Assessment of Pb accumulation in roots of fast-growing trees inoculated with endophytic bacteria: Hydroponic culture

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Abstract

The purpose of this study was to test the effect of *Pseudomonas psychrophila*, isolated from the roots of metallophyte *Pityrogramma calomelanos* (L.) Link collected from the Song Tho abandon Pb mine, Thailand, on Pb accumulation of *Acacia mangium* Willd. and *Eucalyptus camaldulensis Dehnh.* grown in lead (Pb) solution. These plants were inoculated by pruned-root dip method. Further, inoculated plant and un-inoculated plants were treated in 30 mg/L of Pb as $Pb(CH_3COO)_2{\cdot}3H_2O$ and without Pb served as control for 15 days. Pb concentrations in the roots were determined by a flame atomic absorption spectrophotometer (FAAS) after acid digestion. The results showed that the background Pb concentration in the roots grown in the 25% modified Hoagland's nutrient solution without Pb showed no significant difference ($p > 0.05$) ranging from 10-89 mg/kg. In the presence of 30 mg/L Pb, there were no significant changes in Pb contents in roots caused by inoculations. However, *A. mangium* inoculation showed increase trend in Pb concentration in the roots (6,829±697 mg/kg) compared to non-inoculation (6,242±272 mg/kg). While, *E. camaldulensis* inoculation showed decreased trend in Pb content (3,763±592 mg/kg) compared to non-inoculation (4,233±264 mg/kg). These results suggest that *P. psychrophila* seemed to be effective in promoting the phytoremediation potential of *A. mangium*, however, it was not useful for Pb phytoremediation of *E. camaldulensis*.

Keywords: endophytic bacteria, fast-growing tree, hydroponic culture, Pb accumulation.

Introduction

Song Tho Pb mine is one of several Pb mines located in Chalae subdistrict, Thong Pha Phum district, Kanchanaburi province, western Thailand as shown in Fig. 1. The area is known for its large reserve of Pb. It had operated for nearly 25 years since 1977, before the expiry of the concession in 2002. In general, after closure of the mine and the processing facilities, the mine-spoils and ore processing wastes persist, representing a source of potentially toxic heavy metals in the environment [1]. Especially, Pb contamination in soils is one of the most severe problems on a global scale that need to be solved urgently. Once Pb contaminate the soils, it not only persists in soil for long-time, but also accumulates, transfers into the biosystems through the food chain, and is toxic to living organisms even at low concentrations [2]. In addition, Pb is the most abundant heavy metals existing in the soil with the range of 1.00-69,000 mg/kg followed by Cr, As, Zn, Cd, Cu and Hg, respectively [3]. Physiochemical methods can remediate Pb contaminated in soils, however, they mainly cause the negative impacts. They are too costly and cause adverse effects on biological activity, soil structure, and fertility [4,5]. Basically, phytoremediation refers to use of plants and associated soil microbes to reduce the concentrations or toxic effects of contaminants such as heavy metals, radionuclides and organic compounds in the environments [6]. It is an alternatively viable option to address Pb contamination in soils. Phytostabilization using plants to immobilize or reduce the mobility of heavy metals in soils consequently, can prevent pollutant migration to groundwater or entry into the food web [6].

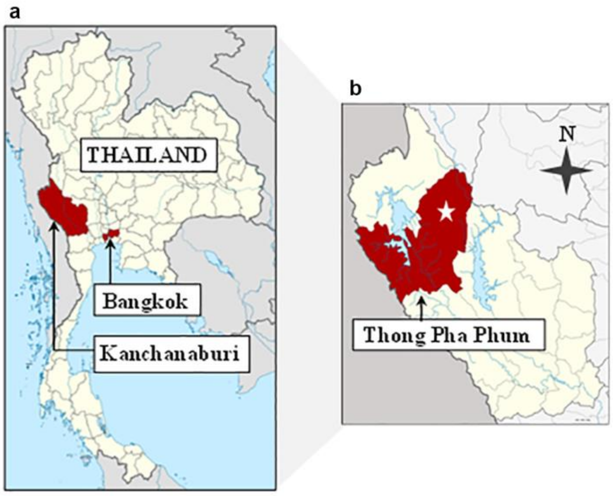

Fig. 1 (a) Map of Thailand showing Kanchanaburi province, and (b) Thong Pha Phum district with star representing the Song Tho Pb mine area in Chalae subdistrict (modified from https://th.wikipedia.org)

