Research article

Enhancing arsenic removal from arsenic-contaminated water by *Echinodorus cordifolius*–endophytic *Arthrobacter creatinolyticus* interactions

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ABSTRACT

In this study, *Echinodorus cordifolius* was the best plant for arsenic removal compared to *Cyperus alternifolius*, *Acrostichum aureum* and *Colocasia esculenta*. Under arsenic stress, the combination of *E. cordifolius* with microbes (*Bacillus subtilis* and *Arthrobacter creatinolyticus*) was investigated. It was found that *A. creatinolyticus*, a native microbe, can endure arsenic toxicity, produce higher indole−3 acetic acid (IAA) and ammonium production better than *B. subtilis*. Interestingly, *E. cordifolius*−endophytic *A. creatinolyticus* interactions showed that dipping plant roots in *A. creatinolyticus* suspension for 5 min had the highest arsenic removal efficiency compared to dipping plant roots in *A. creatinolyticus* suspension for 2 h and inoculating *A. creatinolyticus* with *E. cordifolius* directly. Our findings indicated that under this inoculation condition, the inoculum could colonize from the roots to the shoots of the host tissues in order to avoid arsenic toxicity and favored arsenic removal by the host through plant growth-promoting traits, such as IAA production. Highest levels of IAA were found in plant tissues and the plants exhibited higher root elongation than other conditions. Moreover, low level of reactive oxygen species (ROS) was related to low arsenic stress. In addition, dipping *E. cordifolius* roots in *A. creatinolyticus* for 5 min was applied in a constructed wetland, the result showed higher arsenic removal than conventional method. Therefore, this knowledge can be applied at a real site for improving plant tolerance stress, plant growth stimulation, and enhancing arsenic remediation.

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1. Introduction

Arsenic is a worldwide environmental problem, with more than 150 million people affected by arsenic−contaminated water. The people live in South and Southeast Asia, particularly in Bangladesh, drink water up to 1000 μg/L arsenic, which is a level 100 times higher than arsenic drinking water guideline value (10 μg/L) (WHO, 2011). In Vietnam, ground water is contaminated with up to 3000 μg/L (Christen, 2001). Similarly, the Ron Phibun District (Nakorn Si Thammarat province) in Thailand, this area revealed that it had arsenic concentration in soil ranged 21 to 14,000 μg/g (Visoottiviseth et al., 2002). These affected areas are related to tin-tungsten arsenic mineralization and mining activity, since lies within South and Southeast Asia tin belt (Murcott, 2012).

The presence of arsenic can affect plant, animal, and human health, not only causing skin and nerve damage, but also increasing the chances of lung, bladder, and kidney cancer. Generally, arsenic acid or arsenate (As (V)), arsenious acid or arsenite (As (III)), and the methyl group are the main toxic substances in the environment. Arsenic (V) is predominant and thermodynamically stable state of arsenic in toxic waters, while arsenic (III) is found predominantly in ground water (Pell et al., 2013).

Phytoremediation is a simple, cost−effective, and eco−friendly technology for arsenic removal. There are many reports of hyper-accumulator plants for arsenic remediation from soil, such as *Cyperus* spp., *Colocasia esculenta*, *Mimosa pudica*, and *Melastoma malabrathricum,* especially in fern species belonging to Pteridaceae family such as *Pteris vittata* (Ma et al., 2001; Wang et al., 2011; Visoottiviseth et al., 2002). Furthermore, some macrophyte plants have been reported to accumulate arsenic from contaminated water (Rahman et al., 2011). Nevertheless, searching for a new plant species that has a good potential for arsenic removal such as high

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biomass, rapidly growing and arsenic accumulation is needed to be studied. However, although phytoremediation of arsenic is environmental friendly, if arsenic is at high levels it caused plants to die. Therefore, under environmental stress, the application of microorganisms to enhance arsenic removal by plants could be an effective technology.

Plant−associated microbes are useful for promoting plant growth and enhancing metal resistance in contaminated water (Glick, 2014). *Bacillus subtilis* is an environmentally safe microorganism and an arsenic resistant bacterium (Sato and Kobayashi, 1998; Huang et al., 2015). Many previous studies have suggested that indole−3−acetic acid (IAA) and reactive oxygen species (ROS) play an important role in plant response to harsh conditions (Tognetti et al., 2012; Yuan et al., 2013). IAA is one of the most important auxin−type plant hormones and it is secreted by both microbes and plants. Together, IAA and ROS have been shown to influence plant growth and development, as well as stress-related hormonal networks and signaling molecules that adjust growth, development, and defense pathways to biotic and abiotic stresses in plants (Fu and Harberd, 2003; Laskowski et al., 2002; Mittler et al., 2004). Therefore, endophytic bacteria may play a role in improving plant stress tolerance and enhancing arsenic removal by IAA and ROS production.

