PHYTOREMEDIATION OF LEAD CONTAMINATED SOIL USING FAST-GROWING TREES INOCULATED WITH ENDOPHYTIC BACTERIA
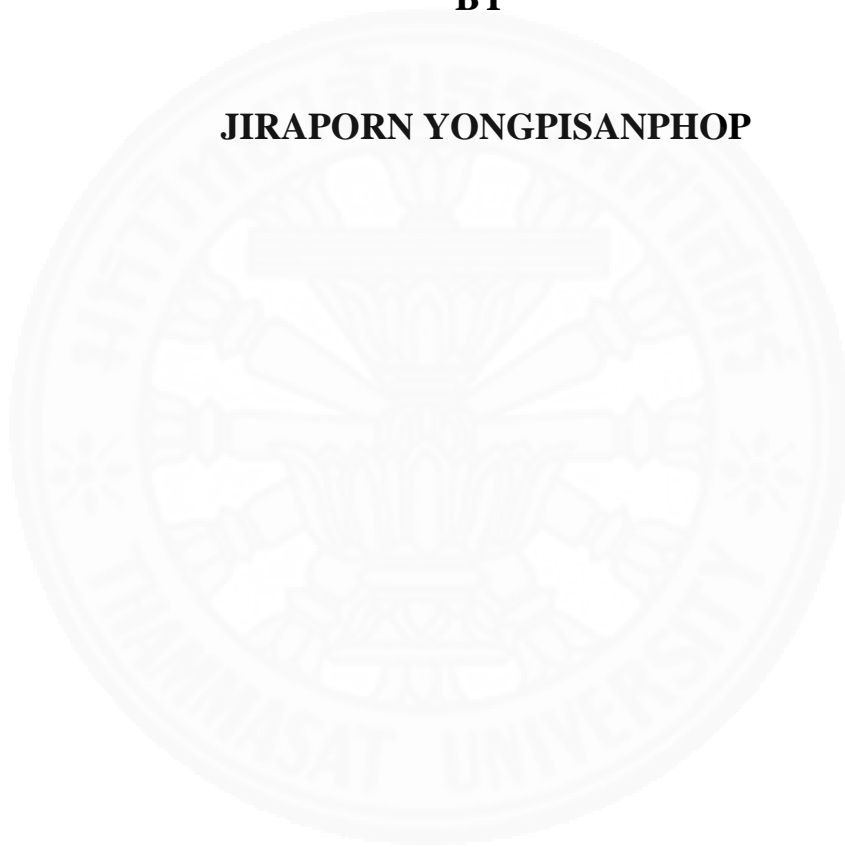
BY

JIRAPORN YONGPISANPHOP

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF
PHILOSOPHY (ENGINEERING AND TECHNOLOGY)
SIRINDHORN INTERNATIONAL INSTITUTE OF TECHNOLOGY
THAMMASAT UNIVERSITY
ACADEMIC YEAR 2017

PHYTOREMEDIATION OF LEAD CONTAMINATED SOIL USING FAST-GROWING TREES INOCULATED WITH ENDOPHYTIC BACTERIA

BY

JIRAPORN YONGPISANPHOP

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF
PHILOSOPHY (ENGINEERING AND TECHNOLOGY)
SIRINDHORN INTERNATIONAL INSTITUTE OF TECHNOLOGY
THAMMASAT UNIVERSITY
ACADEMIC YEAR 2017

PHYTOREMEDIATION OF LEAD CONTAMINATED SOIL USING FAST-
GROWING TREES INOCULATED WITH ENDOPHYTIC BACTERIA

A Dissertation Presented

By

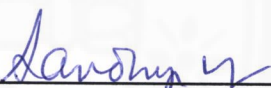JIRAPORN YONGPISANPHOP

Submitted to

Sirindhorn International Institute of Technology

Thammasat University

In partial fulfillment of the requirements for the degree of

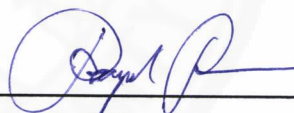DOCTOR OF PHILOSOPHY (ENGINEERING AND TECHNOLOGY)

Approved as to style and content by

Advisor and Chairperson of Thesis Committee

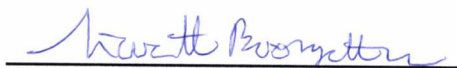(Prof. Dr. Sandhya Babel)

Co-Advisor

(Assoc. Prof. Dr. Prayad Pokethitiyook)

Committee Member and
Chairperson of Examination Committee

(Assoc. Prof. Dr. Alice Sharp)

Committee Member

(Assoc. Prof. Dr. Siwarutt Boonyarattanakalin)

Committee Member

(Prof. Dr. Maleeya Kruatrachue)

External Examiner: Prof. Dr. Helena Maria de Oliveira Freitas

DECEMBER 2017

i

Abstract

PHYTOREMEDIATION OF LEAD CONTAMINATED SOIL USING FAST-
GROWING TREES INOCULATED WITH ENDOPHYTIC BACTERIA

by

JIRAPORN YONGPISANPHOP

Bachelor of Science (Biology), Silpakorn University, 1999

Master of Science (Environmental Biology), Mahidol University, 2003

Master of Science (Bioinformatics and System Biology), King Mongkut's University
of Technology Thonburi, 2011

Doctor of Philosophy (Engineering and Technology), Sirindhorn International
institute of Technology, Thammasat University, 2017

Contamination of Pb in the environment is one of the main problems on a
global scale. Phytoremediation is an emerging green technology to restore the
contaminated sites. Recently, the use of trees as phytostabilizers has become a
popularly powerful tool to remediate heavy metals from the contaminated
environments. However, the ability of trees to uptake Pb is low to moderate level.
Besides, Pb has low bioavailability making it difficult to remove. The high amount of
Pb accumulated in plants causes phytotoxicity reducing the phytoremediation
potential. To overcome these limitations, the use of fast-growing trees assisted by
endophytic bacteria has been recommended as a promising technique to clean up Pb
polluted soils. In this study, the effects of inoculation of the promising plant growth
promoting endophytic bacteria (PGPE) on phytostabilization potential of fast-growing
trees were studied. Four fast-growing trees with high heating value were firstly
screened using hydroponic test. Two species were selected as the new host for
bacterial inoculation based on the highest Pb accumulation in roots such as *Acacia
mangium* (49004 mg/kg) and *Eucalyptus camaldulensis* (40598 mg/kg). To obtain the
potent endophytic bacteria, the Pb accumulation in roots of metallophytes was also

screened by means of field survey. *Pityrogramma calomelanos* containing the highest Pb concentration in roots (32633 mg/kg) was chosen as the host of PGPE. Then, three Pb resistant endophytic bacteria were isolated from the roots of their host. Among them, after characterization of plant growth promoting traits (PGPT) and species identification by 16S rRNA partial gene sequencing analysis, *Pseudomonas psychrophila* Den 03 was chosen as the promising PGPE. It tolerated Pb concentration up to 1850 mg/L. Besides, it exhibited some PGPT such as siderophore production and phosphate solubilization. Moreover, it showed high Pb solubilization from solution and mobilization from soil. After successful inoculation, *P. psychrophila* could colonize in roots tissues of the new plant hosts, *A. mangium* and *E. camaldulensis*. The effect of *P. psychrophila* on growth, phytotoxicity and Pb accumulation in those plants were evaluated with the absence and presence of Pb in soil. Inoculation of *P. psychrophila* showed no influence on plant growth and Pb phytotoxicity. Further, *P. psychrophila* significantly increased Pb accumulation in shoots of *A. mangium* and *E. camaldulensis*. Besides, *P. psychrophila* increased Pb accumulation in roots of *A. mangium*, but slightly reduced that of *E. camaldulensis*. With translocation factor (TF) < 1, *A. mangium* and *E. camaldulensis* were identified as excluders that are suitable for phytostabilization. Inoculation of *P. psychrophila* slightly increased bioconcentration factor (BCF) and TF in *A. mangium*. In addition, inoculation of *P. psychrophila* in *A. mangium* showed higher BCF and lower TF values than those in *E. camaldulensis*. The results of this study suggested that *P. psychrophila* could promote the phytostabilization potential of *A. mangium*, which may be used for remediation of Pb contaminated site.

Keywords: Phytoremediation, Heavy metal, Fast-growing tree, Endophytic bacteria

Acknowledgements

Table of Contents

List of Tables

List of Figures

List of Abbreviations

%	percentage
°C	degree Celsius
BLAST	basic local alignment search tool
bp	base pair
cal/g	calorie/gram
CFU/mL	colony forming units
cm	centimeter
cmol/kg	centimol per kilogram
dS/m	decisiemen per meter
E	longitude East
g	gram or the relative centrifugal force
g/plant	gram per plant
g/pot	gram per pot
h	hour
kg	kilogram
km	kilometer
km^2	square kilometer
m	meter
M	molar
mA	milliampere
mg/kg	milligram per kilogram
mg/L	milligram per liter
min	minute
mL	mililiter
mm	milimeter
mM	milimolar
N	Normal or latitude Northern
No.	Number
Nm	nanometer
p	probability value

pH	potential of hydrogen
pmol/µL	picomolar per microliter
rpm	revolutions per minute
rcf	The relative centrifugal force
V	volt
v/v	volume by volume
w/v	weight by volume
X	times
µg/g	microgram metals per gram
µg/kg	microgram per kilogram
µL	microliter
µm	micrometer
µM	micromolar
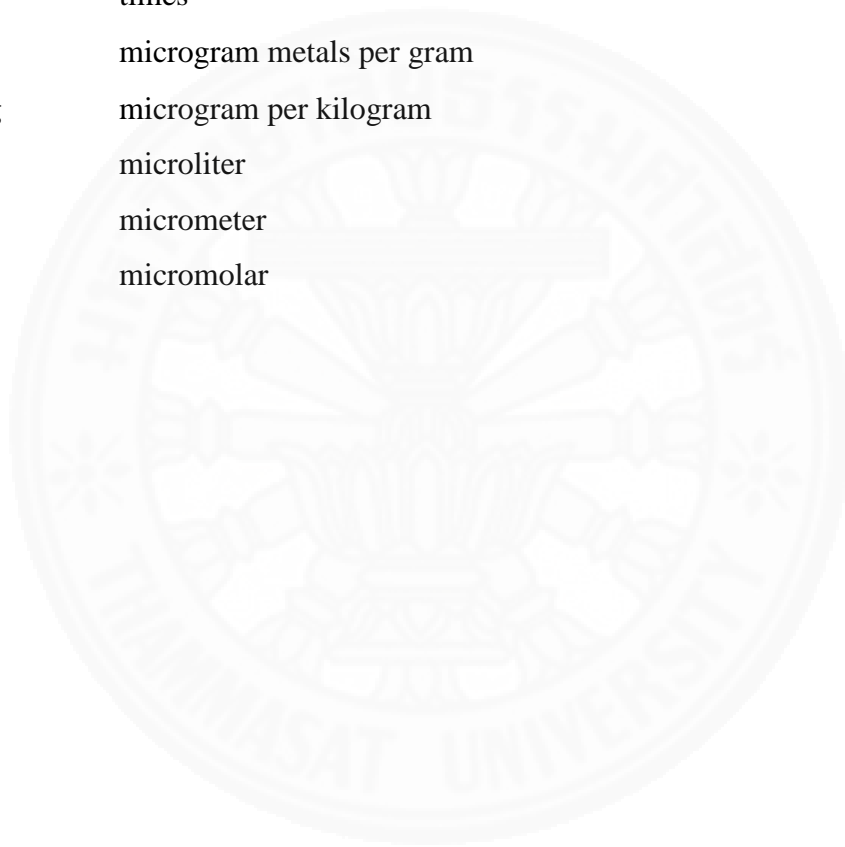
Chapter 1

Introduction

1.1 Background and rationale

Nowadays, quality of the environment such as air, water, and soil for human life is one of the most vital problems throughout the world. Among them, soils are critical components of ecosystems. Soil functions are not only limited to the production of food and fiber, but also are important for the maintenance of environmental quality worldwide. Indeed, whatever occurs in the soil can affect not only the pedosphere itself but also the hydrosphere, atmosphere, and biosphere (González-Oreja et al., 2008). Recently, soil contamination with heavy metals is one of the most serious environmental problems in a global scale due to the anthropogenic activities due to lack of proper management. This problem causes not only negative effects on the ecosystem, but also negative impacts on biota health, economy and society. Among heavy metals contaminated in the soils, Pb is a major concern due to several reasons: (1) Pb has been most extensively used by human for a long time and became the most widespread contaminant in soils, (2) Pb is highly toxic to all organisms even at low concentrations (Fahr et al., 2013), (3) Pb persists in the soils for a long time (150-5000 years) without natural biodegradation (Fahr et al., 2013), and (4) Pb compounds are still indispensable for modern human life (Sharma and Dubey, 2005). Soil contaminated with Pb is also found at Klity creek, Klity village, located in Kanchanaburi province, Thailand. Local people in the area have suffered from Pb toxicity. Therefore, Pb contamination in the soils needs to be managed seriously. These motivated us to find out the suitable method for Pb remediation.

Once Pb is introduced into the soils, it is extremely difficult to remove (Sharma and Dubey, 2005). Even if physico-chemical methods can clean up the soils, most of them are too costly, difficult operation, labor intensive, causes degradation of valuable soil components, change in properties, structure and fertility of the soils, disturbance of biological activity of native soil microorganisms, and generation of the secondary pollution problems (Pulford and Watson, 2003; Niu et al., 2007; Tangahu

1

et al., 2011; Gomes et al., 2016). For these reasons, the sustainable plant-based technology known as phytoremediation has emerged as a potential *in situ* technology to clean up the soils contaminated with heavy metals (Kramer, 2010). In addition, phytoremediation has many advantages such as, cost effective, eco-friendly, *in situ* application, good public acceptance (Ali et al., 2013). Among the sub-categories for Pb removal from soil, phytoextraction and phytostabilization are the most useful phytoremediation techniques adopted for soils contaminated with heavy metals (Handayanto et al., 2014). Additionally, heavy metals accumulated in the aboveground plant tissues for a long term may pose a risk to transfer in the food chain, phytostabilization may be a more feasible approach for the management of contaminated sites than phytoextraction (Handayanto et al., 2014).

There are many factors that need to be considered to achieve a successful Pb phytoremediation. One of them is plant selection (Subhashini et al., 2013). The proper plant for phytoremediation has to present: (1) a high biomass production, (2) a facility for cultivation, and (3) a high ability to tolerate, translocate, accumulate metal(loid)s in its biomass or to exclude these elements from living organs (Sylvain et al., 2016). Hyperaccumulator plants accumulating and hypertolerating high Pb concentration in aerial tissue without exhibiting symptoms of toxicity had been studied in the past decades (Prasad and Freitas, 2003; Wu et al., 2010). However, they are small size, have slow growth and low biomass production (Prasad and Freitas, 2003). To overcome these constraints, the uses of trees that demonstrate a high biomass production and possess a large capacity to store heavy metals in their tissues (dendroremediation) are of great interest in phytoremediation (González-Oreja et al., 2008; Sylvain et al., 2016). For trees, research is preferentially focused on willow, poplar, birch, sugar maple, and black alder (Pulford and Watson 2003; Zhivotovsky et al., 2011). Additionally, the selection of the right tree species should consider other profit making activities such as timber or bioenergy production (González-Oreja et al., 2008). Recently, there has been an increasing interest in using fast-growing tree as energy crops in phytoremediation experiments (Mahar et al., 2016; Pandey et al., 2016). This is not only solving the environmental sustainability, but also achieving economic returns as firewood (Pandey et al., 2016). Based on our knowledge, still little is known on the use of fast-growing trees as energy crop for phytoremediation in

2

Thailand. Fortunately, the Thai government had promoted some fast-growing trees to cultivate for obtaining the economic value as firewood with high heating value (cal/g). Some of them are *Acacia mangium* Willd, *Azadirachta indica* A. Juss, *Eucalyptus camaldulensis* Dehnh, and *Senna siamea* (Lam.) Irwin & Barneby. Their heating values are 4900, 5043, 4800, and 4500 cal/g, respectively (Department of Alternative Energy Development and Efficiency, Thailand). Importantly, they are suitable for cultivation to restore the mining area based on the Department of Primary Industries and Mines, Thailand. So, they can be the promising candidates for phytoremediation in this study.

Unfortunately, the use of fast-growing trees to remediate Pb contaminated soils has many constraints. Firstly, normally, trees accumulate relatively lower amounts of heavy metals (Zárubová et al., 2012). Secondly, Pb has the least bioavailable form due to precipitation with soil components, and difficult to be uptaken by plants (Saifullah et al., 2009; Ali et al., 2013; Jebara et al., 2015). Thirdly, phytotoxicity of Pb which is due to high Pb accumulation in plant tissues (Shin et al., 2012; He et al., 2013; Saifullah et al., 2016). To overcome these limitations, the potential use of fast-growing trees needs to be developed. Although, chemical chelator like ethylenediaminetetraacetic acid (EDTA) can improve phytoremediation efficiency, the use of microorganism-associated plants has attracted attention as environmentally friendly techniques (Karami and Shamsuddin, 2010; Rajkumar et al., 2012). Since bacterial metabolites are biodegradable, less toxic, and have strong influences on metal speciation and transport in the environment (He et al., 2009; Rajkumar et al., 2012). Among microorganisms, currently, plant growth promoting bacteria (PGPB) can help plants to remediate heavy metal contaminated soils (Karami and Shamsuddin, 2010). PGPB can be divided into plant growth promoting rhizospheric bacteria (PGPR), and plant growth promoting endophytic bacteria (PGPE). PGPR are found around the roots of plants, but PGPE are found within the tissues of the plants (Santoyo et al., 2016). In the past, PGPR have been extensively studied in phytoremediation research. At present, PGPE have received an increasing attention (Rajkumar et al., 2012; Ma et al., 2016). Since, PGPE offer advantages over PGPR, especially reduction of competition problems (Doty, 2008). However, their mechanisms to enhance phytoremediation are relatively similar. For example, they are

3

able to tolerate high concentrations of heavy metals. They produce siderophore, solubilize inorganic phosphate, solubilize and mobilize heavy metals, etc. (Lodewyckx et al., 2002; Santoyo et al., 2016). Thus, PGPE could be a better choice to assist in Pb phytoremediation in this study.

Selection of host plant plays a vital role in endophyte metabolic production and endophytic bacterial isolation (Srivastava and Paul, 2016). It is assumed that the good Pb resistant endophytic bacteria may reside in the roots of metallophytes containing the highest Pb concentration (Ali et al., 2013; Sessitsch et al., 2013; Verma and Gange, 2014). Generally, metallophytes accumulating high amount of heavy metals can provide a specific niche (heavy metal-stressed environment) for endophytes where they can develop mechanisms to resist the toxic effects of metals (Ma et al., 2016). In general, Pb metallophytes can be found in Pb contaminated area. There are four abandoned Pb mines, Song Tho, Bo Noy, Bo Yai and Bo Ngam, located in Kanchanaburi province, west of Thailand. Among these Pb mines, Song Tho was chosen because nothing was known about the endophytic bacteria from the Pb-accumulating plants grown in this mine area.

As mentioned above, inoculation of the potent PGPE into fast-growing trees as its new host is very challenging for improving the Pb phytoremediation potential. Importantly, PGPE should successfully colonize in plant tissues. Then, trees associated with PGPE have to be evaluated for the ability of Pb phytoremediation by hydroponic and pot study. Generally, hydroponic test is a rapid and cheap technique (Zhivotovsky et al., 2011). Since, 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 (Zacchini et al., 2009). However, their uptake capacity is different from those in soil due to phytoavailability. These effects are not present in the hydroponic test. Therefore, the promising candidates associated with potent PGPE need to be planted in Pb contaminated soil to obtain the actual information on Pb uptake influenced by soil chemical and microbial interactions.

4

1.2 Objectives of the study

The main objective of this study was to assess the potential of using fast-growing energy trees inoculated with PGPE for Pb uptake from contaminated soils. The specific objectives were:

> ➢ To select the best fast-growing tree species based on their ability to accumulate and tolerate Pb as the new host of endophytic bacteria using hydroponic experiment.
>
> ➢ To select the Pb-metalliferous plants based on the highest Pb accumulation in their roots as the host of endophytic bacteria using field survey.
>
> ➢ To select the best PGPE based on their ability to tolerate, solubilize, and mobilize Pb, solubilize P, and siderophore production using biochemical tests.
>
> ➢ To evaluate Pb phytostabilization of fast-growing trees inoculated with PGPE (*Pseudomonas psychrophila*).

1.3 Scope of work

Four fast-growing energy trees such as *A. mangium*, *A. indica*, *E. camaldulensis*, and *S. siamea* were used as candidates for Pb uptake selection. In addition, Song Tho abandoned Pb mine was selected as the study site. Pb metallophyte host and potent endophytic bacterium were selected. This endophytic bacterium could enhance at least a phytoremediation efficiency via either reduce phytotoxicity or increase Pb uptake. The overall scheme of this study is shown in Figure 1.1. The study was composed of 5 experiments. Firstly, fast-growing energy tree candidates were screened for their phytoremediation potential by growing in nutrient solution added with 0, 10, 30, and 50 mg/L of Pb for 15 d. After Pb analysis, only the species with highest Pb concentration in roots were selected for further experiments. Next, plants and soils collected from Song Tho Pb mine area were analyzed for Pb concentrations. Plant containing the highest Pb concentration in roots was selected as the host of endophytic bacteria. Then, the roots of selected plants were

5

extracted to isolate the PGPE. Then, the PGPE was characterized for their properties and identification strain by 16S rRNA partial gene sequencing. Only the strain showing the best characters such as the ability to solubilize and mobilize Pb in solution and in soil was chosen for inoculation in the selected trees by prune root dip method. After immediate inoculation, the PGPE could be recovered to ensure that they could enter the new host using recovery extraction method. Besides, after 15 d of inoculation, the inoculated PGPE need to be recovered to ensure that they could colonize inside the root tissues of hew host. Finally, trees inoculated with PGPE were grown in pot containing 1500 mg/kg Pb artificial spiked soil. After cultivation for 2 months, plant biomass, phytotoxicity, and Pb concentration in plants and soils were determined.
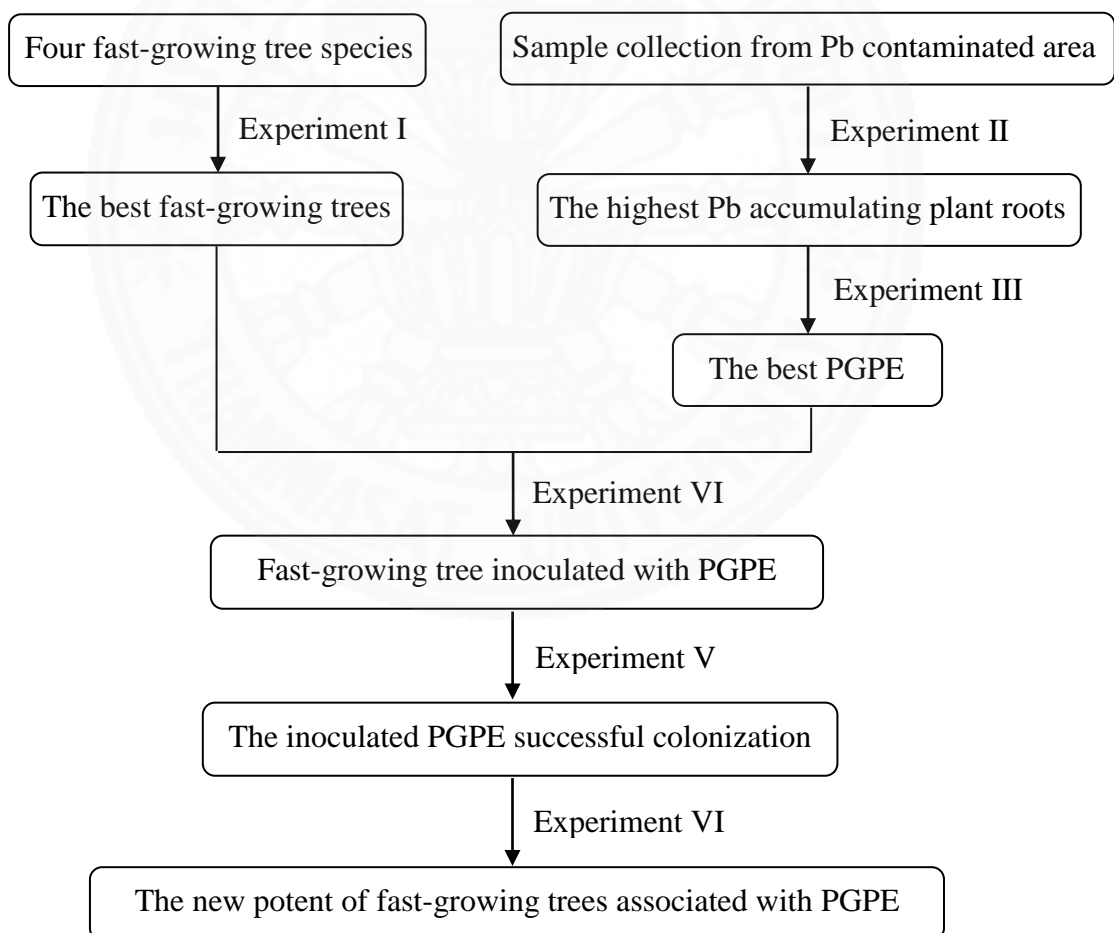
Figure 1.1 Overall method of this study

6

1.4 Significance of work

The main benefit of this study was to sustainably reduce Pb contamination in soil using tree species with high energy value, meanwhile achieving the economic return as bioenergy. The findings obtained from this study would reveal the basic knowledge of plants, endophytic bacteria and phytoremediation. Moreover, if this study is applied in the contaminated site, it will gain many benefits beyond Pb remediation. First, these plants are resource of renewable energy. Second, their wood can be used in many industries such as paper, construction, and furniture. Third, the cost for hazardous waste management after phytoremediation is reduced. Fourth, this cultivation increases the forest area that can mitigate the global climate change as well as creating a quality environment. Fifth, the remediated site becomes a beautiful landscape that can become a public garden. Finally, this bacterial endophyte can be improved for its efficiency by genetic engineering that is easier than modified plants. Importantly, if the modified endophyte is applied in the site, it will not contaminate the environment.

Chapter 2
Literature Review

2.1 Heavy metals

2.1.1 Classification of heavy metals

Heavy metals are naturally occurring elements that are found throughout the earth's crust (Tchounwou et al., 2012). There are 2 definitions of heavy metals. In chemistry, heavy metals are defined as transition metals having specific density higher than 5 g/cm^3, and atomic mass over 20 amu, while in biology, heavy metals are metals and metalloids that can be toxic to both plants and animals even at very low concentrations (Rascio and Navari-Izzo, 2011).

Normally, heavy metals are classified as essential and non-essential heavy metals (Sarwar et al., 2017). Essential elements are needed by organisms in minute quantities for vital physiological and biochemical functions (Ali et al., 2013). They are composed of cobalt (Co), copper (Cu), chromium (Cr), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se) and zinc (Zn). Inadequate supply and an excess amount of these micronutrients results in a variety of deficiency diseases or syndromes (Tchounwou et al., 2012). Non-essential or toxic heavy metals are not needed by organisms for any physiological and biochemical functions (Ali et al., 2013). They are composed of aluminium (Al), antinomy (Sb), arsenic (As), barium (Ba), beryllium (Be), bismuth (Bi), cadmium (Cd), gallium (Ga), germanium (Ge), gold (Au), indium (In), lead (Pb), lithium (Li), mercury (Hg), platinum (Pt), silver (Ag), strontium (Sr), tellurium (Te), thallium (Tl), tin (Sn), titanium (Ti), vanadium (V) and uranium (U). Their toxicity depends on dose, route of exposure, chemical species, age, gender, genetics, and nutritional status of exposed individuals (Tchounwou et al., 2012). Besides, heavy metals are considered as trace elements such as silver (Ag), cerium (Ce), fluoride (F), iodine (I), lanthanum (La), rubidium (Rb), serium (Sr), and tungsten (W) (Sarma, 2011). These heavy metals are present in trace concentrations (ranging from µg/kg to less than 10 mg/kg), or plants require in little amount (Sarma, 2011; Tchounwou et al., 2012).

8

2.1.2 Heavy metal contamination in soils

        Heavy metals have been used by mankind for a long time and contaminated in the environment such as soil, water and air (Alloway, 2013). Among them, soil is the major sink of heavy metals (Wuana and Okieimen, 2011). Heavy metals enter the soils by many sources including natural occurrence and anthropogenic processes (Tangahu et al., 2011). The weathering of minerals, erosion and volcanic activity are the most significant natural sources, while rapid urbanization, industrialization and intensive agriculture are main human sources (Ali et al., 2013; Ma et al., 2016). Additionally, volcanoes have been reported to emit high levels of Al, Zn, Mn, Pb, Ni, Cu and Hg along with toxic and harmful gases (Gill, 2014). The other natural activities such as wind dust, marine aerosol, forest fires, and natural vegetation have been reported to emit heavy metals into the environment. For example, wind dust resulting from Sahara Desert region has high levels of Fe and lesser amounts of Mn, Zn, Cr, Ni and Pb. Marine aerosols and forest fires also exert a major influence in the transport of some heavy metals in many environments. Natural vegetation emits heavy metals into the soil and atmosphere through leaching from leaves and stems, decomposition and volatilization (Gill, 2014).

        There are many sources arising from agriculture activity such as use of phosphate fertilizers pesticides, fungicides, liming, animal manure, sewage sludge, irrigation waters (Gill, 2014). The transportation such as automobiles, diesel-powered vehicles and aircraft, domestic effluents and landfills, are also sources of heavy metals (Gill, 2014). These may be considered as urbanization sources. Industrial sources are mining, refineries (coal burning power plants, petroleum combustion, nuclear power stations and high tension lines), electroplating, processing of plastics, textiles, microelectronics, wood preservation and paper, refuse incineration (Ali et al., 2013; Gill, 2014). Once heavy metals contaminated in soils, they have strong latency because of their colorless and odorless, very long time persisting as well as remediation hardness (Fahr et al., 2013; Su et al., 2014). Moreover, this problem is crucial, since heavy metals are capable of entering the food web leading to increased risks of other serious problems.

        In the past, there are many areas in Thailand where soils have been contaminated with heavy metals (Table 2.1). The average concentrations of heavy

metals including As, Cd, Co, Cr, Cu, Hg, Ni, Pb and Zn concentration were 7.5, 0.03, 6.0, 25.2, 14.1, 0.04, 13.5, 17.5 and 23.9 mg/kg, respectively. These soils were collected from agricultural, forested and uncultivated sites in the central, eastern, northern, northeastern and southern regions of the country (Simmons, 2004). Mainly, Thailand is trying to promote itself as "Kitchen of the World" to produce and export the food for mankind. Especially, many heavy metals have been detected in the polished Thai jasmine rice grown on Mae Sot paddy field (Table 2.2). This is the scientific evidence showing that the soils and crops of Thailand have been contaminated by heavy metals. Importantly, heavy metal problems are primarily chronic, e.g. As-poisoning in Ron Phibun District, Pb-poisoning in Thongphaphum District (Simmons, 2004). Among heavy metals contaminated in the soils, Pb is a major concern due to several reasons: (1) Pb has been most extensively used by human for a long time (Tong et al., 2000), (2) Pb is the most widespread contaminant in soils followed by Cr, As, Zn, Cd, Cu and Hg (Wuana and Okieimen, 2011), (3) Pb is the most contaminant of the urban soils (Alloway, 2013), (4) Pb is highly toxic to all organisms even at low concentrations (Fahr et al., 2013), (5) Pb persists in the soil for a long time (150-5000 years) without natural biodegradation (Fahr et al., 2013), and (6) Pb compounds are still indispensable for modern human life (Sharma and Dubey, 2005).

2.2 Pb

In Thailand, the well-known Pb problem is in Klity village, Thongphaphum District, Kanchanaburi Province. Pb concentration in sediment upstream, onsite and downstream are reported to be 2457, 12941 and 40053 mg/kg, respectively. The vegetables grown on this local area contained Pb in the range of 1.9-9.0 mg/kg. The mean concentrations of Pb in the blood of children aged 0-14 years in the villages of Klity Lang and Klity Bon were 21.3 and 36.3 µg/dl, respectively. With regards to the WHO maximum permissible levels of Pb in blood, 13.3% (Klity Lang) and 81.8% (Klity Bon) of the children sampled had blood Pb levels >25 µg/dL (Simmons, 2004).

Table 2.1 Heavy metal contamination in Thailand

No.	Location	Problem	Year
1	Ron Phibun district Nakhon Si Thammarat province	As contaminated in soil and water	1977-1985
2	Industrial Estate Lamphun province	Pb, Cu and Zn contaminated in soil and ground water	1983
3	Lower Huai Klity village Kanchanaburi province	Pb contaminated in soil and Huai Klity stream	1998
4	Mae Tao subdistrict Mae Sot district, Tak province	Cd contaminated in soil, water and rice	2006

Source: http://www.web.greenworld.or.th/greenworld/local/2311

Table 2.2 Heavy metals in the polished jasmine rice compared to Indian standards

Heavy metals	Heavy metals in rice (mg/kg) Thongsri et al. (2010)	Heavy metals in food (mg/kg) Indian standards
Cd	3.58	1.5
Cr	5.05	20
Cu	1.08	30
Ni	4.08	1.5
Pb	0.27	2.5
Zn	14.77	50

Sources: the data are modified from Thongsri et al. (2010); Nagajyoti et al. (2010)

2.2.1 Natural occurrence

Pb occurs naturally in the earth's crust with abundance of 14.8 mg/kg ranged from 10 to 30 mg/kg (Wuana and Okieimen, 2011; Alloway, 2013). Typical mean Pb concentration for surface soils worldwide averages 32 mg/kg and ranges from 10 to 67mg/kg (Wuana and Okieimen, 2011). Pb is abundant in igneous rocks ranged from 10 to 25 mg/kg, and argillaceous sediments varied from 14 to 40 mg/kg. It is rarely found in ultramafic rocks and calcareous sediments with the ranges of 0.1-8.0 and 3.0-10 mg/kg, respectively (Kabata-Pendias, 2011). Pb can be found in

11

sedimentary rock including shales (22 mg/kg) and sandstones (10 mg/kg) (Alloway, 2013). The most abundant Pb mineral is galena (PbS). Its common minerals are anglesite ($PbSO_4$), cerussite ($PbCO_3$), minium (Pb_3O_4), pyromorphite ($Pb_5(PO_4)_3Cl$), and mimetesite ($Pb_5(AsO_4)_3Cl$) (Kabata-Pendias, 2011).

2.2.2 Physical and chemical properties

Pb belongs to Group IV and Period 6 of the Periodic Table with atomic number 82, atomic mass 207.2, density 11.4 g/cm^3, melting point 327.4°C, and boiling point 1725°C. It is a bluish or silver-grey metal with 4 naturally occurring isotopes with atomic weights of 208, 206, 207 and 204 in decreasing order of abundance (Tangahu et al., 2011; Wuana and Okieimen, 2011). Despite Pb has four electrons on its valence shell, it occurs in the environment mainly as Pb^{2+}. However, its oxidation state +4 is also known (Kabata-Pendias, 2011; Tangahu et al., 2011). Pb^{2+} compounds are predominantly ionic such as Pb sulfate ($PbSO_4$), whereas Pb^{4+} compounds tend to be covalent such as tetraethyl Pb [$Pb(C_2H_5)_4$] (Wuana and Okieimen, 2011). Apart from acetate, nitrate, and chloride, most of the inorganic salts of Pb^{2+} have poor solubility in water (Tangahu et al., 2011).

2.2.3 Production and use

Pb is produced from galena deposits as primary source, and also produced from secondary sources by scraping from spent Pb-acid batteries (Kabata-Pendias, 2011). In 2010, world production of primary Pb is 4100000 tons (Alloway, 2013). It ranks the fifth behind Fe, Cu, Al, and Zn in industrial production (Wuana and Okieimen, 2011). The largest worldwide use of Pb is for Pb-acid batteries. Similarly, about half of the Pb used in the U.S. goes for the manufacture of Pb storage batteries. Other uses include solders, bearings, alloys cables, ammunition, plumbing, pigments, and caulking (Kabata-Pendias, 2011; Wuana and Okieimen, 2011). Many compounds of Pb^{2+} and a few Pb^{4+} compounds are useful. The two most common of these are Pb dioxide and sulfate (Wuana and Okieimen, 2011). Pb is generally used as anti-knock agents in gasoline, pigments in paints and in automobile batteries (Alloway, 2013). But, the use of tetraethyl and tetramethyl Pb [$Pb(CH_3)_4$] as antiknock additives in gasoline had been declined to reduce air pollution (Kabata-Pendias, 2011). In

Ref. code: 25605622300316XKQ

addition, Pb forms several basic salts, such as a basic Pb carbonate [Pb(OH)$_2$·2PbCO$_3$], which was once the most widely used white paint pigment. Pb dioxide (PbO$_2$) and PbSO$_4$ are used in the reversible reaction for the charge and discharge of Pb storage battery (Wuana and Okieimen, 2011).

2.2.4 Pb contamination in soils

Worldwide, the average Pb concentration in uncontaminated soils is estimated to be 17 mg/kg (Alloway, 2013). Generally, Pb in form of ionic including Pb^{2+}, Pb oxides, Pb hydroxides, and Pb-metal oxyanion complexes are released into the soil, groundwater, and surface waters (Wuana and Okieimen, 2011). The maximum amounts permitted of releasing Pb into land in a single site is 20 kg/year (Vamerali et al., 2010). After entering the soils, Pb normally accumulates in the surface soil, which contains Pb ranging from 10 to 67 mg/kg with the average of 32 mg/kg (Wuana and Okieimen, 2011). Then, Pb concentration decreases with depth of the soils (Pourrut et al., 2011). Pb concentration in soil ranges from 1.00-69000 mg/kg (Wuana and Okieimen, 2011). The surface urban soils contaminated with Pb worldwide ranges from 28.6 to 25380 mg/kg with the average of 1976.79 mg/kg, while those in agricultural soil ranged from 18.5 to 213 mg/kg with the average of 68.51 mg/kg. These data are modified from Su et al. (2014). There are many international Pb standards in soils as shown in Table 2.3. Those of Thailand do not exceed 400 mg/kg for habitat and agriculture purpose, and not exceed 750 mg/kg for other purposes issued by the Department of Pollution Control, Thailand (PCD, 2017).

Table 2.3 Standard of Pb concentration in soil

Standard	Value (mg/kg)	References
Indian standard	250-500	Nagajyoti et al. (2010)
European Union standards (EU, 2002)	300	Nagajyoti et al. (2010)
NJDEP (1996)	600	Wuana and Okieimen, (2011)
U.S. EPA (2009)	400 in play areas 1200 in other yard areas	Butcher (2009)

13

2.2.5 Pb behavior in soil

Heavy metals exist in 5 different pools including fraction 1; soluble fraction, (heavy metals in the soil solution as free ions and complexes); fraction 2, exchangeable fraction (metals adsorbed on ion-exchange sites and on inorganic soil constituents); fraction 3, organic fraction (metals bound with the organic matter); fraction 4, insoluble fraction (metals precipitated mainly as oxides, carbonates and hydroxides); and fraction 5, residual fraction (metals incorporated in the silicate minerals) (Mahmood, 2010). Those can be divided into total and available heavy metal concentration. Total concentration includes all fractions. Much of the total concentration is not available for immediate uptake by plants (Alloway, 2013). Meanwhile, available concentration presenting as either in fraction 1 or fraction 2 is readily available for plant uptake. However, fraction 3 and 4 can be released by different soil amendments; whereas, metals in the fraction 5 are potentially non-available (Mahmood, 2010). In general, Pb exists in soils in various forms: (1) Pb may occur as a free metal ion, (2) Pb forms complex with inorganic constituents such as HCO_3^-, CO_3^{2-}, SO_4^{2-}, and Cl^-, (3) Pb may exist as organic ligands such as amino acids, fulvic acids, and humic acids, or (4) Pb may be adsorbed onto particle surfaces such as Fe-oxides, biological material, organic matter, and clay particles (Pourrut et al., 2011). Pb is categorized as having the least bioavailability (Ali et al., 2013).

2.2.6 Pb toxicity to organisms

2.2.6.1 Human

Pb is very highly toxic to all organisms even at low concentrations. The U.S. Department of Health and Human Services, Agency for Toxic Substances and Disease Registry (2007) has given Pb in the second toxicity from 275 hazardous substances (Glick, 2010; Fahr et al., 2013). For human, Pb enters the body by inhalation and ingestion. Exposure to Pb can result in a wide range of biological effects depending on the level and duration of exposure, and age of exposed individual. Acute exposure to Pb induces brain damage, kidney damage, and gastrointestinal diseases, while chronic exposure may cause adverse effects on the blood, central nervous system, blood pressure, kidneys, and vitamin D metabolism (Tchounwou et al., 2012). Children are more sensitive to Pb than adults. Children

14

exposed to Pb are at risk for impaired or delayed neurobehavioral development, lower intelligence quotient (IQ), shortened attention span, hyperactivity, and mental deterioration, decreased hearing acuity, speech and language handicaps, growth retardation, poor attention span, and anti-social and diligent behaviors. While, adults exposed to Pb will suffer from loss of memory, nausea, insomnia, anorexia, weakness of the joints, and decreased sperm count in men and spontaneous abortions in women (Wuana and Okieimen, 2011; Tchounwou et al., 2012).

2.2.6.2 Plants

Pb concentrations in the agricultural crops are in the range of 0.1-10 mg/kg dry weight (Nagajyoti et al., 2010). Plants grown on the uncontaminated soil contains 5 mg/kg of Pb in aerial tissue (Yanqun et al., 2005). The toxic thresholds of Pb in plants are 30-300 mg/kg (Baker et al., 2000). Excess Pb causes a number of toxicity symptoms in plants e.g. stunted growth, decreased dry biomass, chlorosis, necrosis, and blackening of root system (Alkhatib et al., 2013). Pb inhibits root growth because Pb induces inhibition of cell division in the root tip (Sharma and Dubey 2005). Plant growth under heavy metal stress is concerned with the role of plant hormone (Prasad and Hagemeyer, 1999). The primary cause of cell growth inhibition arises from a Pb-induced simulation of phytohormone like indole-3-acetic acid (IAA) oxidation (Gill, 2014). Pb also strongly limits the development and sprouting of seedlings, and causes mitochondrial swelling, loss of cristae, vacuolization of endoplasmic reticulum and dictyosomes, injured plasma membrane and deep colored nuclei (Pourrut et al., 2011). In addition, heavy metals usually decreased the total chlorophyll and chlorophyll a/b ration in higher plant (Prasad and Hagemeyer, 1999). Pb disturbs mineral nutrition, and causes decrease in water potential, change in membrane structure and permeability, decrease in hormone status and electron transport activities, and decrease in germination, root or shoot length, tolerance index, and dry mass, whereas activities of enzymes are either increased or inhibited (Sharma and Dubey 2005). In addition, Pb also decreases photosynthetic and transpiration rates that relate to the distortion of the chloroplast ultrastructure at thylakoid systems (reduction of their number, swelling, and condensation), reduced chlorophyll content of the leaves, CO_2 deficiency because of stomatal closure, the

15

inhibited activities of the Calvin cycle enzymes such as carboxylating enzymes (Alkhatib et al., 2013; Gill, 2014). Inhibition of germination may result from the interference of Pb with important enzymes. Pb also causes irregular radial thickening in roots, cell walls of the endodermis and lignification of cortical parenchyma, induces proliferation effects on the repair process of vascular plants (Gill, 2014). Pb changes in nutrient contents and mineral imbalance, since it blocks the entry of cations (K^+, Ca^{2+} and Mg^{2+}) and anion in the root system (Sharma and Dubey 2005).