The success of phytoremediation depends on plant species, making a suitable plant selection an important step in phytoremediation [7]. Principally, plants having a large amount of biomass, rapid growth and accumulate heavy metals efficiently are required for large-scale phytoremediation [8]. The principle has been changed from single to dual purpose, the promising plant candidate should have both Pb accumulation potential and economic value. Thus, can help in achieving both the environmental sustainability, and also economic gain. Currently, energy crops with high-biomass production such as fast-growing tree are being popularly exploited for phytoremediation [9]. Besides, fast-growing trees are interesting due to their beneficial phytostabilization [4,10,11]. In Thailand, the fast-growing tree having economic value as firewood with high heating value (cal/g) are *A. mangium* (4,900 cal/g), and *E. camaldulensis* (4800 cal/g), recommended by the Department of

Alternative Energy Development and Efficiency, Thailand. Thus, *A. mangium* and *E. camaldulensis* become a promising candidate for this study.

Unfortunately, lower bioavailability of Pb makes it difficult to be uptaken by plants, and the trees show moderate accumulating and low tolerance ability for heavy metals [6,12]. Therefore, this strategy needs to be developed. Recently, use of endophytic bacteria with plant growth promoting trait has been widely studied for enhancing Pb phytoremediation efficiency [13-15]. Normally, plant growth promoting endophytic bacteria (PGPE) improves growth through atmospheric nitrogen fixation, production of phytohormones such as Indole-3-acetic acid (IAA), production of siderophore, solubilisation of phosphorus, synthesis of 1-aminocyclopropane-1-carboxylate (ACC) deaminase [16,17]. In addition, PGPE enhance heavy metal solubilisation and bioavailability by many processes. These processes include reduction/oxidation, methylation, precipitation, biosorption, and production of chelating agents such as siderophore, low molecular weight organic acids and biosurfactants [16]. Based on our knowledge, there is little information on heavy metal-resistant *Pseudomonas* genus, especially *P. psychrophila* to assist Pb phytostabilization making it an interesting candidate. This bacterial endophyte is isolated from the root of Pb metalliferous phytostabilizer plant (*P. calomelanos*). It has ability to tolerate Pb, produce siderophore, solubilizing phosphate, and mobilize Pb in soil and solution (unpublished data).

Generally, the testing of plants for phytoremediation is widely performed using hydroponic test as rapid and cheap technique [7], since the results enables to differentiate among candidate species that has potential tolerate heavy metal tolerance and accumulate [10]. Moreover, it not only reduces the period of plant growth and length of exposure, but also reduces the space needed for experiments, and variability due to other environmental factors [18]. Although, these trees can uptake Pb from Pb solution, but nothing is known about their effect resulting from inoculation with *P. psychrophila* on phytoremediation. Thus, the purpose of this work was to test the effect of *P. psychrophila* on Pb accumulation of *A. mangium* and *E. camaldulensis* gown in Pb solution.

Methodology

Experimental plant acclimatization

Healthy *A. mangium* and *E. camaldulensis* were obtained from the Chatuchak market, Bangkok, Thailand. These plants were acclimatized in 25% modified Hoagland's nutrient solution with low phosphate for 7 days with aeration.

Endophytic bacteria preparation

The Pb resistant and plant growth promoting endophytic bacteria originally isolated from the roots of *P. calomelanos* as shown in Fig. 2 were used. Its colony was grown on a Luria-Bartani (LB) agar plate. After incubation at 30°C for 48 h, a fresh colony was transferred into a LB broth, and incubated on a shaker at 100 rpm, 30±2°C for 48 h. After incubation, 100 µL of pre-culture was grown in new LB broth for 16 h. Bacterial culture was centrifuged at 3500 rcf, 4°C for 20 min. Bacterial density was performed by plant count.

Inoculation by pruned-root dip method

The roots of acclimatized plants were washed with sterile distilled water, and they were cut 50% [19]. Then, pruned root plants were dipped in 300 mL of 25% modified Hoagland's solution added with bacteria inoculum to get final density 10^8 CFU/mL for 48 h. To confirm that the inoculation technique was successful, the inoculated plants and uninoculated plants were extracted by the same method with original isolation of *P. psychrophila* from its host. Plant samples were washed thoroughly with tap water. The roots were cut and weight for 0.5 g. Then, they were cleaned by surface disinfection technique according to Luo et al. [20] with minor modification. Briefly, the roots were washed twice by sterile distilled water for five minutes. Then, 0.5 g of cleaned roots were immersed in 70% ethanol for 40 sec, following 2.5% sodium hypochlorite plus a droplet of polyoxyethylene 80 with gentle shaking for 15 minutes. Then, the samples were rinsed five times with sterile distilled water. Then, 100 μL of the last rinse was spread on LB agar. After incubating, if no colony occurs, this technique is success. The sterilized roots were ground with 5 mL of 0.85% NaCl. Then, these crudes were serially diluted and grown on LB agar supplemented with 20 mg/L Pb as Pb acetate tri hydrate [$Pb(CH_3COO)_2 \cdot 3H_2O$]. After incubation, the morphology of colonies was visually observed. Colonies showing the similar morphological characteristic with those of *P. psychrophila* from each plate were picked to analyse 16S rRNA partial gene sequence.