The aim of our study was to find the best new plant species for arsenic removal. *Echinodorus cordifolius* has been reported for the best plant on organic-contaminated wastewater treatment (Sriprapat et al., 2011), while *Cyperus alternifolius*, *Acrostichum aureum*, and *Colocasia esculenta* were reported to be the best plant for heavy metal removal (Nakwanit et al., 2011; Cheng et al., 2002; Kaewtubtim et al., 2016). Therefore these plants were used to screen for the best arsenic removal. New microbe associated to the best plant for enhancing arsenic removal was studied. A new technique of dipping endophytic inoculum to the plants for arsenic removal was also compared. Then, the plant stress responses in relation to IAA and ROS levels and the inoculum population within the host plant tissues were studied. In addition, this study was also applied to a constructed wetland for remediation of arsenic from arsenic−contaminated water by plant-microbe interactions.

2. Materials and methods

2.1. Plant materials

Echinodorus cordifolius, *Cyperus alternifolius*, *Acrostichum aureum*, and *Colocasia esculenta,* were purchased from a plant shop in Bangkok, Thailand. Age of plants were approximately 3 months. Each plant, particularly the roots, were washed to remove soil particles and other contaminants and then acclimatized in Hoagland's medium for 5 days (Hoagland and Arnon, 1950).

2.2. Arsenic-contaminated water preparation

Due to it is difficult to collect arsenic-contaminated water at a real site because the arsenic concentration was not constant and varied during season. Therefore, arsenic-contaminated water was prepared from arsenic-contaminated soil by using H_2SO_4 to extract arsenic from arsenic-contaminated soil. Arsenic-contaminated soil was obtained from the Ron Phibun District, Nakorn Si Thammarat province in Thailand. This area is related to mineralization and mining activity. Arsenopyrite-rich waste piles from tin mining had been mined for over 100 years (Murcott, 2012). This area had arsenic contamination from water and soil. The soil sample was achieved by digging the soil surface layer in a depth of 0−20 cm, which must be carefully collected and handled. Arsenic-contaminated water was prepared by stirring As-contaminated

soil in distilled water and then pH was adjusted by using H_2SO_4 to control system pH in the range of 3−4 for arsenic leaching. The stock solution sample was filtrated through a filter paper (Whatman No. 5). Thereafter, the filtrated solution was diluted to 2 mg/L and adjusted pH to 7 similar to arsenic-contaminated water at Ron Phibun District, Nakorn Si Thammarat province, Thailand. Arsenic-contaminated water was sterilized by autoclave for 15 min at 15 psi pressure 121 °C.

2.3. Microorganisms and culture conditions

A. creatinolyticus isolated from arsenic−contaminated soil at Nakhon Si Thammarat province, Thailand (Maneesuwannarat et al., 2016) and *B. subtilis*, a high tolerance for arsenic concentration (Adams, 1973; Huang et al., 2015) were selected in this study because both strains can tolerate in arsenic concentration. Before the beginning of the experiment, pure culture from microbial stock of both strains were enriched in 250 mL Erlenmeyer flasks containing 25 mL of nutrient medium (NB). After that flasks were incubated at 30 °C for 16−18 h, shaking at 150 rpm in an orbital incubator until it reached the log phase.

2.4. Pot experiments

2.4.1. Screening plants for arsenic removal

Aquatic plants, such as *E. cordifolius*, *C. alternifolius*, *A. aureum* and *C. esculenta* were screened to find which plant species had the highest arsenic removal efficiency. Due to *C. esculenta* plant had a high biomass, which 1 plant had a weight about 120 g. Therefore, for control the weight of plants, the plants were weighed as follows: 1−2 plants of *E. cordifolius*, 8−10 plants of *C. alternifolius*, 3−4 plants of *A. aureum*, and 1 plant of *C. esculenta*. 120 g of each species were put in glass pots containing 400 mL of 2 mg/L arsenic−contaminated water to investigate arsenic removal efficiency (in triplicate). Plants were treated in glass pots for 5 days until no arsenic left in the solution. The solution levels in each pot were marked with the tape in order to adjust to the same levels due to evaporation and plant respiration before sampling the solution. 10 mL of sample solution were taken and filtered with filter paper (Whatman No.5), the remaining of arsenic in the solution was analyzed by an inductively coupled plasma optical emission spectrometry (ICP-OES), Horiba, JY2000, Japan (arsenic detection limit is 200 µg/L). Arsenic removal efficiency (%) is defined using the following equation:

$$As\ removal(\%) = \left[\frac{C_i - C_f}{C_i}\right] \times 100$$

where C_i and C_f are the initial and final of arsenic concentration in arsenic-contaminated water, respectively.

In addition, the actual leaf areas were determined by grid counting method (Radzali et al., 2016). Density of stomata was determined using nail polish imprints taken from the abaxial leaf. After that stomata were counted under a light microscope (Camargo and Marenco, 2011). Measurements of stomatal conductance were made on the abaxial surface of adult primary leaves by using the leaf porometer SC-1 (Decagon Devices, Pullman, WA, USA) in the morning. Water uptake was determined by the volume of tap water that added in pots to adjust to the same levels before sampling the solution.

2.4.2. Plant−microbe interactions for arsenic removal

400 mL of sterile arsenic−contaminated water was added in sterile glass pots. Then, 120 g of the best plant from the screening experiments (*E. cordifolius*) were put into the glass pots. *B. subtilis*