2.2.6.3 Microorganisms

Pb is toxic to bacterial cells even at low concentrations (Naik and Dubey, 2013). For example, no measurable growth of *Desulfovibrio desulfuricans* G20 was observed at 3 mg/L of Pb(II) chloride ($PbCl_2$) (Sani et al., 2001). Pb enters bacterial cells through the uptake pathways for essential divalent metals such as Mn^{2+} and Zn^{2+}. Pb toxicity occurs as a result of changes in the conformation of nucleic acid and protein inhibition of enzyme activity, disruption of membrane functions and oxidative phosphorylation, as well as alterations of the osmotic balance. Pb^{2+} also shows a stronger affinity for thiol and oxygen groups than essential metals such as Ca and Zn (Jarosławiecka and Piotrowska-Seget, 2014). In addition, Pb affects microbial growth and survival, since Pb causes damage to DNA, protein and lipid as well as replaces essential metal ions such as Zn, Ca and Fe from enzymes (Naik and Dubey, 2013). Recently, Pb^{2+} causes negative effect on growth of *Thermus thermophilus* strain Samu-SA1 at 200 and 300 mg/L. At 100 mg/L, Pb^{2+} reduces protein content and the activity of β-glucosidase and α-maltosidase enzymes. Moreover, the presence of Pb also affects also the secretion of different proteins with molecular weight ranging between 15 and 236 kDa of *T. thermophilus* (Nicolaus et al., 2016).

2.3 Remediation strategies

To protect the environment from heavy metal toxic effects and to conserve the good environment for future human generation, heavy metals must be remediated (Ullah et al., 2015). Normally, there are 3 approaches categorized into physical, chemical and biological approaches as follows:

2.3.1 Physical approach

This approach involves either on-site management or excavation and subsequent disposal to a landfill site (Gomes et al., 2016). On-site management is composed of soil replacement and thermal desorption methods. Soil replacement uses clean soil to replace the contaminated soil to dilute the pollutant concentration. The other technique is soil spading. Briefly, the contaminated soil is deeply dug, making the contaminants spread into the deep sites and achieving the aim of diluting and naturally degrading. Soil capping, the new soil is added, and covered at the surface. This technique is large in working volume, very costly, and is suitable for soil with small area and severely polluted. The thermal desorption technique is suitable for only volatile heavy metals. Briefly, the contaminated soil is heated by steam, microwave or infrared radiation to make the pollutants volatile. Consequently, they are collected using the vacuum negative pressure or carrier gas. The advantages of this method are simple process and the remediated soil being reused. However, this method is expensive and has a long desorption time (Yao et al., 2012). In soil excavation with disposal to a landfill site, this is only shifts the contamination problem to elsewhere and causes additional risk hazards associated with the transportation and migration of the contaminants to adjacent environmental compartments (Gomes et al., 2016).

2.3.2 Chemical approach

This approach is composed of chemical leaching, chemical fixation, electrokinetic remediation and vitrify technology. Chemical leaching is washing the contaminated soil using fresh water, reagents, or gas that can leach the pollutant from the soil via the ion exchange, precipitation, adsorption and chelation. Consequently, heavy metals in soil were transferred from soil to liquid phase, and then recovered from the leachate (Yao et al., 2012). This technique is very costly, produces metal-rich residues that require further treatment, and usually renders the land unusable for plant growth, since it removes all biological activities (Gomes et al., 2016). Chemical fixation is adding the reagents into the contaminated soil to form insoluble with heavy metals. This process decreases the migration of heavy metals to water, plant and other environmental media and achieving the remediation of soil. However, this technique can remediate the soil with low concentration contaminant. Also, the bioavailability

of fixed heavy metals may be changed with the environmental condition changing. Additionally, the use of chemical agents at some level could change the soil structure and have effects on the microbes in soil. A new technique is electrokinetic remediation. It is mainly applying voltage at the 2 sides of soil and then forming electric field gradient. The contaminant is carried to 2 poles treatment room via electromigration, electroosmotic flow or electrophoresis and then further treated (Yao et al., 2012). In addition, chemical approach always creates secondary pollution problems, generate large volumetric sludge and increase costs (Gomes et al., 2016).

2.3.3 Biological approach

This approach is considered to be the most adequate, since it is natural ecological process that does not impact the environment (Gomes et al., 2016). It consists of bioremediation (using microorganisms such as bacteria, yeast, algae and fungi) and phytoremediation (using plant) (Bhatnagar and Kumari, 2013). There are many methods for bioremediation such as compositing, land formation, bioreactors, bioventing, biofilters, biostimulation, bioaugmentation (Ullah et al., 2015). Although, microorganisms cannot degrade and destroy heavy metals, however, they can affect the migration and transformation through changing their physical and chemical characterizations. The remediation mechanisms include extracellular complexation, precipitation, oxidation-reduction reaction and intracellular accumulation (Yao et al., 2012). The methods in this approach have advantages over physical and chemical approaches because they preserve natural soil properties, low cost and have high public acceptance (Ullah et al., 2015). Currently, lower animal such as earthworm shows the characteristics of adsorbing, degrading, migrating the heavy metals and thus removing and inhibiting heavy metal toxicity (Yao et al., 2012).

Phytoremediation basically refers to the use of plants to remediate a contaminated medium (Vamerali et al., 2010). In this case, plants can be both naturally occurring and genetically engineered (Pandey et al., 2016). In addition, the meaning of phytoremediation includes plants and associated soil microbes to reduce or clean up the concentrations or toxic effects of contaminants in the environment such as soil, water, and air (Ali et al., 2013; Ma et al., 2016). In biological processes, plants are used to uptake vast quantities of heavy metals, and store these metals in a

18

harvestable component (Ullah et al., 2015). Normally, plants generally handle the contaminants without affecting topsoil, thus conserving its utility and fertility, and improving soil fertility with inputs of organic matter (Ali et al., 2013). Finally, contaminated plants can be recycled or must be treated as hazardous waste by applicable regulations to eliminate and prevent potential risk (Gomes et al., 2016). Besides heavy metals, plants can be used for removal of radionuclides, and organic pollutants such as, polynucleic aromatic hydrocarbons, polychlorinated biphenyls, and pesticides (Ali et al., 2013). Among 3 approaches, phytoremediation is one of the safest, most innovative, and effective tools for the remediation of heavy metals (Ullah et al., 2015). Significantly, phytoremediation has low installation and maintenance cost, making it very suitable for developing country like Thailand.

2.4 Phytoremediation

The concept of phytoremediation has started from the past 300 years on wastewater discharge, but the idea of using plants to remove heavy metals and other compounds was first introduced in 1983 (Pandey et al., 2016). The term phytoremediation is formed by Greek word "phyto" (meaning plant) and Latin word "remedium" (meaning balance, able to cure or restore) (Vamerali et al., 2010; Gomes et al., 2016). This term was coined by Dr. Ilya Raskin in 1989 (Pandey et al., 2016). The main phytoremediation techniques are described as follows:

2.4.1 Techniques

There are many different techniques for cleaning up the contaminated environment, herein categorization based on the environmental media. For contaminated water, phytofiltration is used. It is the removal of pollutants from contaminated surface waters or waste waters by plants, which the contaminants are absorbed or adsorbed from contaminated water, minimizing their movement (Ali et al., 2013). It is divided into rhizofiltration (use of plant roots), blastofiltration (use of seedlings), caulofiltration (use of excised plant shoots) (Gomes et al., 2016). In rhizofiltration, contaminants surrounding the root zone are adsorbed or precipitated onto the roots, or absorbed into and accumulated in the roots (Tangahu et al., 2011).

19

For soil remediation, phytoremediation essentially comprises 5 different techniques as shown in Figure 2.1 (Favas et al., 2014).
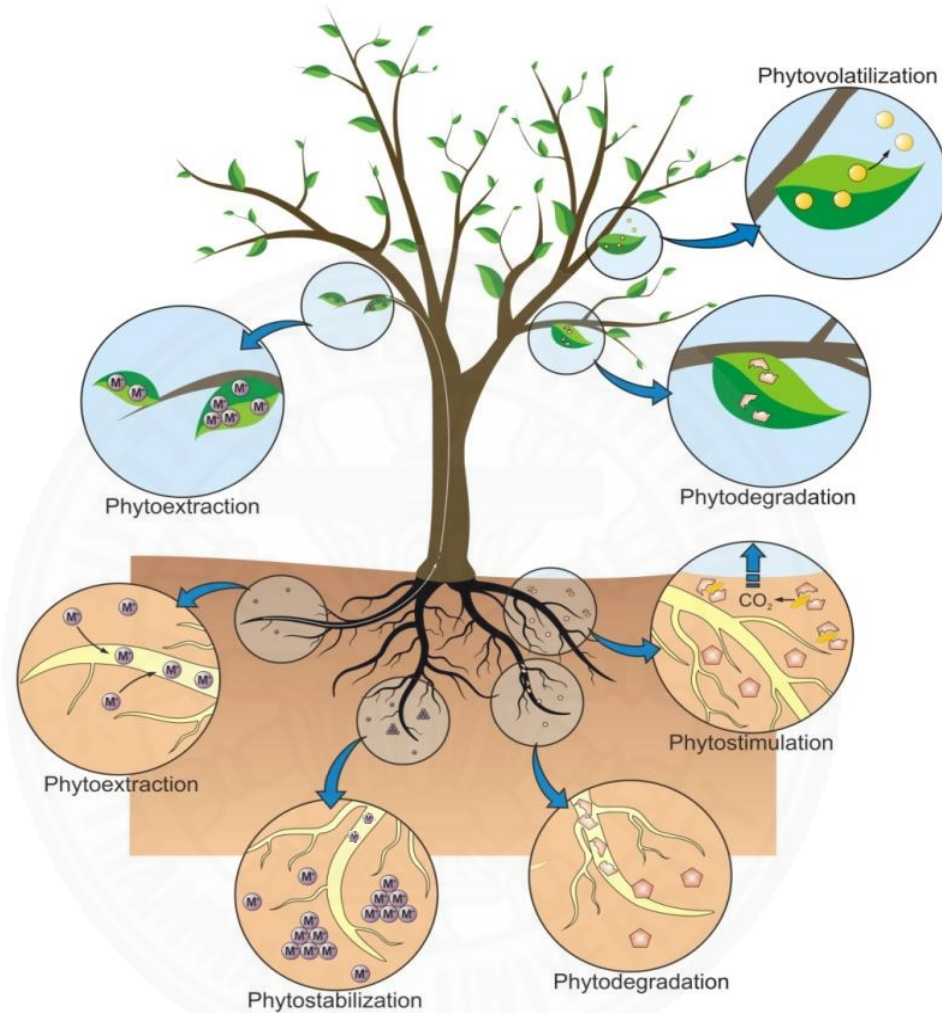
Figure 2.1 Phytoremediation techniques, modified from Favas et al. (2014).

2.4.1.1 Phytovolatilization (phytoevaporation)

This technique relies on the ability of some plants to absorb and volatilize both organic compounds and heavy metals having high volatility characteristic. Specifically, Hg, Se and As are absorbed by roots, converted into non-toxic forms, and then released into the atmosphere (Favas et al., 2014; Gomes et al., 2016). It is the most controversial of phytoremediation technologies (Ali et al., 2013).

2.4.1.2 Phytodegradation (Phytotransformation)

This technique uses to degrade (metabolize) or mineralize organic contaminants inside plant cells by specific enzymes. For instance, nitroreductases degrade nitroaromatic compounds (Favas et al., 2014). Hence, plants can be regarded as ''Green Liver'' for the biosphere. However, some plants can transform toxic heavy metal Cr^{6+} to less toxic substance Cr^{3+} (Gomes et al., 2016).

2.4.1.3 Phytostimulation (Rhizodegradation)

This technique is the breakdown of only organic contaminants in the rhizosphere where extends about 1 mm around roots by rhizosphere microorganisms. However, these organisms utilize exudates and metabolites of plants as a source of carbon and energy. In addition, plants may exude biodegrading enzymes themselves (Favas et al., 2014; Gomes et al., 2016).

2.4.1.4 Phytoextraction (Phytoaccumulation)

Other names are phytosequestration and phytoabsorption. It involves the absorption of contaminants (organics and heavy metals) by roots followed by translocation and accumulation in the aerial parts. In addition, translocation is desirable in an effective phytoextraction. This technique preferentially uses hyperaccumulator plants that have the ability to store high concentrations of specific metals in their aerial parts (Favas et al., 2014; Gomes et al., 2016).

2.4.1.5 Phytostabilization (Phytoimmobilization)

This technique uses plants to stabilize or reduce the mobility and bioavailability of organic and inorganic contaminants in the contaminated soil. These contaminants are incorporated into the lignin of the cell wall in root cells or into humus. Plants can immobilize heavy metals in soils through sorption by roots, precipitation as insoluble forms by direct action of root exudates, and subsequently trapped in the soil matrix (Favas et al., 2014; Gomes et al., 2016). Additionally, Pb accumulates in large quantities in the root tissue, which can be considered as a form of phytostabilization. In other cases, phytostabilization can be accomplished by metal immobilization outside the roots due to root exudates (Brunner et al., 2008).

21

Considering the impact to environment, phytostabilization may be more a feasible strategy for cleaning up the contaminated sites in the long period than phytoextraction because of lesser accumulation of toxic contaminants in aboveground biomass leading to reduce the risk for transferring to food chain (Handayanto et al., 2014). Furthermore, phytostabilization cost is generally lower than that of many remediation options, and it particularly suits to the large contamination area. As a bonus, soil organic carbon levels may eventually increase, thereby improving soil physical and chemical properties (Pierzynski et al., 2002).

2.4.1.6 Dendroremediation

The term of dendroremediation comes from the Ancient Greek dendron meaning "tree" and Latin remediare meaning "reuse". It is an emerging phytoremediation technology for cleaning up environment contaminated with organic or inorganic pollutants by using living trees (woody plant) to remove, sequester, or chemically decompose the pollutant (Mackova et al., 2006). A tree may be considered as a solar driven pump with treat system, which appears of great potential for the phytoextraction and phytostabilization of heavy metals polluted soils (Mackova et al., 2006; González-Oreja et al., 2008). Its efficiency has been proven in cleaning up the soils contaminated with heavy metals. Namely, tree has the lowest tolerant ability and moderate accumulating capacity, compared to herbaceous and bushes (Yanqun et al., 2004). Despite tree accumulates relatively lower amounts of heavy metals, it can be very effective for heavy metals phytoextraction and phytostabilization (Favas et al., 2010; Zárubová et al., 2012).

Currently, the cultivation of tree, especially fast-growing tree as a vegetation cover has been an increasing interest in phytostabilization with some advantages over herbaceous ground cover due to its properties: (1) a large biomass both above and below ground to accumulate high heavy metal contents; (2) a stable root system with deeper and more integrated for decreasing the risk of soil loss by wind and water erosion and increasing stabilization directly; (3) a permanent central self-supporting stem with wood for water and nutrient transport and storage of compounds and gases that may be important for efficient dendroremediation; (4) a highly efficient competitor for light, nutrients, and water and tend to dominate the

22

vegetation wherever conditions are favorable for plant growth; (5) a long lifespan and had evolved mechanisms to cope with variable biotic and abiotic stresses. For example, formation of wood can be viewed as an adaptive mechanism that enables trees to secure a dominant position in ecosystems; (6) leaf fall, dead roots, and root exudates to improve soil physical characteristics by adding organic matter to the soil for promoting nutrient cycling, soil aggregation, and water holding ability; and (7) the large amount of water removed from soil by transpiration decreases the downward flow through the soil, reducing leaching losses; (8) grow easily and resistant to drought or insects; and (9) the opportunity for input into the terrestrial food chain is less with trees, and they differentially accumulate metals in specific plant organs (Pierzynski et al., 2002; Pulford and Watson 2003; Mackova et al., 2006; Wang et al., 2014; Liu et al., 2015). However, establishing trees on mine spoil was affected by compaction, infertility (especially N deficiency), acidity, salinity and poor water-holding capacity (Pulford and Watson, 2003). For fast-growing trees, research is preferentially focused on poplars, birches, sugar maples, black alders, willow, English oaks, black locusts, (Pulford and Watson 2003; González-Oreja et al., 2008; Malá et al., 2010; Zhivotovsky et al., 2011).

Globally, there are many concerns such as increasing the contaminated sites, bioenergy demands, global warming, and associated climate change effects. The interest in energy crop has increased globally due to its potential use as carbon-neutral, clean and eco-friendly source of renewable energy. The promising energy crops for phytoremediation are *Miscanthus* species, *Ricinus communis* L., *Jatropha curcas* L., and *Populus* species (Pandey et al., 2016). Not surprisingly, linking dendroremediation with energy crop offers many benefits. Direct benefits are remediation of contaminants and bioenergy production. Indirect benefits are forestry, substrate quality enhancement, esthetically pleasant landscape, carbon sequestration, pulp and paper (which would help take pressure off natural forests), solid wood products (structural lumber, boxes, furniture components, interior trims, etc.), composite wood products, feed products, biochar, and biofortified products and so on (González-Oreja et al., 2008; Favas et al., 2010; Pandey et al., 2016). The bioenergy production may be used directly as heat or processed into gases or liquids (e.g., ethanol, biodiesel). However, these economic revenue opportunities should be

23

analyzed carefully considering and evaluating all possible environmental and health risks (Favas et al., 2010).

There are many fast-growing trees as energy crops in Thailand, but seven promising candidates with their properties have been promoted by the Government of Thailand as shown in Table 2.4.

Table 2.4 Fast growing trees with heating value and planting time

Species	Heating value (firewood) (cal/g)	Planting time (year)
Acacia auriculiformis Cumn.	4000-5000	2
Acacia mangium Wild	4800-4900	2
Senna siamea (Lam.)	4500	1
Pterocarpus macrocapus Kurz.	5022	5
Eucalyptus camaldulensis Dehnh	4500-4800	2
Azadirachta indica A.Juss	4244-5043	1-2
Azadirachta excelsa (Jack) Jacobs	4000-4500	2

Sources: Pothisat (2000)

2.4.2 Variables affecting success of phytoremediation

The success of phytoremediation depends on plant species, soil characteristics, form of heavy metals as well as the presence of microorganisms.

2.4.2.1 Plant species

The major tool of phytoremediation is plant, and the uptake of a compound is affected by plant species characteristics (Tangahu et al., 2011). In addition, heavy metals concentrated in plants growing in the same soil vary between species and even between genotypes of a species (Laghlimi et al., 2015). Therefore, the judicious selection of plant species is an important step for success (Zhivotovsky et al., 2011). Principally, plant's ability to survive in the climate of geographic region at a given site is an absolute requirement. Hence, a native species to the site should be firstly considered (Gerhardt et al., 2017). Since native species require less management, eliminate eco-logical risks associated with introducing an exotic plants

to an ecosystem, and well adapt to the climate and soil conditions, as well as pests and other stressors at the site (Sarma, 2011; Gerhardt et al., 2017). However, some exotic plants may perform better in remediation of specific metals and can be safely used where the possibility of invasive behavior has been eliminated (Sarma, 2011). Furthermore, other criteria in selecting plants are ability to tolerate, accumulate, translocate, and uptake the contaminants and co-contaminants, ability to grow in the poor soils as well as badly conditions such as water logging and drought, and preference of habitat such as terrestrial or aquatic (Sarma, 2011; Zhivotovsky et al., 2011; Gerhardt et al., 2017). Moreover, plant roots can influence heavy metal phytoavailability by modifying the soil properties in the rhizosphere that facilitate uptake by plant (Laghlimi et al., 2015).

2.4.2.2 Characteristics of soil

The amount of Pb absorbed by plants is affected by pH, organic matter, soil texture, macronutrient levels, phosphorus (P), clay and available water content, cation exchange capacity (CEC), redox status, and temperature and $CaCO_3$ of the soil (Alloway, 2013; Tangahu et al., 2011; Laghlimi et al., 2015). In addition, microbial conditions, amount of Pb present, inorganic ligand levels, competing cation levels, and plant species involved. Such factors may act individually or in combination with each other and may alter the soil behavior of the Pb present, as well as the rate of uptake by plants (Pourrut et al., 2011). Soil pH, heavy metal cations are the most mobile under acidic conditions, while anions tend to sorb to oxide minerals. At low pH, heavy metal bioavailability increases as more heavy metals are released into the soil solution due to competition with H^+ ions. At high pH, cations precipitate or adsorb to mineral surfaces and metal anions are mobilized. At neutral or alkaline pH, most of the metals in soil are not available to plants. Soil texture reflects the particle size distribution, and the content of fine particles like oxides and clay. Fine particles (< 100 μm) are more reactive and have a higher surface area for containing heavy metals than coarser material. For example, the fine textured soils contain 3889 mg/kg of Pb, while coarse textured soil contains 530 mg/kg of Pb. Soil organic matter is frequently reported to have a dominant role in controlling the behavior of trace metals in the soil. The organic matter may reduce the ability of metals to be

25

phytotoxic in the soil due to metal-organic complexation. The presence of organic carbon increases the cation exchange capacity of the soil which retains nutrients assimilated by plants. Increasing the amount of organic matter in the soil helps to minimize the absorption of heavy metals by plants. Land rich in organic matter actively retains metallic elements. Soils with relatively low organic matter concentration are more susceptible to contamination by trace elements. Compost amendments to contaminated soils containing labile elements reduce the overall bioavailabilities of metals due to sorption processes. Redox potential is established by oxidation-reduction reactions resulting from microbial activity. It converts heavy metal speciation to non-hazardous that is more stable, less mobile and/or inert (Laghlimi et al., 2015).

2.4.2.3 Characteristics of heavy metals

The chemical speciation of heavy metals determines their bioavailability. It is related to the different natures of the metals, their bonding strength, and either in free ionic form or complexes by organic matter, or incorporated in the mineral fraction of the sample. Forms of occurrence of heavy metals in soil significantly influence their mobility. The most mobile elements include Cd, Zn and Mo, while the least mobile are Cr, Ni and Pb. In soils with low pH, heavy metal mobility decreases in the order: $Cd > Ni > Zn > Mn > Cu > Pb$ (Laghlimi et al., 2015). Since, the absorption of Pb may become significant at approximately pH 3-5 (Rieuwerts et al., 1998).

2.4.3 Management of toxic plants after phytoremediation

The contaminated plants of phytoremediation are classified as hazardous waste that needs to be managed in compliance with applicable regulations to prevent potential risk (Gomes et al., 2016). There are many options relied on contaminants type. If toxic plants accumulate the economically valuable heavy metals like Au, Pt and Tl, they are recycled and operated via smelting, pyrolysis of biomass, or phytomining process (Sheoran et al., 2013; Gomes et al., 2016). The process of phytomining is shown in Figure 2.2 But if not, the contaminated plant can be disposed as hazardous waste safely in specialized dumps (Ali et al., 2013).

26
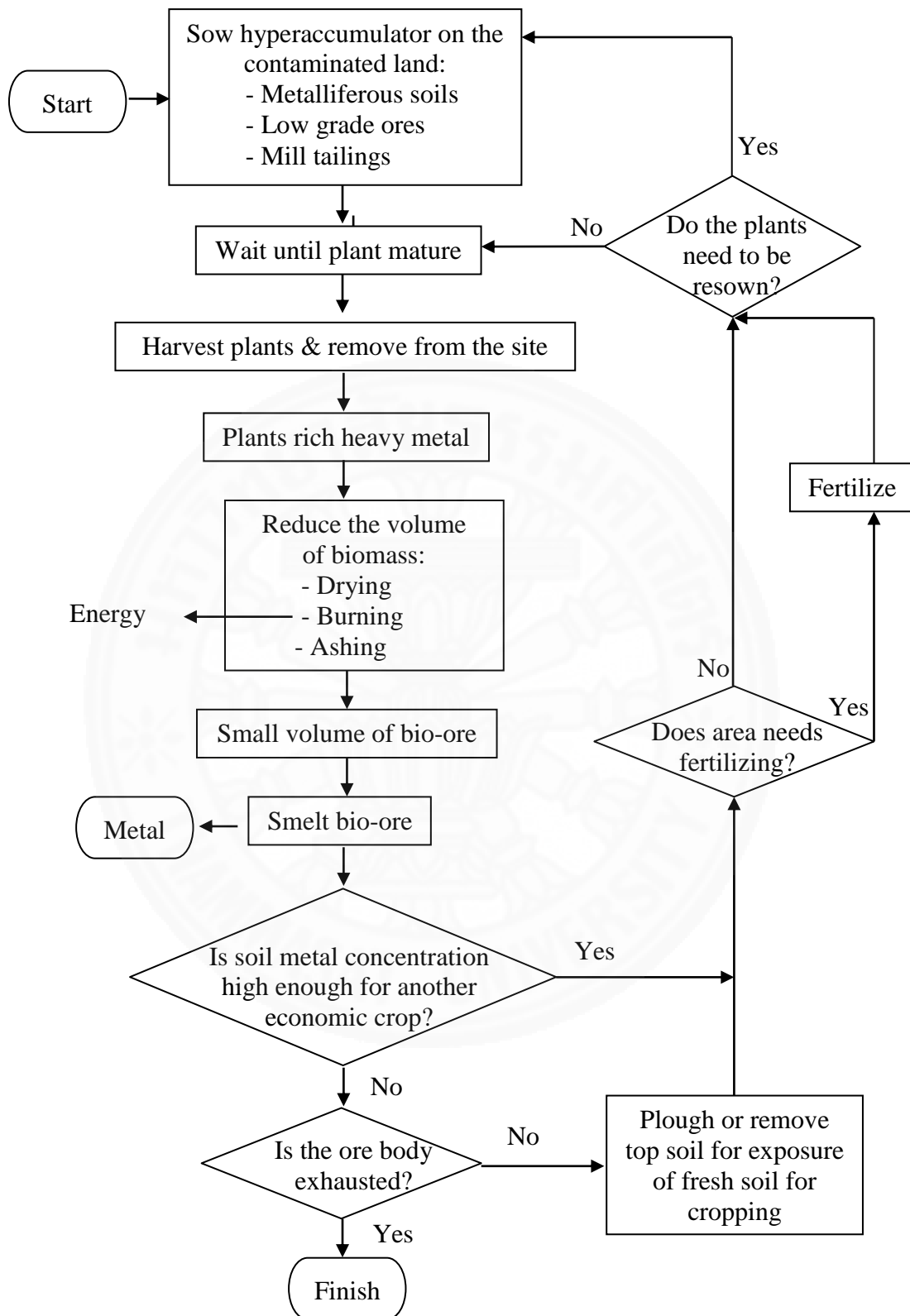
Figure 2.2 Phytomining process, modified from Brooks et al. (1998)

Source: Sheoran et al. (2009)

Importantly, the volume of these toxic plants needs to be reduced through various techniques such as composting, compaction, combustion, incineration, and gasification in order to handle it safely such as disposing in a hazardous waste landfill (Gomes et al., 2016; Mohanty, 2016). If plants are contaminated by radioactive compounds, they must be disposed as radioactive waste. Moreover, importantly, knowledge about the life cycle of plant needs to be cared for proper disposal of the toxic biomass, otherwise they will become the secondary source of heavy metal pollution (Gomes et al., 2016).

2.4.4 Advantages and limitation of phytoremediation

Phytoremediation has several advantages, but it also has many limitations as shown in Table 2.5.

Table 2.5 Summary of advantages and limitations of phytoremediation

No.	Advantages	Limitations
1	Eco-friendly and collateral impacts	Long time remediation
2	Increase vegetation growth, provide habitat for animal life	Limited to shallow soils or where contamination is localized to a surface
3	Reduction in dispersal of contaminants by wind, reduction of surface runoff	Still under development and so not accepted by many regulatory agencies
4	Reduction of leaching, mobilization of contaminants in soil	There is little knowledge of genetics, diseases of phytoremediation plants
5	*In situ* application in many ecosystems contained more contaminants	To increase the mobility of the metals, these can be leached into groundwater
6	High acceptance by the public	Management of toxic plant waste
7	Harvesting of the treated plants is easy to achieve with existing technology, and they can be economically valuable	Contamination may spread through the food chain if accumulator plants are ingested by animals
8	Plant process more easily controlled than those of microorganisms	Toxicity and bioavailability of degradation products remain unknown

All data are modified from Ali et al. (2013); Favas et al. (2014); Gomes et al. (2016)

28

Generally, phytoremediation cost is accepted in the range 25-100 USD/ton for phytoremediation, while that is 150-350 USD/ton for excavation and landfill (Gerhardt et al., 2017). González-Oreja et al. (2008) reviewed that the total cost for some phytoremediation schemes would be between 50% and 80% lower than other options. The cost of excavating and landfilling Pb-polluted soils increased to $500/acre, whereas a 50% to 65% saving ($150-250/acre) for phytoextraction.

2.5 Heavy metal removal by plants

Generally, plants can be compared to a solar driven pump that can extract and concentrate several elements from their environment (Memon et al., 2001). This process involves many mechanisms such as mobilization, uptake, translocation and sequestration (Figure 2.3). The uptake and translocation of a pollutant in the trees depends on many factors: (1) the pollutant's concentration in the soil solution, (2) its efficiency to enter the root system, and (3) the rate of transpiration in the plants. Trees are known to take up large amounts of water loss from the leaf surface in the transpiration stream. For example, mature poplar trees can transpire 200-1000 L of water per day (Mackova et al., 2006).

2.5.1 Metal mobilization

Efficacy of dendroremediation strongly depends on the bioavailability of the pollutant. Metal ions are mobilized by secretion of chelators and by acidification of the rhizosphere. Pb has low solubility and availability for plant uptake, because it forms precipitates with phosphates, sulfates, and chemicals in the rhizosphere. These forms of Pb in soils affect its solubility that directly influences its mobility (Fahr et al., 2013). Nevertheless, plants have certain mechanisms for solubilizing heavy metals in soil. Plants secrete metal-mobilizing substances such as phytosiderophore, H^+, root exudates, and several metabolites (Fahr et al., 2013; Ali et al., 2013). H^+ can acidify the rhizosphere and increase metal dissolution by displacing heavy metal cations adsorbed to soil particles. Root exudates can lower the rhizosphere pH generally by one or two units over that in the bulk soil thereby promoting heavy metals desorption (Ali et al., 2013). Citric, fumaric, uronic acids,

29

and polysaccharides can form complexes and to chelate Pb becoming soluble metal-organic complexes (Fahr et al., 2013). In addition, the rhizospheric microorganisms may significantly increase the bioavailability of heavy metals in soil. Interactions of microbial siderophores can increase labile metal pools and uptake by roots (Ali et al., 2013). Also, Pb availability is increased by the presence of Zn and Cu due to the antagonistic interaction between Cu and Zn, which made Pb more available for plant uptake (Fahr et al., 2013).
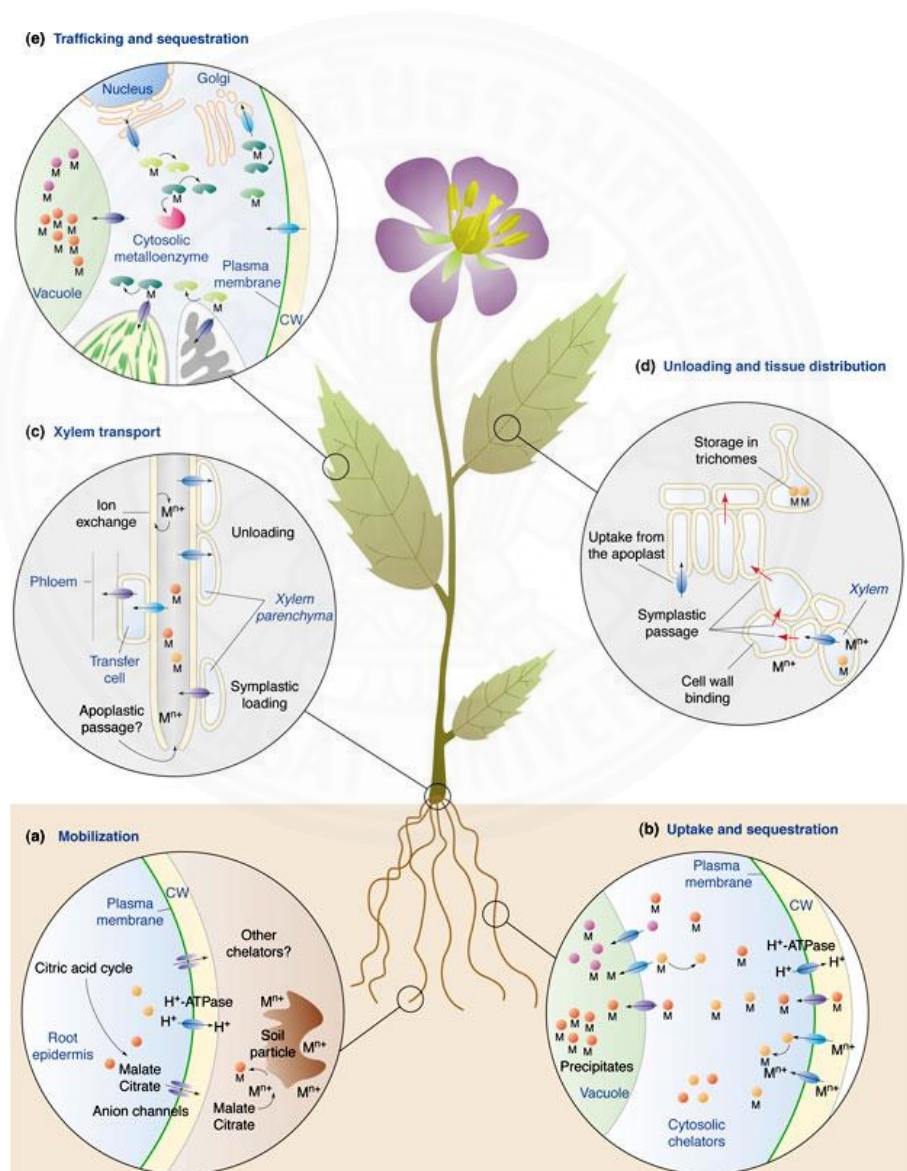
Figure 2.3 Mechanisms involved in heavy metal uptake and accumulation by plants. CW, cell wall; M, metal; filled circles, chelators; filled ovals, transporters; bean-shaped structures, metallochaperones. Source: Clemens et al. (2002)

30

2.5.2 Metal uptake

Plant uptake is the first step in whole plant metabolism of contaminants (Weyens et al., 2009). Uptake of hydrated metal ions or metal-chelate complexes is mediated by various uptake systems residing in the plasma membrane. Inside the cell, metals are chelated and excess metal is sequestered by transport into the vacuole (Clemens et al., 2002). Following mobilization, heavy metal ions must be captured by root cells by first binding at cell wall acting as ion exchanger. Then, these ions are transported across the root membrane by passive uptake (apoplastic pathway; driven only by the concentration gradient across the membrane). The other one is an active uptake (symplastic pathway; inducible substrate-specific and energy dependent uptake mediated by membrane protein with transport functions such as a proton pump) (Ghosh and Singh, 2005). Pb uptake is a non-selective phenomenon (Pourrut et al., 2011). Soluble Pb from soil transported to root cells has to cross the root cell plasma membrane through a channel (Shamar and Dubey, 2005). Plants possess various families of plasma membrane transporters involved in metal uptake and homeostasis such as P_{1B}-ATPase, the natural resistance-associated macrophage protein (NRAMP), cation diffusion facilitator (CDF) family, and ZRT, IRT-like protein (ZIP) families (Furini, 2012). But, Pb has no specific transporters. It enters the cells through cation transporters normally through Ca^{2+} channels (Shamar and Dubey, 2005; Furini, 2012). In addition, at the root surface, available Pb in the soil solution is adsorbed onto the roots by binding to carboxyl groups of mucilage uronic acid, or directly to the polysaccharides of the rhizoderm cell surface. Following adsorbing, Pb may enter the roots passively and follow translocating water streams (Pourrut t et al., 2011). Pb uptake and tolerance depends on root system conditions. The adventitious root system is more tolerant to Pb than the primary root systems because of its mechanism protect them against Pb penetration and Pb-induced oxidative stress (Fahr et al., 2013).

2.5.3 Pb translocation

From the roots, transition metals are transported to the shoot via the xylem. Presumably, the larger portion reaches the xylem via the root symplast. Apoplastic passage might occur at the root tip. Inside the xylem, metals are present as

hydrated ions or as metal-chelate complexes (Clemens et al., 2002). Normally, heavy metal translocation involves proton pump, co-transporters and channels (Gomes et al., 2016). Following uptake, the next step is either localization in root or translocation to other parts mainly by the xylem. Generally, only a small fraction of Pb is translocated to aerial plant parts. When entering the root, Pb mainly moves by apoplast, and follows water streams. Until Pb reaches the endodermis where Pb is blocked in this layer by the Casparian strip and must follow symplastic transport. Thus, the endodermis acting as a physical barrier plays a vital role in this phenomenon (Pourrut et al., 2011). Because the Casparian strip is a waxy coating which is impermeable to solutes (Ghosh and Singh, 2005). After penetrating the central cylinder of the stem, Pb can be transported via the apoplastic pathway again. Once heavy metal is loaded into the xylem, the translocation process will occur via vascular flow driven by transpiration (Pourrut et al., 2011). This process makes the uptake system different from others. Because it acts as a phytopumping or life pump which is responsible for moving compound into the shoots as well as leading to automatic cycle of uptake (Tangahu et al., 2011). After translocating to leaves, heavy metals must be loaded into the leaf cells by crossing membrane again, where metals can be stored in various cell types. After reaching the apoplast of the leaf, metals are differentially captured by different leaf cell types and move cell-to-cell through plasmodesmata. Uptake into the leaf cells again is catalyzed by transporters. Intracellular distribution of essential transition metals or trafficking is mediated by specific metallochaperones and transporters localized in endomembranes (Clemens et al., 2002).

2.5.4 Pb accumulation

The content of Pb in plant organs tends to decrease in the following order: roots > leaves > stem > inflorescence > seeds (Shamar and Dubey, 2005). About 95% of Pb accumulates in roots (Fahr et al., 2013). Amount of Pb deposits are present mainly in the intercellular space, cell wall, and vacuoles, whereas small deposits of Pb are seen in the endoplasmic reticulum, dictyosomes, and dictyosome derived vesicles. The cell wall and vacuole together account for about 96% of absorbed Pb (Shamar and Dubey, 2005). Storage appears to occur preferentially in trichome (Clemens et al., 2002). As this biomass consist amount of heavy metals, fate

of these metals should be studied before using it for various aspects. Recently, the phytoaccumulated biomass has been suggested to produce the synthetic natural gas and bio-energy (Pandey et al., 2016). Mechanisms of Pb accumulation are associated with Pb tolerance. Hence, the mechanisms of accumulation are described in Pb tolerance section.

2.6 Mechanisms of Pb resistance in plants

The other factor influencing the success of phytoremediation is the specific mechanism of stress avoidance and stress tolerance in plants resulting from the presence of high concentrations of heavy metals in soil (Małachowska-Jutsz and Gnida, 2015). Avoidance is an organism's ability to prevent excessive metal uptake into its body, while tolerance is an organism's ability to cope with heavy metals that are excessively accumulated within its body (Shaw, 1990).

2.6.1 Avoidance mechanisms

These mechanisms involve in reducing of plant uptake. The earliest mechanism is a synthesis of polysaccharide such as callose (β-1, 3 glucan) deposited on the outside of the cell membrane, thereby reducing the diffusion of heavy metal ions into the plant cell. As a first line of defense against heavy metals, plant roots secrete exudates into the soil matrix to chelate metals and to prevent their uptake inside the cells (Furini, 2012). The root exudates may contain several substances such as organic acids, simple sugars, phenols, amino acids and polysaccharide gels, that can bind with heavy metal ions, thereby reducing their absorption by plants. (Małachowska-Jutsz and Gnida, 2015). For example, oxalate compound can bind with Pb (Sharma and Dubey, 2005). Moreover, microorganisms in the rhizosphere such as arbuscular mycorrhizae fungi can immobilize heavy metals within the mycelium and inhibit their movement to plant tissues (Małachowska-Jutsz and Gnida, 2015). Also, Pb binding with carboxy groups of mucilage uronic acids restricts uptake of Pb into the root (Sharma and Dubey, 2005). Synthesis of small molecules such as carboxylic acids: malate, citrate, or oxaloacetate that bind heavy metals with their acid groups.

33

These carboxylic acids may bind heavy metals outside the roots or in the root apoplast which may prevent uptake of heavy metals (Pourrut et al., 2011).

2.6.2 Tolerance mechanisms

In some cases, heavy metal ions may overcome the plants protective barrier and penetrate the cells (Małachowska-Jutsz and Gnida, 2015). In this situation, plants can cope with heavy metals by accumulating or detoxifying them within plant cells through many mechanisms. These are divided into passive and inducible mechanism, and antioxidant systems (Pourrut et al., 2011).

2.6.2.1 Passive mechanisms

A sequestration of Pb in plant cell wall is one of the most important defense strategies of plants to cope with Pb. Cell wall acting as a barrier which limits the amount of Pb entering the protoplast. Also, it may accumulate metal ions which have already entered the cell and have been removed from the protoplast by secretion pathway and exocytosis. To capture Pb in the cell wall, plant uses 2 mechanisms. Firstly, Pb binds with negatively charged pectins on the cell wall (Pourrut et al., 2011). Pectin is component of plant cell walls considered as the main compounds responsible for binding Pb^{2+} in cell wall. It is a polysaccharide containing galacturonic acid molecules and many of the methylated ($COOCH_3$) carboxyl groups, hemicelluloses and cellulose. These compounds contain numerous negatively charged carboxyl groups which are typically saturated with calcium. Heavy metals (Cd^{2+}, Pb^{2+}, Cu^{2+}) can competitively replace calcium ions (Małachowska-Jutsz and Gnida, 2015). Secondly, extracellular Pb precipitation that mainly is in the form of Pb carbonate can deposit Pb in the cell wall (Pourrut et al., 2011).

2.6.2.2 Inducible mechanisms

This mechanism involves in exiting Pb in cell induces express of biomolecules to cope with Pb (Pourrut et al., 2011). Thiol compounds such as glutathione (GSH), phytochelatins (PCs), and metallothioneins (MTs) contain sulfhydryl (-SH) groups for binding a variety of metals (Anjum et al., 2015). GSH (γ-glu-cys-gly) is the most common low molecular weight thiol compound in nature

34

(Małachowska Jutsz and Gnida, 2015). It is known to be non-enzymatic antioxidants in plants. GSH protects plants from Pb stress by quenching Pb-induced ROS. In addition, as the precursor of PC, GSH-related proteins play an important role in heavy metal detoxification and homeostasis (Pourrut et al., 2011).

