile, the fresh weight of *Ceratophyllum* sp. grown under non-contaminated water without using any plant growth regulators was 14.74±0.20g. In the arsenic-contaminated water, the application of salicylic acid and salicylic acid + calcium chloride tended to increase the fresh weight of *Ceratophyllum* sp. to 17.12±0.17 and 17.05±0.35g, respectively. The dry weight of *Ceratophyllum* sp. grown under arsenic-contaminated water with the application of salicylic acid, salicylic acid + calcium chloride and indole butyric acid + calcium chloride was higher than that grown without receiving any plant growth regulator (Table 01).

The changes in the *Ceratophyllum* sp. fresh weight were different between those grown in arsenic-contaminated and non-contaminated water. In non-contaminated water, the application of plant growth regulator decreased the plant fresh weight, while salicylic acid application increased the *Ceratophyllum* fresh weight in arsenic-contaminated water (Table 01). There are previous reports indicating that both salicylic acid and calcium chloride could alleviate the toxic effect of arsenic on the plant by increasing the antioxidant system, stimulating plant growth under environmental stress and reducing the arsenic accumulation in plants (Rahman *et al*., 2015; Singh *et al*., 2017; Maghsoudi *et al*., 2020). Salicylic acid has been reported to improve the growth of both terrestrial and aquatic plants grown under arsenic stress, including *Trigonella foenum-graecum* L. (Mabrouk *et al*., 2019), *Oryza sativa* (Singh *et al*., 2017) and *Lepidium sativum* (Nouri and Haddioui, 2021). Calcium also improves the growth of *Oryza sativa* (Boorboori *et al*., 2021) and *Brassica juncea* (Singh *et al*., 2020) under arsenic stress. Arsenic also disturbed the indole acetic acid biosynthesis and its transport in a plant (Ronzan *et al*., 2018), and exogenous auxin has been reported to alleviate arsenic stress and simulate plant growth under arsenic contamination (Piacentini *et al*., 2020). The positive effect of auxin on plant growth, especially root growth, under arsenic stress was reported in rice (Piacentini *et al*., 2020); however, the positive effect of auxin on the growth of *Ceratophyllum* sp. was not obviously detected in this study when either using indole butyric acid alone or in combination with calcium chloride. Based on the fresh weight and dry weight of the plant, using some plant

growth regulators could increase the fresh weight of *Ceratophyllum* sp. in a short period, but the plant died after five days of the experiment. The leaves of *Ceratophyllum* sp. fell out after arsenic exposure and the plant died after five days of the experiment when observed by the naked eye. In general, the toxicity of arsenic on an aquatic plant is usually from membrane damage and plant stress from reactive oxygen species (de Campos *et al*., 2019). Visual symptoms of plants when exposed to high concentrations of arsenic, were chlorosis, darkening and reduction of root volume (de Campos *et al*., 2019). Moreover, the acidity of the contaminated water when using an arsenic standard solution diluted in nitric acid to prepare arsenic-contaminated water may be another possible factor affecting the death of *Ceratophyllum* sp. The time of arsenic exposure is another reason for arsenic toxicity in plants. For example, exposure of *Ceratophyllum demersum* to 40µM for 48 hours could reduce biomass production and arsenic accumulation in this plant, while there were no symptoms of toxicity when observed by the naked eye after 24 hours of arsenic exposure by this plant, *Ceratophyllum* sp.

Potential of Ceratophyllum sp. on Arsenic Phytoremediation

The application of *Ceratophyllum* sp. tended to remove arsenic from water because only 1.47±0.05mg/L of arsenic remained in the water, while about 2.50mg/L of arsenic remained in the unplanted control. However, the arsenic accumulation by *Ceratophyllum* sp. in this study was low. This may be caused by the high concentration of arsenic in the water. The accumulation of arsenate and arsenite within the shoots of *C. demersum* decreased when the arsenic concentration in water was higher than 10µM (Xue *et al*., 2012). The application of salicylic acid and indole butyric acid did not stimulate the removal of arsenic by *Ceratophyllum* sp. as the amount of arsenic remaining in the water was not significantly different from that without plant growth regulator application. Using salicylic acid or indole butyric acid in combination with calcium chloride also did not stimulate the removal of arsenic from the water by *Ceratophyllum* sp. as the amounts of arsenic remaining in the water were 2.85±0.24 and 2.70±0.06mg/L, which were not significantly different from the unplanted control (Table 02).

Table 01: Fresh and dry weight of Ceratophyllum sp. grown in non-contaminated and arsenic-contaminated conditions in the presence of plant growth regulator (Mean ± SE).