Fig. 2 *Pityrogramma calomelanos* grown on the Song Tho abandon Pb mine, Thailand

Hydroponic test

Inoculated and un-inoculated pruned roots plants were treated in 25% modified Hoagland's solution added with 30 mg/L Pb. Plants grown in nutrient solution without Pb served as controls. The solutions were not aerated. Each treatment had 3 replicates. After 15 days, plants were harvested and rinsed twice with deionized water, and the roots were separated. Roots were oven dried at 60°C for 3 days. Dried roots were macerated and sieved before wet digestion. 0.5 g of dried plant tissues was extracted with 5 mL of 69% HNO_3 in digestion block until solution become clear. Samples were then filtered using a Whatman No.42 filter paper, and the filtrates were adjusted to 25 mL in a volumetric flask with deionized water. Total Pb content in the extracts was determined by a FAAS (SpectrAA 55, Varian) with a hollow cathode lamp of Pb (10 mA, wavelength 217 nm). Quantification was carried out with a calibration curve made from series of diluted standard solution with the coefficient of determination (r^2) higher than 0.995.

Statistical Analysis

Data were expressed as the means of triplicate ± standard deviation (SD). They were subjected to analysis of variance (ANOVA), and means were compared using least significant difference method (LSD) at ($p \leq 0.05$) with Windows SPSS version 17.

Results and Discussion

The result of the surface root disinfection technique was effective in removing epiphytic microorganisms making inoculated *P. psychrophila* become the true endophytic bacteria of its new hosts [14]. The colony appearance of each treatment is presented in Fig. 3. The morphological colonies of pure *P. psychrophila* were circle and white milk (Fig. 3a). Some colonies of inoculated *A. mangium* (Fig. 3b) and *E. camaldulensis* (Fig. 3c) were circle and white milk that related to colony morphology of pure *P. psychrophila*. This may imply that endophytic bacteria, *P. psychrophila,* can enter and colonize inside the roots of the new hosts. The results of 16S rRNA gene analysis confirmed that these colonies were *P. psychrophila*. This indicates that *P. psychrophila* was successfully colonized in the roots of new hosts. This step is necessary to assist phytoremediation, since survival under metal stress is very important factor to produce the beneficial substances for the activity of endophytic bacteria [21]. In turn, these circle and white milk colonies did not appear on the plates of un-inoculated *A. mangium*, (Fig. 3d) and *E. camaldulensis* (Fig. 3e). This indicates that *P. psychrophila* was not local species of new hosts, while, the circle and dark brown colonies (Fig. 3d, and e) of native plants were identified as *P. extremaustralis* by 16S rRNA gene analysis. This ensures that the change of Pb content in roots totally cause from the ability of alien species.

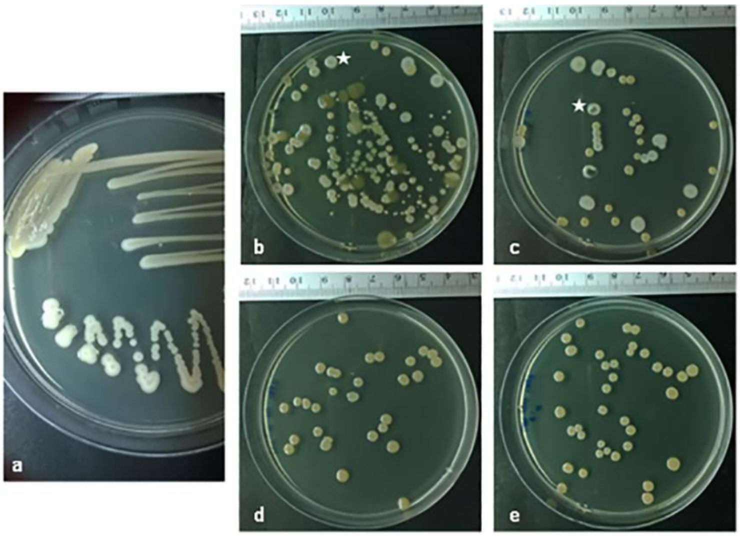

Fig. 3 Morphology of colonies isolated from each treatment: (a) pure colonies of *P. psychrophila*; (b) *A. mangium* inoculated with the *P. psychrophila*; (c) *E. camaldulensis* inoculated with the *P. psychrophila*; (d) un-inoculated *A. mangium* and (e) un-inoculated *E. camaldulensis*