PCs and MTs are the best characterized metal-binding ligands involved in the cellular detoxification and accumulation of Pb in plant cells. They belong to different classes of cysteine-rich heavy metal binding protein. These thiols are biologically active compounds, whose function is to prevent oxidative stress in plant cells. Their general structure is (γ-glutamyl-cys)n-Gly where n = 2-11, and they are synthesized by the action of γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase; PCS) from GSH. PCs are low molecular weight, and can form stable mercaptide bonds with Pb and sequester soluble Pb in the cytoplasm before transporting it to vacuoles and chloroplasts. This process can reduce the deleterious effect of Pb^{2+} in the cells. However, the mechanism regulating the passage of the Pb-PCs complex through the tonoplast is not yet known (Pourrut et al., 2011). In plants MT may participate in metal homeostasis, metal detoxification senescence and protection against abiotic stress (Małachowska-Jutsz and Gnida, 2015). Besides, Pb can induce gene of transporter proteins. These proteins play an important role in metal detoxification, by allowing the excretion of metal ions into extracellular spaces (Pourrut et al., 2011). Chaperones are special proteins that transport ions into places in the cell, where they are incorporated into molecules such as enzyme (Małachowska-Jutsz and Gnida, 2015). In addition, the human divalent metal transporter 1 (DMT1), expressed in yeast, has been shown to transport Pb via a pH-dependent process in plants. Simultaneously, several ATP-binding cassette (ABC) carriers, at ATP-binding sites in *Arabidopsis*, were involved in resistance to Pb (Pourrut et al., 2011).

2.6.2.3 Antioxidant enzyme

To cope with the increased production of reactive oxygen species (ROS) and to avoid oxidative damage due to heavy metal, plants have a system of antioxidant enzymes that scavenge the ROS. There are several types of antioxidants such as non-protein thiol (NP-SH), cysteine, glutathione, ascorbic acid, proline. Antioxidant enzymes are also used, such as superoxide dismutase (SOD), ascorbate

peroxidase (APX), guaiacol peroxidase (GPX), catalase (CAT), and glutathione reductase (GR) (Pourrut et al., 2011).

2.6.2.4 Other mechanisms

Another strategy is the ability of plants to remove excess heavy metals in the form of crystals by salt glands of the leaf epidermis, hydathodes, or ectoderms. Heavy metals may also be transported to the ageing leaves and removed along with them (Małachowska-Jutsz and Gnida, 2015).

2.7 Metallophytes

Metallophytes can be defined by their ability to survive and reproduce on metal-rich soils without suffering toxicity (Alford et al., 2010). They evolve biological mechanisms to permit them to tolerate toxic concentrations of metals (Whiting et al., 2004; Batty, 2005). They are indicator, excluder, and (hyper) accumulators classified by a response strategy of them to heavy metal concentration in soils (Ali et al., 2013; Hunt et al., 2014). Shaw (1990) suggests that there are three patterns of heavy metals uptake related to metallophyte types as shown in Figure 2.4. Firstly, indicator strategy, where uptake of metals and transport to the shoot are regulated or passive uptake occurs so that internal concentrations accurately reflect external concentrations. Secondly, excluder strategy, where uptake data for plant sampled over a wide range of soil metal concentrations, suggest shoot concentrations are maintained at a constant low level until a critical soil concentration is reached when toxicity ensues and unrestricted metal transport results. Thirdly, hyperaccumulator strategy, where metals are actively concentrated with in plant tissues over the full range of soil concentration, implies a highly specialized physiology.
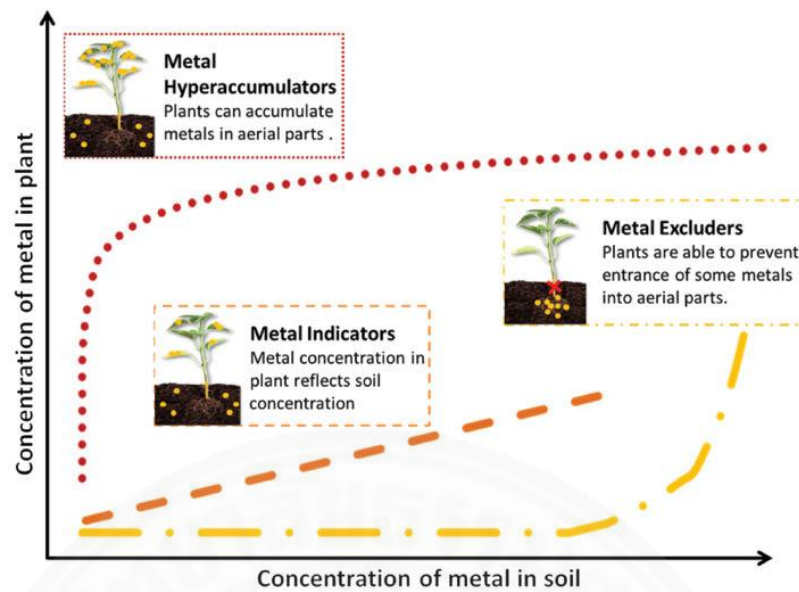
Figure 2.4 Conceptual strategies of plants to an increasing metal concentration in soil: hyperaccumulation (red line), metal indication (orange line) and exclusion (yellow line). Source: Hunt et al. (2014).

2.7.1 Indicator

Indicator accumulates heavy metals in above ground tissues to levels similar to those in the surrounding soil making the relationship between the concentration of metal in the plant and soil generally linear (Hunt et al., 2014). The heavy metal uptake and transport to the shoot by indicators are regulated so that internal concentrations accurately reflect external concentrations (Shaw, 1990). The results from plant survey showed that a Pb indicators are *Agave sisalana* Perr., *Ludwigia stolonifera* (Ghill. and Pers) Raven, *Pluchea dioscoridis* (L) DC., and *Sphaeranthus kirkii* Oliv. (Mganga et al., 2011). Accumulators accumulate in their above ground tissue to levels far exceeding those present in the soil, or in the non-accumulating species growing nearby (Memon et al., 2001; Ali et al., 2013).

2.7.2 Excluder

Excluder avoids importing metals to aerial part, however, they may still contain relatively high amounts of metals in roots (Hunt et al., 2014). They uptake heavy metals over a wide range of heavy metal concentration in the soil, until a critical soil concentration is reached and unrestricted metal transport results (Shaw,

37

1990). In addition, the certain conditions for defining the excluders are high BCF ($\geq$ 1), low BAC and TF ($\leq$ 1), and they have potential for phytostabilization as stabilizers (Nirola et al., 2015). Mendez and Maier (2008) reviewed that plants showing potential for Pb phytostabilization from plant survey are *Schinus molle* L., *Bidens humilis* H.B.K., *Isocoma veneta* (Kunth) Greene, *Teloxys graveolens* (Willd.) W.A. Weber, *Atriplex canescens* (Pursh) Nutt., *Euphorbia* sp., *Dalea bicolor* Humb. & Bonpl. ex Willd., *Lygeum spartum* L., *Piptatherum miliaceum* (L.) Coss. In addition, *Cyperus exaltatus* L. and *Hygrophylla auriculata* (Schumach) Heine also are Pb excluder from plant survey (Mganga et al., 2011).

2.7.3 Hyperaccumulator

Currently, there are many definitions of hyperaccumulator. They are generally defined as a plant growing in nature and containing metals in leaves at a concentration over a specified threshold. The threshold is 2-3 orders of magnitude higher than in leaves of most species growing on normal soils. Also, the threshold is at least one order of magnitude greater than the usual range found in plants growing on metalliferous soils (Pollard et al., 2014). In addition, the classic criteria of hyperaccumulators with unit of (μg metals/g of dry leaf tissue) are 100 for Cd, Se and Tl; 300 for Co, Cr and Cu; 1000 for As, Ni and Pb; 3000 for Zn and 10000 for Mn (van der Ent et al., 2013; Pollard et al., 2014). Moreover, hyperaccumulators are plants that can be satisfied by certain conditions, such as bioconcentration factor (BFC: root:soil ratio > 10, but BCF > 1 identified as accumulator), biological absorption coefficient (BAC: shoot:soil ratio > 1), and translocation factor (TF: shoot:root ratio > 1)   (Yanqun et al., 2005; Vamerali et al., 2010; van der Ent et al., 2013; Salazar and Pignata, 2014). Additionally, they are normally used in phytoextraction (Vamerali et al., 2010; Ali et al., 2013). For example, plants are suitable for phytoextraction such as *Brassica campetris* L.; *B. carinata* A. Braun; *B. juncea* L.; *B. napus* L.; *B. nigra* (L.) Koch.; *Helianthus annuus* L.; *Pisum sativum* L.; *Zea mays* L. (Prasad and Freitas, 2003). Particularly, Pb hyperaccumulators are quite rare (about 14 species out of 500 known hyperaccumulators) when compared to those of Ni (450 species). However, approximate number of Pb hyperaccumulators is similar with Cu (32), Co (30), Se (20), Zn (12), Mn (12), As (5), Cd (2), and Tl (2)

(van der Ent et al., 2013). The examples of Pb hyperaccumulators studied by field survey are listed in Table 2.6.

Table 2.6 Pb hyperaccumulators from field survey

No.	Species	Pb contents (g/kg)	References
1	*Ambrosia artemisiifolia* L.	> 2	Mahmood (2010)
2	*Armeria maritima* (Mill.) Willd.	1.3	Baker and Brooks (1989)
3	*Arrhenatherum elatius* (L.) P. Beauv. ex J. Presl & C. Presl	24	Mahmood (2010)
4	*B. juncea*	> 100	Mahmood (2010)
5	*Brassica pekinensis* (Lour.) Rupr.	> 7	Mahmood (2010)
6	*Euphorbia cheiradenia* Boiss. & Hohen.	1.1	Ali et al. (2013)
7	*Fagopyrum esculentum* Moench	4.2	Mahmood (2010)
8	*H. annuus*	> 100	Mahmood (2010)
9	*Minuartia verna* (L.) Hiern [excluded]	>1	Prasad (2004)
10	*P. sativum*	> 6	Mahmood (2010)
11	*Polycarpaea synandra* F.Muell.	1.0	Mahmood (2010)
12	*Sedum alfredii* Hance	Up to 1.2	Mahmood (2010)
13	*Sesbania drummondii* (Rydb.) Cory	> 40	Sahi et al. (2002)
14	*Solidago bicolor* L.	> 2	Mahmood (2010)
15	*Thlaspi alpestre* J. et C. Presl	2.7	Mahmood (2010)
16	*Thlaspi rotundifolium* L. Gaudin subsp. cepaeifolium (Wulf.) Rouy et Fouc	8.2	Mahmood (2010)
17	*Z. mays*	> 2	Mahmood (2010)
18	*Epilobium denticulatum* Ruiz & Pav.	14	Bech et al. (2014)
19	*Taraxacum officinalis* (L.) Weber ex F.H. Wigg	2.5	Bech et al. (2014)
20	*Trifolium repens* L.	2.8	Bech et al. (2014)

2.8 Strategies for improving phytoremediation efficiency

Although some of hyperaccumulators, such as *Alyssum serpyllifolium* Desf, *Phytolacca americana* L., *Thlaspi caerulescens* J.Presl & C.Presl, *Solanum nigrum* L. and *Sedum plumbizincicola* X.H. Guo et S.B. Zhou ex L.H. Wu are known to take up high concentrations of heavy metals, most of them are not suitable for heavy metal phytoremediation because of their very low biomass production, slow growth rates and preference for selected metal (Ma et al., 2016). Thus, the ability of plants needs to be developed. Otherwise, the contaminated soils should be modified so that contaminants can be easily removed by plants. The following approaches used to improve phytoremediation can be performed alone or in combination. For example, Pb extraction by bacteria (*Desulfuromonas palmitatis*) and EDTA increased 10%, compared to using EDTA alone (Karami and Shamsuddin, 2010).

2.8.1 Increasing the bioavailability of heavy metals

Some chemicals (surfactants and ligands) may increase phytoextraction or phytodegradation of pollutants through the enhancement of bioavailability of organic and inorganic compounds in media. Decreasing the pH to values below 5.5 can enhance availability of Pb (Butcher, 2009). Normally, decreasing pH of soils can be done by adding chelating reagents (EDTA). However, they cause not only many potential risks, but also toxic to plants and to soil microorganisms (Saifullah et al., 2009; Rajkumar et al., 2012). Charcoal, a wood biochar which is a carbonaceous porous substance synthesized in result of pyrolysis of organic feed stocks such as plant materials, organic manures and sludges, is the most common biochar used for sorption of metals (Sarwar et al., 2017). Increasing the electrode potential (Eh) can also enhance the bioavailability of heavy metals in soil solution (Karami and Shamsuddin, 2010).

2.8.2 Increasing plant growth

Appropriate application of fertilizers and irrigation also is beneficial to phytoremediation. The more biomass, the more metal accumulation (Karami and Shamsuddin, 2010).

40

2.8.3 Decreased phytoremediation period

This could also be achieved by transferring the seedlings to the field so as to decrease the duration of phytoremediation (as the accumulation of heavy metal in plant shoots at flowering stage is high). Phytoremediation cycle can be decreased by providing specific demands of respective species such as the adjustment of light, temperature, and CO_2 (Karami and Shamsuddin, 2010).

2.8.4 Genetic engineering

Genetic engineering can enhance plant's phytoremediation ability towards the removal or detoxification of hazardous pollutants in the environment. This technique is based on the overexpression of specific genes involved in uptake, translocation, sequestration and plant tolerance of xenobiotic compounds in transgenic plants (Sarwar et al., 2017).

2.8.5 Microorganism-assisted phytoremediation

Among strategies for enhancing phytoremediation, using of microorganism is one of the most environmentally friendly techniques considered as a useful process in phytoremediation (Karami and Shamsuddin, 2010). Mycorrhizal fungi are major component of living organisms in the root zone and live in associations with most of the higher plants in different forms. Especially, arbuscular mycorrhizal fungi associations with the roots of terrestrial plants being the most widespread used (Sarwar et al., 2017). The principal role of mycorrhizal fungi is to improve the uptake of P and mineral nutrients for plants and enhance the number and length of root branch. Plant growth promoting bacteria (PGPB) is another important microbial community helpful for plants to remediate heavy metal contaminated soils (Karami and Shamsuddin, 2010). A number of limiting factors with respect to phytoremediation such as heavy metal solubility, level of contamination, and soil chemistry can be reduced by PGPB as shown in Figure 2.5. This indicates that PGPB assisted plant to accumulate higher amount of heavy metals and grow better (Ullah et al., 2015). PGPB also exhibit the capacity to tolerate high concentration of contaminants (Santoyo et al., 2016).

41
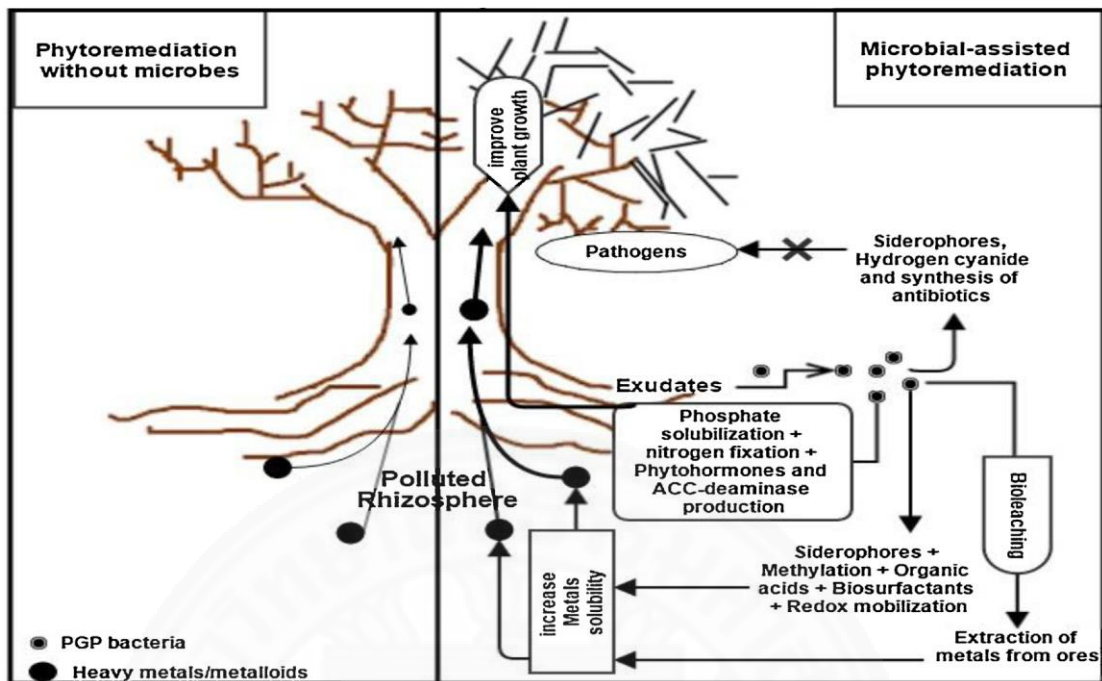
Figure 2.5 Phytoremediation comparison between with and without microbial PGPB

Source: Ullah et al. (2015).

PGPB normally are *Acinetobacter* sp., *Azospirillum amazonense*, *Bradyrhizobium* sp., *Enterobacter asburiae*, *Klebsiella* sp., *Mesorhizobium* sp., *Ochrobactrum cytisi*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *P. fluorescens*, *P. jessenii*, *P. putida*, *Psychrobacter* sp. SRS8, *Ralstonia metallidurans*, *Serratia marcescens* and *Rhizobium* sp. (Ahemad, 2015). PGPB can be divided into plant growth promoting rhizospheric bacteria (PGPR) and plant growth promoting endophytic bacteria (PGPE). PGPR are found around the plant roots, but PGPB are found within the plant tissues (Santoyo et al., 2016). In the past, PGPR have been extensively studied in phytoremediation, however, at present PGPE have received an increased attention (Rajkumar et al., 2012; Ma et al., 2016). However, their mechanisms to enhance phytoremediation are relatively similar (Lodewyckx et al., 2002; Santoyo et al., 2016). PGPE offer many advantages over PGPR. A rhizospheric population is difficult to control, and competition occurs between microbes which often reduce the number of the desired strains unless metabolism of the pollutant is selective. Meanwhile, an endophytic population seems to be selected or controlled by the plant. Therefore, the use of endophytes that naturally inhabit the plant would

42

reduce the problem of competition (Doty, 2008). In any case, the advantages of using rhizobacteria or endophytic bacteria will depend on the type of contaminant and the accumulation ability of bacterial species (Segura et al., 2009). Importantly, plant and bacteria have their own mechanism for dealing with heavy metal contaminants. The interaction of plants and microorganisms may increase or decrease heavy metal accumulation in plants, depending on the nature of the plant-microbe interactions (Stout and Nüsslein, 2010). Recently, PGPE containing PGP traits have been confirmed to enhance phytoremediation as listed in Table 2.7.

2.9 Endophytic bacteria-assisted phytoremediation

2.9.1 Definition and classification

PGPE are endophytic bacteria having the capacity of promoting plant growth (Santoyo et al., 2016). Therefore, endophytic bacteria can be defined as bacteria colonizing the internal tissues of plants without causing symptoms of infection or negative effects on their host (Weyens et al., 2009). Moreover, they can be isolated from surface-disinfected plants or extracted from within plant (Luo et al., 2011). Even if, the origin of endophyte is still not agreed because of diversity of host's living environment and complex relationship between endophytes and their host. However, there are 2 hypotheses. Firstly, endophytes are evolved from mitochondria and chloroplast in the plant cells, so they have similar genetic backgrounds to the host. Secondly, endophytes come from outside of the plant and enter the host from surface, root wound or induced channels (Zhenhua et al., 2012).

Endophytic bacteria have been classified into 82 genera within Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Firmicutes Actinobacteria, and Bacteroidetes. Most of them belong to the third first group (Chen et al., 2012). They span a significant range of Gram-positive and Gram-negative bacteria. Pseudomonaceae, Burkholderiaceae and Enterobacteriaceae are among the most common cultivatable endophytic species isolated from a wide variety of hosts, including woody trees, herbaceous crops, and grass species (Segura et al., 2009).

Table 2.7 Endophytic bacteria containing plant growth promoting traits

PGPE	Host plant	PGP traits
Pseudomonas fluorescens VI8L1, II8L4 and VI8R2 *Bacillus pumilus* VI8L2 *Acinetobacter calcoaceticus* II2R3	*S. alfredii*	▪ Fixation of nitrogen ▪ Solubilization of $ZnCO_3$ and $Zn_3(PO_4)_2$ ▪ Production of IAA and siderophores
Pseudomonas sp. A3R3	*A. serpyllifolium*	▪ Production of IAA, ACCD and siderophores ▪ P solubilization ▪ Excreted cellulose and pectinase
Pseudomonas monteilii PsF84, *Pseudomonas plecoglossicida* PsF610	*Pelargonium graveolens* L'Her	▪ Production of IAA and siderophores, ▪ P solubilization
Bacillus sp. MN3-4	*Alnus firma* Siebold & Zucc and *B. napus*	Production of IAA and siderophores
Staphylococcus, Bacillus Curtobacterium, Leifsonia Microbacterium, Arthrobater Paenibacillus, Pseudomonas	*Alyssum bertolonii* Desv.	Siderophores production
Pseudomonas sp. LK9	*S. nigrum*	
Bacillus sp. SLS18	*Sorghum bicolor* (L.) Moench	
P. fluorescens G10 *Microbacterium* G16	*B. napus*	
Microbacterium sp. NCr-8 *Arthrobacter* sp. NCr-1 *Bacillus* sp. NCr-5 and NCr-9 *Kocuria* sp. NCr-3	*Noccaea caerulescens* (J.Presl & C.Presl) F.K.Mey *Thlaspi perfoliatum* L.	▪ Production of IAA ▪ Production of ACCD ▪ Production of siderophores
Acinetobacter sp. Q2BJ2 *Bacillus sp.* Q2BG1	*Commelina communis* L.	

Table 2.7 *(continued)*

PGPE	Plant host	PGP traits
Bacillus thuringiensis GDB-1	*A. firma*	
B. pumilus E2S2		
Bacillus sp. E1S2 and E4S1	*S. plumbizincicola*	
Achromobacter sp. E4L5		
Stenotrophomonas sp. E1L		
Serratia nematodiphila LRE07		
Enterobacter aerogenes LRE17		
Enterobacter sp. LSE04		
Acinetobacter sp. LSE06		
Serratia marcescens LKR01	*S. nigrum*	
Arthrobacter sp. LKS02		
Flavobacterium sp. LKS03		▪ Production of IAA, ACCD
Chryseobacterium sp. LKS04		and siderophores
Rahnella sp. JN6	*Polygonum pubescens* Blume	▪ P solubilization
Rahnella sp. JN27	*Amaranthus hypochondriacus* L. and *A. mangostanus* L.	
Ralstonia sp. J1-22-2	*B. napus*	
Pantoea agglomerans Jp3-3		
Pseudomonas thivervalensis Y1-3-9		
Actinobacterium	*Salix caprea* L.	Production of ACCD and siderophores

Sources: Modified from Ma et al. (2016)

45

2.9.2 Biology

In general endophytic bacteria occur at lower population densities than rhizospheric bacteria or bacterial pathogens (Rosenblueth and Martínez-Romero, 2006). In addition, there are approximately 300,000 plant species living on the Earth, and each individual plant ranged from monocots to dicots can be the host to one or even more kinds of endophytes. Within plants, endophytic bacteria can reside in all parts (roots, stems, leaves, flower, and seed), and the highest density of them has been found in the roots and decreasing from root to stem and leaves (Hallmann et al., 1997; Sura-de Jong et al., 2015). They may form symbiotic, mutualistic and commensalistic relationships with their host (Khan et al., 2013). Based on life cycle, endophytic bacteria can be obligative or facultative. Obligate endopytes are strictly dependent on the host plant for their growth and survival, and transmission from one generation to the next occurs vertically through the seed. Meanwhile, facultative endophytes have a stage in their life cycle, where they exist outside their host plants. Normally, facultative endophytes can survive in the rhizosphere and colonize the plant through the roots (Cherian et al., 2012). Currently, several studies confirmed that endophytic bacteria mostly derive from the rhizosphere, e.g., endophytes represent a subgroup of the rhizobacterial communities, which have the ability to enter the root interior of their hosts once the rhizoplane is colonized (Compant et al., 2010).

2.9.3 Colonization

Colonization goes through many vital stages. Precolonization as shown in Figure 2.6 includes (1) bacterial movement toward the root, (2) attachment to the root surface, (3) plant-bacterial recognition process at the root surface, and (4) root penetration by the bacterium. After entering to the plant cell, endophytic bacteria have to multiply and localize as shown in Figure 2.7 within the root tissue including potential plant benefit effects (Jeger and Spence, 2001). The processes of colonization depend on many biotic and abiotic factors such as physical and biological characteristics of the host plant, temperature and humidity conditions, and seasonal fluctuations of other cohabiting microorganisms (Stępniewska and Kuźniar, 2013).
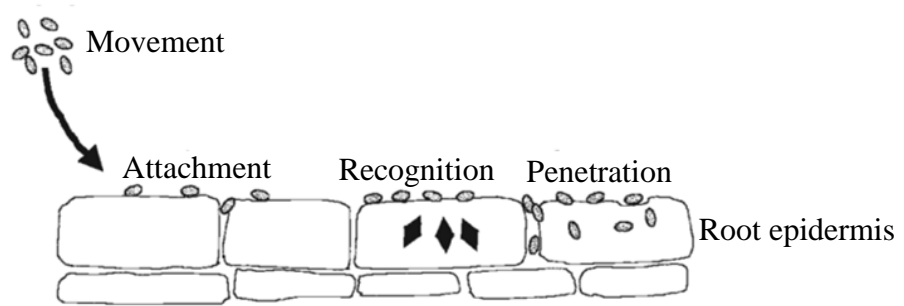
Figure 2.6 Endophytic bacterial precolonization occurring at the root surface

Source: Jeger and Spence (2001).
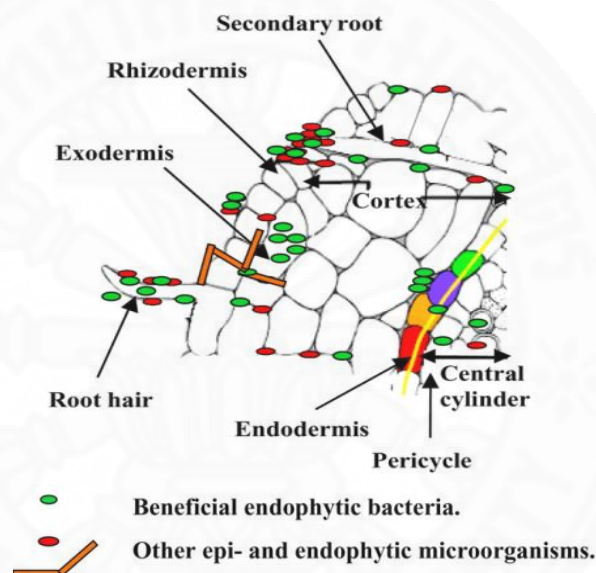
Figure 2.7 Endophytic bacterial movement in the root tissue

Source: Compant et al. (2010).

2.9.4 General roles of endophytic bacteria (PGPE) on plant

　　　　Endophytic bactria, especially PGPE can supply nutrients and essential vitamins to plant (Cherian et al., 2012,). Other benefits are adjust osmotic pressure, regulate stomata and modify root morphology (Ryan et al., 2008). PGPE can protect the plant from infection of pathogen (acting as biocontrol agent). PGPE can compete for the space and nutrient, because endophytic bacterial can colonize in an ecological niche similar to that of phytopathogens (Cherian et al., 2012, Ryan et al., 2008). PGPE can also produce some metabolites such as hydrolytic enzymes and antibiotic compound, inhibit the pathogen-produced enzymes or toxin and induce

47

plant defense mechanisms (Cherian et al., 2012). In addition, PGPE can improve the ability of host plant to tolerate to abiotic stresses such as draught, flood and salinity, as well as to resist to insect and mammalian herbivores by producing a range of natural products (Jha et al., 2013).

2.9.5 Roles of endophytic bacteria (PGPE) on phytoremediation

PGPE are highly beneficial and play a vital role in enhancing phytoremediation by enhancing plant growth and heavy metal tolerance (heavy metal phytotoxicity reduction), as well as altering of heavy metal concentration in plants, and translocation of heavy metal to the aboveground plant parts (Weyens, 2009; Ma et al., 2016;). In order to achieve in assisting plants for phytoremediation, PGPE uses many mechanisms as follows:

2.9.5.1 Mechanisms for promoting plant growth

Normally, PGPE improve plant growth by direct and indirect way as shown in Figure 2.8. In direct way, PGPE use several mechanisms such as atmospheric nitrogen fixation, mineral solubilization as well as phytohormone (IAA), specific enzymatic (ACC deaminase), and siderophore production. While, indirect way, PGPE can produce antibiotics, cell wall-degrading enzymes, lowering plant ethylene levels, induced systemic resistance, decreasing the amount of iron available to pathogens, and the synthesis of pathogen-inhibiting volatile compounds (Sanyato et al., 2016). Additionally, PGPE can also alleviate heavy metal toxicity by one or more of these mechanisms (Ma et al., 2016).

For mineral solubilization, P is one of the major micronutrients as it plays a crucial role in various enzymatic reactions responsible for normal functioning of living organisms. Unfortunately, more than 75% of applied P form complexes and are unavailable for plant uptake. PGPE can solubilize precipitated phosphates in soil by acidification, chelation, ion exchange and release of organic acid (Nautiyal et al., 2000), or to mineralize organic P in soil by secreting extracellular acid phosphatase (Ma et al., 2016), thereby enhancing the P availability to plant. Fe is one of the important elements for life. Most Fe in soils exists in highly insoluble ferric

(Fe^{3+}) form that is not available for plant uptake, such as oxides, hydroxides, phosphates and carbonates (Rajkumar et al., 2009).
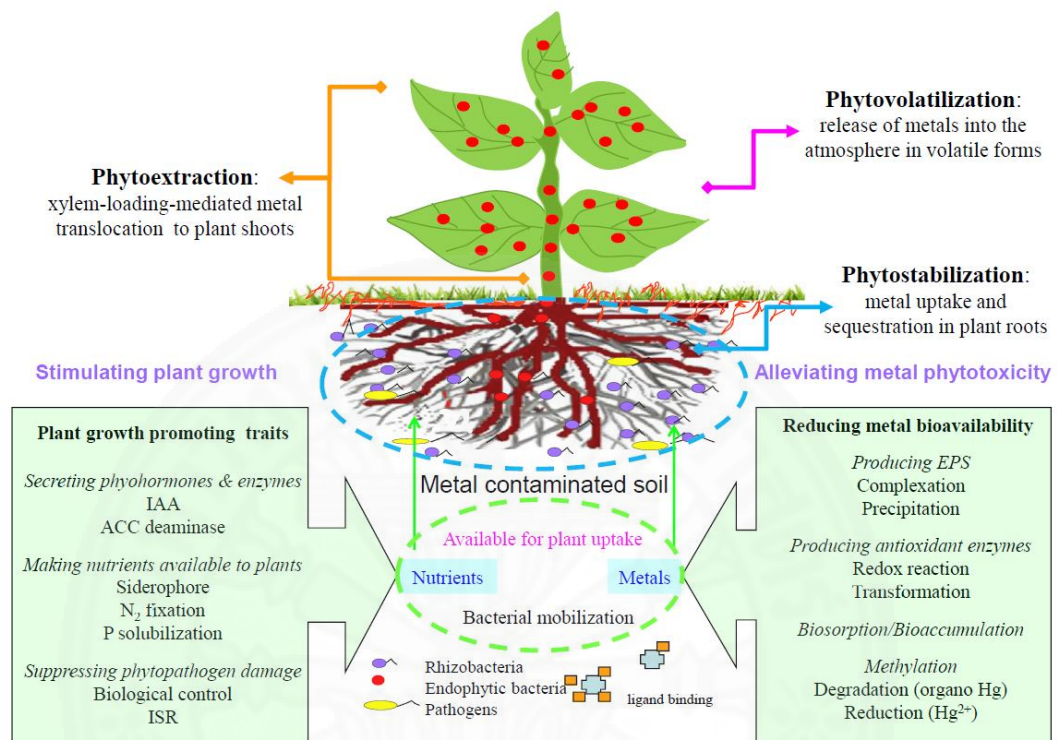
Figure 2.8 Mechanisms of endophytic bacteria in heavy metal contaminated soils. Source: Ma et al. (2016).

Fe availability to plant roots may be modified by the microbial production of chelating agent, which can solubilize Fe under conditions of Fe deficiency. Siderophores are low molecular weight organic compounds (500-1500 Da) with an affinity for Fe^{3+}, and can also bind other bivalent metal ions or Fe^{2+} that can be assimilated by the plant (Rajkumar et al., 2009). Fe acquisition by higher plants takes place by two basic strategies. The first is by microbial siderophores where plants can uptake Fe from Fe siderophore complexes through root mediated chelate degradation. The second strategy involves the solubilization of unavailable forms of Fe by the release of phytosiderophores. Since the microbial siderophores typically have higher affinity for Fe than phytosiderophores, the plants growing in metal contaminated soils are able to accumulate high amounts of Fe with the help of siderophore producing bacteria.

49

Endophytic bacteria produce siderophore which is organic molecule and shows high affinity for Fe^{3+} that can be absorbed by plant not only for promote plant growth under Fe deficient condition, but also for reduce heavy metal toxicity under heavy metal condition (Rajkumar et al., 2010). In soil, Fe bioavailability is low, as Fe prevails in insoluble form (Fe^{3+} hydroxides at neutral pH). Fortunately, the secreted siderophore can form high affinity complex with Fe^{3+}. This complex are recognized and scavenged from soil by membrane receptor proteins. However, siderophore can bind less stability complex with Fe^{2+} and other divalent cation like a Zn^{2+}, Cd^{2+}, Cu^{2+} and Pb^{2+} etc. Siderophore-divalent metal complex cannot compete for membrane passage with nutrient cation and are likely not recognized by siderophore receptor due to conformational mismatching (Sessitsch et al., 2013). This is advantage for phytoimmobilization technique. However, focus on phytoextraction, this mechanism will decrease the uptake of heavy metal by plants and thereby decrease phytotoxicity and increase plant biomass as well.

2.9.5.2 Mechanisms for altering heavy metal phytotoxicity

Under heavy metal stress, plants synthesize ethylene, but the over level of ethylene causes damage to plants. Endophytic bacteria can produce 1-aminocyclopropane-1-carboxylic (ACC) deaminase to modulate the ethylene levels leading to improve plant growth and indirectly reduce toxicity (Cherian et al., 2012). Moreover, endophytic bacteria can reduce the Cd toxicity by increasing the uptake of trace element such as Zn and Fe by plant. Besides, endophytic bacteria produce siderophore that is not only for promote plant growth under Fe deficient condition, but also for reduce heavy metal toxicity under heavy metal condition (Rajkumar et al., 2010). In short, endophytic bacteria reduce phytotoxicity caused from heavy metal concentration leading to healthy plant which can tolerate heavy metal and increase duration of exposure time. These reasons increase phytoremediation potential.

2.9.5.3 Mechanisms for altering heavy metal bioavailability

In soils, heavy metals exist in non-soluble form especially Pb, causing a difficult uptake by plant. PGPE can change form of heavy metal to bioavailable form by producing and releasing the bacterial siderophore and organic

50

acids. Bacterial siderophore acts as solubilizing agent by forming complex with other metals such as Al, Cd, Cu, Ga, In, Pb and Zn. Consequently, heavy metals are dissolved and released from soil particles, thereby increase bioavailability and increase heavy metal uptake (Rajkumar et al., 2010; Li et al., 2012). Importantly, siderophores are observed to both promote and reduce heavy metal uptake (Sessitsch et al., 2013). In addition, PGPE can produce and secrete low-molecular-mass organic acids such as 5-ketogluconic acid, citric acid, formic acid and oxalic acid to adjust the soil pH to acid soil leading to the increase of heavy metal solubility (Li et al., 2012).

2.9.5.4 Mechanisms of Pb resistance in bacteria

Pb resistant bacteria have evolved several mechanisms to survive at high Pb exposure as shown in Figure 2.9. Pb precipitation is a general process of bacteria to lower the concentration of free Pb^{2+} by sequestering Pb in the form of phosphate salts outside and inside the cell (Jarosławiecka and Piotrowska-Seget, 2014). The form of Pb precipitates are Pb(II) hydrogen phosphate ($PbHPO_4$), Pb(II) phosphate [$Pb_3(PO_4)_2$], Pb apatite [$Pb_9(PO_4)_6$], pyromorphite, or PbS depending on the strain (Nalik and Dubey, 2013). Extracellular sequestration is composed of surface biosorption and exopolysaccharides (EPS) at the cell wall. Surface biosorption can prevent the entry of Pb inside the bacterial cell. It is composed of several mechanisms such as ion exchange, chelation, adsorption, and diffusion (Nalik and Dubey, 2013). Normally, the cell wall acts as the natural barrier for Pb^{2+}, since the functional groups of macromolecules bind with Pb (Jarosławiecka and Piotrowska-Seget, 2014).

In Gram-negative bacteria, the negative charges of phosphate group of lipopolysaccharide at the cell wall are key metal binding site. The carboxyl groups of the peptidoglycan are main binding site of Pb in Gram-positive bacteria (Nalik and Dubey, 2013). Normally, EPS bind with Pb^{2+} to protect the cellular component. This phenomenon is found in many bacterial strains such as *Bacillus firmus*, *Halomonas sp*. *Paenibacillus jamilae*, and *Pseudomonas sp*. The composition of EPS is very complex, including proteins, humic acids, polysaccharides and nucleic acids that chelate heavy metals with different specificity and affinity (Jarosławiecka and Piotrowska-Seget, 2014). EPS are high molecular weight biopolymers, chemically diverse, and mostly acidic heteropolysaccarides with functional groups

51

such as hydroxyl, carboxyl, amides, and phosphoryl that exhibit high affinity to heavy metals by electrostatic interaction (Nalik and Dubey, 2013).
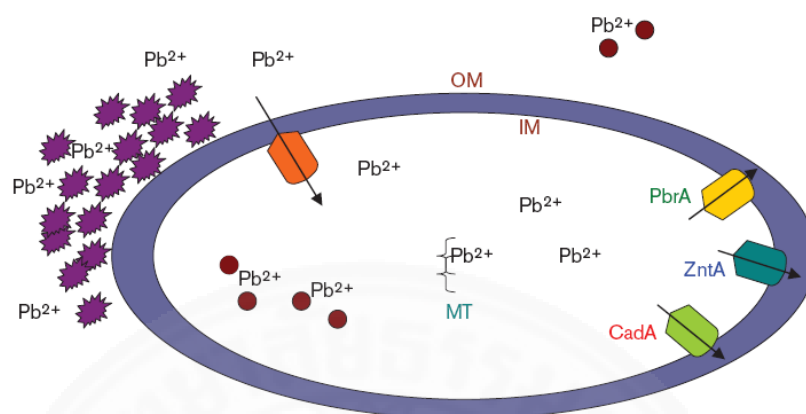
Figure 2.9 Mechanisms of Pb resistance by bacteria, Pb precipitation as insoluble phosphates (brown circles), adsorption on extracellular polysaccharides (violet stars). After entering the cell through the essential metal transporters, Pb^{2+} can be further inactivated by binding to the metallothioneins (MT), sequestered as insoluble phosphates or removed from the cell via transporters such as CadA, ZntA or PbrA. OM, outer membrane; IM, inner membrane; MT, metallothioneins. Source: Jarosławiecka and Piotrowska-Seget (2014).

Intracellular bioaccumulation is one of the common mechanisms that microorganisms have evolved to withstand the toxic effects of heavy metals. This mechanism involves specific metal binding proteins, especially metallothioneins (MTs). This cysteine-rich protein has low molecular weight ranged from 3.5 to 14 kDa, and they demonstrate induction in response to specific heavy metal including, Cd, Cu, Pb and Zn. MTs facilitate the sequestration of toxic metal inside the cell to protect bacterial metabolic process catalyzed by enzyme (Nalik and Dubey, 2013).

Ions that escape from extra- and intracellular binding can be excluded from the cell through the activity of efflux system. This system is the most effective mechanism to maintain heavy metal homeostasis that provides resistance to toxic metals. Efflux of Pb^{2+} is mediated by P-type ATPases from the P_{IB} family (Jarosławiecka and Piotrowska-Seget, 2014). P-type ATPases belong to the family of transmembrane transporters responsible for movements of ions and small organic

molecules in and out of the cell membranes. The subfamily of transmembrane transporters such as P_{IB}-type ATPases encoded by genes *cadA*, *zntA*, and *pbrA*, regulates efflux of toxic heavy metals (Pb^{2+}, Cd^{2+} and Zn^{2+}) outside the cell membrane using ATP as the energy source. This mechanism prevents the over accumulation of highly toxic and reactive metal ions. In addition, superoxide dismutase enzyme can also be engaged in Pb^{2+} binding (Jarosławiecka and Piotrowska-Seget, 2014). Moreover, Pb resistant bacteria exhibit significant alterations in cell morphology to cope up with heavy metal stress such as reduction in cell size and shrinkage (Nalik and Dubey, 2013).

2.9.6 Advantages and limitation of using endophytic bacteria

There are many advantages of using endophytic bacteria in phytoremediation. Quantitative gene expression of bacterial pollutant catabolic genes can be used to assess the efficiency of the remediation process. Genetic engineering of a bacterial catabolic pathway is easier to manipulate than plant catabolic pathway and toxic pollutants taken up by plant may be degraded in plant by endophytic degraders which reduces the toxic effects of contaminants in environmental soil on flora and fauna (Khan and Doty, 2011). Also, there are many disadvantages which are the choice of plant can mean that it is only seasonally effective and it is associated with phytotoxicity effects of contaminants. Contaminants or their metabolites also have a potential to enter the food chain if contaminants are not completely detoxified and if the plants are consumed by local fauna (Khan and Doty, 2011).