Treatment	Day 0 Fresh Weight (g)	Day 5 Fresh weight (g)	Relative fresh weight (%)	Day 5 Dry weight (g)
Non-contamination				
No plant growth regulator	15.28±0.07	14.74±0.20a	97.4±1.18a	1.05±0.03a
Salicylic acid	15.37±0.06	13.72±0.69ab	89.3±4.67ab	0.84±0.07c
Salicylic acid + Calcium chloride	15.52±0.05	13.90±0.50ab	90.3±3.30ab	0.94±0.03bc
Indole butyric acid	15.40±0.06	13.12±0.61b	88.1±1.68b	0.90±0.07bc
Indole butyric acid + Calcium chloride	15.45±0.07	12.86±0.36b	83.2±2.25b	0.96±0.03b
Arsenic-contamination				
No plant growth regulator	15.41±0.04	11.20±0.59c*	72.8±3.99c*	0.62±0.03c*
Salicylic acid	15.35±0.02	17.12±0.17a*	111.5±1.11a*	0.92±0.02b
Salicylic acid + Calcium chloride	15.37±0.03	17.05±0.35a*	110.9±2.36a*	1.10±0.03a*
Indole butyric acid	15.38±0.03	12.13±0.43c	78.9±2.82c*	0.73±0.04c*
Indole butyric acid + Calcium chloride	15.41±0.04	14.99±0.35b*	97.2±2.18b*	0.92±0.02b

*Different lower-case letters show significant differences between plant growth regulators within the same arsenic concentration; * shows a significant difference between non-contaminated and arsenic-contaminated conditions between the same plant growth regulator.*

Table 02: Arsenic remaining in water and plant material under various plant growth regulators (Mean ± SE).

Treatment	As remaining in water (mg/l)	As in plant tissue (mg/kg)	Bioconcentration factor
Plant + No As	-	21.46	-
No plant + As	2.50±0.36a	-	-
Plant + As + No plant growth regulator	1.47±0.05b	93.9	49.33
Plant + As + Salicylic acid	1.44±0.03b	70.65	34.17
Plant + As + Salicylic acid + Calcium chloride	2.85±0.24a	62.57	14.40
Plant + As + Indole butyric acid	1.33±0.07b	112.6	68.72
Plant + As + Indole butyric acid + Calcium chloride	2.70±0.06a	121.9	39.23

Different lower-case letters show significant differences between plant growth regulators within same arsenic concentration.

The pH of arsenic-contaminated water in this study is quite acidic and the study by Khang *et al*. (2012) revealed of a slightly acidic pH stimulates arsenic accumulation in *C. demersum*. However, acidic conditions in water in this study did not support the accumulation of arsenic by *Ceratophyllum* sp. This may be because the pH in this study (between 3.3-4.1) was more acidic than the optimum pH (pH 5.0) for arsenic accumulation in *C. demersum* as reported by Khang *et al*. (2012).

The arsenic in the plant biomass was also determined, and the background level of arsenic in *Ceratophyllum* sp. was 21.46mg/kg and the *Ceratophyllum* sp. accumulated 93.9mg/kg with a bioaccumulation factor of 49.33 in the absence of a plant growth regulator. Based on the arsenic concentration in the biomass of plants in our results, *Ceratophyllum* sp. was not an arsenic hyperaccumulator because arsenic hyperaccumulators usually accumulate arsenic with a unit of grams per kilogram of dry biomass (Abou-Shanab *et al*., 2020). Moreover, the previous study revealed that the shoots of *C. demersum* could accumulate arsenic as high as 862 and 963µg of arsenic / g dry weight after exposure to arsenate and arsenite at 10µM for four days (Xue *et al*., 2012).

The presence of salicylic or salicylic + calcium chloride decreased the arsenic accumulation in the biomass of *Ceratophyllum* sp. to be 70.65 and 62.57mg/kg, respectively. Salicylic acid has been reported to decrease metal accumulation in terrestrial plants via stimulating stomatal closure (Acharya and Assmann, 2009) and may decrease arsenic accumulation in submerged aquatic plants with other mechanisms. The bioconcentration factor of arsenic also decreases when using salicylic or salicylic + calcium chloride as a plant growth regulator. Meanwhile, indole butyric acid or indole butyric acid + calcium chloride increased the arsenic accumulation in the biomass of *Ceratophyllum* sp. to 112.6 and 121.9mg/kg, respectively. In general, the application of salicylic acid helped to decrease arsenic accumulation in plants, especially in a study on *Oryza sativa* (Singh *et al*., 2015). In addition, the antagonistic effect of calcium on arsenic removal was seen in this experiment, in both the indole butyric acid + calcium chloride and salicylic + calcium chloride treatments. It may be relevant as the Ca-As precipitate has been reported in soil and slurries (Moon *et al*., 2004). The effects on growth and arsenic remediation in this study seem to come from only the salicylic addition because in the treatment using salicylic acid in combination with calcium chloride the result did not differ from salicylic acid alone.

CONCLUSIONS

In conclusion, this *Ceratophyllum* sp. was not appropriate to be used in arsenic phytoremediation under the conditions in this study because it can only accumulate a small amount of arsenic in its biomass and dies after exposure to 2.50mg/L arsenic for five days. The application of plant growth regulators either alone or in combination did not stimulate the growth of and arsenic removal by *Ceratophyllum* sp.

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