The results of Pb content in roots are presented in Fig 4. Without Pb in solution, all treatments showed no significant difference ($p > 0.05$) in Pb contents ranging from 10-89 mg/kg (background concentration). In the presence of 30 mg/L Pb, there were no significant changes in Pb contents caused by inoculations. However, changes in tendencies of Pb contents were observed. *A. mangium* inoculation showed increasing trend in Pb concentration in the roots (6,829±697 mg/kg) compared to non-inoculation (6,242±272 mg/kg). While, *E. camaldulensis* inoculation showed decreased trend in Pb concentration in the roots (3,763±592 mg/kg) compared to non-inoculation (4,233±264 mg/kg). The results from this study are in line with the results of Sheng et al. [13] who found that inoculation of *Brassica napus* with Pb resistant endophytic bacteria did not show significant increase in Pb contents in roots at high Pb contaminated soils. In addition, He et al. [15] indicated that Pb resistant endophytic bacteria *Rahnella sp*. JN6 significantly increase Pb content in roots of *B. napus* grown in soil (113.9 mg/kg) compared to non-inoculation (77.9 mg/kg). These can be due to bacterial mechanisms. In general, endophytic bacteria increase metal accumulation by siderophore production and phosphate solubilization leading to improve efficiency of phytoremediation [21]. Similarly, *P. psychrophila* also possess these properties. Furthermore, the metal bioaccumulation process of endophytic bacteria also improves efficiency of phytoremediation [22]. In contrast, there are some opposing viewpoints suggesting that the presence of metal-resistant endophytes can decrease plants' metal uptake and accumulation [23]. Additionally, Xu et al. [24] found that heavy metal resistant bacteria, *Pseudomonas putida* CZ1, amended in Cu solution not significantly reduced Cu contents in roots of *Elsholtzia splendens* compared to non-amendment. Madhaiyan et al. [25] found that endophytic bacteria *Methylobacterium oryzae*, and *Burkholderia sp.* reduce metal contents in roots of *Lycopersicon esculentum* due to bacterial immobilization of metals in rhizosphere. This information supports the result of Pb content reduction in *E. camaldulesis* from this study. The opposite trend of Pb content in roots of plants from this study were found. This can be explained that the effect of microbial inoculation on metal extraction capacity of plants depends on the plant species, metal concentration, as well as the microbial species and strains used [26].

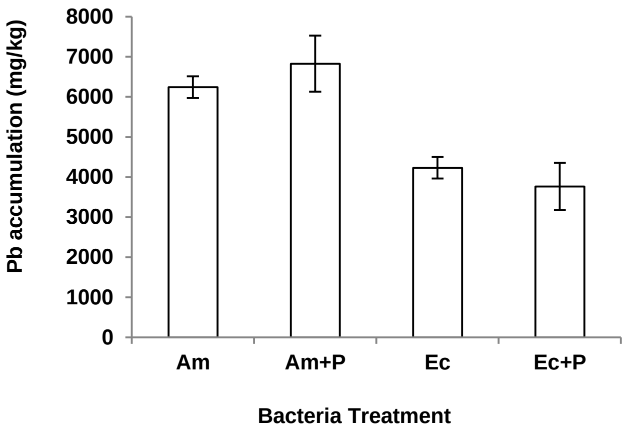

Fig. 4 Pb concentration in the roots of plants grown in Pb solution for 15 days. Each value is the mean of triplicates. Error bars represent standard deviation. Different letters indicate significant differences at *p* value ≤ 0.05, according to Fisher's LSD test

Conclusions

To our knowledge, this is the first study reporting Pb multifunctional endophytic bacteria, *P. psychrophila*, assisted phytoremediation potential of *A. mangium* and *E. camaldulensis*. The study demonstrated that *P. psychrophila* seemed to be effective in promoting the phytoremediation potential of *A. mangium* by enhancing Pb concentration in roots, but it was not useful for Pb phytoremediation of *E. camaldulensis* based on reduction of Pb content in roots. However, pot experiments of these inoculations need to be investigated to confirm the efficiency of Pb phytostabilization in soil.

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