2.9.7 Research on endophytic bacteria-assisted phytoremediation

Currently, a wide range of PGPE has been recognized that successfully aid in phytoremediation via promoting plant growth, reducing phytotoxicity, accumulating metals, improving plant's metal tolerance, altering metal bioavailability in soil, and translocating metal in plants (Li et al., 2012; Ma et al., 2016). Some endophytic bacteria were found to improve plant growth. PGPE isolated from Cd hyperaccumulator (*S. nigrum*) increased plant growth depending on the initial Cd concentration. In low Cd contaminated soil (about 12.1 mg/kg), *Enterobacter* sp. LSE04 enhanced shoot length (13.7%), fresh weight (28.2%) and

dry weight (41.4%) of *S. nigrum*. At the moderate Cd concentration (about 63.7%), *Acinetobacter* sp. LSE06 showed the most excellent growth effect, which the increases were up to 10.9% for shoot length, 15.7% for fresh weight and 23.1% for dry weight. At high Cd concentration (116.5%), *Serratia nematodiphila* LRE07 was the best and the plant shoot length, fresh and dry weight were increased by 18.9%, 23.1% and 19.8%, respectively compared to un-inoculated plants (Chen et al., 2010). Ma et al. (2011a) found that inoculation with the plant growth promoting endophytic bacterium *Pseudomonas* sp. A3R3 significantly increased the fresh and dry weight of *B. juncea* by 50% and 45%, respectively compared to un-inoculated plants. In addition, *Psychrobacter* sp. SRS8 could stimulate growth of energy crops. In case of *Ricinus communis*, the percent increase was 32% for fresh weight and 38% for dry weight and for *Helianthus annuus* 39% and 41%, respectively. The endophytic *Bacillus* sp. MN3-4 increased root elongation of *B. napus* seedlings by 46.25% compared to the un-inoculated control (Li et al., 2012; Ma et al., 2016). Pb-resistant with ACC deaminase producing endophytic bacteria (*Acinetobacter* sp. Q2BJ2 and *Bacillus* sp. Q2BG1) were isolated from metal-tolerant plant (*C. communis*) grown on Pb/Zn mine tailing. They can increase the dry weight of above-ground tissues of *B. napus* grown on quartz sand containing 100 mg/kg of Pb (ranging from 39% to 71%) and roots (ranging from 35% to 123%) compared to the un-inoculated controls (Zhang et al., 2011). *B. napus* inoculated with the *Rahnella* sp. JN6 showed a significant increase in chlorophyll content, plant height, root length, aboveground weight and root weight compared with the un-inoculated controls, which were 10%, 27%, 26%, 38% and 52% in Cd-added soil, 22%, 17%, 27%, 31% and 34% in Pb-added soil, 24%, 28%, 19%, 26% and 41% in Zn-added soil, and 20%, 30%, 13%, 27% and 41% in un-amended soil, respectively (He et al., 2013).

PGPE could potentially improve phytoremediation by altering the solubility and availability of heavy metal. For example, Pb resistant endophytic bacteria *P. fluorescens* G10 and *Microbacterium* sp. G16 could significantly increase water solubility of Pb after 60 h of inoculation about 20-fold (7540 µg/L) and 17-fold (6250 µg/L), respectively compared to un-inoculated controls (381 µg/L) (Sheng et al., 2008). *Gluconacetobacter diazotrophicus* dissolved various Zn sources such as zinc oxide (ZnO), zinc carbonate ($ZnCO_3$), or zinc phosphate [$Zn_3(PO_4)_2$], via

releasing of 5-ketogluconic acid thus making Zn available for plant uptake. Endophyte *Actinobacterium* could mobilize Zn and Cd via releasing of metal-mobilizing metabolites (Li et al., 2012). *Rahnella* sp. JN6 significantly ($p < 0.05$) increased Cd, Pb and Zn solubilization in the metal-amended soil, which were 1.46, 1.25 and 1.30 folds higher than those in the un-inoculated soil (He et al., 2013).

The efficiency of phytoremediation is mainly dependent on metal accumulation. It has been demonstrated that some heavy metal resistant/plant growth promoting endophytic bacteria can improve metal uptake and accumulation in plants (Li et al., 2012). For instance, *Psuedomonas sp*. A3R3 significantly increased Ni content in the shoot of *A. serpyllifolium* and *B. napus* by 10% and 15%, respectively, grown in soil amended with 450 mg/kg of Ni compared to un-inoculated plants (Ma et al., 2011a). Inoculation of *B. napus* with Pb resistant endophytic bacteria (*P. fluorescens* G10 and *Microbacterium sp*. G16) could increase Pb uptake in shoot from 76% to 131% by *Pseudomonas fluorescens* and from 59% to 80% by *Microbacterium* sp. compared to the un-inoculated controls (Sheng et al., 2008). In addition, *Acinetobacter* sp. Q2BJ2 and *Bacillus* sp. Q2BG1 significantly ($p < 0.05$) increased total Pb concentration in shoots of *B. napus* (ranging from 3.4-fold to 5.6-fold) and roots (ranging from 2.1-fold to 3.5-fold) compared to the un-inoculated control (Zhang et al., 2011). One particular strain, *Rahnella* sp. JN6 isolated from Mn hyperaccumulator (*P. pubescens*) significantly increased metal concentrations in tissues of *B. napus*. Metal (Cd, Pb and Zn) concentrations in above-ground tissues of un-inoculated control were 49%, 47%, 28%, and of inoculated plants were 106%, 97%, 62%, respectively. Meanwhile, those metals concentrations in root tissues of un-inoculated control were 58%, 46%, 33%, and of inoculated plants were 140%, 95%, 89%, respectively (He et al., 2013). Similarly, *Bacillus pumilus* E2S2 significantly increased plant's Cd uptake, and increased plant root and shoot length, as well as fresh and dry biomass compared with un-inoculated plants. Some endophytic bacteria such as *Bulkholderia capacia* and *Herbaspiillum seropedicae*, could enhance Ni accumulation in root of yellow lupin leading to enhance phytoremediation potential (Lodewyckx et al., 2001; Cherian et al., 2012). Recently, *Pseudomonas* sp. LK9, inoculated into Cd hyperaccumulator (*S. nigrum*) growing in multi-heavy metal contaminated soil significantly increased metal uptake (Chen et al., 2014). Similarly,

multi-metal resistant endophytic bacterium *Bacillus* sp. L14 isolated from *S. nigrum* increased the uptake 76%, 80%, and 21% of Cd^{2+}, Pb^{2+}, and Cu^{2+}, respectively (Zaidi et al., 2012). However, there were some opposite viewpoints that the presence of metal-resistant endophytic bacteria decreased the uptake of heavy metals by plants and thereby increased plant biomass (Rajkumar et al., 2009). Madhaiyan et al. (2007) found that endophytic bacteria *Methylobacterium oryzae*, and *Burkholderia* sp. isolated from rice tissues reduced Ni and Cd contents in roots and shoots of *Lycopersicon esculentum* Mill.

Translocation factor was calculated to assess the efficiency of PGPB inoculation on the translocation of heavy metals from roots to shoots. Ma et al. (2015) reported that PGPB *Sanguibacter* sp. decreased TF of Zn in *Nicotiana tabacum* L. grown in Cd- and Zn-enriched soil. Ma et al. (2016) reviewed that Cu-resistant endophytic bacteria enhanced Cu transfer from root to shoot of *B. napus*, thus improving the overall phytoextraction potential. Ma et al. (2015) found that the inoculation of PGPB strain (*Pseudomonas* sp. A3R3) slightly increased the TF of Ni in *B. juncea* and *R. communis* with the increasing proportions of multi-metal polluted serpentine soil. This indicates that the inoculated PGPB played an important role on Ni accumulation in plant shoots. In turn, *Pseudomonas* sp. A3R3 significantly decreased the TF of Zn in both plant species.

Phytoremediation can be inhibited because of phytotoxicity. Some endophytic bacteria can reduce the heavy metal toxicity. For instance, *Psychrobacter* sp. SRS8 can protect the plants (*R. communis* and *H. annuus*) against the inhibitory effect of Ni by increasing both protein and chlorophyll contents in leaf tissue (Ma et al., 2011b). *Methylobacterium oryzae* and *Burkholderia sp*. isolated from rice reduced the toxicity of Ni and Cd in tomato plants and promoted plant growth under pot experiment. Besides, *Bacillus* sp. isolated from roots of hyperaccumulator *A. firma* could potentially reduce heavy metal phytotoxicity and increase Pb accumulation in *A. firma* (Shin et al., 2012).

As the above-mentioned cases, removal of heavy metals via phyto-bacterial systems is more efficient than phytoremediation alone. Therefore, it is clear from the mentioned cases that PGPB play a key role in the enhancement of heavy metal phytoremediation.

Chapter 3

Methodology

3.1 Material preparation

3.1.1 Experimental plant preparation

The experimental plants such as *Acacia mangium, Azadirachta indica, Eucalyptus camaldulensis*, and *Senna siamea* (Appendix A) were obtained from several sources as described in each experiment. They were acclimatized in a container containing 3 L of 25% modified Hoagland's solution under laboratory conditions (27-28°C, 4600 lux light intensity, 12 h photoperiod) for 7 days with aeration before test.

3.1.2 Chemical preparation

All reagents used in the experiments were analytical grades. Deionized water was used for all solution preparation. The 25% modified Hoagland's nutrient solution was prepared as described in Appendix B. Very high concentration of Pb stock solution and standard solution for calibration were prepared as described in Appendix C. The chemicals involved in microbial test were also prepared as described in Appendix C.

3.1.3 Glassware preparation

The glassware were cleaned with washing detergent, immersed in 10% HNO_3 at least overnight, rinsed with deionized water and dried before use. All digestion vessels were acid washed with the same process as for the glassware.

3.2 Methods

3.2.1 Experiment I: Hydroponic screening of fast-growing tree species for Pb phytoremediation potential

3.2.1.1 Experimental plants

Healthy plants, *A. mangium*, *A. indica*, *E. camaldulensis*, and *S. siamea* were obtained from Bangkok, and Nakhon Nayok province, Thailand. They were approximately 30 cm in height and selected for uniformity based on the diameter and height. Selected plants were washed several times with tap water to remove soil and rinsed with deionized water. They were acclimatized as described in section 3.1.1.

3.2.1.2 Hydroponic procedures

After acclimatization, plants were harvested, washed with deionized water, weighed for initial fresh weight. Then, a plant was placed in each plastic container (capacity 660 mL) which contained 500 mL of 25% modified Hoagland's solution supplemented with 10, 30, and 50 mg/L Pb as $Pb(CH_3COO)_2 \cdot 3H_2O$. Plants grown in nutrient solution without Pb served as controls. The pH of the solutions was adjusted to 5.0. Each plastic container was covered with a plastic cap to prevent contamination without aeration. All containers were arranged on the shelf by means of complete randomized block design. A total of 12 plants of each species (48 plants) were used for each treatment in triplicate. The solutions were changed every 5 d to maintain the desired level of nutrients. The toxicity symptoms of plants were observed throughout the experiment. After 15 d, plants were harvested and weighed for final fresh weight. Plants were rinsed twice with deionized water, and separated into shoots and roots. Then, samples were oven dried at 60°C for 3 d to a constant weight. Dried plants were weighed, homogenized with a mortar (IKA: A11 basic, Japan), and passed through a 0.28 mm nylon mesh. According to APHA (2012), 0.5 g of each sample was added into each digestion tube with 5 mL of concentrated nitric acid (69% HNO_3). All tubes were heated in digestion block at 80°C until the clear solution was obtained. The homogenized solution was cooled before filtering through a Whatman No. 42 filter paper to remove the impurities. The

58

filtered samples were adjusted to 25 mL volume with deionized water prior to Pb analysis by a flame atomic absorption spectroshotometer (FAAS: SpectrAA 55B, Varian, Australia) with a hollow cathode lamp of Pb (10 mA, wavelength 217 nm).

3.2.2 Experiment II: Phytoremediation potential of plants growing on the Pb-contaminated soil at the Song Tho Pb mine, Thailand

3.2.2.1 Field survey study

A field survey was conducted at the Song Tho Pb mine area. This mine is located in Chalae subdistrict, Thong Pha Phum district, Kanchanaburi province, situated about N 14.849787, E 98.797600 (Figure 3.1). It contained open-pit mining (in the area of secondary minerals) and underground mining (in the area of primary minerals). It had been operated for nearly 25 years since 1977, before the expiry of concession in 2002.

3.2.2.2 Site description

Samples were collected from 3 different sites (site 1-3) as shown in Figure 3.2. Site 1 was an ore dressing plant area (N 14.85328, E 98.79664), site 2 was a stockpile area (N 14.85268, E 98.79598), and site 3 was a waste pond area (N 14.85629, E 98.79633).
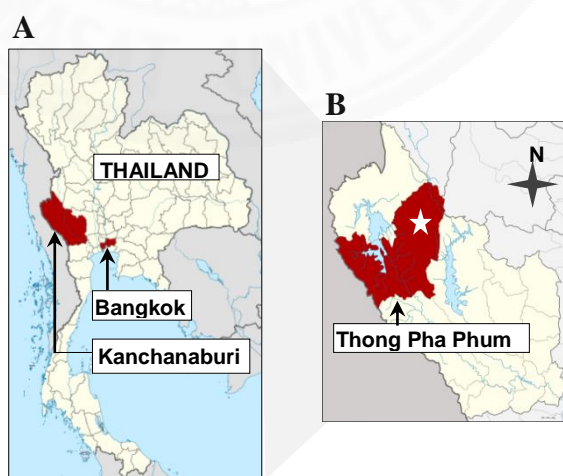
Figure 3.1 (A) Map of Thailand showing Kanchanaburi province, (B) Thong Pha Phum district, star representing the Song Tho Pb mine area in Chalae subdistrict (modified from https://th.wikipedia.org)

59
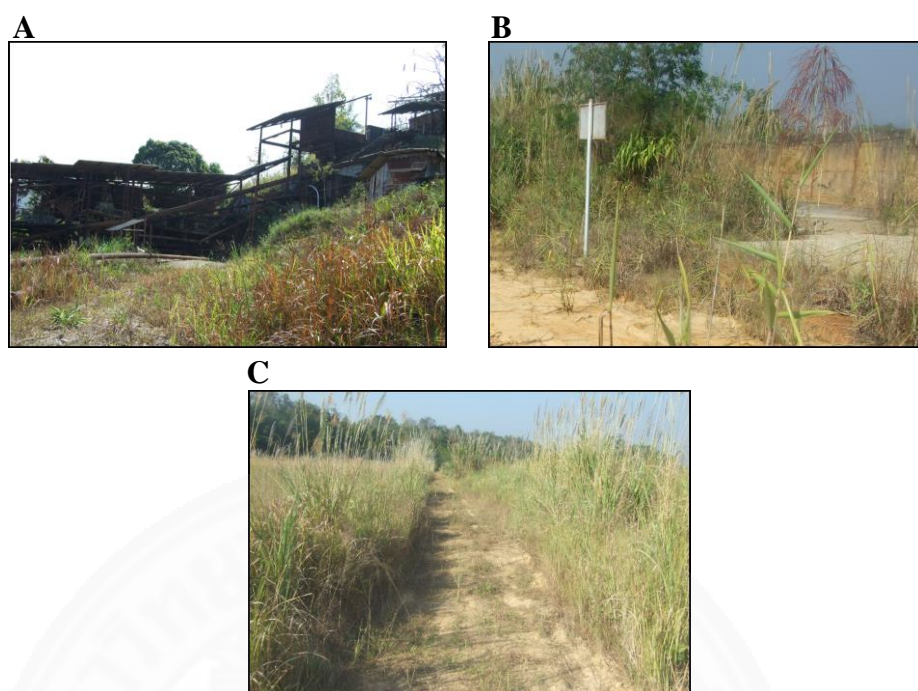
Figure 3.2 Three sampling sites (A) Site 1, (B) Site 2, and (C) Site 3

3.2.2.3 Plant and soil sample collection

All samples were collected in January 2015, where the mean annual temperature was 27.4°C, 76% mean annual relative humidity, and 1376 mm total annual rainfall with the highest in June (315.6 mm) and the lowest in December (1.9 mm). Five samples of top soil in 4x4 m^2 (10 cm in depth) were collected from each site using a shovel. Then, soils were put in polyethylene bags. Plant samples were collected from each site including the area in front of the tunnel. Seven plants of each species with the soil surrounding plant roots were randomly collected around the sampling areas. They were separately kept in the polyethylene bags. After collection, all samples were immediately transported for analysis.

3.2.2.4 Plant and soil sample analysis
(1) Plant species identification

Plant species were identified by (1) the Plant Variety Protection Division, Department of Agriculture, and (2) the Office of the Forest Herbarium, Department of National Parks, Wildlife and Plant Conservation, Ministry of Agriculture and Cooperatives, Thailand.

60

(2) Physico-chemical properties

Five samples of top soil from each site were mixed and sieved through a 0.28 mm nylon sieve. Soil texture and the physico-chemical properties were analyzed by the Land Development Department, Ministry of Agriculture and Cooperatives of Thailand. The soil texture was analyzed using the pipette method, electrical conductivity (EC) by electrical conductivity meter after mixing soil and water in a ratio of 1:5 (w/v) at 25°C (Peverill et al., 1999), cation exchange capacity (CEC) by ammonium saturation method with NH_4OAc 1 N, pH 7.0 (Black, 1965), organic matter (OM) by Walkley-Black titration (Walkley and Black, 1947), available P by spectrophotometer after extracting with Bray II method (Bray and Kurtz, 1945), available K by atomic absorption spectrophotometer after extracting with NH_4OAc 1N pH 7.0 (Jackson, 1958), and total Pb concentration (Lisle et al., 1993). Meanwhile, soil pH was measured using a pH meter after mixing soil and deionized water in a ratio of 1:2.5 w/v at room temperature in triplicate (Badr et al., 2012).

(3) Total Pb concentration in plant tissue

Plant samples were cleaned as described in section 3.2.1.2. 0.5 g of plant dried sample was digested with 10 mL of concentrated nitric acid (69% HNO_3) (Enamorado-Báez et al., 2013) in a microwave (ETHOS PRO, Milestone Inc: Italy). The operational conditions and the heating program were set to a ramp time of 10 min to reach 200°C, then a hold time of 10 min according to manufacturer's instruction. After digestion, the homogenized solutions were filtered using a Whatman No. 42 filter paper, and the volume adjusted to 50 mL with deionized water. Pb concentrations were determined by a FAAS (SpectraAA 220FS, Varian) with a hollow cathode lamp of Pb (10 mA, wavelength 217 nm).

(4) Total Pb concentration in soil

Soil was sieved and dried at 60°C for 3 days. 0.5 g of dried soil was digested with 10 mL of concentrated nitric acid (69% HNO_3) according to the method of US-EPA 3051A (2007) in a microwave. The operational conditions and the heating program were set to a ramp time of 10 min to reach 200°C, then a hold time of 15 min according to manufacturer's instruction. After digestion, the homogenized

solutions were analyzed for Pb concentration as described in section 3.2.2.4, and subsection 3.

(5) Available Pb concentration in soil

The extractable Pb from top soil and soil around plant roots were extracted by diethylene triamine pentaacetic acid (DPTA) solution (Lindsay and Norvell, 1978). Briefly, 10 g of soil were sieved through 0.28 mm nylon sieve, and air dried for 2 days. Pb was extracted with 20 mL of DTPA solution containing 0.005 M of DTPA, 0.01 M of $CaCl_2 \cdot 2H_2O$, and 0.1M of TEA (triethanolamin). The pH was adjusted to 7.30±0.05 with 1N HCl. Samples were horizontally shaken approximately at 150 rpm for 2 h. After extraction, solutions were analyzed for Pb concentration as described in section 3.2.2.4, and subsection 3.

3.2.3 Experiment III: Isolation and characterization of PGPE from the roots of *Pityrogramma calomelanos*

3.2.3.1 Isolation of endophytic bacteria
(1) Root surface disinfection

To remove the epiphytes, the surface disinfection technique was performed (Chen et al., 2010; Luo et al., 2011). Roots were washed by running tap water until clean, and rinsed with distilled water for five minutes twice. Then, 0.5 g fresh weight of cleaned roots was immersed in series of 70% ethanol for 40 seconds, and 2.5% sodium hypochlorite (NaOCl) plus a droplet of polyoxyethylene 80 with gentle shaking for 15 minutes. Then, samples were rinsed five times in sterilized distilled water to remove surface disinfection agents. The disinfection technique was indirectly confirmed by taking 100 μL of the last rinse, plating on LB agar (CulGenex™), and incubating at 30ºC for 48 h. If no colony occurred, it indicated that the surface disinfection was successful.

(2) Root extraction and bacterial isolation

These procedures were modified from Shin et al. (2012). The sterilized samples were ground with a sterile mortar and pestle in 5 mL of 0.85%

NaCl to make plant slurry. Then, this crude sample was transferred into a 15 mL centrifuge tube, and kept at 4ºC until further use. The suspension of each crude sample was serially diluted ten-fold with sterile 0.85% NaCl until the 10^{-6} fold was obtained. After that, 100 µL of appropriate dilution, was spread on LB agar plate supplemented with 20 mg/L as $Pb(CH_3COO)_2 \cdot 3H_2O$ (Wako). Then, all plates were incubated at 30ºC for 48 h in order to observe the colony appearance. This step was done in duplicate. After incubation, the morphologically different Pb-resistant colonies were selected, purified by streaking on LB agar plate twice, and incubated at 30°C for 48 h. Finally, these plates were stored at 4°C for further study.

3.2.3.2 Endophytic bacteria characterization

(1) Pb resistance

The ability of isolate to tolerate Pb was determined as the minimal inhibitory concentration (MIC) value using agar dilution method modified from Shin et al. (2012). MIC is the lowest concentration of Pb which completely inhibits the growth of the isolate. In the preliminary test, each pure isolate after fresh growth for 24 h was streaked on LB agar plate supplemented with increasing concentration of Pb ranging from 50 to 3200 mg Pb/L, as $Pb(CH_3COO)_2 \cdot 3H_2O$. The plates were incubated at 30°C for 48 h and bacterial growth was observed. These concentrations were employed as broad range. The specific concentrations of 1800, 1825, 1850, 1875, 1900 and 2000 mg Pb/L were tested with the same method. This experiment was done in duplicate.

(2) Siderophore production

The qualitative assay of siderophore production by the isolates was determined on chrome azurol S (CAS) agar plate modified from Schwyn and Neilands (1987). Each pure isolate after fresh growth for 24 h was stabbed using sterile toothpicks. These plates were incubated at 30°C for 3 days. This part was done in triplicate. The appearance of orange halos around the colonies indicated the positive result.

(3) Phosphate solubilizing activity

The qualitative assay of phosphate solubilizing activity of the isolate was determined using National Botanical Research Institute Phosphate (NBRIP) medium belonging to Nautiyal (1999). Three strains per plate were stabbed using sterile toothpicks. These plates were incubated at 30°C for 7 days. This part was done in triplicate. The appearance of clear zone indicated the positive result.

(4) Bacterial inoculum preparation

Each colony was grown by streaking on a LB agar plate, and incubating at 30°C for 48 h. Then, the colonies were transferred into a LB broth, and incubated on a shaker at 100 rpm, 30±2°C for 48 h. 100 μL of pre-culture was transferred into 30 ml of LB broth, and incubated on a rotary shaker at 150 rpm, 30±2°C for 16 h. Bacterial cells were harvested by centrifugation (Megafuge 1.0R: Heraeus) for 20 min at 3500 rcf at 4°C, and suspended in 30 mL sterile 0.85% NaCl. Bacterial density of final suspension was performed by plate count.

(5) Pb solubilization in solution culture

This method was modified from He et al. (2013). 1 mL of each inoculum was added to a 125 mL Erlenmeyer flask containing 30 mL of LB broth supplemented with 250 mg/L basic Pb carbonate [$(PbCO_3)_2Pb(OH)_2$]. Then, all flasks were autoclaved at 121°C for 15 min. Un-inoculated solution was used as the control. Three replicates were done in this experiment. All flasks were incubated on a rotary shaker at 150 rpm, 30±2°C for 48 h. After 0 and 48 h, the spent culture was filtered through a 0.22 μm Millipore filter. The water soluble Pb concentrations in the supernatants were determined with FAAS (SpectrAA 55B, Varian).

(6) Pb mobilization in soil

This method was modified from Liang et al. (2014). 1 g of natural Pb contaminated soil, collected from Song Tho Pb mine, was put into 50-mL centrifuge tube, and autoclaved at 121°C for 15 min. Then, soil was incubated at room temperature for 24 h, and autoclaved at 121°C for another 15 min to kill other microorganisms. 1 mL of each suspension was added to a tube, whereas, 1 mL of

64

sterile water was used as an un-inoculated control. All tubes were wrapped by paraffin to protect water evaporation, and placed on a rotary shaker at 150 rpm, at $30 \pm 2°C$ for 48 h. Three replicates were done in this experiment. After 2 days, 10 mL of sterile water was added to each tube to extract the water-extractable metal. The soil suspension was centrifuged at 3500 rcf, 25°C for 10 min, and the supernatant was filtered through a 0.22 μm Millipore filter. The water-soluble Pb concentration was determined by FAAS.

3.2.3.3 Identification of endophytic bacteria
(1) Bacterial genomic DNA extraction

The pure colony of endophytic bacteria was cultivated in LB broth at 30ºC, 150 rpm for 12 h. 1 mL cell suspension was centrifuged at 13000g for 10 min, 4ºC to pellet cells. After removing the supernatant, the cells were washed with 400 μL of 10 mM Tris-HCl (pH 8.0). Then, they were centrifuged at 13000g for 10 min, 4ºC. Subsequently, the pellets were resuspended in 200 μL of 10 mM Tris-HCl (pH 8.0). Then, these tubes were boiled at 100ºC for 15 min using heat block to lyse cells. Samples were centrifuged at 13000g for 10 min at 4ºC to separate the aqueous phase from the organic phase. 100 μL of aqueous phase containing purified DNA was transferred to a new 1.5 mL tube and stored at -20ºC.

(2) Measurement of DNA concentration and purity

The concentration and purity of bacterial genomic DNA was measured using NanoDrop® (ND-1000) spectrophotometer. The ratio of A260/A280, and A260/230 were analyzed to determine protein impurities.

(3) PCR amplification of partial 16S rRNA gene

The partial 16S rRNA gene amplification was done using the forward and reverse primer of 8F (5'-AGAGTTTGATCCTGGCTCAG-3'), and 534R (5'-ATTACCGCGGCTGCTGG-3'). The 50 μL of PCR mixture was composed of 49 μL of reaction master mix and 1 μL of sample bacterial genomic DNA. The master mix (50 μL) was composed of 40.6 μL RNase free water, 5.0 μL 10 x PCR Buffer, 4.0 μL 10 mM dNTP Mix, 0.1 μL of Primer 8f (100 pmol/μL), 0.1 μL of Primer 534r

(100 pmol/µL), and 0.25 µL TaKaRa Taq. The PCR amplification was performed in an automated thermocycler (Eppen-dorf, Germany) with the cycling conditions as: 94ºC for 5 min (initial denaturation) followed by 30 cycles of 94ºC for 1 min (denaturation), 60ºC for 1 min (annealing), 72ºC for 1 min (extension) and then a final extension step of 72ºC for 15 min. A blank that contained all the components of the reaction mixture without the bacterial genomic DNA sample was used as a negative control. The PCR products about 500 base pairs were analyzed using 1.5% (w/v) agarose gel electrophoresis.

(4) PCR product analysis by gel electrophoresis

1.5% (w/v) agarose was prepared by adding 1.2 g of agarose into an Erlenmeyer flask 300 mL. Then, 80 mL of 1X TAE buffer was added with gentle swirl. Then, the agarose content was melted by heating in a microwave. At 60 s intervals, the flask was removed and the content swirled to mix well. The process was repeated until the agarose had completely dissolved. Meanwhile the flask was cooled (50-60°C) on the benchtop, the gel mold was prepared by placing the gel tray into the casting apparatus and placing a comb to create the wells. Then, the cooled molten gel was poured into the gel mold, and allowed the agarose to set at room temperature. Then, the comb and gel were removed from the tray. The gel was kept in a plastic box containing 1X TAE buffer and stored at 4°C until use. After that, aliquots (5 µL) of different amplified PCR product were mixed well with 1 µL of DNA ladder (100 bp, Dye plus, Takara). Each aliquot was loaded in each well of 1.5% agarose gel in a gel box. A DNA ladder was used as the molecular size marker. They were electrophoresed in 1X TAE buffer at 100 V for 25 min. The gels were stained by immersing in a solution of Cyber safe 0.5 mg/L for 30 min. The bands were visualized and photographed on a Stratagene Eagle Eye Imaging System.

(5) Partial 16S rRNA gene sequencing analysis

The PCR products were purified using MinElute®PCR product purification kit (Cat. No. 28004) to remove the primers and dNTPs. 50 µL of PCR product were added to 250 µL of buffer PB (binding buffer), then mixed well. A MinElute column was prepared. Sample was applied to the MinElute column, and

centrifuged at 17900g, for 1 min, at room temperature. Solution sample was discarded, because DNA was attached at the column. 750 µL of buffer PE (wash buffer) was added to MinElute column and centrifuged again under the same condition. Solution sample was discarded and centrifuged again. A MinElute column was placed into a 1.5 mL of new microcentrifuge tube. 10 µL DNA was eluted using 40 µL of buffer EB (elution buffer). After PCR product purification, the 1.5% agarose gel electrophoresis was performed. The amount of the products was estimated by comparing the band intensity with marker band. The purified 16S rRNA gene was sequenced by FASMAC Company, JAPAN. To sequence gene, the mixture reaction was prepared following the manufacture condition. In this study, 1 µL of purified PCR product of each isolate was mixed with 1 µL of each 100-fold diluted primer (8F, 534R), and 12 µL of water.

(6) Strain identification

16S rRNA gene sequences were matched against nucleotide sequences in GenBank using BLAST tool (blastn) belonged to NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The first hit sequence of each isolate was chosen with at least 99% homology. There were many sequences which showed the same % homology. Thus, the phylogenetic tree was analyzed (Appendix D).

3.2.3.4 Selection of the best PGPE strain

Considering bacterial resistance to Pb, siderophore production and phosphate solubilization, Pb solubilization and mobilization, a strain with the best-performing endophytic bacterium was selected for further experiment.

3.2.4 Experiment IV: Fast-growing trees inoculation with *P. psychrophila*

3.2.4.1 Bacterial inoculum preparation

The colony of selected endophytic bacteria was grown by streaking on a LB agar plate, and incubating at 30°C for 48 h. Then, colonies were transferred into a LB broth, and incubated on a shaker at 180 rpm, 30 ± 2°C for 48 h. 100 µL of pre-culture was transferred into a flask containing 50 mL of LB broth, and

67

incubated on a rotary shaker at 180 rpm, 30 ± 2°C for 16 h. Bacterial cells were harvested by centrifugation (Megafuge 1.0R: Heraeus) for 20 min at 3500 rcf at 4°C, and suspended in 50 mL sterile 0.85% NaCl. The final suspension of bacterial strain had a cell density of approximately 10^8 colony forming units (CFU)/mL.

3.2.4.2 Inoculation of *P. psychrophila* strain into new host plants by pruned-root dip method

The selected new plant hosts were acclimatized for 7 d as described in section 3.1.1. Plants were washed twice with deionized water. The method for inoculation was performed according to Bressan and Borges, (2004) with minor modification. Briefly, 50% of roots were mechanically cut. The pruned roots were dipped in the 25% modified Hoagland's solution supplemented with 10% of bacterial inoculum prepared as described in section 3.2.4.1 for 48 h.

3.2.4.3 Recovery of *P. psychrophila*

The endophytic bacteria which colonized well inside the inoculated plants were isolated by the same method as described in section 3.2.3.1. After incubation, the colonies showing the morphological characteristic similar to the inoculated endophytic bacteria were selected and purified by streaking on LB agar plates, and incubated at 30°C for 24 h. Finally, these plates were sent to the Mahidol University-Osaka University Collaborative Research Center for Bioscience and Biotechnology (MU-OU: CRC) for the partial 16S rRNA gene sequencing analysis. Colonies belonged to the new host plants were also gene analyzed.

3.2.4.4 Hydroponic test

This study was carried out to indirect check the colonization of endophytic bacteria inside the new plant hosts, *A. mangium* and *E. camaldulensis* after 0 d of inoculation. Plants were acclimatized for 7 d as describe in section 3.1.1. The uniform plants (40 cm height for *A. mangium*, 60 cm height for *E. camaldulensis*) were used. Selected plants were washed several times with tap water to remove soil and rinsed with deionized water. Plants were inoculated with *P. psychrophila* as described in section 3.2.4.2. After inoculation, inoculated plants were extracted and

appeared colonies were identified as described in section 3.2.4.3. Finally, inoculated plants were tested in hydroponic and only Pb accumulation in root was analyzed as described in section 3.2.1.2.

3.2.5 Experiment V: Colonization of *P. psychrophila* after 15 d of inoculation

The bacterial colonization was carried out to check the survival of bacteria inside the tissues of the new plant hosts after 15 d of inoculation. The uniform plants (*A. mangium* and *E. camaldulensis*) were obtained from Chatuchak market, Bangkok, Thailand. Plants were acclimatized for 7 d as describe in section 3.1.1. Plants were washed several times with tap water to remove soil and rinsed with deionized water. The inoculation method was performed according to section 3.2.4.2 with minor modification to keep plant fresh. Briefly, 10% of roots were mechanically cut. The pruned root plants were dipped in the 25% modified Hoagland's solution supplemented with 4% of bacteria inoculum for 3 h.

After 0 d of inoculation, inoculated and un-inoculated plants were recovery extracted to isolate the inoculated *P. psychrophila* by the same method of endophytic bacterial isolation as described in section 3.2.3.1. The appeared colonies were observed by culturing on King's B agar without adding Pb. These plates were incubated at 30°C for 48 h. This ensures that *P. psychrophila* could enter inside the plant root. At the same time, after inoculation, inoculated and un-inoculated plants were tested in hydroponic as described in section 3.2.1.2, except Pb concentration used only 30 mg/L. After 15 d of hydroponic test, the roots of all treatments were recovery extracted and the appeared colonies were observed as described in day 0. This not only ensures that *P. psychrophila* could dwell inside the plant root, but also implies that *P. psychrophila* could colonize inside the plant root for a long time (60 d) of pot experiment.

69

3.2.6 Experiment VI: Inoculation of *P. psychrophila* strain for the assessment of Pb phytostabilization by fast-growing trees using pot study

3.2.6.1 Pb spiked soil preparation

Uncontaminated soil was obtained from Suphanburi province, Thailand. Soil was crushed to remove rocks and debris and passed through a 2x2 mm sieve. The soil was thoroughly mixed with $Pb(CH_3COO)_2 \cdot 3H_2O$ solution to reach 1500 mg/kg soil. The soil without Pb was used as control (uncontaminated soil). These soils were equilibrated in plastic containers for 15 days. The physico-chemical characteristics of soils were analyzed by Kasesart University, Thailand: soil texture using hydrometer, pH by a pH meter after mixing soil and deionized water in a ratio of 1:1, electrical conductivity (EC) by saturation water extract with electric conductivity meter, cation exchange capacity (CEC) by leaching with $1N$ NH_4OAc and distillation, organic matter (OM) by Walkley and Black method, available P by spectrophotometer after extracting with Bray II method, exchangeable K, Ca and Mg by atomic absorption spectrophotometer after extracting with $1N$ NH_4OAc pH 7.0, total N by Semi microkjeldahl method with distillation, and total Pb concentration by FAAS.

3.2.6.2 Plant inoculation

The selected new plant hosts were acclimatized for 7 d as described in section 3.1.1. Plants were washed twice with deionized water. The method for inoculation was performed according to section 3.2.5. After inoculation, inoculated and un-inoculated plants were extracted to isolate the inoculated *P. psychrophila* by the same method as for the endophytic bacterial isolation as described in section 3.2.3.1. This ensures that the *P. psychrophila* strain could dwell inside the plant root before planting in the pot.

3.2.6.3 Plant cultivation in pot experiment

The inoculated plants were washed with deionized water, and weighed for initial fresh weight before transplanting into plastic pots (9 inch in diameter, 7 inch in height). Each pot contained 2 kg of equilibrated soil. A piece of

filter paper was put at the bottom of the pot to retain the soil and to protect Pb and endophytic bacteria from leaking out. A pot with 1 plant represented 1 replicate. Each treatment was done in triplicate. There were 8 treatments as follows:

Treatment 1: Uncontaminated soil, *A. mangium*, un-inoculated

Treatment 2: Uncontaminated soil, *A. mangium*, inoculated

Treatment 3: Uncontaminated soil, *E. camaldulensis*, un-inoculated

Treatment 4: Uncontaminated soil, *E. camaldulensis*, inoculated

Treatment 5: Pb soil, *A. mangium*, un-inoculated

Treatment 6: Pb soil, *A. mangium*, inoculated

Treatment 7: Pb soil, *E. camaldulensis*, un-inoculated

Treatment 8: Pb soil, *E. camaldulensis*, inoculated

Plants were acclimatized under laboratory conditions for 3 days before transferring to the greenhouse for another 60 d (July-September 2016) under natural conditions. The average temperature was 35°C and 46% mean relative humidity. All pots were watered by tap water every 2 days to maintain 60% of water-holding capacity (200 mL). Soils were collected at day 0 and day 60. Plants were harvested after 60 d. Then they were weighed for initial fresh weight. Phytotoxicity symptoms were observed throughout the experiment. Pots were arranged in complete randomized block design. In each box, all pots were alternated every 2 d, but between boxes all pots were alternated after 1 month to ensure that they obtained similar environmental conditions (Figure 3.3).
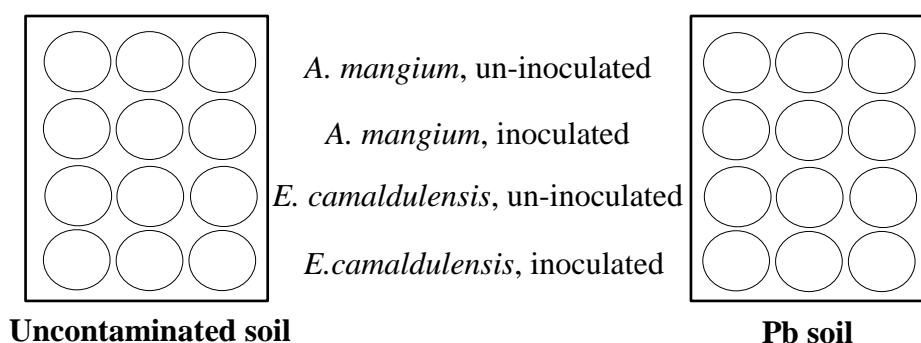
Figure 3.3 Plants arrangement in pot experiment

71

3.2.6.4 Pb concentration analysis

For total Pb concentration analysis, plants and soils were cleaned and digested as described in section 3.2.2.4, sub-section 3 and 4, respectively. Then samples were digested in a microwave (Mar 6, USA). The operational conditions and the heating program were set according to manufacturer's instruction. For plant digestion, a ramp time of 10 min was set to reach 200°C, then a hold time of 10 min according to manufacturer's instruction. Meanwhile, for soil digestion, a ramp time of 10 min was set to reach 200°C, then a hold time of 15 min. After digestion, the homogenized solutions were filtered using a Whatman No. 42 filter paper, and the volume was adjusted to 100 mL with deionized water. Pb concentrations were determined by an inductively coupled plasma optical emission spectroscopy (ICP-OES: Optima 8000: PerkinElmer, USA) with a hollow cathode lamp of Pb (10 mA, wavelength 220.35 nm, pressure: 280.0 kPa, flow: 0.55 L/min).

For available Pb concentration in soil, extractable Pb from top soil and soil around plant roots were extracted by DPTA solution as described in section 3.2.2.4 and sub-section 5. After extraction, samples were filtered and Pb concentration was analyzed as described in section 3.2.5.4.

3.3 Data analysis

In response to Pb exposure, the survival rate, tolerance index, relative growth, the percentage metal removal, bioconcentration factors (BCF) for whole plant and root part, biological absorption coefficient (BAC), translocation factor (TF) and metal uptake were calculated according to the following equations (Meeinkuirt et al. 2012; Zhivotovsky et al., 2011; Yaowakhan et al., 2005; Tanhan et al., 2007; Niu et al., 2007; Vamerali et al., 2010; Meeinkuirt et al., 2013, respectively):

$$\text{Survival rate (\%)} = \frac{\text{Number of plant at the end of experiment}}{\text{Number of plant at the beginning of experiment}} \times 100 \quad (1)$$

$$\text{Tolerance index (\%)} = \frac{\text{Dry weight of plant grown in Pb treatment (g)}}{\text{Dry weight of plant grown in control treatment (g)}} \times 100 \quad (2)$$

72

$$\text{Relative growth (\%)} = \frac{\text{Final fresh biomass (g) or height (cm)}}{\text{Initial fresh biomass (g) or height (cm)}} \times 100 \quad (3)$$

$$\text{The percentage heavy metal removal} = \frac{C_0 - C_1}{C_0} \times 100 \quad (4)$$

Where, C_0 is initial concentrations of heavy metal in medium (mg/L).

C_1 is remaining concentrations of heavy metal in medium (mg/L).

The BCF can be calculated by either whole or part of plant (Kim et al., 2003). The BCF was calculated from the whole plant for hydroponic test as shown in equation 5, while BCF in field survey was calculated from the roots as shown in equation 6.

$$\text{Bioconcentration factor} = \frac{\text{Heavy metal concentration in whole plant (mg/kg)}}{\text{Heavy metal concentration in solution (mg/L)}} \quad (5)$$

$$\text{Bioconcentration factor for root} = \frac{\text{Heavy metal concentration in root (mg/kg)}}{\text{Heavy metal concentration in soil (mg/kg)}} \quad (6)$$

$$\text{Biological absorption coefficient} = \frac{\text{Heavy metal concentration in shoot (mg/kg)}}{\text{Heavy metal concentration in soil (mg/kg)}} \quad (7)$$

$$\text{Translocation factor} = \frac{\text{Heavy metal concentration in shoot (mg/kg)}}{\text{Heavy metal concentration in root (mg/kg)}} \quad (8)$$

$$\text{Pb uptake (mg/plant)} = \text{Pb concentration in plant (mg/kg)} \times \text{dry biomass (g/plant)} \quad (9)$$

In response to bacterial selection, siderophore production and phosphate solubilization index, Pb solubilization and mobilization were calculated according to the following equations: (Searle et al., 2015; Edi Premono et al., 1996; Sheng et al., 2008, respectively):

73

$$\text{Siderophore production index} = \frac{\text{Colony diameter (mm)} + \text{Halo zone (mm)}}{\text{Colony diameter (mm)}} \quad (10)$$

$$\text{Phosphate solubilization index} = \frac{\text{Colony diameter (mm)} + \text{Clear zone (mm)}}{\text{Colony diameter (mm)}} \quad (11)$$

$$\text{Pb solubilization or mobilization} = \frac{\text{Pb concentration (mg/L) in isolates}}{\text{Pb concentration (mg/L) in control}} \quad (12)$$

3.4 Quantification analysis

Pb concentrations in all experiments were carried out with a calibration curve obtained from series of diluted standard solution with the coefficient of determination (r^2) higher than 0.995. The detection limit of Pb for FAAS is 0.05 mg/L, but for ICP-OES is 0.002 mg/L

3.5 Statistical analysis

All data were expressed as the mean ± standard deviation of three replicates. All experiments were statistically tested. Analysis of variance (ANOVA) followed by the Fisher Least Significant Difference (LSD) ($p \leq 0.05$) and independent sample t-test ($p \leq 0.05$) was used to compare treatment means. All the statistical analyses were carried out using SPSS for Window.

74

Chapter 4

Results and Discussion

4.1 Experiment I: Hydroponic screening of fast-growing tree species for Pb phytoremediation potential

4.1.1 Survival and growth performance

Throughout the experiment, 100% of *A. indica* and *E. camaldulensis* survived in all treatments. While *S. siamea* did not survive at 10 mg/L Pb (92% survival), and *A. mangium* did not survive at 30 and 50 mg/L Pb (83% survival). Leaves showed necrosis in *A. mangium* and chlorosis in *E. camaldulensis* (Figure 4.1). In addition, the common toxicity symptoms of all species were wilting and drooping of leaves. However, production of new roots was found in *A. mangium* and *E. camaldulensis* grown in low Pb concentration (10 mg/L) (Figure 4.2).
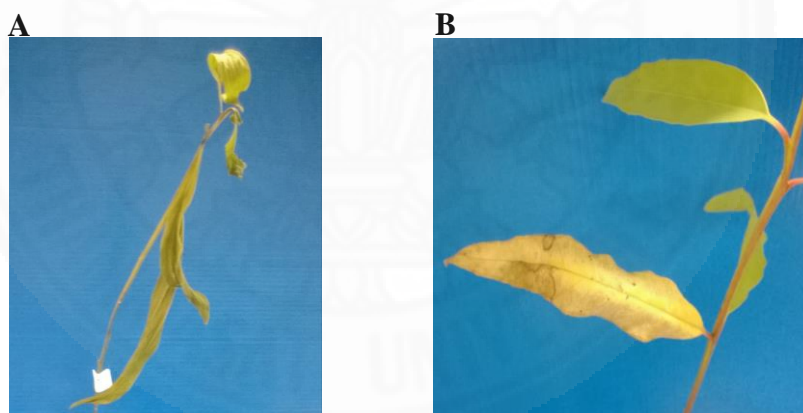
Figure 4.1 Pb phytotoxicity in plants: (A) *A. mangium*, (B) *E. camaldulensis*
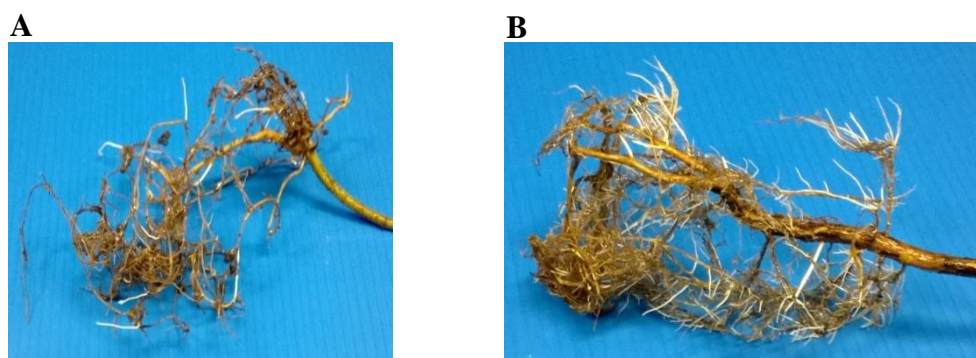
Figure 4.2 New root production: (A) *A. mangium*, (B) *E. camaldulensis*

75

Pb causes many effects on various levels such as physiology, biochemical and structural changes, enzyme activity, photosynthesis, nutrient uptake, oxidative metabolisms and water status (Sharma and Dubey, 2005). In this study, the visible symptoms of Pb toxicity including chlorosis and necrosis were found. Similarly, willows exposed to 241 μM Pb for 2 weeks also showed Pb phytotoxicity in as chlorosis and necrotic regions were observed along the ribs. Some leaves had veinal and interveinal chlorosis at the basal section of the leaf, following the midrib and then expanding out to the leaf margins and tips (Zhivotovsky et al., 2011). Chlorosis and necrosis could be due to reduction of chlorophyll, disruption of thylakoid and stromal membranes (Sharma and Dubey, 2005; Almeida et al., 2007). In addition, Pb phytotoxicity depends on the concentration, salt type, pH and plant species involved (Almeida et al., 2007). Pb can induce a decline in water content by lowering the level of compounds that are associated with maintaining cell turgor and cell wall plasticity, thereby reducing the water potential within the cell (Sharma and Dubey, 2005). This could explain the wilting and drooping of leaves in the present study.

Importantly, only *A. mangium* and *E. camaldulensis* could produce the new white roots at lower Pb concentration. This can be explained that Pb is able to stimulate metabolic processes at low concentrations (Almeida et al., 2007). Additionally, Cheng (2003) reported that plants treated by low Pb concentration, the cell division exponent rose from 16% to 20%, while increasing the Pb concentration, the cell division exponent declined. In addition, the process involves root production by plant hormones such as abscisic acid and auxin. Abscisic acid involves in regulating the production of lateral roots and controlling root elongation (Harris, 2015). Auxin is known to induce root growth by enhancing cell division, cell extension and inducing lateral root growth (Fässler et al., 2010). Sadeghipour (2017) also reported an increase in the level of abscisic acid under exposure to Pb. Plants treated with Pb and exogenous auxin showed an significant increase of root growth when compared to those treated with Pb alone (Fässler et al., 2010).

The effect of Pb concentrations on the fresh weight of plants is shown in Table 4.1 and Figure 4.3. These values were used to calculate relative plant growth.

76

Table 4.1 Fresh weight of plants grown in various Pb concentrations for 15 d

[Pb] (mg/L)	*A. indica*	*A. mangium*	*E. camaldulensis*	*S. siamea*
Initial fresh weight (g/plant)				
Control	5.94 ± 0.81	5.01 ± 2.66	5.21 ± 0.87	7.05 ± 0.76
10	5.66 ± 0.52	4.98 ± 1.23	5.25 ± 0.64	6.80 ± 0.97
30	6.00 ± 1.09	4.78 ± 1.60	5.72 ± 1.00	7.24 ± 2.65
50	6.88 ± 1.40	5.14 ± 0.61	5.29 ± 0.87	7.44 ± 1.12
Final fresh weight (g/plant)				
Control	5.11 ± 0.60	6.10 ± 2.48	5.36 ± 1.14	7.43 ± 1.21
10	5.30 ± 0.55	5.05 ± 1.04	5.15 ± 0.40	7.30 ± 1.15
30	4.60 ± 1.13	4.94 ± 2.00	4.94 ± 0.97	6.84 ± 2.48
50	5.66 ± 1.21	4.15 ± 0.04	4.59 ± 0.87	7.11 ± 1.11
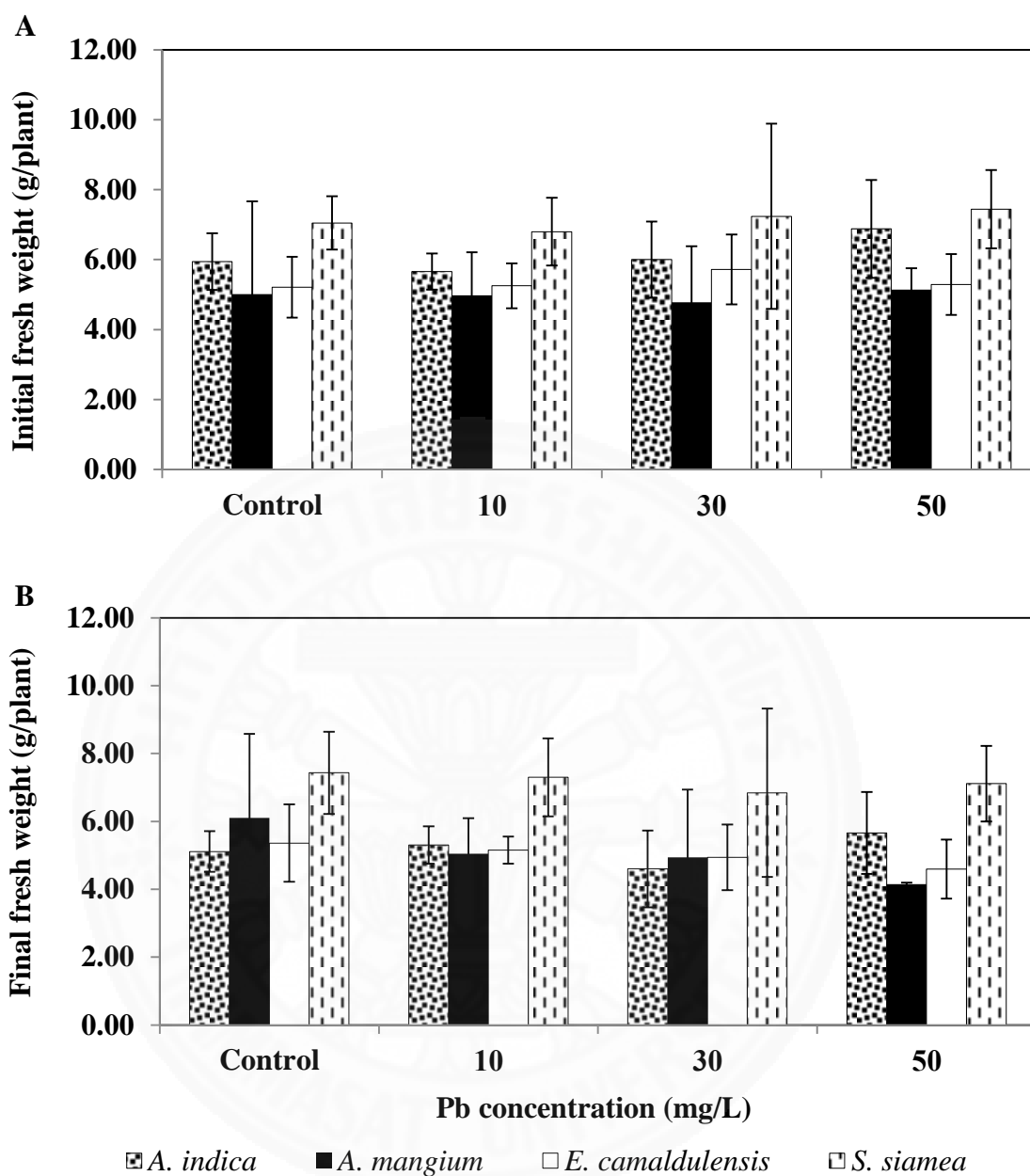
Figure 4.3 Fresh weight of plants grown in various Pb concentrations for 15 d: (A) initial fresh weight, (B) final fresh weight. Values are the mean of triplicate samples. Error bars are standard deviations.

78

The effect of Pb concentrations on the relative growth (RG) of plants is shown in Table 4.2 and Figure 4.4. There were positive and negative effects on relative growth of plants depending on species and Pb concentrations. The RGs of *E. camaldulensis* and *S. siamea* were not significantly decreased ($p > 0.05$) for all Pb concentrations. The RG of *A. mangium* was significantly decreased ($p \leq 0.05$) at the highest Pb concentration (50 mg/L) compared to other concentrations investigated. The RGs for *A. indica* significantly decreased ($p \leq 0.05$) at Pb concentration of 30 mg/L while increased at 10 mg/L. At the highest Pb concentration, *S. siamea* showed the best growth compared to other species. Moreover, most plants showed no significant difference ($p > 0.05$) in the RG values at the highest Pb concentration (50 mg/L), except for *A. mangium* when compared with the control. The greatest reduction of growth was recorded for *A. mangium* treated with Pb, as the RG was reduced from $102 \pm 5.17\%$ (10 mg Pb/L) to $86.5 \pm 6.30\%$ (50 mg Pb/L).

Table 4.2 Relative growths of plants grown in various Pb concentrations for 15 d

[Pb] (mg/L)	*A. indica*	*A. mangium*	*E. camaldulensis*	*S. siamea*
Control	$86.3 \pm 2.52^{a,A}$	$93.9 \pm 9.48^{ab,A}$	$103 \pm 11.8^{b,A}$	$105 \pm 7.49^{b,A}$
10	$93.6 \pm 2.14^{a,B}$	$102 \pm 5.17^{b,A}$	$98.6 \pm 4.82^{ab,A}$	$103 \pm 1.29^{b,A}$
30	$76.3 \pm 5.95^{a,C}$	$97.3 \pm 1.51^{b,A}$	$87.0 \pm 13.7^{ab,A}$	$94.6 \pm 5.99^{b,A}$
50	$82.1 \pm 2.01^{a,A}$	$86.5 \pm 6.30^{a,B}$	$86.7 \pm 6.36^{a,A}$	$95.5 \pm 0.59^{b,A}$

Each value is mean of triplicate samples ± standard deviation. Different small letters indicate significant difference of RG among plant species for each treatment, while different capital letters indicate significant difference of RG among treatments of each plant species at $p \leq 0.05$ according to Fisher's LSD test. *A. indica* (Ai); *A. mangium* (Am); *E. camaldulensis* (Ec) and *S. siamae* (Ss).

79
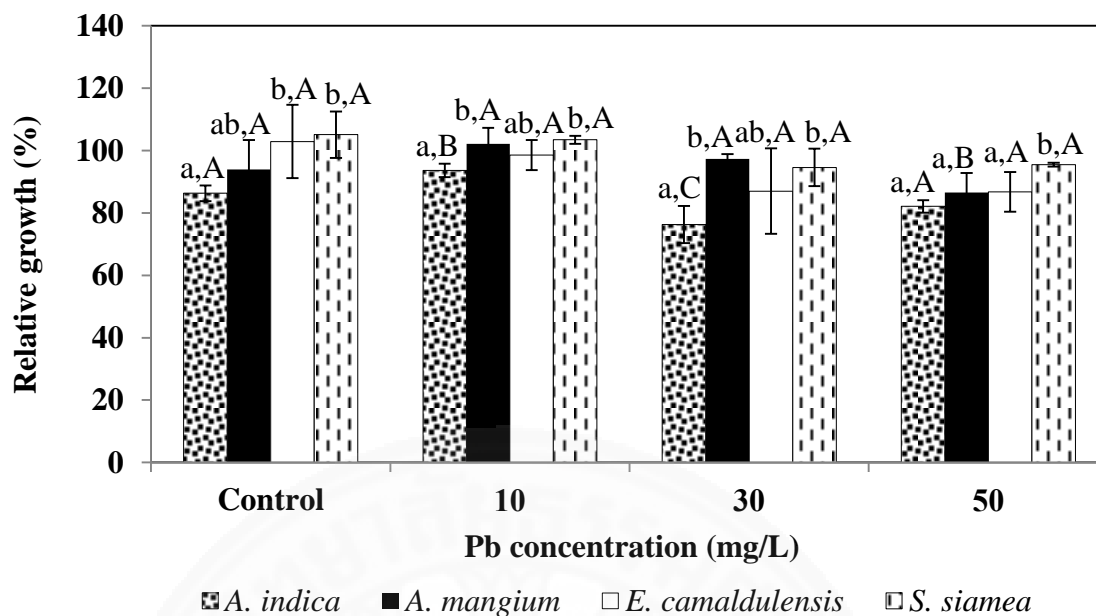
Figure 4.4 Relative growth of plants grown in various Pb concentrations for 15 d. Values are the mean of triplicate samples. Error bars are standard deviations. Different small letters indicate a significant difference of RG among plant species for each treatment, while different capital letters indicate significant difference of RG among different treatments of each plant at $p \leq 0.05$, according to Fisher's LSD test.

The effect of Pb concentrations on biomass production is shown in Table 4.3 and Figure 4.5. The dry weights (DW) of all plant species were not significantly affected ($p > 0.05$) by different Pb concentrations investigated. At the highest Pb concentration, the dry weight of *A. mangium* showed a significant difference as compared with *A. indica* and *S. siamae*. The greatest reduction of biomass production was recorded for *E. camaldulensis* treated with Pb, as the dry weight was reduced from 1.84 g (10 mg Pb/L) to 1.46 g (50 mg Pb/L).

Table 4.3 Dry weight and Pb accumulation of plants grown in various Pb concentrations for 15 d

Pb (mg/L)	Plant	Dry weight (g/plant)	Pb accumulation (mg/kg)	
			Root	Shoot
0	Ai	$1.72 \pm 0.17^{a,A}$	-	-
	Am	$1.47 \pm 0.41^{a,A}$	-	-
	Ec	$1.77 \pm 0.31^{a,A}$	-	-
	Ss	$1.98 \pm 0.08^{a,A}$	-	-
10	Ai	$1.73 \pm 0.30^{a,A}$	$7413 \pm 2537^{a,B}$	$1284 \pm 98.8^{a,B}$
	Am	$1.27 \pm 0.27^{a,A}$	$17956 \pm 3225^{b,B}$	$401 \pm 92.3^{b,B}$
	Ec	$1.84 \pm 0.07^{a,A}$	$17797 \pm 3139^{b,B}$	$278 \pm 55.1^{b,A}$
	Ss	$1.81 \pm 0.23^{a,A}$	$8750 \pm 114^{a,B}$	$302 \pm 85.2^{b,B}$
30	Ai	$1.62 \pm 0.44^{a,A}$	$11723 \pm 2936^{a,BC}$	$1397 \pm 179^{a,B}$
	Am	$1.42 \pm 0.48^{a,A}$	$30830 \pm 6852^{b,C}$	$668 \pm 13.4^{a,C}$
	Ec	$1.86 \pm 0.30^{a,A}$	$31210 \pm 5461^{b,C}$	$1117 \pm 512^{a,B}$
	Ss	$1.88 \pm 0.77^{a,A}$	$16799 \pm 594^{a,C}$	$1374 \pm 256^{a,C}$
50	Ai	$1.91 \pm 0.52^{ac,A}$	$15158 \pm 6046^{a,C}$	$4097 \pm 782^{a,C}$
	Am	$1.16 \pm 0.03^{b,A}$	$49004 \pm 6149^{b,D}$	$764 \pm 10.1^{b,C}$
	Ec	$1.46 \pm 0.17^{bc,A}$	$40598 \pm 2694^{bc,C}$	$1840 \pm 125^{c,C}$
	Ss	$1.68 \pm 0.22^{c,A}$	$26722 \pm 8557^{ac,D}$	$2339 \pm 198^{c,D}$

Each value is mean of triplicate samples ± standard deviation. Different small letters indicate significant difference of each column among plant species for each treatment, while different capital letters indicate significant difference of each column among treatments of each plant species at $p \leq 0.05$ according to Fisher's LSD test. *A. indica* (Ai); *A. mangium* (Am); *E. camaldulensis* (Ec); *S. siamae* (Ss).

81
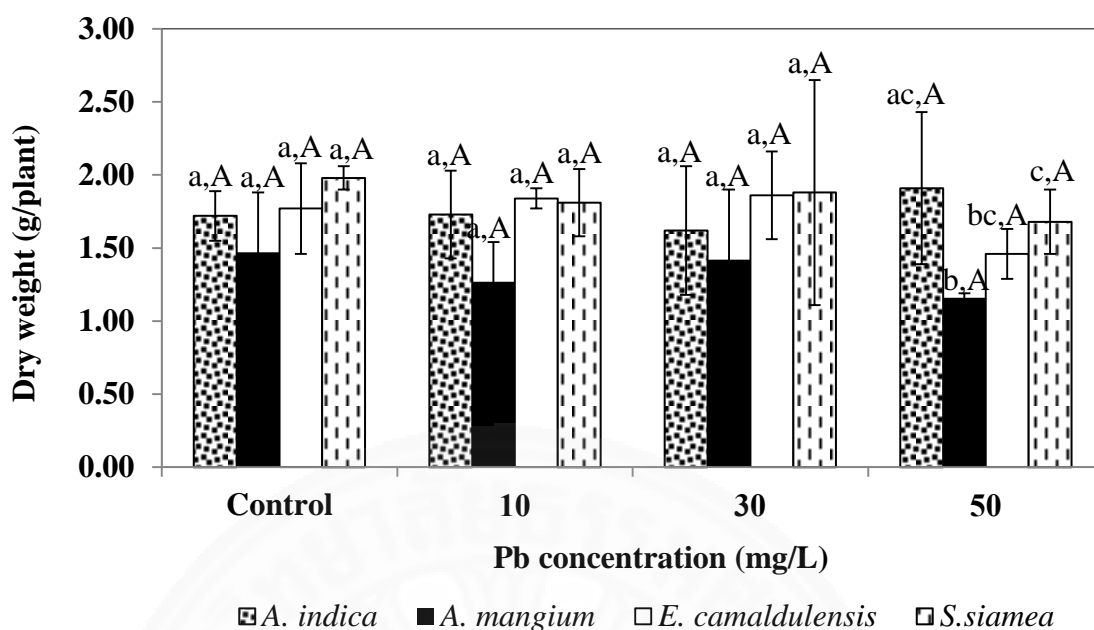
Figure 4.5 Dry weight of plants grown in various Pb concentrations for 15 d. Values are the mean of triplicate samples. Error bars are standard deviations. Different small letters indicate a significant difference of DW among plant species for each treatment, while different capital letters indicate significant difference of DW among different treatments of each plant at $p \leq 0.05$, according to Fisher's LSD test.

The results of growth reduction under Pb toxicity are in line with previous studies (Zhivotovsky et al., 2011; Karimi et al., 2012). This can be due to low water potential, hampered nutrient uptake, secondary stress, disturbed microtubule organization in meristematic cells, reduction in leaf photosynthetic rate, and drooping of leaves which are the main photosynthetic organ, and increased non-photosynthetic biomass of roots (Karimi et al., 2012; Aini Syuhaida et al., 2014). In addition, the dry weight reduction relates to high Pb accumulation, since plants may have to use energy to cope with the high Pb concentration in their tissues (Karimi et al., 2012). In this study, *A. indica* treated with 50 mg/L showed the highest dry weight, but the lowest Pb content in plants. Moreover, the biomass yields of *A. indica* and *E. camaldulensis* at 10 mg/L of Pb were higher than that of the control. This phenomenon might be due to the increased synthesis of cell wall polysaccharides in the nutrient solution (Liu et al., 2015). Karimi et al. (2012) also reported that, in some cases, increased plant biomass is observed at low concentrations of heavy metals.

82

4.1.2 Pb accumulation in plant organs

The effect of Pb concentrations on Pb accumulation in plant tissues is shown in Table 4.3 and Figures 4.6A and B. The results showed that both Pb concentration and species of plant candidate have a significant effect on Pb accumulation both in shoots and roots. There was an increased trend of Pb content in shoots and roots of all species. Moreover, all species had higher Pb concentrations in roots than in shoots in all treatments. In addition, *A. indica* showed the best Pb accumulation in shoots (4097 mg/kg) (Figure 4.6A), while *A. mangium* showed the highest Pb accumulation in roots (49004 mg/kg) (Figure 4.6B) at 50 mg/L of Pb.

Figure 4.6A shows that Pb content in shoot of *A. indica* had a significant difference ($p \leq 0.05$) with the remaining species. *E. camaldulensis* and *S. siamae* showed a significant increase ($p \leq 0.05$) of Pb in plant shoots as Pb concentrations were increased from 10 to 50 mg/L. *A. indica* showed a significant increase ($p \leq 0.05$) of Pb in plant shoots as Pb concentrations were increased from 10 and 30 to 50 mg/L. *A. mangium* showed a significant increase ($p \leq 0.05$) of Pb in plant shoots as Pb concentrations were increased from 10 mg/L to 30 and 50 mg/L.

Figure 4.6B also shows clearly a significant difference ($p \leq 0.05$) between *A. mangium* and *E. camaldulensis*, and *A. indica* and *S. siamae*. However, Pb content in roots of each member of the same group did not show any significant difference ($p > 0.05$).

Pb content in plant tissues from this study showed difference with previous works. At 10 mg/L Pb, the candidate fast-growing trees could accumulate Pb in the range of 278-1284 mg/kg in shoots, and 7413-17956 mg/kg in roots. These values are higher than those in willow species (114-637 mg/kg in shoots, and 4164-5567 mg/kg in roots) grown in hydroponics (48 µM for 14 d) (Zhivotovsky et al., 2011). This implies that if willow has been accepted as a popular plant for contaminated soil remediation with a high ability to accumulate heavy metals (Malá et al., 2010), the trees of this study can also be employed for phytoremediation as well.
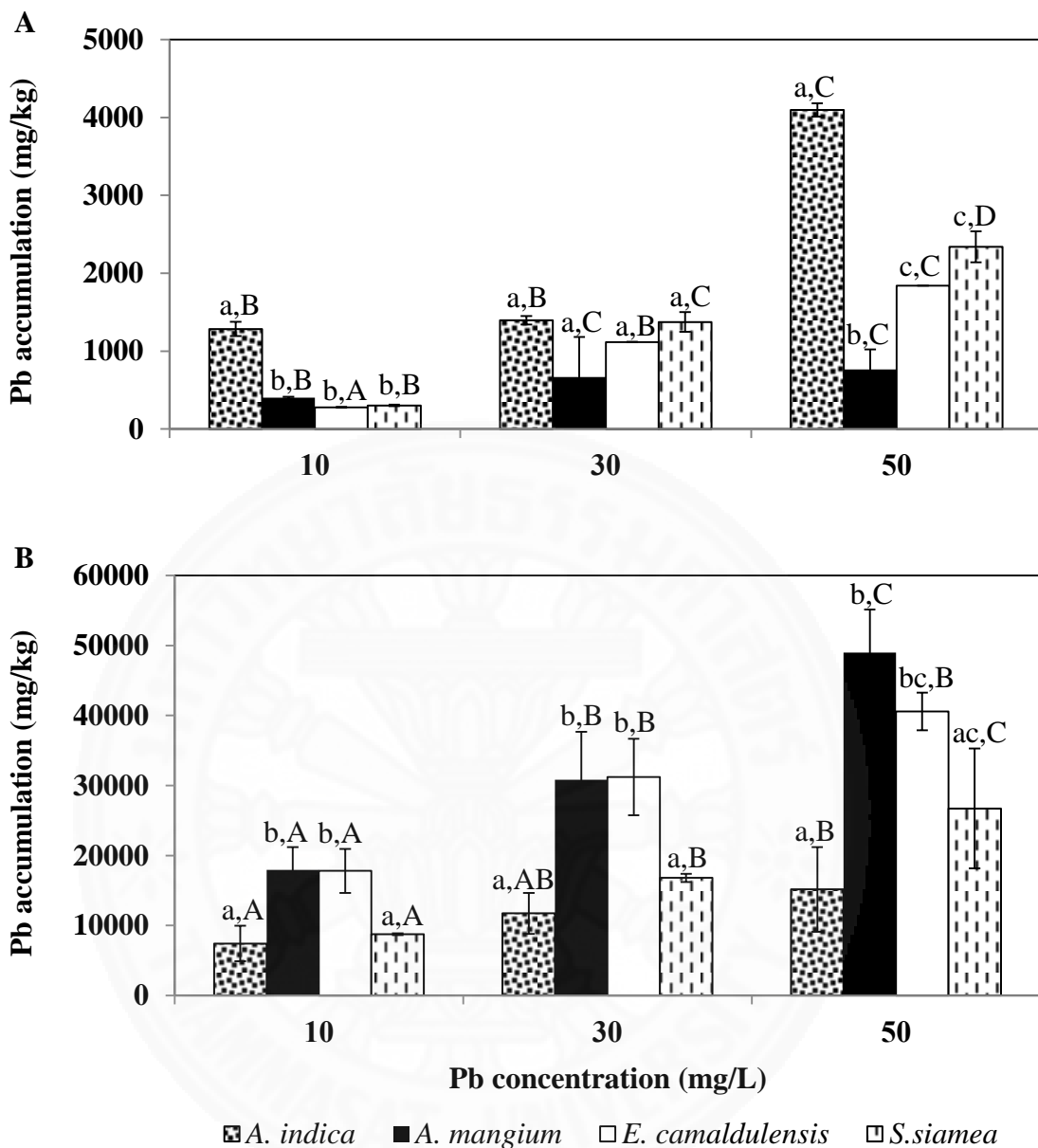
Figure 4.6 Pb accumulation in plant organs: (A) in shoot, (B) in root of plant grown in various Pb concentrations for 15 d. Values are the mean of triplicate samples. Error bars are standard deviations. Different small letters indicate a significant difference of Pb accumulation among plant species for each treatment, while different capital letters indicate significant difference of Pb accumulation among different treatments of each plant at $p \leq 0.05$, according to Fisher's LSD test.

The highest Pb accumulation values (1284 mg/kg in shoots, and 49004 mg/kg in roots) from this study were lower than those in the shrubs, *Chromolaena odorata* (L.) R.M.King & H.Rob. with Pb in shoots 1721 mg/kg, and in roots 51493 mg/kg. In addition, Pb accumulation values of this study were higher than those in herbs such as *B. juncea* and *Medicago sativa* L. They accumulated Pb in their tissues approximately 400 mg/kg at 50 mg/L Pb in solution (Niu et al., 2007). Similarly, Yanqun et al. (2004) indicated that the heavy metal accumulation ability of trees is lower than that of shrubs, but higher than that of herbs.

The present investigation showed that Pb accumulations in shoots and in roots were positively increased with increasing Pb concentrations. A similar pattern can be found in other hydroponic studies despite different plant types such as monocots (*Phyllostachys pubescens* Mazel ex H. De Lehale); perennial herb (*Cynara scolymus* L.); crop (*H. annuus*, *B. juncea*, *M. sativa*, and *R. communis*); and shrub (*C. odorata*, and *S. drummondii*) (Sahi et al., 2002; Romeiro et al., 2006; Niu et al., 2007; Tanhan et al., 2007; Karimi et al., 2012; Liu et al., 2015). Additionally, the accumulation in plant tissues has an upper limit. If Pb contents exceed this point, the accumulation will reduce (Sahi et al., 2002; Zhivotovsky et al., 2011). The results follow similar trend as some other studies. Sahi et al. (2002) found that the highest Pb accumulation in shoots of *S. drummondii* was found in plants grown in modified Hoagland's medium added with 1000 mg/L of Pb. Accumulation of Pb was slightly reduced when plants were grown in 1500 mg/L. Zhivotovsky et al. (2011) also found that Pb accumulation in roots of *Salix lucida* Muhl. (4164, 10064 and 11807 mg/kg) and *Salix serissima* (L.H. Bailey) Fernald (4243, 9573 and 11247 mg/kg) increased as Pb concentration increased from 48, 121 and 169 μM, respectively. But when Pb concentration increased up to 241 μM, their Pb accumulation in roots was reduced for *S. lucida* (11535 mg/kg) and for *S. serissima* (7036 mg/kg).

The other common phenomenon is Pb accumulation in roots (about 95% or more) more than that in shoots. Several previous studies including this study show the same pattern. Zhivotovsky et al. (2011) found that at the highest Pb concentration 241 μM, *S. lucida*, *Salix nigra* Marshall and *S. serissima* had Pb concentration in roots (11535, 14091 and 7036, respectively) higher than those in aerial tissue; in wood (126, 249 and 133, respectively), in shoot (55.1, 149 and 117,

85

respectively), and in leaves (184, 25.1 and 47.1, respectively). Liu et al. (2015) found that *P. pubescens* grown in nutrient solution supplemented with 200 µM Pb contained higher Pb in the root 1221 mg/kg as compared to that in the stem (351 mg/kg) and in leaf (165 mg/kg). An efficient barrier of Pb translocation is the blockage by Casparain strips acting as a physical barrier within the endodermis, where the major part of Pb is sequestered or excreted by plant detoxification systems. Besides this barrier, the other potential reasons are immobilization by negatively charged pectins within the cell wall, precipitation of insoluble Pb salts as Pb carbonate in intercellular spaces, accumulation in plasma membranes, or sequestration in the vacuoles of rhizodermal and cortical cells (Pourrut et al., 2011). With these reasons, TF values of plants were less than 1 for field or hydroponic experiments (Yanqun et al., 2004; Malar et al., 2014).

4.1.3 Percentage of Pb removal

The effect of Pb concentrations on the percentage of Pb removal is shown in Table 4.4 and Figure 4.7. Plants and Pb concentrations showed a significant difference ($p > 0.05$) in Pb removal after 5 d. The percentage of Pb removal of all treatments showed the same trend, namely it was the highest (94.0-100%) in the first 5 d for all treatments. In addition, the percentage of Pb removal decreased with increasing exposure time. *A. indica* showed the best removal efficiency (93.1-95.1%, respectively) at higher Pb concentrations after 10 and 15 d of exposure time.

The results from this study indicated that all plants had high Pb removal capacity. In other words, the trees in this study can remove almost all of Pb from the solutions, which is consistent with the removal of *R. communis* exposed to 100 and 200 µM of Pb (Romeiro et al., 2006). In addition, similar result from hydroponic culture was found in *C. odorata* at 10 mg/L of Pb, the highest Pb removal was almost 100% after the first 3 d of exposure (Tanhan et al., 2007). Similarly, Pb accumulation by *S. drummondii* was the highest on day 5 after grown on modified Hoagland's medium (Sahi et al., 2002). This can be due to the saturation state. When saturation state is achieved, it is difficult for plants to absorb more metals (Aini Syuhaida et al., 2014).

86

The maximum Pb removal at the first 5 d involved the pH of solution. The removal of Pb from hydroponic solutions was strongly related to solution pH. Pb can be easily uptaken at low pH (3.7-4.7) (Sahi et al., 2002). The initial pH solution of this study was adjusted to 5.0. In addition, once Pb enters the root, it normally precipitates with phosphate or carbonate. This helps the plant to reduce the uptake of Pb to prevent damage to the plant tissues (Aini Syuhaida et al., 2014). Additionally, mechanisms of Pb uptake are passive (apoplastic transport) pathway followed by translocation water streams (symplastic transport). Also, Ca^{2+} permeable channels are the main pathway of Pb entering into roots (Pourrut et al., 2011).

Table 4.4 Effect of Pb concentration and exposure time on Pb removal by fast-growing trees grown in hydroponic culture

[Pb] (mg/L)	Plant	Exposure time (day)		
		5	10	15
10	Ai	99.0 ± 1.25^{abd}	94.1 ± 0.99^{acd}	74.9 ± 10.0^{be}
	Am	98.3 ± 1.60^{abe}	89.8 ± 5.35^{abcde}	68.6 ± 11.0^{e}
	Ec	100 ± 0.00^{a}	95.7 ± 3.61^{a}	89.3 ± 4.23^{ac}
	Ss	99.6 ± 0.38^{a}	95.6 ± 4.80^{a}	92.8 ± 5.31^{ad}
30	Ai	96.7 ± 2.01^{be}	89.1 ± 7.18^{abcde}	89.2 ± 6.54^{ac}
	Am	94.0 ± 2.89^{c}	88.2 ± 1.29^{bde}	79.2 ± 1.67^{b}
	Ec	98.4 ± 1.63^{ab}	83.7 ± 3.35^{be}	80.2 ± 2.40^{bc}
	Ss	98.5 ± 1.42^{ab}	87.3 ± 7.03^{bde}	84.5 ± 6.55^{bcd}
50	Ai	95.7 ± 2.11^{ce}	93.1 ± 5.23^{abd}	95.1 ± 5.03^{a}
	Am	96.6 ± 1.76^{bcd}	93.4 ± 1.69^{abd}	92.6 ± 1.23^{ad}
	Ec	97.8 ± 1.37^{abe}	86.4 ± 3.13^{b}	84.5 ± 2.63^{bcd}
	Ss	98.8 ± 0.37^{ab}	93.2 ± 1.13^{abd}	90.4 ± 2.89^{ad}

Each value is mean of triplicate samples ± standard deviation. Different small letters indicate significant difference of Pb removal among plant species for each treatment in each exposure time at $p \leq 0.05$ according to Fisher's LSD test. *A. indica* (Ai); *A. mangium* (Am); *E. camaldulensis* (Ec) and *S. siamae* (Ss).
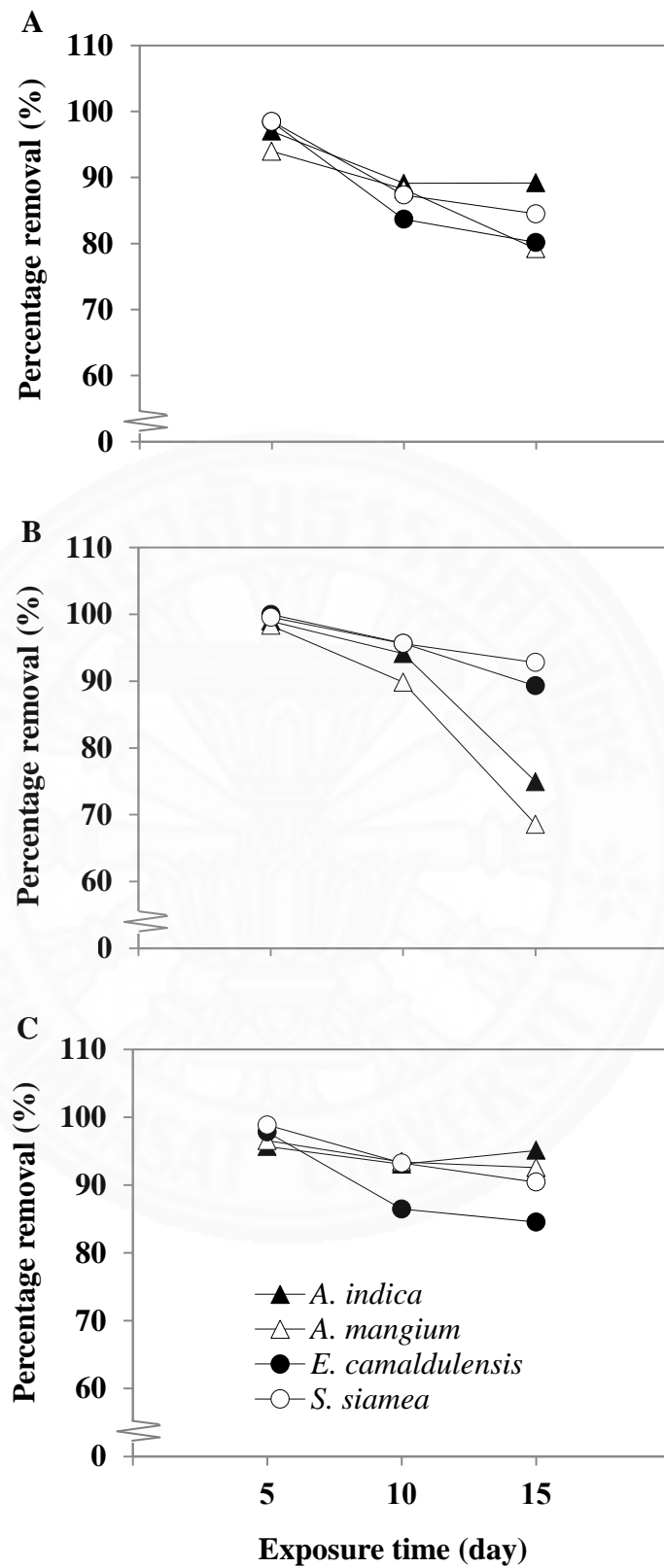
Figure 4.7 Percentage of Pb removal by plants at different concentrations of Pb: (A) 10 mg/L, (B) 30 mg/L, (C) 50 mg/L. Values are the means of triplicate samples.

88

4.1.4 Tolerance index

One of the most general parameters used to evaluate plant's ability to tolerate heavy metals is the tolerance index (TI). In this study, the plant's ability to tolerate Pb is presented in Table 4.5 and Figure 4.8. Both Pb concentrations and plant species did not significantly affect the tolerance index ($p > 0.05$). Also, the ability to tolerate Pb in these plant species showed no clear trend. However, at the highest Pb concentration (50 mg/L), *A. mangium* showed the lowest Pb tolerance.

The evaluation of a plant's ability to tolerate Pb toxicity is of supreme importance for plant screening used in phytoremediation (Wang et al., 2014). All trees in this study showed high tolerance to Pb with TI values over 75%. The criterion value is 60% (Zhivotovsky et al., 2011; Wang et al., 2014) which is defined as high tolerance ability. Moreover, the lowest Pb tolerance from this study was 79.0% (50 mg/L Pb) for *A. mangium* which indeed is higher compared to other tree species investigated by other researchers. For example, *S. lucida* and *S. nigra* treated in 241 µM of Pb showed TI values of 39% and 31%, respectively (Zhivotovsky et al., 2011). *Salix integra* Thunb. with 3 different varieties, such as Yizhibi, Weishanhu, and Dahongtou, treated in 196 µM of Pb showed TI values of 67%, 73%, 63%, respectively (Wang et al., 2014). The reason why TI values of plants in this study are higher than those of the other studies may be explained by the initial lower Pb concentration used. Since, TI values for plants decreased as the heavy metal concentration increased (Aini Syuhaida et al., 2014). Normally, TI values vary among plant species and variety (Zhivotovsky et al., 2011; Wang et al., 2014). Different plant species may have developed different mechanisms to tolerate excess levels of metals (Aini Syuhaida et al., 2014).

The effect of Pb concentrations on BCF and TF values is shown in Table 4.5 and Figures 4.9 and 4.10. The BCFs of all plant species decreased when Pb concentrations were increased. *A. mangium* showed the highest BCF value (1836) after treated with 10 mg/L of Pb. All plant species have TFs < 1 in all Pb solutions. The highest TF value (0.30) was observed in *A. indica*, whereas the lowest (0.01) was found in *A. mangium* and *E. camaldulensis*.

Table 4.5 TI, BCF, and TF of plants grown in various Pb concentrations for 15 d.

[Pb] (mg/L)	Plants	TI (%)	BCF	TF
0	A. indica	100 ± 9.7[a,A]	-	0.90 ± 0.08
	A. mangium	100 ± 28[a,A]	-	0.71 ± 0.21
	E. camaldulensis	100 ± 17[a,A]	-	0.83 ± 0.18
	S. Siamae	100 ± 4.0[a,A]	-	0.27 ± 0.09
10	A. indica	101 ± 18[a,A]	869.7 ± 258	0.19 ± 0.06
	A. mangium	86.5 ± 19[a,A]	1836 ± 323	0.02 ± 0.01
	E. camaldulensis	104 ± 4.4[a,A]	1808 ± 308	0.02 ± 0.01
	S. Siamae	91.6 ± 12[a,A]	905.2 ± 2.84	0.03 ± 0.01
30	A. indica	94.1 ± 25[a,A]	437.3 ± 92.3	0.13 ± 0.05
	A. mangium	96.9 ± 33[a,A]	1050 ± 220	0.02 ± 0.00
	E. camaldulensis	105 ±17[a,A]	1078 ± 167	0.04 ± 0.01
	S. Siamae	94.9 ± 39[a,A]	605.8 ± 11.5	0.08 ± 0.02
50	A. indica	111 ± 30[a,A]	385.1 ± 125	0.30 ± 0.13
	A. mangium	79.0 ± 2.2[a,A]	995.4 ± 123	0.01 ± 0.00
	E. camaldulensis	82.1 ± 9.4[a,A]	848.8 ± 52.4	0.05 ± 0.01
	S. Siamae	84.7 ± 11[a,A]	581.2 ± 173	0.09 ± 0.03

Each value is mean of triplicate samples ± standard deviation. Different small letters indicate significant difference of each column among plant species for each treatment, while different capital letters indicate significant difference of each column among treatments of each plant species at $p \leq 0.05$ according to Fisher's LSD test.
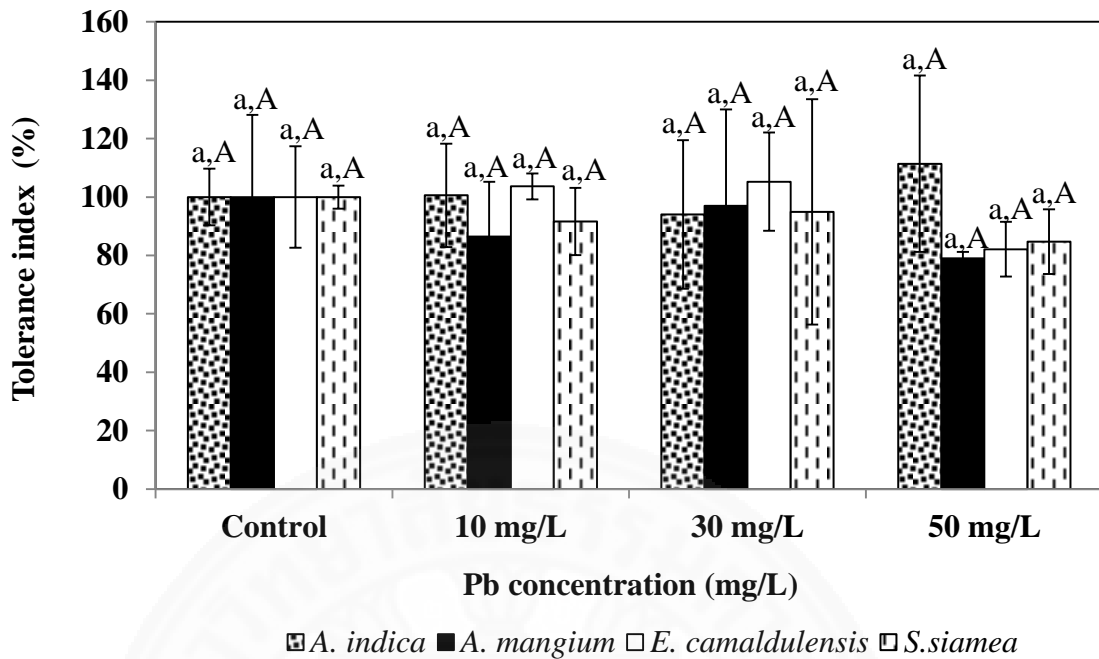
Figure 4.8 Pb tolerance of plants grown in various Pb concentrations for 15 d. Values are the mean of triplicate samples. Error bars are standard deviations. Different small letters indicate a significant difference of Pb tolerance among plant species for each treatment, while different capital letters indicate significant difference of Pb tolerance among different treatments of each plant at $p \leq 0.05$, according to Fisher's LSD test.
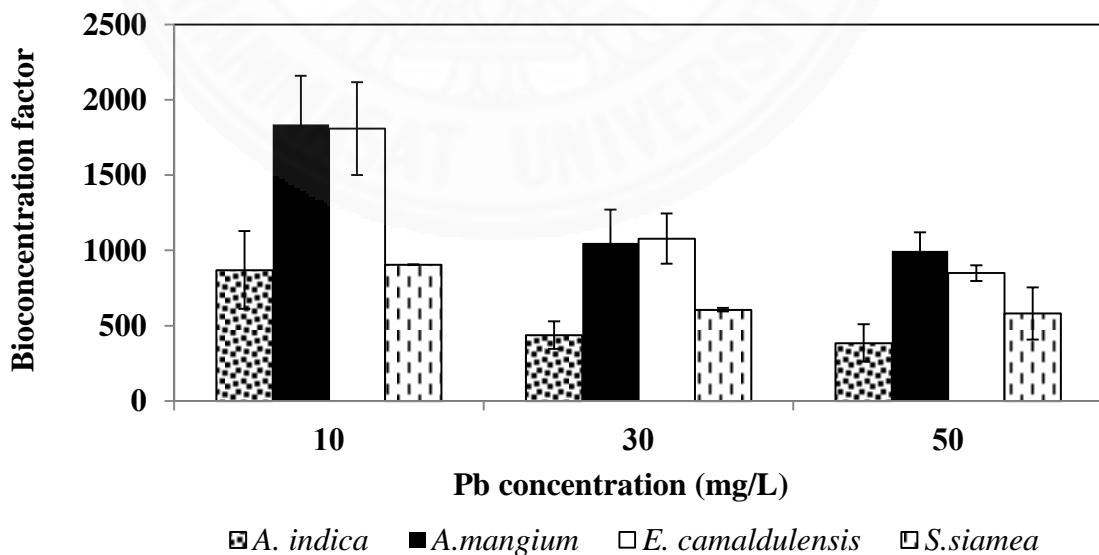
Figure 4.9 Bioconcentration factor of plants grown in various Pb concentrations for 15 d. Values are the mean of triplicate samples. Error bars are standard deviations.

91
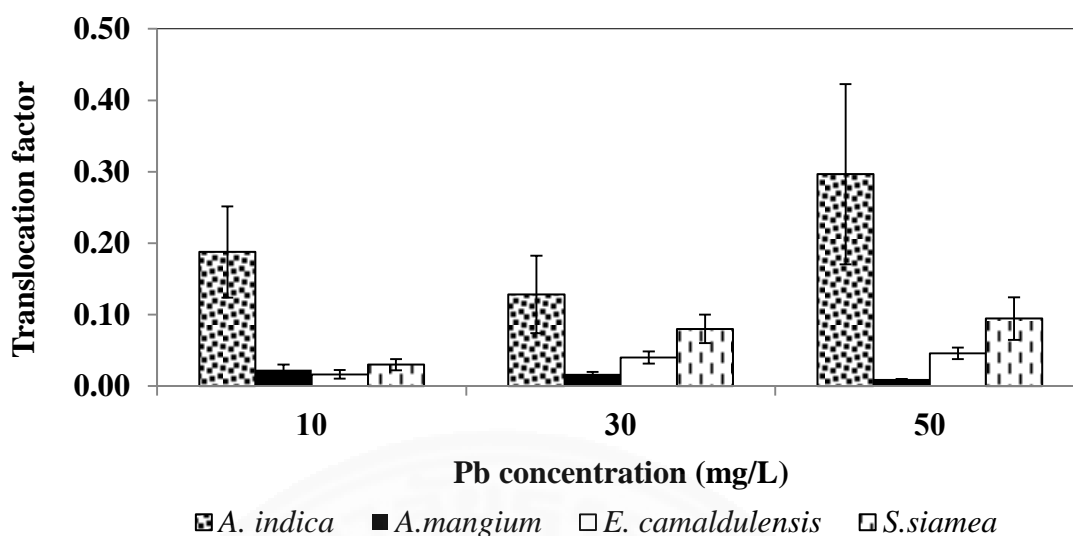
Figure 4.10 Translocation factor of plants grown in various Pb concentrations for 15 d. Values are the mean of triplicate samples. Error bars are standard deviations.

A negative relationship between BCFs and Pb concentrations is found in many works including this study (Niu et al., 2007; Tanhan et al., 2007; Liu et al., 2015). At low metal concentrations, there is low competition between ions at the uptake sites, while the opposite trend occurs at high metal concentration (Prasad and Hagemeyer, 1999). The BCF of *H. annuitaus*, *B. juncea*, *M. sativa*, *R. communis* was < 12 at 50 mg/L of Pb concentration (Niu et al. 2007). Conversely, the BCF of *C. odorata*, classified as a moderate hyperaccumulator, was 5320 at 10 mg/L of Pb (Tanhan et al., 2007). An extremely low BCF belongs to herb *Hibiscus cannabinus* (1.92 to 3.21 at 100-400 mg/L of Pb) (Ho et al., 2008). However, in order to indicate the efficiency of accumulation ability, BCFs of trees tested with similar experiments need to be compared. The BCF of *S. nigra* is 641 (calculated from the result of Zhivotovsky et al., 2011). These imply that the accumulation ability of all fast-growing trees from this study is higher than that of willow species, which is a prospective candidate for phytoremediation. Interestingly, the accumulation ability of plants cannot be related with tolerance and uptake ability (Pulford and Watson, 2003; Niu et al., 2007). In this study, *A. mangium* showed the highest accumulation ability (BCF), but it had the lowest Pb tolerance index (86.5%). This may be due to differences in physiological and morphological characteristics (Niu et al., 2007).

92

Since, BCF and TF values are used to evaluate a plant's ability to accumulate and translocate heavy metals, and identify the suitability of plants for phytoextraction and phytostabilization (Niu et al., 2007; Ali et al., 2013; Wang et al., 2014), values > 1 indicates that the plant has the potential for phytoextraction (Ali et al. 2013). However, BCF value varies in environmental media. In soil with high heavy metal concentration could result in BCF value < 1 (Ali et al., 2013). Meanwhile, in hydroponic test, a BCF value ≥ 1000 is used to identify the capacity of plant to accumulate heavy metals (Aini Syuhaida et al., 2014). In this study, the average BCF values of *A. mangium* and *E. camaldulensis* were 1294 and 1245, respectively, whereas those in *A. indica* and *S. siamea* were 564 and 697, respectively. With hydroponic BCF criterion, only *A. mangium* and *E. camaldulensis* were useful for phytoremediation. However, to reduce the effect resulting from substrate, TF value could be used to identify. Normally, TF > 1 is identified as an accumulator, while TF < 1 is classified as an excluder (Baker, 1981). In this study, all fast-growing trees showed TF values < 1. Hence, they were classified as excluders having the ability to accumulate heavy metal from substrate into the roots, but restrict the transport into the shoot. The excluders have a low potential for phytoextraction, but may be efficient for phytostabilization strategy (Ali et al., 2013).

4.1.5 Selection of the best-promising plant for phytoremediation

The major goal of this study was to select the proper plant as the new host of endophytic bacteria. The normal criteria used to choose the promising plant are plant tolerance, accumulation, and translocation of heavy metals (Zhivotovsky et al., 2010). In this study, the tolerance index could not be used as a criterion for comparison in spite of its popular use in numerous studies (Zacchini et al., 2009; Ribeiro de Souza et al., 2012). Since all plant candidates showed high Pb tolerance, parameters such as Pb content in roots and production of new roots are used as the criteria for selecting the best plant in this study. Only *A. mangium* and *E. camaldulensis* could produce the new roots. This criterion is very important, because roots are the first organ to come into contact with Pb, and provide the primary route for the penetration of metal ions (Liu et al., 2015). The more new roots, the higher uptake or stabilize Pb, because the uptake of Pb is based mainly on the plant species

93

and the interaction between roots (structures and synthesizes exudates) and the rhizosphere (biochemical properties). The first defense strategy is to stop the metal entering the root tissues by excluding it. Roots rapidly respond to the presence of Pb by forming mechanical barrier. This newly formed structure functions as a barrier against stress factors including heavy metals. The higher Pb concentration in roots, the better for alien endophytic bacteria. Clearly, *A. mangium* and *E. camaldulensis* showed significantly higher Pb accumulation capacity than *A. indica* and *S. siamae*. Hence, *A. mangium* and *E. camaldulensis* were selected as the new host of endophytic bacteria.

4.2 Experiment II: Phytoremediation potential of plants growing on the Pb-contaminated soil at the Song Tho Pb mine, Thailand

4.2.1 Physico-chemical properties of Pb contaminated soils

The physico-chemical properties of Pb contaminated soils collected from 3 different sites at Song Tho Pb mine are presented in Table 4.6. All soil samples were silt loam classified by the USDA soil texture triangle. Soil from site 1 (ore dressing area) had neutral pH, low EC (0.10 dS/m), relatively higher organic matter (OM), cation exchange capacity (CEC), and nutrients than those of site 2 (stockpile area) and 3 (tailing pond area). Soil from site 3 had the lowest pH (acidic), OM, and nutrients. Table 4.6 also presents the total and DTPA-extractable Pb concentrations. While soils from sites 1 and 3 were moderately contaminated (Pb 4881-5255 mg/kg), soil from site 2 was highly contaminated (Pb 16720 mg/kg). The DTPA-extractable Pb concentrations ranged from 115-1624 mg/kg.

The results of soil pH and salinity indicated that they are in the normal range for plant growth. Plants can grow and survive in a pH range of 5-7, and an EC range of 0-2 dS/m (Shu et al., 2001). Additionally, pH plays a vital role in balancing nutrient and heavy metal availability for uptake by plants, and maintaining the soil fertility that affects plant growth. Although, at low pH value, solubility of micronutrients is high, however, bioavailability of heavy metal can also be increased (Ali et al., 2013; Tale and Ingole, 2015). While, at high pH, solubility and availability of micronutrients to plant is declined (Tale and Ingole, 2015). However, there are

94

many plants that can thrive in alkaline soils due to the evolutionary process. Plants can adapt for surviving in alkaline soil by adjusting the internal and intracellular pH values (Gao et al., 2014). All soil samples had low CEC, OM, and nutrients, especially phosphorus (< 10 mg/kg). The available phosphorus concentration which is suitable for plant growth is more than 10 mg/kg (Tale and Ingole, 2015).

Table 4.6 Physico-chemical properties of Pb contaminated soils at Song Tho Pb mine

Soil properties	Study site		
	Site 1	Site 2	Site 3
pH	7.58	7.72	5.49
Organic matter (%)	1.50	0.42	0.26
Cation exchange capacity (cmol/kg)	1.39	0.20	0.60
Electrical conductivity (dS/m)	0.10	0.49	0.10
Available phosphorus (mg/kg)	6.7	2.1	2.1
Available potassium (mg/kg)	30	24	4.0
Particle size distribution			
Sand (%)	26.4	21.2	25.9
Silt (%)	57.0	54.2	54.1
Clay (%)	16.6	24.6	20.0
Soil texture	Silt loam	Silt loam	Silt loam
Total Pb concentration (mg/kg)	5255	16720	4881
Extractable Pb concentration (mg/kg)	1446	1624	115

Total Pb concentrations in this study were in range of 4881 to 16720 mg/kg. These concentrations are much higher than the global baseline level of Pb in uncontaminated surface soils (27 mg/kg; Kabata-Pendias, 2011), and the standard level of the Department of Pollution Control, Thailand (< 400 mg/kg). However, these concentrations are lower than those in the other mine. Extremely high Pb concentrations were reported in soils from Bo Ngam Pb mine area, about 40 km from Song Tho Pb mine. They were 142400 mg/kg in the ore dressing plant area, 65000 mg/kg in the stockpile area, and 6420 mg/kg in the tailing pond area (Rotkittikhun et

al., 2006). As expected, the total Pb concentrations in this study were higher (3.6-42.4 X) than those of DTPA-extractable concentrations at all sites. This is consistent with the results of Erika-Andrea et al. (2010) who found that the total Pb concentration was 11X higher than that of DTPA-extractable Pb concentration. Since Pb forms complex with inorganic constituents (HCO_3^-, CO_3^{2-}, SO_4^{2-}, and Cl^-), and adsorbs on particle surfaces such as Fe-oxides, organic matter, and clay particles (Pourrut et al., 2011). In addition, Pb has the least bioavailability (Ali et al., 2013).

4.2.2 Plant identification

Although soils at Song Tho Pb mine, Thailand is not a suitable habitat for a normal plant growth due to high Pb concentrations, low nutrients and organic matter, however, 80% of the surface area affected by the mining activities had a vegetation cover. Thus, this soil could be an ecological site containing viable Pb-tolerant plant biodiversity. Plant species and type identification are presented in Table 4.7 and Figure 4.11. More species were found at sites 1 and 3, while at site 2 (with the highest Pb concentration) only *T. latifolia* was found. Each area had different species and family, except for Gramineae which could be found in all areas. There were 7 herbs (46.69%) and 4 each (26.68%) of grasses and shrubs.

Some identified species in this study also showed roles in phytoremediation of heavy metals. *B. pilosa* and *C. crepidioides* were Cd-hyperaccumulators (Sun et al., 2009; Khaokaew and Landrot, 2015). *P. calomelanos* was an As hyperaccumulator (Francesconi et al., 2002), while *L. philippensis* had shown an ability to tolerate and grow well under high Cu concentration in the Zn smelting sediment (Kangwankraiphaisan and Suntornvongsagul, 2013). In addition, *M. malabathricum* accumulated 13800 mg/kg of Pb in the roots by phytostabilisation (Selamat et al., 2014). *B. asiatica* and *M. pudica* have Pb accumulation in shoots > 1000 mg/kg dry weight and also have TF > 1 (Thangavel and Sridevi, 2015). *L. clavatum* can accumulate Se in the shoot and in the root and TF value is 1.17 (Yuan et al., 2012). Moreover, *L. cernuum* and *M. pudica* can accumulate Pb in the roots about 688 and 641 mg/kg, respectively. In addition, their BCF values are 0.22 and 0.76, respectively (Ashraf et al., 2013). However, in this study, *T. triquetrum* and *D. auricomum* are rarely studied in the field of phytoremediation.

96

Table 4.7 List of plant species collected from 3 sites at the Song Tho Pb mine area

No.	Site	Family name	Scientific name	Type
1	1	Compositae	*Bidens pilosa* L.	A/H
2	1	Compositae	*Crassocephalum crepidioides (*Benth.) S. Moore	A/H
3	1	Solanaceae	*Solanum* sp.	P/S
4	1	Gramineae	Unidentified	Grass
5	1	Pteridaceae	*Pityrogramma calomelanos* (L.) Link	P/H
6	1	Plantaginaceae	*Lindenbergia philippensis* (Cham. & Schltdl.) Benth.	P/H
7	2	Gramineae	*Thysanolaena latifolia* (Roxb. ex Hornem.) Honda	Grass
8	3	Lycopodiaceae	*Lycopodium clavatum* L.	P/H
9	3	Leguminosae	*Mimosa pudica* L.	P/H
10	3	Gramineae	*Axonopus* sp.	P/H
11	3	Leguminosae	*Desmodium auricomum* Graham ex Benth	P/H
12	3	Leguminosae	*Tadehagi triquetrum* (L.) H.Ohashi	P/S
13	3	Melastomatace	*Melastoma* sp.	P/S
14	4	Scrophulariace	*Buddleja asiatica* Lour.	P/S
15	4	Gramineae	*Chrysopogon* sp.	Grass

A/H is annual/herb, P/H indicates perennial/herb, and P/S indicates perennial/shrub

Most of plant species from this study, except for *Melastroma* sp., *M. pudica* and *L. cernuum* that could not detect Pb in shoots, still accumulated higher Pb concentration than that of the normal limits in shoots, 5 mg/kg, (Malik et al., 2010; Yanqun et al., 2004). Also, most of plant species from this study still accumulated higher Pb concentration in plant than that of the normal levels of Pb content ranged of 0.5-10 mg/kg or phytotoxic levels ranged of 30-300 mg/kg (Yoon et al., 2006; Shi et al., 2011). These might imply that these plants were Pb-tolerant, as they accumulated Pb content higher than that of phytotoxic level either in roots or shoots. Many plant species have become metal tolerant due to the adaptive responses of plant to heavy metals, and they are growing in contaminated sites for a long time (Badr et al., 2012).

Bidens pilosa.   *Crassocephalum crepidioides*   *Solanum* sp.

Unidentified   *Pityrogramma calomelanos*   *Lindenbergia philippensis*

Thysanolaena latifolia   *Lycopodium clavatum*   *Mimosa pudica*

Axonopus sp.   *Desmodium auricomum*   *Melastoma* sp.

Tadehagi triquetrum   *Chrysopogon* sp.   *Buddleja asiatica*
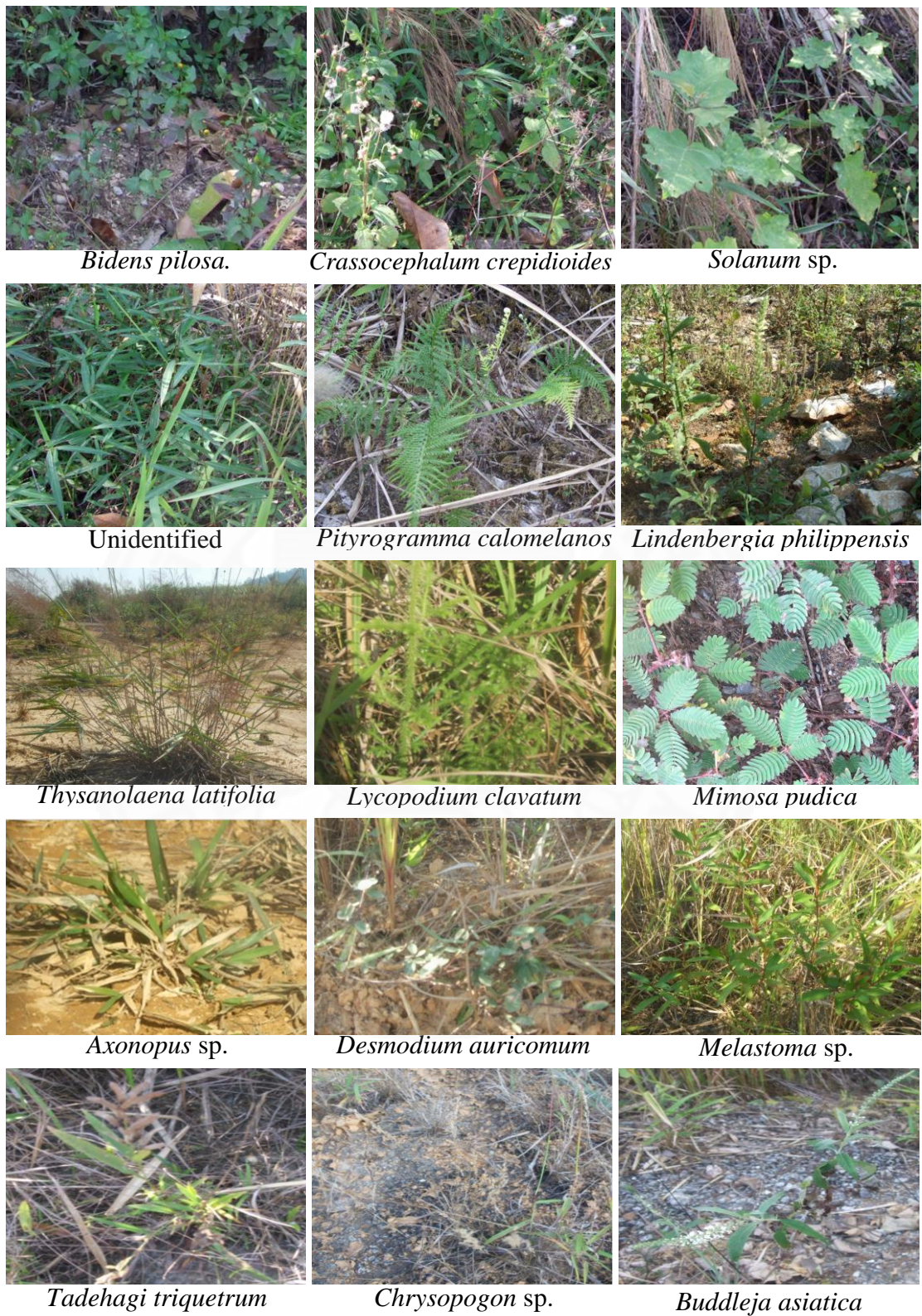
Figure 4.11 Plant species collected from the Song Tho Pb mine.

98

Plant species found belong to 9 families. There were 4 Gramineae (26.7%), 3 Leguminosae (20.0%), 2 Compositae (13.3%) and one each (6.67%) of Solanaceae, Pteridaceae, Lamiaceae, Lycopodiaceae, Melastomataceae and Scrophulariaceae as shown in Figure 4.12. Interestingly, families which were Compositae, Gramineae, and Leguminoseae, Lamiaceae, Poaceae and Scrophulariaceae have been found in the calamine soil as well (JÍdrzejczyk-KoryciÒska, 2006). Calamine soil is the soil containing high Zn, Pb and Cd concentrations (Prasad and Hagemeyer, 1999). Moreover, they were metal tolerant and capable of growing on elevated concentrations of toxic metals (Prasad and Freitas, 2003). This might imply that these families are common plant species in Pb contaminated soil.
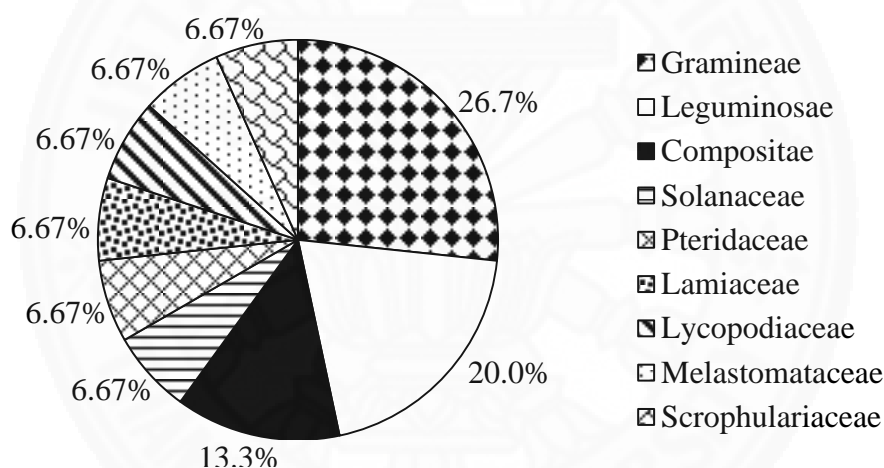
Figure 4.12 Percentage of family of plants collected from the Song Tho Pb mine

4.2.3 Pb concentration in soils

Total Pb concentrations of soil around plant roots are presented in Table 4.8. While plants from sites 1 had very high Pb concentrations (6500-48883 mg/kg), those from site 2 (534 mg/kg) and 3 (421-968 mg/kg) had relatively low Pb concentration. Especially, Pb concentration of soil around roots of *B. asiatica* collected form another place (site 4) was high (21100 mg/kg) which that in *Chrysopogon* sp. was low (1306 mg/kg).

Table 4.8 also shows the DTPA-extractable Pb concentrations in soils around plant roots which ranged from 43-1560 mg/kg. The lowest extractable Pb was

99

found in *Melastoma* sp. from site 3 which corresponded with total Pb concentration. Surprisingly, the highest total Pb concentration was found in *C. crepidioides* from site 1, and the highest extractable Pb was found in *L. philippensis* from site 1. As expected, the total Pb concentrations in this study were higher than those of DTPA-extractable concentrations in all plants.

Table 4.8 Total Pb concentration in plant parts and soils around plant roots and the DTPA-extractable Pb concentration

Plant species	Total Pb concentrations (mg/kg)			Extractable Pb (mg/kg)
	Shoots	Roots	Soils	
B. pilosa	**1270 ± 82.9*[a]**	709 ± 0.53[a]	28517 ± 3225[a]	829 ± 8.92[a]
C. crepidioides	235 ± 32.9[b]	1217 ± 67.6[a]	48883 ± 6255[b]	753 ± 3.06[a]
Solanum sp.	242 ± 95.2[b]	589 ± 136[a]	6500 ± 278[c]	597 ± 15.2[b]
Unidentified	200± 37.5[b]	802± 40.3[a]	31237 ±1207[a]	450 ± 36.6[c]
P. calomelanos	431 ± 56.9[c]	32633 ± 1077[b]	45500 ± 6801[b]	612 ± 22.0[b]
L. philippensis	**1489 ± 70.5*[d]**	2780 ± 966[c]	43567 ± 8003[b]	1560 ± 94.6[d]
T. latifolia	22.7 ± 8.62	647 ± 37.3	534 ± 9.81	111 ± 2.23
L. clavatum	ND	414 ± 2.00[b]	621 ± 163[b]	335 ± 9.90[a]
M. pudica	ND	826 ± 86.3[a]	496 ± 34.6[c]	198 ± 5.41[b]
Axonopus sp.	85.0 ± 7.00[a]	396 ± 248[b]	968 ± 36.8[a]	363 ± 14.1[c]
D. auricomum	39.0 ± 1.73[b]	92.7 ± 2.08[c]	598 ± 5.66[b]	112 ± 1.51[d]
T. triquetrum	32.0 ± 7.00[b]	424 ± 9.71[b]	612 ± 24.0[b]	130 ± 1.50[e]
Melastoma sp.	ND	47.3 ± 1.53[c]	421 ± 3.54[c]	43.2 ± 1.22[f]
B. asiatica	226 ± 0.58 [a]	2534 ± 30.0 [a]	21100 ± 1342[a]	916 ± 4.21[a]
Chrysopogon sp.	22.0 ± 10.5 [b]	1660 ± 40.0[b]	1306 ± 213[b]	565 ± 16.3[b]

Each value is mean of triplicate samples ± standard deviation. ND represents not detected; detection limit for Pb = 0.1 µg/kg. Bold values indicate that the plants have properties of hyperaccumulators[*]. Means with the same letter of each column are not significantly different among plant species from the same sites. Site 1 and 3 analyzed by Fisher's LSD test, while site 4 used *t*-test at $p \leq 0.05$.

100

4.2.4 Pb concentration in plants

Total Pb concentrations in plants vary with plant species as presented in Table 4.8. These values ranged from non-detectable to as high as 1489 mg/kg in shoots, and 47.3-32633 mg/kg in roots. In addition, all species except for *B. pilosa* accumulated Pb in roots more than in shoots.

According to the absolute Pb concentration in plant tissues, 2 herbaceous plants, *B. pilosa* and *L. philippensis* showed Pb concentration above 1000 mg/kg in the shoots. They could be identified as hyperaccumulators. This is based on the update criteria of heavy metal hyperaccumulators defined by van der Ent et al. (2013) and Pollard et al. (2014). These criteria (with unit of μg metals/g of dry leaf tissue) are 100 for Cd, Se and Tl; 300 for Co, Cr and Cu; 1000 for As, Ni and Pb; 3000 for Zn and 10,000 for Mn. Normally, in this study, Pb concentrations in roots were greater than those in shoots. This is in line with the results of Yoon et al. (2006) and Fahr et al. (2013) who showed that more that 90% of the plant species accumulated more Pb in roots than in shoots. Moreover, they pointed that there was low mobility of Pb from roots to shoots and immobilization of Pb in roots. Since most Pb in roots is localized in the insoluble fraction of cell walls and nuclei, which is linked with the detoxification mechanism. The capacity of cell walls to bind divalent metal cations mainly depends on the amount of polysaccharides with many carboxyl groups. Clearly, Pb-galacturonic acid fragments were detected in root of *Arabidopsis thaliana* (L.) Heynh. treated with Pb. In addition, Pb^{2+} was also shown to bind to carboxyl groups of pectin in cell walls of *Raphanus sativus* L. (Fahr et al., 2013).

4.2.5 Accumulation indices

The BCF, BAC, and TF values in plants are presented in Table 4.9. These values can be used to estimate a plant's potential for phytoremediation. By comparing these values, we can compare the ability of different plant species in taking up metals from soils and translocating them into shoots (Yoon et al. 2006). Plants with high BCF, BAC, and TF (> 1) are suitable for phytoextraction as accumulators (Vamerali et al., 2010). While those with high BCF ($\geq$ 1) but low BAC and TF ($\leq$ 1) have potential for phytostabilization as stabilizers (Nirola et al., 2015).

Table 4.9 Pb accumulation indices: BCF, BAC and TF

Site	Plant species	BCF	BAC	TF
1	*B. pilosa*	0.03 ± 0.00^b	0.04 ± 0.01^a	**1.79 ± 0.12^{a*}**
1	*C. crepidioides*	0.03 ± 0.00^b	0.00 ± 0.00^b	0.19 ± 0.04^{cd}
1	*Solanum* sp.	0.09 ± 0.02^b	0.04 ± 0.01^a	0.41 ± 0.11^{bc}
1	Unidentified	0.01 ± 0.02^b	0.03 ± 0.00^a	0.25 ± 0.04^{cd}
1	*P. calomelanos*	0.73 ± 0.12^a	0.01 ± 0.00^b	0.01 ± 0.00^d
1	*L. philippensis*	0.06 ± 0.02^b	0.04 ± 0.01^a	0.58 ± 0.21^b
2	*T. latifolia*	**1.21 ± 0.09^{**}**	**0.04 ± 0.02^{**}**	**0.04 ± 0.01^{**}**
3	*L. clavatum*	0.68 ± 0.12^b	-	-
3	*M. pudica*	**1.66 ± 0.05^{a**}**	-	-
3	*Axonopus* sp.	0.41 ± 0.27^c	0.09 ± 0.01^a	0.32 ± 0.27^{ab}
3	*D. auricomum*	0.15 ± 0.00^d	0.07 ± 0.00^{ab}	0.42 ± 0.02^a
3	*T. triquetrum*	0.69 ± 0.04^b	0.05 ± 0.01^b	0.08 ± 0.01^b
3	*Melastoma* sp.	0.11 ± 0.00^d	-	-
4	*B. asiatica*	0.12 ± 0.00^a	0.01 ± 0.00^a	0.09 ± 0.00^a
4	*Chrysopogon* sp.	**1.27 ± 0.00^{a**}**	**0.02 ± 0.00^{b**}**	**0.01 ± 0.00^{a**}**

Each value is mean of triplicate samples ± standard deviation. Bold values indicate that the plants have properties of hyperaccumulators[*] or excluders[**]. Means with the same letter of each column are not significantly different ($p \leq 0.05$) among plant species from the same sites. Site 1 and 3 analyzed by $p \leq 0.05$, according to Fisher's LSD test, while site 4 used *t*-test at $p \leq 0.05$.

In this study, none of the plant species showed BAC values > 1. However, the BCF $\geq$ 1 was found in *M. pudica* (1.66), *Chrysopogon* sp. (1.27) and *T. latifolia* (1.21) indicating their ability to stabilize Pb in their rhizosphere (Yoon et al., 2006). These plant species could be considered as excluders because they can extract more Pb from soil, retain Pb in roots, and accumulate very little Pb in their shoots (0-23 mg/kg). Another indicator, the TF, indicates a plant's ability to translocate metals from roots to shoots (Yoon et al., 2006). Only *B. pilosa* showed a TF value > 1 (1.79).

102

In addition, *B. pilosa* also accumulated > 1000 mg/kg of Pb in its shoots (1270 mg/kg). Both criteria indicated the hyperaccumulating ability of *B. pilosa* for Pb (Baker, 1981; van der Ent et al., 2013).

Several previous studies reported on Pb accumulation in shoots by means of a field survey. The highest values of shoot Pb concentrations from these studies varied from 1138-28370 mg/kg. The highest Pb accumulation in shoots belonged to *Spermacoce mauritiana* (28370 mg/kg), followed by *Echinophora platyloba* (10126 mg/kg), *Thlaspi rotundifolium* subsp. cepaeifolium (8200 mg/kg), *Buddleja asiatica* (4336 mg/kg), *Viola baoshanensis* (1902 mg/kg), *Sedum alfredii* (1182 mg/kg), and *Euphorbia cheiradenia* (1138 mg/kg) (Baker and Brooks, 1989; Yang et al., 2002; Rotkittikhun et al., 2006; Chehregani and Malayeri, 2007; Waranusantigul et al., 2008; Wu et al., 2010; Cheraghi et al., 2011). In comparison, *B. pilosa* in this study can be classified as a moderate hyperaccumulator.

Even though soil in the Song Tho Pb mine is not suitable for plant growth due to its low nutrient and high Pb concentrations, this soil is an ecological site containing viable Pb-tolerant plant biodiversity. Among 15 species screened, all plants can be ecologically and economically valuable candidates for sustainable phytoremediation of Pb-contaminated soils as Pb-tolerant species. In addition, some potential plant candidates for phytoextraction (*B. pilosa* as a hyperaccumulator and *L. philippensis* as an accumulator) and for phytostabilization (*T. latifolia* and *M. pudica* as excluders) are identified. The 2 species identified as phytoextractors are herbs with high biomass which is favorable for Pb removal. Interestingly, *L. philippensis* could mobilize Pb in soils higher than other plants in this study.

This is the first report to indicate that *B. pilosa* can be a potential candidate for phytoextraction as a new Pb hyperaccumulator. *B. pilosa* is an annual fast-growing herb which has better characteristics of hyperaccumulator than shrubs and tree, since (1) relative biomass of herbs is higher than trees or bushes in the same space and time, (2) herbs are easy to cultivate, and (3) herbs have stronger ability to adapt to a stressed environment (Yanqun et al., 2005). Annual plants are suitable candidates in the aspect of avoiding potential risk to the environment, since they accumulate heavy metals in their shoots for a shorter time. *B. pilosa* can be found in areas all over Thailand, and the entire plant can be easily harvested mechanically,

which is important for phytoextraction. In addition, *T. latifolia* and *M. pudica* are suitable for phytostabilization as excluders.

More importantly, the aim of this study was to select the host plant species for Pb-resistant endophytic bacteria. There were many heavy metal resistant endophytic bacteria isolated from different plants growing well in metal-contaminated soils (Verma and Gange, 2014). All 15 collected plant species from this study can be candidates. In order to reduce the number of candidates, 2 constraints need to be considered. One is the area of plant tissue where there is high density and diversity of endophytic bacteria. Several studies found that roots have high density and diversity of endophytic bacteria more than other plant tissues. The highest bacterial densities are usually observed in the roots and decrease progressively from stem to leaves. The fact that colonization is especially abundant in root tissue may reflect the fact that the root is the primary site where endophytes gain entry into plants with the exception of seed transmitted bacteria (Lodewyckx et al., 2002). Also a higher number of strains are found in the roots than in the stem and leaf tissues (Verma and Gange, 2014). The other constraint is Pb concentration. The higher Pb concentration, the higher number of Pb-resistant endophytic bacteria. The high concentrations of heavy metals in the plant might directly select endophytes which are able to resist the contaminated environmental conditions. It is also possible that plants accumulating high concentration of heavy metals may be colonized by heavy metal-resistant endophytic bacteria (Verma and Gange, 2014). Considering these constraints together, plants accumulating extremely high Pb content in roots could be the best candidates. From the results, only *P. calomelanos* which contained the highest Pb concentration in roots at 32633 mg/kg was selected.

4.3 Experiment III: Isolation and characterization of PGPE from the roots of *Pityrogramma calomelanos*

4.3.1 Isolation of Pb-resistant endophytic bacteria

The surface root disinfection technique was effective in removing epiphytic microorganisms, thus these isolates can be considered as true endophytic bacteria (Shin et al., 2012). The total viable bacteria extracted from *P. calomelanos* were 24 x 10^6 CFU/mL, while those from *A. mangium* and *E. camaldulensis* were 1×10^4 and 10^2 CFU/mL, respectively. This is not surprising, because *P. calomelanos* grew in high Pb contaminated soil (5255 mg/kg) and contained high content Pb in roots (32633 mg/kg). However, in this study, the endophytic bacterial diversity was small. This may be due to the death of bacteria from extraction with detergent. No proper protocol of the surface sterilization method results in the complete killing of surface bacteria on samples without penetrating interior tissues, and thereby killing internal colonies (Lodewyckx et al., 2002).

After isolation, colonies with different morphology were selected as Pb resistant endophytic bacteria designated as Pc, Pd, Pe, Ai, Aj, and El (Table 4.10). Isolates Pc, Pd and Pe were extracted from *P. calomelanos*. Ai and Aj were extracted from *A. mangium*, and El was isolated from *E. camaldulensis*.

Table 4.10 Colony morphology of endophytic bacteria after 48 h of incubation

Isolate	Morphological characteristics (form, elevation, margin, surface, and color)	Size (ϕ, mm)
Ai	Circular, raised, entire, smooth and glistening, ivory	1
Aj	Circular, convex, entire, smooth and glistening, pale yellow	2
El	Circular, flat, erose, dull, ivory	120
Pc	Circular, raised, entire, smooth and glistening, ivory	2
Pd	Irregular, flat, curled, concentrically ringed and dull, ivory	5
Pe	Circular, raised, entire, smooth and glistening, dark-ivory	1

105

4.3.2 Identification of endophytic bacteria

The yield and purity of genomic DNA are shown in Table 4.11. The quality of the extracted DNA was evaluated by the A260/280 ratio, and values close to 1.8 indicated a good DNA extract with little protein contamination. The bands of each isolate are shown in Figure 4.13.

Table 4.11 Yield and purity of DNA extracted from endophytic bacteria

Isolate	A260/A280	A260/A230	DNA concentration (ng/µL)
Ai	2.03	1.55	166.4
Aj	2.06	1.95	243.6
El	2.14	2.32	314.0
Pc	2.08	2.00	264.8
Pd	2.00	1.70	98.4
Pe	2.06	2.00	204.9
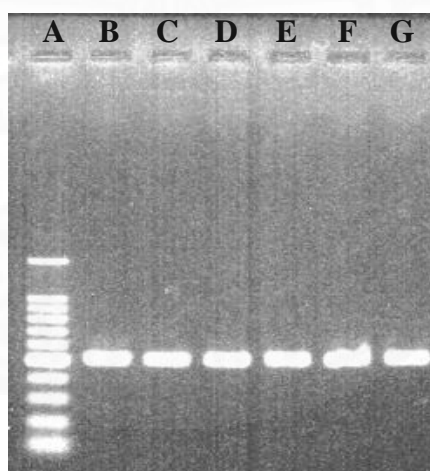
Figure 4.13 Electrophoresis results of each isolate and DNA ladder: (A) DNA ladder, (B) Pc, (C) Pd, (D) Pe, (E) Ai, (F) Aj, and (G) El

The partial 16S rRNA sequenced fragments were about 500 base pair in length. After comparison, bacterial sequences from this study shared very high similarities (99%) with the first match of their recognized relative species in NCBI database as shown in Table 4.12. These endophytic bacteria mainly belonged to γ-proteobacteria (83%), the other one was firmicutes (17%) based on NCBI

106

classification. Most of them were identified as genus *Pseudomonas* (66%) belonging to Family Pseudomonadaceae. The remaining isolates were *Serratia* (16%) belonging to Enterobacteriaceae and *Bacillus* (16%) belonging to Bacillaceae.

Table 4.12 Closest species to isolates based on partial 16S rRNA gene analysis. In bold: strain selected for further experiments.

Isolate	Related species	% Identity	Accession No.
Pc	***Pseudomonas psychrophila***	99	JQ782901.1
Pd	*Pseudomonas* sp.	99	HQ841030.1
Pe	*Pseudomonas* sp.	99	HQ841030.1
Ai	*Serratia* sp.	99	JF312984.1
Aj	*Pseudomonas* sp.	99	HQ841030.1
El	*Bacillus* sp.	99	KM817248.1

The results of this study are in conformity with many previous studies which indicated that *Pseudomonas* was one of the most common genera of cultivatable endophytic bacterial species isolated from root tissues (Sheng et al., 2008; Weyens et al., 2009; Long et al., 2011; Cherian et al., 2012). Besides, endophytic bacteria are closely related to common soil bacteria such as *Pseudomonas, Enterobacter, Bacillus, Arthrobacter, Burkholderia*, and *Methylobacterium* (Verma and Gange, 2014). Moreover, *Pseusomonas* belongs to γ-Proteobacteria that are mostly found in the terrestrial environment (Chen et al., 2012). Li et al. (2012) also found that the metal-resistant endophytic bacteria were *Pseudomonas*. Importantly, *Pseudomonas* has been promoted for Pb phytoremediation (Sheng et al., 2008; Zhang et al., 2011; Shin et al., 2012).

However, there are many hit sequences with the same homology (data not shown). To identify the exact related species, a phylogenetic tree was reconstructed by MEGA6 (Tamura et al., 2013), and the representative bacteria of related taxa are shown in Figure 4.14. The constructed phylogenetic tree also indicated that Pc was closest to *P. psychrophila*; Pd to *P. proteolytica*, Pe to *P. extremaustralis*, Ai to *Serratia* sp., Aj to *Pseudomonas* sp., and El to *Bacillus* sp.
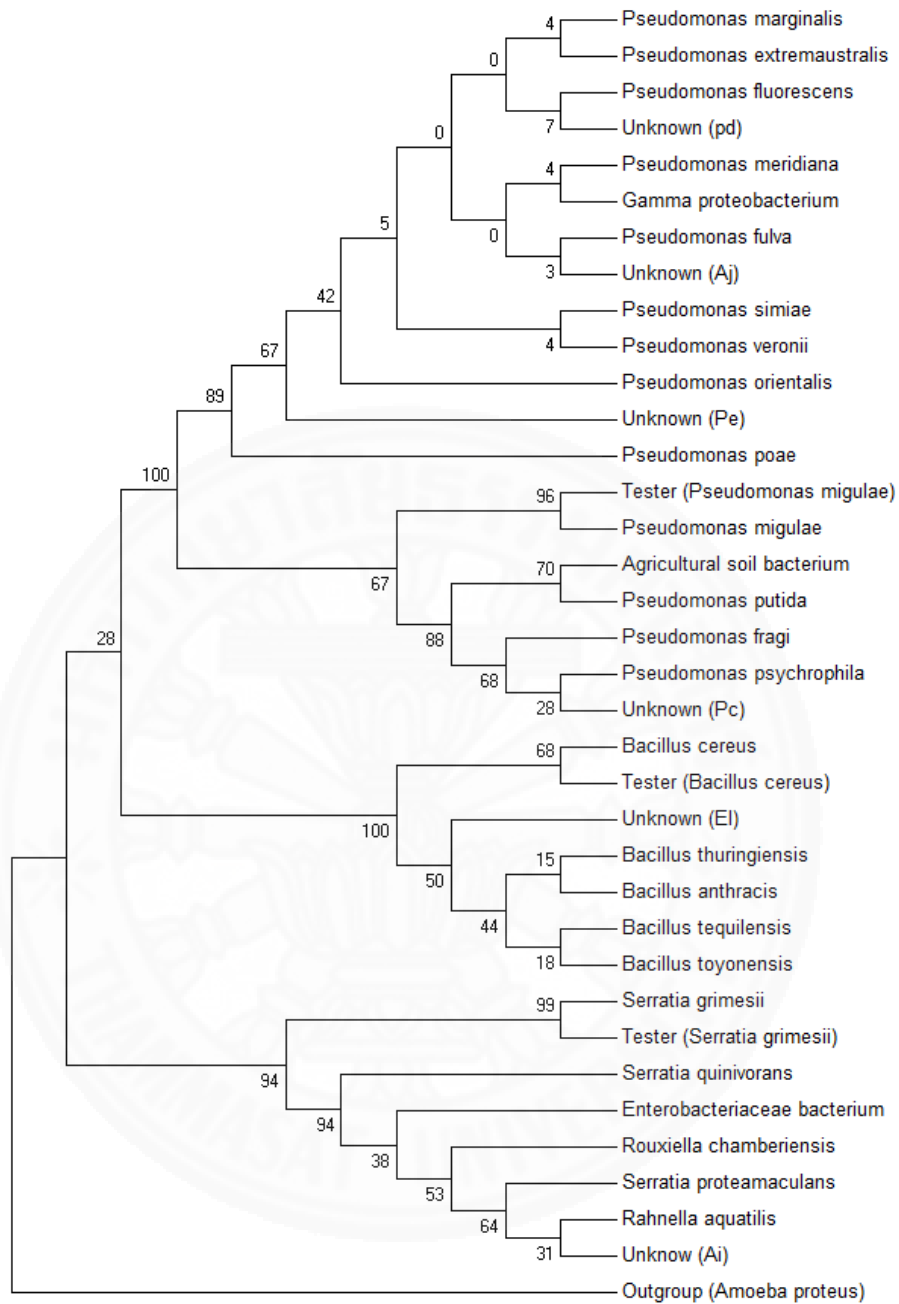
Figure 4.14 Phylogenetic tree of each isolate. Reconstruction was done by the Maximum likelihood method with the Kimura 2-parameter model on the basis of partial 16S rRNA sequences. Bootstrap numbers indicated the value of 1000 replicates. Branches corresponding to partitions reproduced in less than 80%, bootstrap replicates are removed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

4.3.3 Characterization of Pb-multifunctional endophytic bacteria

Bacterial cell density (CFU/mL) of isolate Ai, Aj, El, Pc, Pd and Pe was 4×10^{11}, 4×10^{6}, 4×10^{5}, 1×10^{10}, 4×10^{6} and 3×10^{10}, respectively. In this study, all isolates could tolerate Pb equally with the MIC value of 1875 mg/L. This is not surprising, since Li et al. (2012) reported that heavy metal resistant endophytes can be isolated from hyperaccumulating plants, non-hyperaccumulating plants and non-heavy metal polluted plants. In addition, the same genera of endophytic bacteria isolated from different plants in different locations (heavy metal polluted and non-polluted soil) show the same metal resistance.

Normally, Pb resistance is one of the important functions whether to use these bacteria in Pb phytoremediation (Navarro-Torre et al., 2016). However, it is very difficult to compare MIC value with those of other studies, because the conditions used in tolerance test were different such as type of Pb salts and type and concentration of medium. For example, *P. koreensis* AGB-1 showed high tolerance with MIC value for Pb 1800 mg/L on SLP agar that is similar to the results of the other study (Babu et al., 2015).

The possible mechanisms that help bacteria to resist high Pb concentration are binding of Pb^{2+} at cell wall, releasing of extracellular polysaccharides to adsorb Pb^{2+} outside the cell, precipitating Pb^{2+} as insoluble phosphates both outside and inside the cell. After entering the cell, Pb^{2+} can be further inactivated by binding to metallothioneins (intracellular Pb sequestration), sequestered as insoluble phosphates or removed from the cell (efflux) via transporters such as CadA, ZntA or PbrA, extracellular Pb sequestration in exopolysaccharide, cell surface adsorption of Pb, biosorption of Pb in the cell wall and periplasmic space (bioaccumulation), and Pb precipitation (Naik et al., 2013; Jarosławiecka and Piotrowska-Seget, 2014). The success of obtaining Pb-resistant endophytic bacteria from this study is similar to that of the previous researches that obtained Pb-resistant endophytic bacteria from roots of accumulator plants grown on contaminated areas (Sheng et al., 2008; Zhang et al; 2011; Shin et al., 2012; He et al., 2013).

The abilities of endophytic bacteria to produce siderophore and solubilize phosphate are presented in Table 4.13. Most isolates could produce siderophore, except El (*Bacillus* sp). This is in line with the previous works which

indicated that *Pseudomonas* sp., *Rahnella* could produce siderophore, while *Bacillus* could not (Chen, 2010; Sheng et al., 2008; Rajkumar et al., 2010; Ngamau et al., 2012). Also, most isolates could solubilize inorganic phosphate, except Ai and El.

Table 4.13 Some plant growth promoting traits of each isolate

Isolate	Siderophore production index	Phosphate solubilization index
Ai	1.00 ± 0.00^a	-
Aj	1.93 ± 0.19^b	8.7 ± 1.2^a
El	-	-
Pc	1.63 ± 0.24^{cd}	8.7 ± 1.5^a
Pd	1.86 ± 0.17^{bd}	9.0 ± 1.7^a
Pe	1.50 ± 0.07^c	8.0 ± 1.0^a

Each value is mean of triplicate samples ± standard deviation. Mean of each columns indexed by the same letter are not significantly different according to Fisher's LSD test ($p \leq 0.05$).

Generally, Pb in soil precipitates with phosphates (fluoropyromorphite $Pb_5(PO_4)_3F$), carbonates ($PbCO_3$), and sulfate (PbS), hence Pb has very low bioavailability for root uptake even for hyperaccumulator (Park et al., 2011; Rajakumar 2013). Pb-mobilizing features such as siderophore production and phosphate solubilizing activity of endophytic bacteria play a vital role in phytoremediation, since bacterial siderophore can form stable complex with other metals such as Al, Cd, Cu, Ni, Pb and Zn. This complex increases the soluble metal concentration in soil. Moreover, isolates from this study are similar with those from the previous works. *Serratia nematodiphila*, *Serratia proteamaculans*, *P. fluorescencens* G10, and *Pseudomonas protegens* can produce siderophore (Chen, 2010; Sheng et al., 2008; Rajkumar et al., 2010; Ngamau et al., 2012).

Phosphate solubilizing endophytic bacteria are very important to the plants under phosphorus stress such as Song Tho Pb mine. Because phosphorus is essential nutrient that plays a vital biochemical role in photosynthesis, respiration, cell division, cell enlargement, and other processes in the living plant (Karpagam and Nagalakshmi, 2014). Nevertheless, phosphorus is insoluble form in soil (Rajkumar et

110

al., 2009; Karpagam and Nagalakshmi, 2014). These bacterial species such as *P. aeruginosa*, *P. putida*, *P. fluorescens*, and *P. chlororaphis* are defined as phosphate-solubilizing bacteria using secretion of organic acid and phosphatase (Rajkumar et al., 2009; Ahemad, 2015). Meanwhile, *Brevibacterium halotolerans*, *Serratia plymuthica* and *Serratia proteamaculans* cannot convert insoluble inorganic tricalcium phosphate to soluble forms (Sgroy et al., 2009; Ngamau et al., 2012). Moreover, phosphate-solubilizing endophytic bacteria have been proved in many studies that they can increase the bioavailability of heavy metal leading to enhancing of heavy metal uptake by plants (Jeong et al., 2012). Additionally, inoculating of phosphate-solubilizing bacteria simultaneously increases phosphorus uptake by plant such as maize, green gram, sorghum, wheat, potato, bean and tomato, resulting in higher yields (Surapat et al., 2013).

The abilities of endophytic bacteria to solubilize and mobilize Pb in soils are presented in Table 4.14. The results of Pb solubilization in solution, water-soluble Pb concentration of un-inoculated control significantly increased ($p \leq 0.05$) with the increase in exposure time. Similarly, Sheng et al. (2008) found that water-soluble Pb concentration was 305 µg/L at 0 h and it increased to 375 µg/L after 48 h. In addition, water-soluble Pb concentrations of all endophytic bacteria were significantly higher ($p \leq 0.05$) than that of control. This clearly indicated that different endophytic bacteria had different effects on the mobility of Pb in soil. For Pb mobilization, most bacterial isolates except Pd could mobilize Pb in soil, compared to un-inoculated control. Besides, water-soluble Pb in soil of isolated Ai and Pc were significantly ($p \leq 0.05$) higher than that of the remaining isolates as well as control.

The results of Pb mobilization in soil revealed that the highest water-soluble Pb concentration (21.2 mg/L) was found in isolates Pc on day 0. This value showed significant difference ($p \leq 0.05$) with the remaining isolates including control. However, water-soluble Pb concentration of isolate Pc on day 2 was not significantly decreased ($p > 0.05$) on day 0. The lowest water-soluble Pb concentration (1.07 mg/L) was found in isolate Aj on day 2, which significantly decreased ($p \leq 0.05$) from day 0. The remaining isolates showed the opposite trend. Water-soluble Pb concentrations were expressed as solubilizing index relative to that of control. The maximum solubilizing index was found in isolate Pc on day 0 and 2. The minimum solubilizing

111

index was found in isolate Pe on day 0, and isolate Aj on day 2. As expected, isolates Pc, Pd and Pe extracted from plants grown on Pb contaminated soil showed abilities to produce siderophore, solubilize P, solubilize and mobilize Pb. This can be associated with its habitats in the contaminated area (He et al., 2013).

Table 4.14 Some key traits of endophytic bacteria for phytoremediation

| Isolates | Pb solubilization | | | | Pb mobilization | |
| | 0 h | | 48 h | | | |
	[Pb] mg/L	Index	[Pb] mg/L	Index	[Pb] mg/L	Index
Control	5.46 ± 1.54^{ab}	1.00	14.4 ± 3.03^{a}	1.00	0.93 ± 0.12^{a}	1.00
Ai	5.77 ± 1.53^{ab}	1.06	2.46 ± 0.33^{de}	0.17	2.07 ± 0.15^{b}	2.23
Aj	8.53 ± 1.18^{b}	1.56	1.07 ± 0.02^{d}	0.07	1.43 ± 0.31^{c}	1.54
El	7.71 ± 4.37^{b}	1.41	5.19 ± 2.99^{e}	0.36	1.57 ± 0.15^{cd}	1.69
Pc	21.2 ± 1.33^{c}	3.88	18.2 ± 0.89^{b}	1.26	2.23 ± 0.45^{b}	2.40
Pd	4.35 ± 0.76^{a}	0.80	1.39 ± 0.06^{d}	0.10	1.00 ± 0.00^{a}	1.08
Pe	8.01 ± 2.52^{b}	1.47	9.28 ± 2.64^{c}	0.26	1.93 ± 0.12^{bd}	2.08

Each value is mean of triplicate samples ± standard deviation. Mean of each columns indexed by the same letter are not significant difference according to Fisher's LSD test ($p \leq 0.05$).

4.3.4 Selection of the best-performing PGPE strain

P. psychrophila was selected as the best-performing PGPE strain based on the highest Pb mobilization and solubilization. Moreover, it could also produce siderophore and solubilize inorganic phosphate. There are a few strains in the genus *Pseudomonas* which are defined as Pb-mobilizing endophytic bacteria. For example, *P. koreensis* AGB-1 isolated from the roots of *Miscanthus sinensis* Andersson grown in mine-tailing soil did not produce siderophore, but it produced biosurfactant and strongly solubilized inorganic phosphate leading to the increase in Pb bioavailability (Babu et al., 2015). In addition, *P. fluorescens* G10 isolated from the roots of *B. napus* grown in heavy metal contaminated soil also produced siderophore, solubilized insoluble Pb, and mobilized Pb in soil (Sheng et al., 2008).

4.4 Experiment IV: Fast-growing trees inoculation with *P. psychrophila*.

4.4.1 Recovery of *P. psychrophila*.

    The surface root disinfection technique was effective in removing epiphytic microorganisms making inoculated Pc isolate the true endophytic bacteria in its new hosts (Shin et al., 2012). The colony appearance of each treatment is presented in Figure 4.15. The morphological colonies of pure Pc were circular and milky white (Figure 4.15A). Some colonies of inoculated *A. mangium* (Figure 4.15B) and *E. camaldulensis* (Figure 4.15C) were circular and milky white that were related to colony morphology of pure Pc. This may imply that endophytic bacteria Pc can enter and colonize inside the roots of the new hosts as alien species. 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 (Ma et al., 2011a). In turn, these circular and milky white colonies did not appear on the plates of un-inoculated plants which showed only circular and dark brown colonies as native species (Figures 4.15D and E). This indicates that isolate Pc was not local species of new hosts (*A. mangium* and *E. camaldulensis*). This ensures that the change of Pb content in roots of new plant hosts after inoculation and grown in Pb contaminated soil totally results in the ability of inoculated *P. psychrophila*.

    The results of 16S rRNA partial gene analysis indicated that alien species (circular and milky white colony) from successful inoculation were *Pseudomonas fragi*, but native species (circular and dark brown colony) of *A. mangium* and *E. camaldulensis* were *Psedumonas extremaustralis* based on 100% identity. However, the results from strain identification as described in section 4.3.2 indicated that PGPE (Pc) was closest to *P. psychrophila* more than *P. fragi*. Besides, *P. psychrophila* was very similar to *P. fragi*. This is consistent with the result from Yumoto et al. (2001) who reported that the phylogeny of *P. psychrophila* was closest to *P. fragi*. Based on the physiology results, *P. psychrophila* from this study showed positive siderophore production. This result is consistent with Ngamau et al. (2012), while *P. fragi* is non-siderophore producing bacteria (Champomier-Verges et al., 1996). As expected, the result from 16S rRNA gene full length sequencing analysis confirmed that Pc isolate was *P. psychrophila*.

113
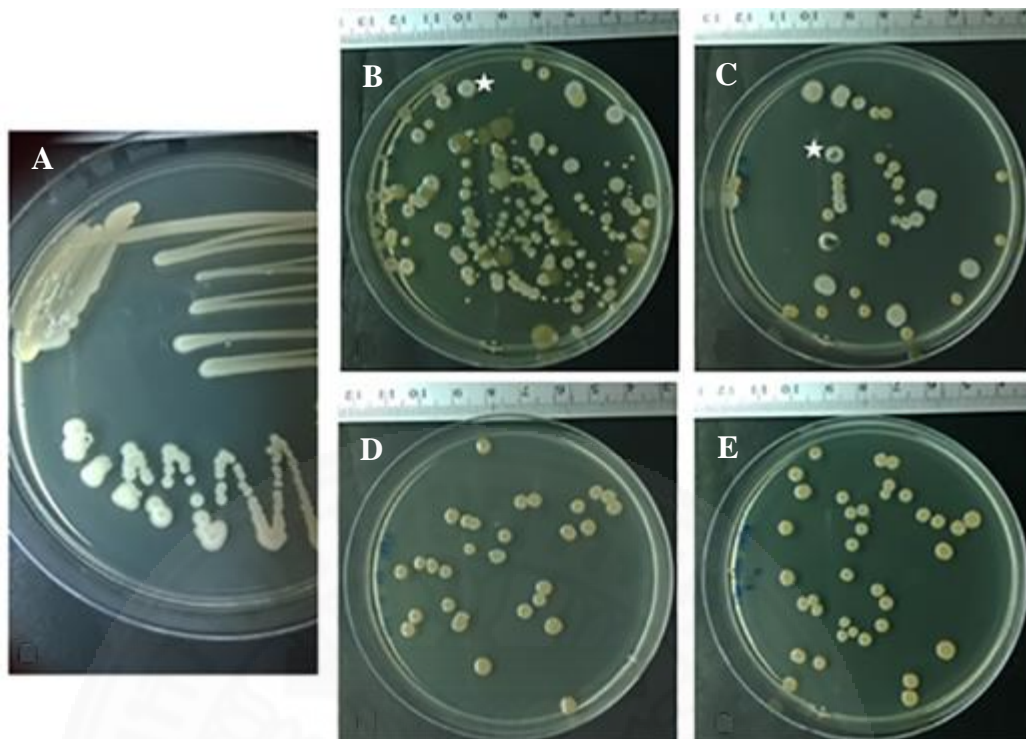
Figure 4.15 Different morphology of each colony on LB media added with 20 mg/L of Pb for 3 weeks: (A) pure colonies of Pc; (B) *A. mangium* inoculated with *P. psychrophila*; (C) *E. camaldulensis* inoculated with *P. psychrophila*; (D) un-inoculated *A. mangium* and (E) un-inoculated *E. camaldulensis*. White stars represent colonies of inoculated *P. psychrophila*.

4.4.2 *P. psychrophila* application

Although, *Pseudomonas* sp. causes some phytotoxicity symptoms such as leaf spots, galls, canker and bud blast, wilt, and blight (Schaechter, 2004), *P. psychrophila* is not plant pathogenic bacteria (Bull et al., 2012). Especially, this strain was not involved in the industrial and biotechnology processes, and was also not involved in human and animal diseases (Dworkin et al., 2006). Moreover, using *P. psychrophila* to inoculate into interior plant tissues will pose less risk with other organisms in the natural environment. Therefore, this strain can be safely applied in the phytoremediation of heavy metals.

114

4.4.3 Effect of *P. psychrophila* on Pb accumulation in roots of fast-growing trees: Hydroponic test

The results of Pb content in roots are presented in Table 4.15 and Figure 4.16. Without Pb in the 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 Pb contents were observed in *A. mangium* inoculation which showed increasing trend in Pb concentration in the roots (6829 mg/kg) compared to un-inoculation (6242 mg/kg). While, *E. camaldulensis* inoculation showed decreased trend in Pb concentration in the roots (3763 mg/kg) compared to non-inoculation (4233 mg/kg).

The results from this study are in line with the results of Sheng et al. (2008) who found that inoculation of *B. 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. (2013) indicated that Pb resistant endophytic bacteria *Rahnella* sp. JN6 significantly increased Pb content in roots of *B. napus* grown in soil (114 mg/kg) compared to non-inoculation (77.9 mg/kg). These can be due to bacterial mechanisms. In general, endophytic bacteria increases metal accumulation by siderophore production and phosphate solubilization leading to improved efficiency of phytoremediation (Ma et al., 2011a). The metal bioaccumulation of endophytic bacteria also improves the efficiency of phytoremediation (Ma et al., 2016).

Table 4.15 Pb accumulation (mg/kg) in roots of un-inoculated and inoculated plants grown in Pb solution (30 mg/L) and control solution (0 mg/L) for 15 d

Treatments	Control solution	Pb solution
A. mangium (-Pc)	10.2 ± 4.76^a	6242 ± 272^a
A. mangium (+Pc)	89.3 ± 3.67^a	6829 ± 697^a
E. camaldulensis (-Pc)	16.6 ± 8.26^b	4233 ± 264^b
E. camaldulensis (+Pc)	16.6 ± 1.11^b	3763 ± 592^b

Each value is mean of triplicate samples ± standard deviation. Mean of each columns indexed by the same letter are not significant difference according to Fisher's LSD test ($p \leq 0.05$).
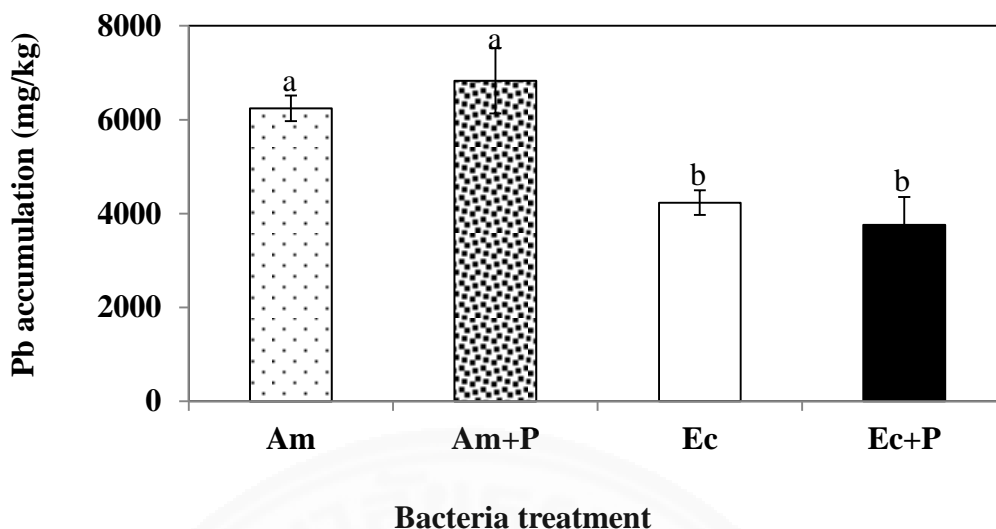
Figure 4.16 Effect of *P. psychrophila* inoculation on Pb accumulation of fast-growing trees treated with 30 mg/kg for 15 d. Each value is the mean of triplicates. Error bars represent standard deviation. The different small letter above the bar graph denotes a significant difference ($p \leq 0.05$) according to Fisher's LSD test. Am is *A. mangium*, Am+P is *A. mangium* inoculated with *P. psychrophila*, Ec is *E. camaldulensis*, and Ec+P is *E. camaldulensis* inoculated with *P. psychrophila.*

In contrast, there are some opposing viewpoints suggesting that the presence of metal-resistant endophytes can decrease both metal uptake and accumulation by plant (Li et al., 2012). For example, Xu et al. (2015) found that heavy metal resistant bacteria, *Pseudomonas putida* CZ1, amended in Cu solution did not significantly reduce Cu contents in roots of *Elsholtzia splendens* compared to non-amendment. Madhaiyan et al. (2007) found that endophytic bacteria *Burkholderia* sp. and *Methylobacterium oryzae* not only reduced the toxicity and accumulation of Ni and Cd in roots and shoots, but also reduced translocation to shoots of *L. esculentum*. These are consistent with the reduction Pb content in roots of *E. calmaldelensis* inoculated with *P. psychrophila* in this study. The declined accumulation of metals might be due to endophytic bacterial immobilization of metals in plant rhizosphere via secretion of extracellular polymeric substance (Ma et al., 2016). Additionally, the effects of microbial inoculation on metal extraction capacity depend on the plant species, metal concentration, and the microbial strains used (Sessitsch et al., 2013).

116

In addition, the results of partial difference in Pb accumulation in roots of all treated plants could also imply that the inoculated *P. psychrophila* could colonize inside the roots of new plant hosts.

4.5 Experiment V: Colonization of *P. psychrophila* after 15 d of inoculation

The result of root surface disinfection technique was successful for removing epiphytic microorganisms in all treatments. This indicates that the inoculated *P. psychrophila* could be considered as true endophytic bacteria of new hosts. The colony appearance is presented in Figure 4.17. Pure *P. psychrophila* colonies did not show a yellow to greenish-yellow zone on LB agar (Figure 4.17A) compared to King's B agar (Figure 4.17B). Certainly, *P. psychrophila* also did not show fluoresces under UV light on LB agar (Figure 4.17C), but it showed fluoresces under UV light on King's B agar (Figure 4.17D).
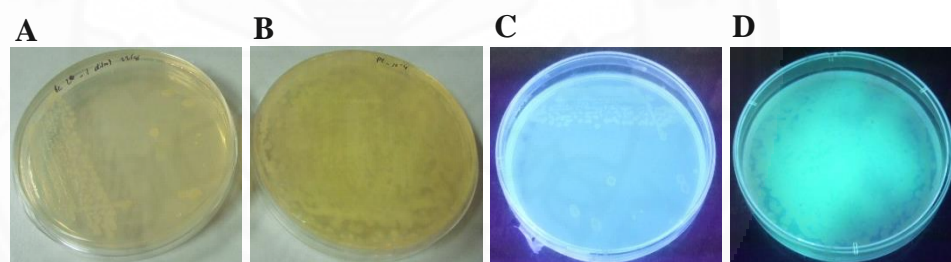
Figure 4.17 Pure *P. psychrophila* grown on different media: (A) LB agar under normal light; (B) King's B under normal light; (C) LB agar under UV light and (E) King's B under UV light.

Since, *Pseudomonas* sp. can produce a fluorescein showing a yellow to greenish-yellow zone on King's B agar under normal light, which fluoresces under UV light. These indicate the positive result (King et al., 1954). Moreover, Ramı́rez-Bahena et al. (2015) also found that strain produced a fluorescent pigment on King's B agar, closet to *P. psychrophila*. Thus, the positive result indicates that the inoculated *P. psychrophila* could be found and it could colonize inside the *A. mangium* and *E. camaldulensis* after 15 d of inoculation. As expected, the positive results were found on the plates culturing the 100 µL aliquots (10^{-4} dilution) that extracted from the roots of *A. mangium* (Figure 4.18A) and *E. camaldulensis* (Figure

117

4.18B) after 15 d of inoculation with *P. psychrophila* grown in Pb solution. The negative results were also found on the plates culturing the aliquots of un-inoculated *A. mangium* (Figure 4.18C) and *E. camaldulensis* (Figure 4.18D). These indicate that *P. psychrophila* was not the native species of *A. mangium* and *E. camaldulensis*.
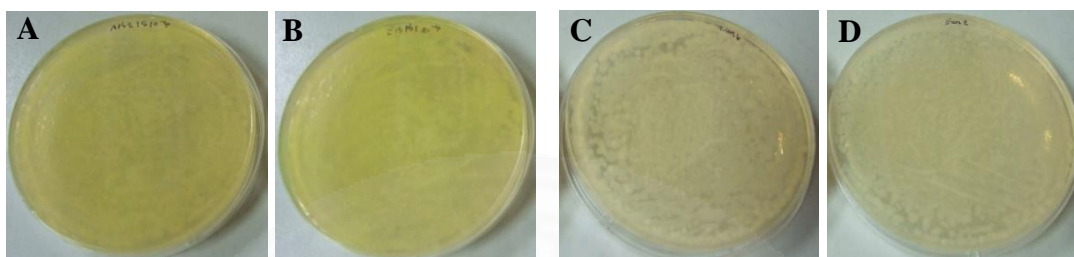
Figure 4.18 Fluorescein production by *P. psychrophila* cultured on King's B agar: (A) inoculated *A. mangium* grown in 30 mg/L of Pb for 15 d; (B) inoculated *E. camaldulensis* grown in 30 mg/L of Pb for 15 d; (C) un-inoculated *A. mangium* and (D) un-inoculated *E. camaldulensis*.

Since, the numerous colonies were observed, so the dilution was increased. This was to indicate that which colonies between native strain and inoculated strain produce fluorescein. The results after increasing dilution are presented in Figure 19. As expected, the negative results were found on the plates culturing the 100 μL aliquots (10^{-5} dilution) that extracted from the roots of *A. mangium* (Figure 19A) and *E. camaldulensis* (Figure 19B) inoculated with *P. psychrophila*. This indicates that some colonies producing fluosescein now lost. This confirms that the inoculated *P. psychrophila* could colonize inside the root tissue of *A. mangium* and *E. camaldulensis* approximately 10^5 CFU/mL.
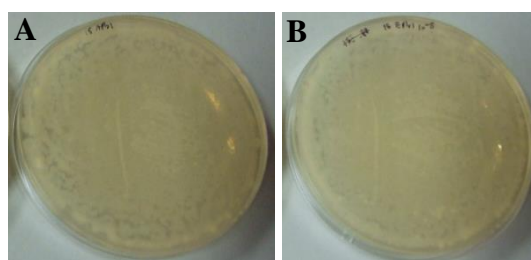
Figure 4.19 Characteristic of colonies after increasing dilution to 10^{-5}: (A) inoculated *A. mangium* grown in 30 mg/L of Pb for 15 d; (B) inoculated *E. camaldulensis* grown in 30 mg/L of Pb for 15 d.

118

4.6 Experiment VI: Inoculation of *P. psychrophila* for the assessment of Pb phytostabilization by fast-growing trees using pot study

4.6.1 Physico-chemical properties of Pb spiked soils

    The physico-chemical properties of the artificial Pb-spiked soils are presented in Table 4.16. These soil properties had no effect on plant growth, since *A. mangium* and *E. camaldulensis* can grow well on all soil types. However, soils have direct effect on Pb movement in the soil. Soil texture plays an important role in mobility of metals in soil. Fine particles like oxides and clay are important adsorption media for heavy metals in soils (Sherene, 2010). The strong affinity of Pb and other metals to the clay fraction is demonstrated by the ranking in term of adsorption of clay > silt > sand (Rieuwerts et al., 1998). The experimental soil of this study is clay loam, so this soil may adsorb Pb^{2+} resulting in Pb immobilization. The soils pH is the main factor affecting the mobility and bioavailability of heavy metals (Ngorwe et al., 2014). The pH also plays a vital role in balancing nutrient and metal availability for plant uptake, and maintaining the soil fertility that affects plant growth.

Table 4.16 Physico-chemical properties of artificial Pb spiked soil

Soil properties	Values
Soil texture	Clay loam
Particle size distribution	38% sand, 34% silt, 28% clay
pH	6.8 (neutral)
Organic matter (%)	6.07 (high)
Cation exchange capacity (cmol/kg)	31.6 (high)
Electrical conductivity (dS/m)	3.27 (high)
Phosphorus (mg/kg)	563 (very high)
Potassium (mg/kg)	1092 (very high)
Total nitrogen (%)	0.14 (low)
Calcium (mg/kg)	6503 (high)
Magnesium (mg/kg)	545 (high)
Total Pb concentration (mg/kg)	1470

119

At low pH value, the solubility of micronutrients is high, and the bioavailability of heavy metals can also be increased (Ali et al., 2013; Tale and Ingole, 2015). *A. mangium* grows well in optimal pH 4.0-6.0, while *E. camaldulensis* grows on less acidic soil with pH 5.0-6.0. In theory, at pH 6 or 7, very little metal is likely to be found in soil solution, and soil pH 6-7 is also involved in precipitation of Pb (Rieuwerts et al., 1998).

In general, plants can grow and survive in an EC range of 0-2 dS/m (Shu et al., 2001). The results showed that *A. mangium* and *E. camaldulensis* could grow well in this artificial soil with high salinity (3.27 dS/m). However, the higher the EC value, the lower the uptake of heavy metals such as Cd and Pb because at higher EC, there are more adsorption sites for exchange of heavy metal which are eventually immobilized leading to the low uptake by plants (Ngorwe et al., 2014). OM makes strong complexes with heavy metals (Ngorwe et al., 2014). High OM (> 2.0%) in soils is conducive for heavy metal chelation formation (Amos-Tautua et al., 2014). High CEC can reduce the mobility and bioavailability of metal in soils (Yoon et al., 2006; Vamerali et al., 2010). It is likely that the physico-chemical properties of the experimental soil such as clay texture, neutral pH 6-7, high OM, CEC and EC could enhance Pb immobilization.

4.6.2 Plant inoculation with endophytic bacteria

The results from recovery extraction before planting of endophytic bacteria from plant roots showed that inoculation of *P. psychrophila* into *A. mangium* and *E. camaldulensis* was successful as well as the results of recovery extraction for hydroponic test. The colonies isolated from plants were compared with pure colony of *P. psychrophila* on LB agar supplemented with 20 mg/L of Pb (Figure 4.20). Morphologically, the colony of *P. psychrophila* was circular and milky white (Figure 4.20A). These colonies were also found in 4 plates at serial dilution 10^{-4} (Figures 4.20B-E). Meanwhile, *P. psychrophila* colony did not appear on *A. mangium* and *E. camaldulensis* without inoculation (Figures 4.20F and G). Thus, *P. psychrophila* was not a native species of the new host plants for pot experiment.
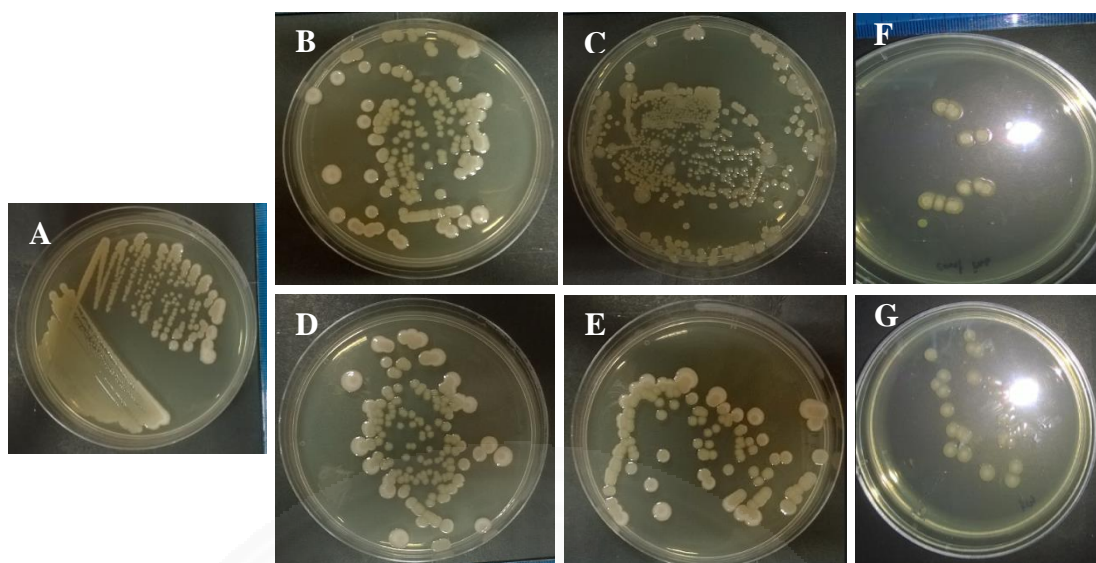
Figure 4.20 Colony morphology of endophytic bacteria on LB media supplemented with 20 mg/L of Pb: (A) Pure *P. psychrophila* colony, (B) *A. mangium*, uncontaminated soil, (C) *A. mangium*, Pb soil (D) *E. camaldulensis*, uncontaminated soil, (E) *E. camaldulensis*, Pb soil, (F) *A. mangium*, un-inoculation, and (G) *E. camaldulensis*, un-inoculation

4.6.3 Overall vitality and phytotoxicity

Throughout the experiment, all plants could survive without Pb phytotoxicity symptoms such as chlorosis and necrosis as shown in Figure 4.21. The phytotoxicity of metal is a critical factor affecting the success of phytoremediation. Some study suggested that PGPE *Bacillus* sp. MN3-4 can reduce Pb phytotoxicity via adsorption, intracellular accumulation, intracellular sequestration, or extracellular precipitation (Shin et al., 2012). However, the results from this study showed opposite viewpoint. Namely, throughout the experiment, all treatments could survive without the visible symptoms of Pb toxicity. This might be due to high Pb tolerance ability of *A. mangium* and *E. camaldulensis* as described in section 4.1.4. In addition, the physico-chemical properties of this experimental soil could induce Pb immobilization. However, the common toxicity symptoms were observed including wilting and drooping of leaves in all treatments. Normally, endophytic bacteria can also contribute to a decrease in metal toxicity by producing ACC deaminase and sequestration of metals (Cherian et al., 2012). Khan and Doty (2011) reported that endophytes could reduce Cd toxicity by increasing uptake of nutrient such as Zn and Fe.

121
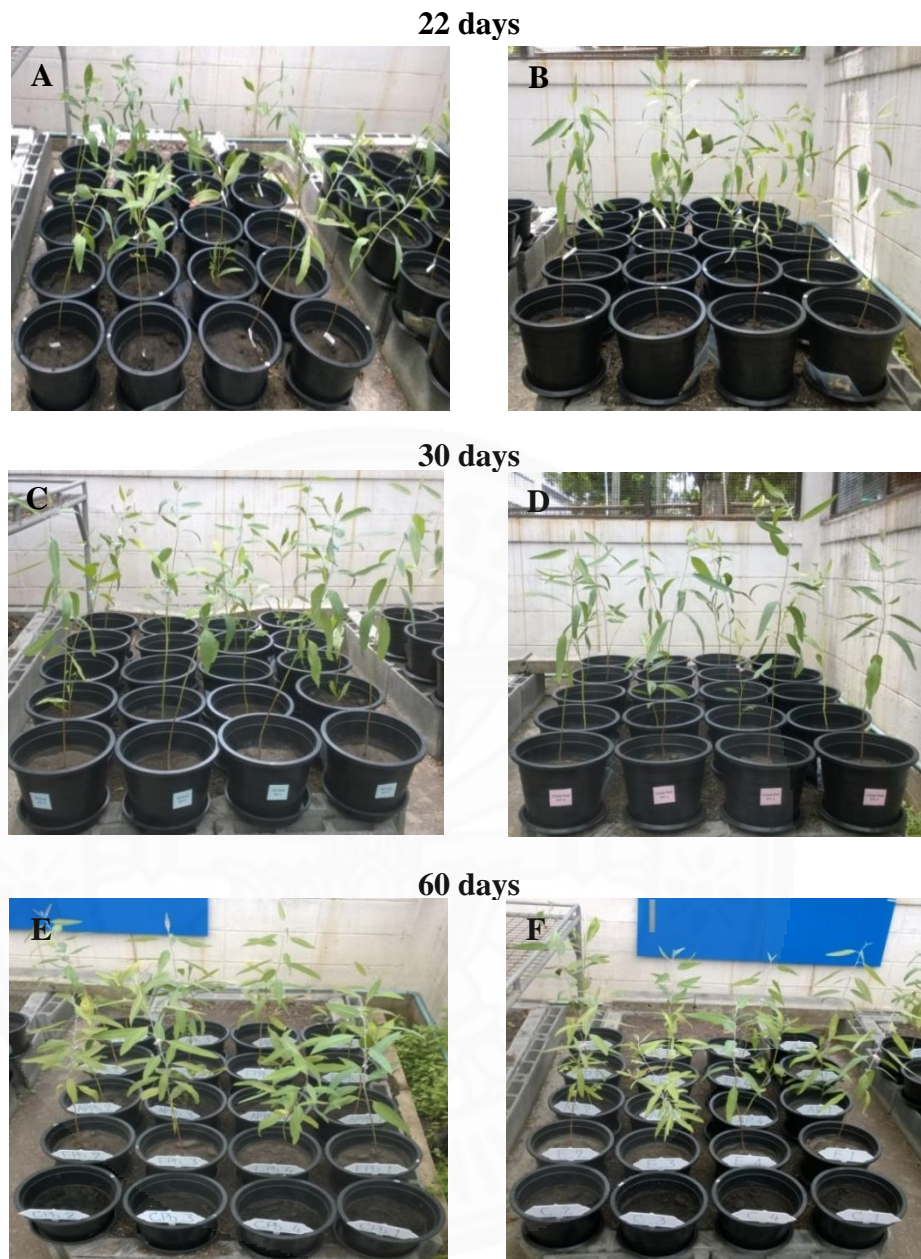
Figure 4.21 Plants grown on uncontaminated soil (B, D, F) and Pb soil (A, C, E) after 22-60 d of planting

4.6.4 Effect of *P. psychrophila* on plant growth

The ability of endophytic bacteria to promote plant growth in heavy metal contaminated soil is the preferred choice for phytoremediation. In this study, the effects of *P. psychrophila* inoculated and un-inoculated trees subjected to 1500 mg/kg Pb in the soil for 60 d on plant growth in fresh weight and height are shown in Table 4.17

122

and Figures 4.22 and 4.23. After 60 d, the fresh weight and height of all plants increased very little. In uncontaminated soil, inoculation of *P. psychrophila* did not influence the initial and final fresh weight of *A. mangium* and *E. camaldulensis* (Figures 4.22A and B). Inoculation of *P. psychrophila* did not affect the initial and final height of plants (Figures 4.23A and B). *P. psychrophila* did not show a great influence on fresh weight and height of *E. camaldulensis* in Pb contaminated soil compared to un-inoculated plant. While, *A. mangium* inoculated with *P. psychrophila* showed a significant reduction ($p \leq 0.05$) in plant initial and final fresh weight, but did not affect plant height. As individual plants are different, the biomass parameter should be changed to relative biomass growth.

Table 4.17 Effects of *P. psychrophila* inoculation and Pb concentration on fresh weight and height of *A. mangium* and *E. camaldulensis*

Treatments	Initial fresh weight (g)		Final fresh weight (g)	
	Control soil*	Pb soil	Control soil*	Pb soil
A. mangium (-Pc)	$4.22 \pm 2.69^{a,A}$	$11.0 \pm 2.15^{a,B}$	$5.92 \pm 4.62^{a,A}$	$13.3 \pm 0.96^{a,B}$
A. mangium (+Pc)	$5.32 \pm 2.92^{a,A}$	$5.50 \pm 0.62^{b,A}$	$7.92 \pm 4.78^{a,A}$	$5.98 \pm 0.79^{b,A}$
E. camaldulensis (-Pc)	$6.32 \pm 1.51^{a,A}$	$6.31 \pm 0.71^{a,A}$	$10.7 \pm 2.39^{a,A}$	$11.5 \pm 2.45^{a,A}$
E. camaldulensis (+Pc)	$7.11 \pm 0.80^{a,A}$	$7.82 \pm 0.78^{a,A}$	$12.1 \pm 1.80^{a,A}$	$14.0 \pm 1.28^{a,A}$
	Initial height (cm)		**Final height (cm)**	
	Control soil*	Pb soil	Control soil*	Pb soil
A. mangium (-Pc)	$33.3 \pm 13.6^{a,A}$	$36.8 \pm 5.30^{a,A}$	$37.8 \pm 17.9^{a,A}$	$39.3 \pm 4.16^{a,A}$
A. mangium (+Pc)	$41.2 \pm 11.5^{a,A}$	32.8 ± 6.83^{a}	47.3 ± 16.2^{a}	35.0 ± 7.21^{a}
E. camaldulensis (-Pc)	$45.5 \pm 1.04^{a,A}$	$47.7 \pm 5.51^{a,A}$	$67.2 \pm 2.57^{a,A}$	$71.2 \pm 4.25^{a,A}$
E. camaldulensis (+Pc)	$42.5 \pm 2.65^{a,A}$	$45.5 \pm 2.65^{a,A}$	$69.0 \pm 8.50^{a,A}$	$70.2 \pm 1.89^{a,A}$

Each value is mean of triplicate samples ± standard deviation. Mean of each columns indexed by the different small letters denote a significant difference ($p \leq 0.05$) between inoculation and un-inoculation. The different capital letters indicate significant difference of biomass between absence and presence of Pb concentration (1500 mg/kg) as determined by the *t*-test at $p \leq 0.05$. (-Pc) is un-inoculation with *P. psychrophila* and (+Pc) is *P. psychrophila* inoculation. * represent uncontaminated soil
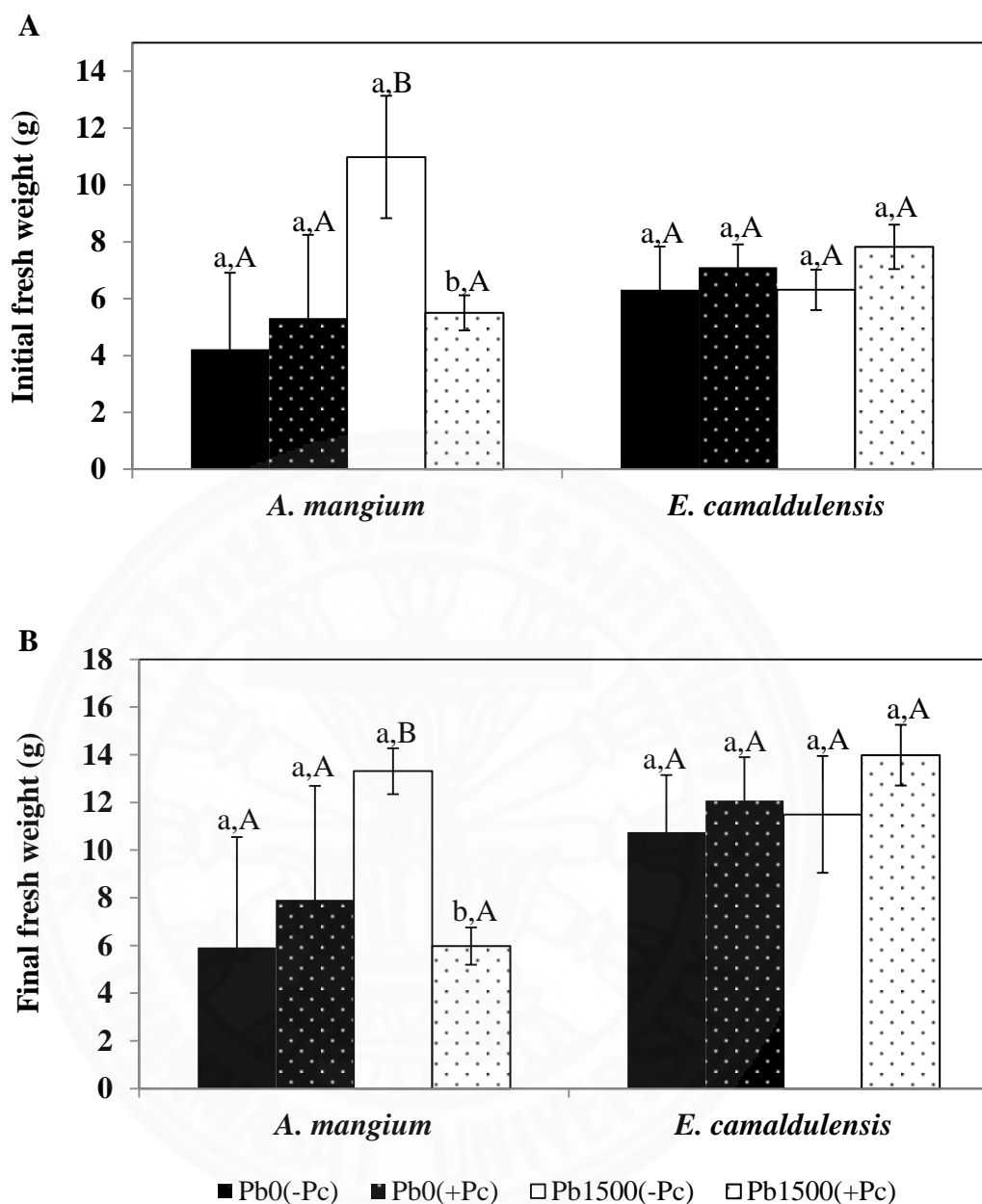
Figure 4.22 Effects of *P. psychrophila* inoculation and Pb concentration on fresh weight of fast-growing trees: (A) initial fresh weight, (B) final fresh weight. Each value is the mean of triplicates. Error bars represent standard deviation. The different small letters above the bar graph denote a significant difference of fresh weight ($p \leq$ 0.05) between inoculation and un-inoculation. The different capital letters indicate significant difference of fresh weight between absence and presence of Pb concentration (1500 mg/kg) as determined by the *t*-test at $p \leq 0.05$. (-Pc) is un-inoculation with *P. psychrophila* and (+Pc) is *P. psychrophila* inoculation.

124

Figure 4.23 Effects of *P. psychrophila* inoculation and Pb concentration on plant height of fast-growing trees: (A) initial height, (B) final height. Each value is the mean of triplicates. Error bars represent standard deviation. The different small letters above the bar graph denote a significant difference ($p \leq 0.05$) of plant height between inoculation and non-inoculation. The different capital letters indicate significant difference of plant height between absence and presence of Pb concentration (1500 mg/kg) as determined by the *t*-test at $p \leq 0.05$. (-Pc) is un-inoculation with *P. psychrophila* and (+Pc) is *P. psychrophila* inoculation.

The effects of inoculation and Pb concentration on the relative growth based on fresh weight and plant height are shown in Table 4.18 and Figure 4.24, respectively. In uncontaminated soil, there were no significant changes ($p > 0.05$) in growth caused by inoculation. Inoculation of *A. mangium* with *P. psychrophila* hardly increased the growth (144%), when compared with un-inoculated plant (143%). In *E. camaldulensis*, the relative growth (170%) did not change compared with un-inoculated plant (170%). The results indicated that *P. psychrophila* had no ability to increase plant growth. However, *A. mangium* and *E. camaldulensis* are fast-growing species by themselves.
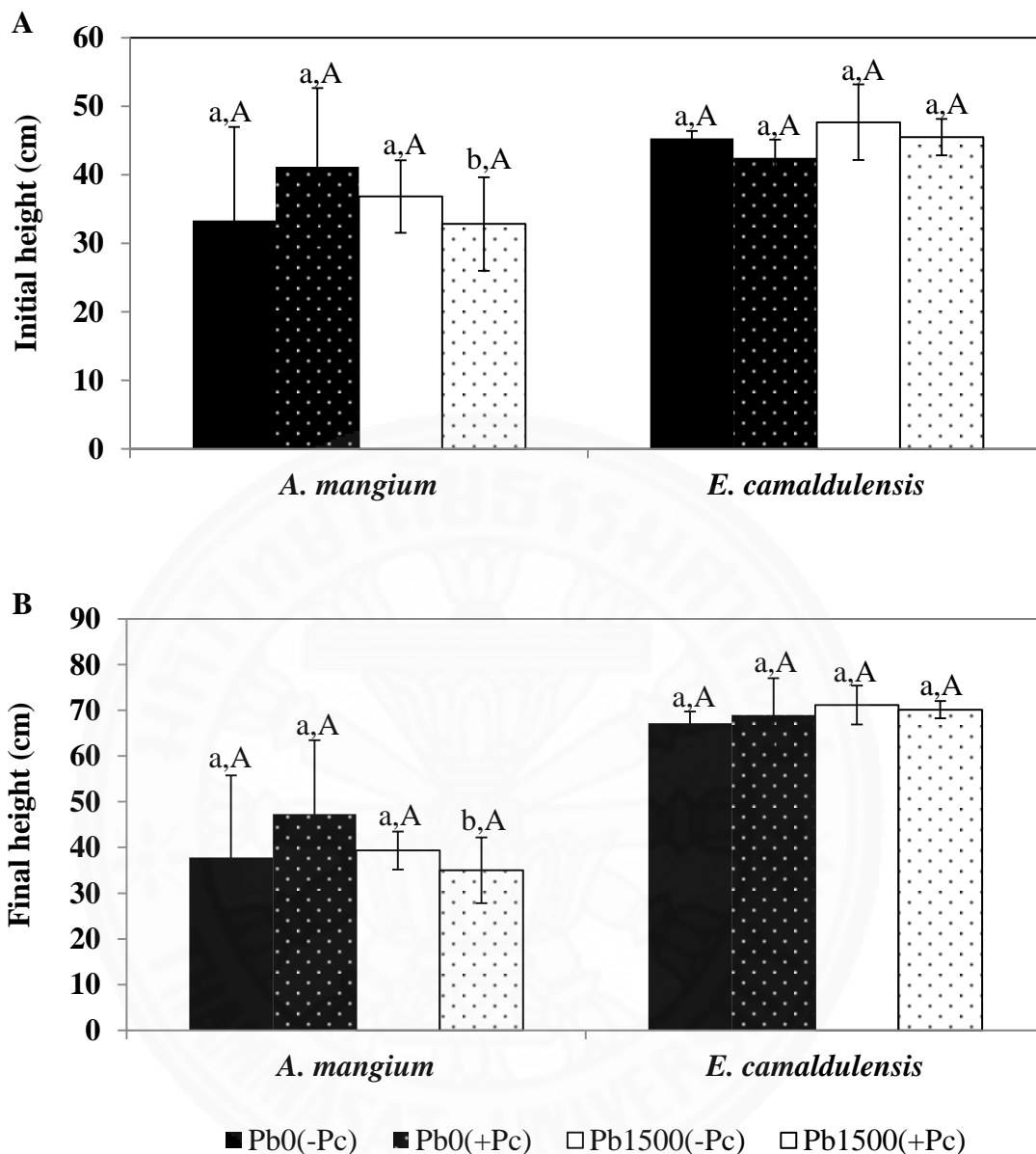
Table 4.18 Effects of *P. psychrophila* inoculation and Pb concentration on relative growth of *A. mangium* and *E. camaldulensis*

Treatments	Relative weight growth (%)		Relative height growth (%)	
	Control soil[*]	Pb soil	Control soil[*]	Pb soil
A. mangium (-Pc)	$143 \pm 45.3^{a,A}$	$124 \pm 28.1^{a,A}$	$112 \pm 9.06^{a,A}$	$107 \pm 4.70^{a,A}$
A. mangium (+Pc)	$144 \pm 21.8^{a,A}$	$110 \pm 18.4^{b,A}$	$113 \pm 9.86^{a,A}$	$107 \pm 1.95^{a,A}$
E. camaldulensis (-Pc)	$171 \pm 4.66^{a,A}$	$181 \pm 20.9^{a,A}$	$148 \pm 6.54^{a,A}$	$150 \pm 10.3^{a,A}$
E. camaldulensis (+Pc)	$170 \pm 14.5^{a,A}$	$179 \pm 5.10^{a,A}$	$162 \pm 11.7^{a,A}$	$154 \pm 5.29^{a,A}$

Each value is mean of triplicate samples ± standard deviation. Mean of each columns indexed with different small letters denote a significant difference ($p \leq 0.05$) of relative growth between inoculation and non-inoculation. The different capital letters indicate significant difference of relative growth between absence and presence of Pb concentration (1500 mg/kg) as determined by the *t*-test at $p \leq 0.05$. (-Pc) is un-inoculation with *P. psychrophila* and (+Pc) is *P. psychrophila* inoculation. * represent uncontaminated soil.

126
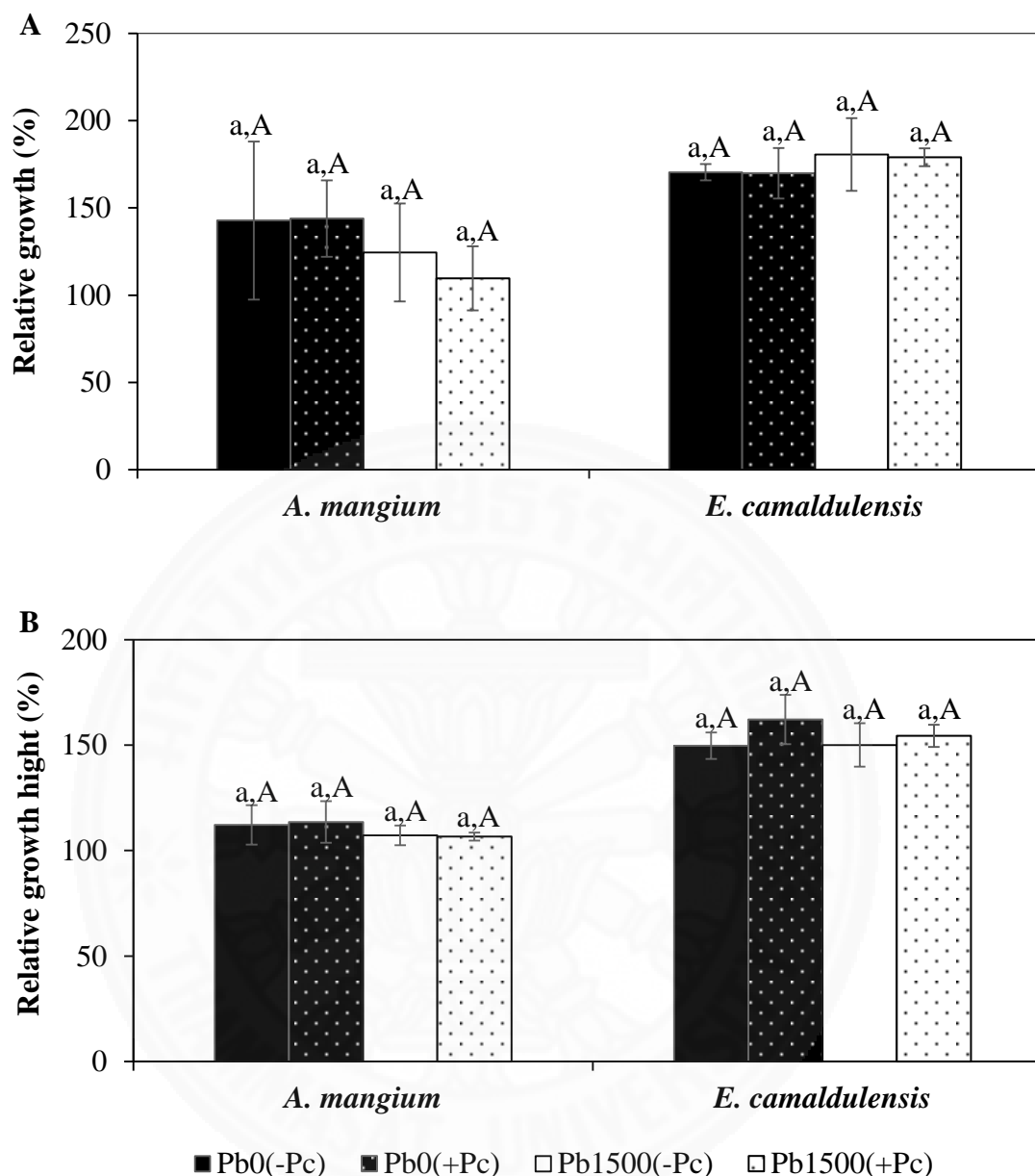
Figure 4.24 Effects of *P. psychrophila* inoculation and Pb concentration on the relative growth of fast-growing trees: (A) fresh weight, (B) height. Each value is the mean of triplicates. Error bars represent standard deviation. The different small letters above the bar graph denote a significant difference ($p \leq 0.05$) of relative growth between inoculation and non-inoculation. The different capital letters indicate significant difference of relative growth between absence and presence of Pb concentration (1500 mg/kg) as determined by the *t*-test at $p \leq 0.05$. (-Pc) is un-inoculation with *P. psychrophila* and (+Pc) is *P. psychrophila* inoculation.

127

In Pb contaminated soil, there were no significant changes ($p > 0.05$) in growth caused by inoculation. *A. mangium* inoculated with *P. psychrophila* had reduced relative growth (110%), compared with un-inoculation (124%). Similarly, *E. camaldulensis* inoculation showed reduced relative growth (179%) compared with un-inoculated plant (181%). The results indicated that *P. psychrophila* inoculation did not exhibit a great influence on growth of *A. mangium* and *E. camaldulensis* as compared with non-inoculated trees. These results are in agreement with Ma et al. (2011a), who found that *Pseudomonas* sp. A3R3 inoculations did not exhibit great influence on plant growth of *A. serpyllifolium* in Ni contaminated soils as compared with un-inoculated plants.

4.6.5 Effect of *P. psychrophila* on Pb concentration in soil

Total Pb concentrations in soil of *A. mangium* and *E. camaldulensis* at different exposure time are shown in Table 4.19 and Figures 4.25A and B, respectively. There were no significant differences ($p > 0.05$) of total Pb concentration in all treatments of *A. mangium* (1326-1426 mg/kg). At initial and 30 d of exposure, all treatments of *E. camaldulensis* did not show any significant difference in total Pb concentration (1275-1458 mg/kg).

The effects of *P. psychrophila* on DTPA-extractable Pb concentration in spiked soil of *A. mangium* and *E. camaldulensis* are shown in Table 4.19 and Figures 4.26A and B respectively. The extractable Pb concentration of *A. mangium* and *E. camaldulensis* with and without inoculation were not significantly different when the exposure time was increased (2 months). The exposure time in this study was considered as short time. Marcos et al. (2016) reported that the positive effects of bacterial inoculation in sugarcane plants grown under field conditions occurred after 6 months. As expected, inoculation of plants with *P. psychrophila* could promote and reduce heavy metals bioavailability depending on the combination of plant, bacterium and metal used (Sessitsch et al., 2013). Henning et al. (2016) also explained that host physiological response to endophyte inoculation may vary with bacterial strain, plant host and plant ontogeny. In addition, the available Pb concentration was lower than total Pb concentration in all treatments for all exposure time.

128

Table 4.19 Effect of *P. psychrophila* inoculation on total and the DTPA-extractable Pb concentration in soil of plants grown on Pb soil (1500 mg/kg)

Treatment	0 d	30 d	60 d
	Total Pb concentration (mg/kg)		
A. mangium (-Pc)	$1390 \pm 32.0^{a,A}$	$1357 \pm 48.9^{a,A}$	$1326 \pm 60.6^{a,A}$
A. mangium (+Pc)	$1395 \pm 52.1^{a,A}$	$1426 \pm 74.4^{,A}$	$1371 \pm 85.1^{b,A}$
E. camaldulensis (-Pc)	$1390 \pm 45.0^{a,A}$	$1458 \pm 68.3^{a,A}$	$1288 \pm 14.7^{a,A}$
E. camaldulensis (+Pc)	$1358 \pm 44.4^{a,A}$	$1334 \pm 33.6^{a,A}$	$1275 \pm 18.3^{a,A}$
	DTPA-extractable Pb concentration (mg/kg)		
A. mangium (-Pc)	$456 \pm 40.8^{a,A}$	$394 \pm 21.9^{a,A}$	$436 \pm 65.7^{a,A}$
A. mangium (+Pc)	$449 \pm 29.9^{a,A}$	$517 \pm 81.2^{b,A}$	$458 \pm 51.0^{a,A}$
E. camaldulensis (-Pc)	$429 \pm 36.6^{a,A}$	$486 \pm 51.3^{a,A}$	$452 \pm 15.3^{a,A}$
E. camaldulensis (+Pc)	$458 \pm 26.7^{a,A}$	$473 \pm 33.5^{a,A}$	$425 \pm 20.3^{a,A}$

Each value is mean of triplicate samples ± standard deviation. Mean of each columns index the different small letters denote a significant difference ($p \leq 0.05$) Pb concentration between inoculation and non-inoculation as determined by the *t*-test at $p \leq 0.05$. The different capital letters indicate significant difference ($p \leq 0.05$) of Pb concentration among exposure time according to Fisher's LSD test. (-Pc) is un-inoculation with *P. psychrophila* and (+Pc) is *P. psychrophila* inoculation. * represent uncontaminated soil
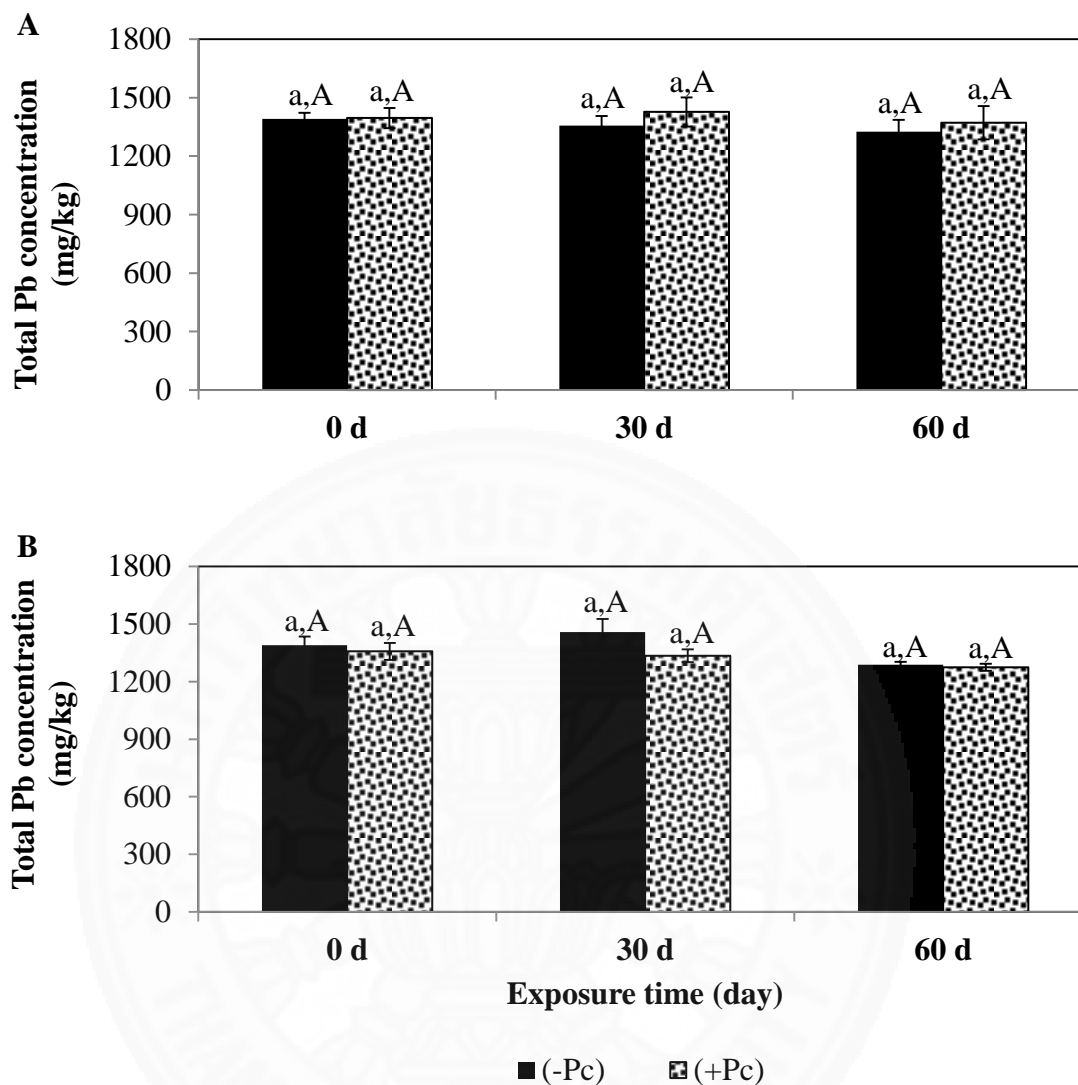
Figure 4.25 Effect of *P. psychrophila* inoculation on total Pb concentration in soil. (A) *A. mangium*, (B) *E. camaldulensis*. Each value is the mean of triplicates. Error bars represent standard deviation. The different small letters above the bar graph denote a significant difference of Pb concentration between inoculation and un-inoculation by *t*-test at $p \leq 0.05$, whilst different capital letters denote a significant difference of Pb concentration among exposure time according to Fisher's LSD test at $p \leq 0.05$. (-Pc) is un-inoculation with *P. psychrophila* and (+Pc) is *P. psychrophila* inoculation.

130
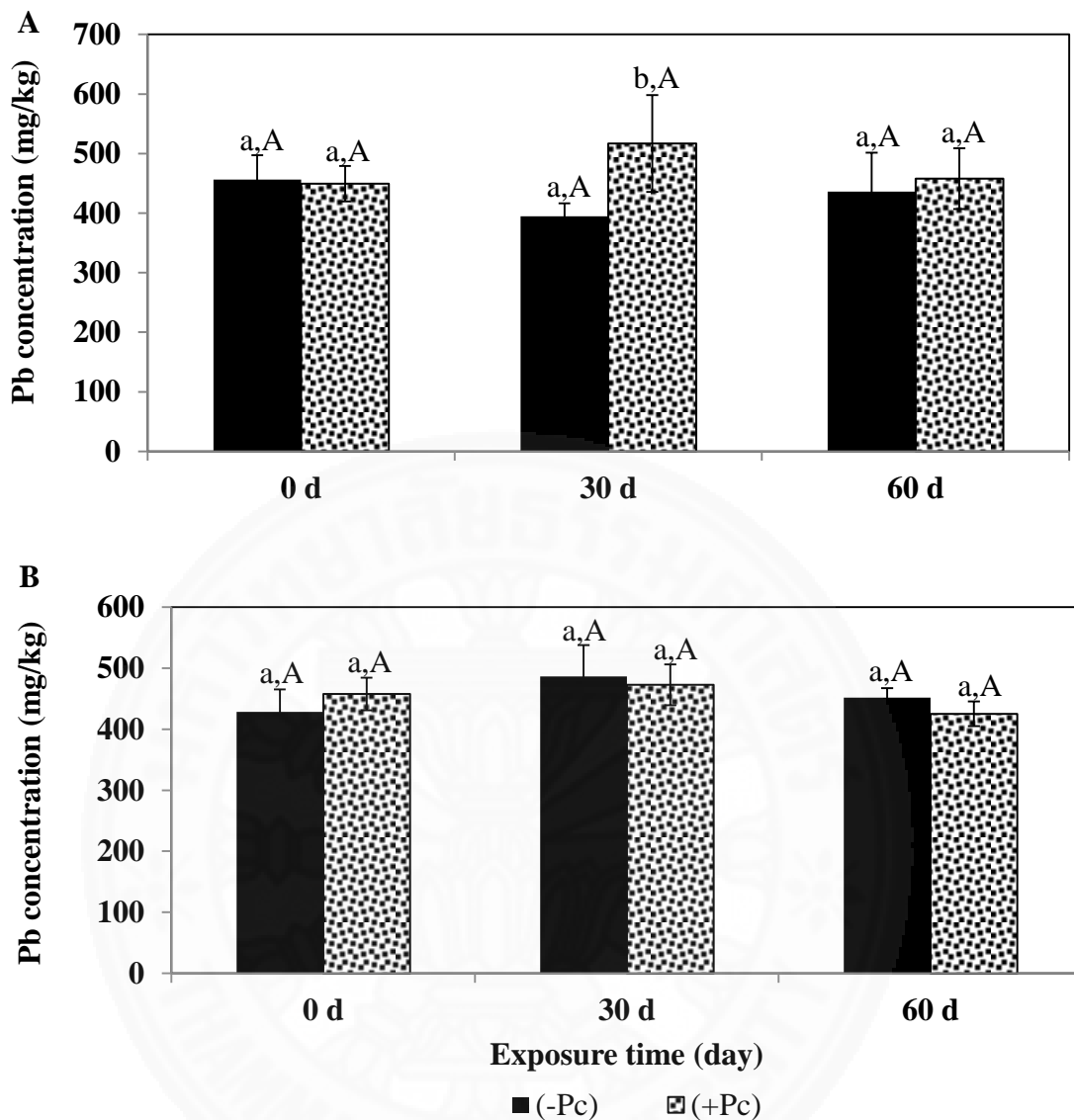
Figure 4.26 Effect of *P. psychrophila* inoculation on the DTPA-extractable Pb concentration in soil: (A) *A. mangium*, (B) *E. camaldulensis*. Each value is the mean of triplicates. Error bars represent standard deviation. The different small letters above the bar graph denote a significant difference between inoculation and un-inoculation by *t*-test at $p \leq 0.05$, whilst different capital letters denote a significant difference of available Pb concentration among exposure time according to Fisher's LSD test at $p \leq 0.05$. (-Pc) is un-inoculation with *P. psychrophila* and (+Pc) is *P. psychrophila* inoculation.

131

4.6.6 Effect of *P. psychrophila* on Pb accumulation

The effects of bacterial inoculation on Pb accumulation in shoots of *A. mangium* and *E. camaldulensis* are shown in Table 4.20 and Figure 4.27A. Inoculation in *A. mangium* had significantly increased ($p \leq 0.05$) Pb concentration in shoot (16.6 mg/kg) compared to un-inoculation (4.08 mg/kg). Similarly, inoculation in *E. camaldulensis* also resulted in a significant increase ($p \leq 0.05$) in Pb concentration in shoot (11.3 mg/kg) compared to un-inoculation (3.83 mg/kg). Similarly, Ma et al. (2011a) found that inoculation of PGPE *Pseudomonas* A3R3 significantly increased the accumulation of Ni in shoots of *A. serpyllifolium* and *B. juncea* compared with un-inoculated control. In this study, the increase of Pb accumulation in shoots could be due to PGPE *P. psychrophila*'s ability to transfer Pb from root to shoot (Ma et al., 20016).

The effect of bacterial inoculation on Pb accumulation in roots of *A. mangium* and *E. camaldulensis* are shown in Table 4.20 and Figure 4.27B. There were no significant changes ($p > 0.05$) in Pb contents in roots caused by inoculations. However, *A. mangium* inoculation showed an increased trend in Pb root concentration (988 mg/kg) compared to un-inoculation (790 mg/kg). While *E. camaldulensis* inoculation showed a decreased trend in Pb content (341 mg/kg) compared to un-inoculation (388 mg/kg). The results of Pb accumulation in roots from pot experiment are in accordance with the results from hydroponic experiment of this study. In addition, Pb accumulation in shoot and root from this study were similar with the results of Sheng et al. (2008) who found that there was no significant difference in root Pb concentrations of *B. napus* between endophytic bacterial inoculation and un-inoculation, while, Pb concentrations in shoot was significantly increase.

P. psychrophila inoculation had more influence on *A. mangium* than *E. camaldulensis*. For un-inoculation, they had similar Pb concentration in shoots (about 4 mg/kg). After inoculation, Pb concentration in shoot of *A. mangium* increased 4-fold, while that of *E. camaldulensis* increased almost 3-fold. Similarly, Jiang et al. (2008) found plants (Indian mustard, maize and tomato) inoculated with metal mobilizing PGPB, vary in their ability to accumulate heavy metals, and tomato accumulated heavy metals higher than other plant species from the soil.

132

Table 4.20 Effect of *P. psychrophila* inoculation on Pb accumulation in plant tissues grown in Pb contaminated soil for 2 months

Treatments	Pb accumulation (mg/kg)	
	Root	**Shoot**
A. mangium (-Pc)	790 ± 274^a	4.08 ± 1.65^a
A. mangium (+Pc)	988 ± 16.6^a	16.6 ± 4.00^b
E. camaldulensis (-Pc)	388 ± 95.3^a	3.83 ± 1.78^a
E. camaldulensis (+Pc)	341 ± 65.8^a	11.3 ± 3.00^b

Each value is mean of triplicate samples ± standard deviation. Mean of each columns indexed by the same letter are not significant difference according to *t*- test ($p \leq 0.05$).

In this study, the increase in Pb accumulation in *A. mangium* by *P. psychrophila* inoculation could be due to its efficiency of mobilizing Pb from soil particle via siderophore production and P solubilization. In contrast, there are some opposing viewpoints suggesting that the presence of metal-resistant endophytes can decrease plants' metal uptake and accumulation (Li et al. 2012). Xu et al. (2015) found that heavy metal resistant bacteria, *Pseudomonas putida* CZ1, amended in Cu solution did not significantly reduce Cu contents in roots of *Elsholtzia splendens* compared to non-amendment. Madhaiyan et al. (2007) found that endophytic bacteria *Methylobacterium oryzae* and *Burkholderia* sp. reduced metal contents in roots of *Lycopersicon esculentum* due to bacterial immobilization of metals in rhizosphere. This information supports the result of a little reduction of Pb content in root of *E. camaldulesis* from this study. It is likely that in the present study, the decrease in the accumulation of Pb by the inoculated plants may be associated with other factors, including microbial populations and their response to environmental conditions in the rhizosphere, plant, and soil type, since the plants growing in metal polluted soils may modulate their growth and metal accumulation response to various physico-chemico-biological properties of the environment (Sessitsch et al., 2013; Ma et al., 2015).

Considering plant species, *P. psychrophila* seemed to be effective in promoting the phytoremediation potential of *A. mangium* by enhancing Pb

concentration, but it was not useful for Pb phytoremediation of *E. camaldulensis* based on reduction of Pb accumulation.
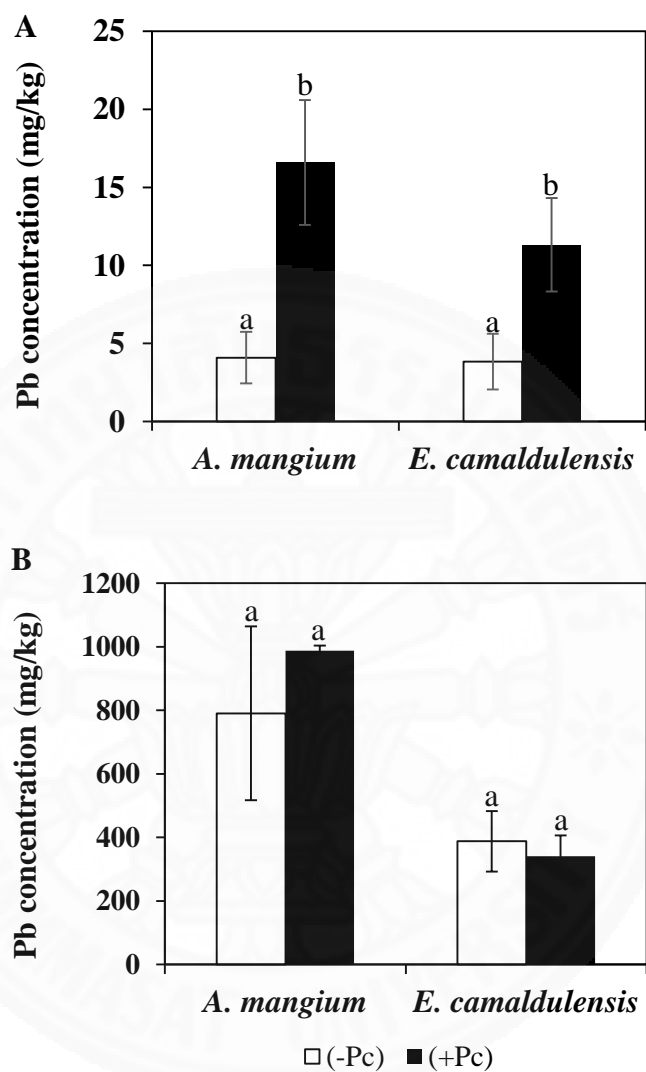
Figure 4.27 Effect of *P. psychrophila* inoculation on Pb accumulation: (A) in shoot, (B) in root of plants grown on Pb contaminated soil (1500 mg/kg). Each value is the mean of triplicates. Error bars represent standard deviation. The different letters above the bar graph denote a significant difference between inoculation and non-inoculation as determined by the *t*-test at $p \leq 0.05$. (-Pc) is un-inoculation with *P. psychrophila* and (+Pc) is *P. psychrophila* inoculation.

134

4.6.7 Effect of *P. psychrophila* on Pb uptake

The effect of bacterial inoculation on Pb accumulation in shoots of *A. mangium* and *E. camaldulensis* are shown in Table 4.21 and Figure 4.28. All treatments showed no significant difference ($p > 0.05$) in Pb uptake. The results of Pb uptake were also similar with those of Pb accumulation.

Table 4.21 Effect of *P. psychrophila* inoculation on Pb uptake in whole plants grown in Pb contaminated soil for 2 months

Treatments	Pb uptake (mg/plant)
A. mangium (-Pc)	2.11 ± 0.25^{a}
A. mangium (+Pc)	3.20 ± 1.27^{a}
E. camaldulensis (-Pc)	2.00 ± 0.77^{a}
E. camaldulensis (+Pc)	2.02 ± 0.09^{a}

Each value is mean of triplicate samples ± standard deviation. Mean of each columns indexed by the same letter are not significant difference according to *t*- test ($p \leq 0.05$).

Figure 4.28 Effect of *P. psychrophila* inoculation on Pb uptake of plants grown on Pb contaminated soil (1500 mg/kg). Each value is the mean of triplicates. Error bars represent standard deviation. The different letters above the bar graph denote a significant difference between inoculation and non-inoculation as determined by the *t*-test at $p \leq 0.05$. (-Pc) is un-inoculation with *P. psychrophila* and (+Pc) is *P. psychrophila* inoculation.

135

4.6.8 Effect of *P. psychrophila* on phytostabilization potential

    The BCF, BAC and TF values of Pb in plants are presented in Table 4.22 and Figure 4.29. The BCF, BAC and TF values of all treated plants are < 1. *P. psychrophila* slightly increased the BCF of Pb in *A. mangium*, but reduced the BCF of Pb in *E. camaldulensis* compared with un-inoculated plants. Similarly, Ma et al. (2014) also found that BCF of Ni < 1 in all treatments. *Pseudomonas* sp. A3R3 increased the BCF values both in *B. juncea* and *R. communis* compared to un-inoculated plants. Certainly, these results could also support that *P. psychrophila* plays an important role in enhancing Pb accumulation (Table 4.20 and Figure 4.27) and uptake (Table 4.21 and Figure 4.28) only in *A. mangium*. Since, *P. psychrophila* increased bioavailability of Pb as DTPA-extractable Pb concentration in soil of *A. mangium* (Table 4.19 and Figure 4.26A).

    Besides, *P. psychrophila* slightly increased ($p > 0.05$) the BAC and TF values of Pb in *A. mangium* and *E. camaldulensis* compared to un-inoculated plants. Similarly, Ma et al. (2015) found that *Pseudomonas* sp. A3R3 slightly increased TF values of Ni in *B. juncea* and *R. communis* compared to un-inoculated plants. Besides, Sun et al. (2010) found that endophytic bacteria enhanced Cu transfer from root to aboveground tissue of *B. napus* growing in Cu-contaminated substrate. Namely, Cu concentration in aerial part of rape increases (125%) compared to the un-inoculated control (63%). This indicates that *P. psychrophila* plays an important role in transferring Pb from roots leading to increase Pb accumulation in shoots. This is consistent with Ma et al. (2016). It was reported that endophytic bacteria can alter heavy metal bioavailability and its translocation in plant.

    Normally, plants with BAC and TF values < 1 can be identified as a metal excluder and suitable for phytostabilization (Yoon et al., 2006). In this study, although the inoculation of PGPE *P. psychrophila* could alter Pb accumulation in plants, both *A. mangium* and *E. camaldulensis* had BCF, BAC and TF values <1 indicating that these plant species with inoculation with *P. psychrophila* could be used for the phytostabilization purpose. Actually, *A. mangium* showed higher BCF and lower TF values that those in *E. camaldulensis*. Therefore, it has a higher potential for phytostabilization than *E. camaldulensis*.

Table 4.22 Effect of *P. psychrophila* inoculation on Pb accumulation indices: BCF, BAC and TF in plants grown in Pb contaminated soil.

Treatments	BCF	BAC	TF
A. mangium (-Pc)	0.46 ± 0.18^a	$0.00 \pm 0.00^*$	0.01 ± 0.00^a
A. mangium (+Pc)	0.59 ± 0.04^a	$0.01 \pm 0.00^*$	0.02 ± 0.00^a
E. camaldulensis (-Pc)	0.24 ± 0.06^a	$0.00 \pm 0.00^*$	0.01 ± 0.00^a
E. camaldulensis (+Pc)	0.21 ± 0.04^a	$0.01 \pm 0.00^*$	0.04 ± 0.02^a

Each value is mean of triplicate samples $\pm$ standard deviation. The different small letters indicate significant difference between inoculation and un-inoculation as determined by the *t*-test $p \leq 0.05$. (-Pc) is un-inoculation with *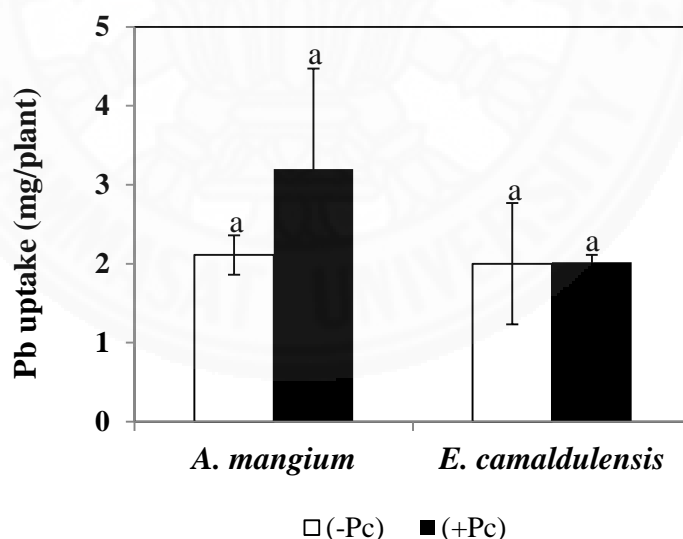P. psychrophila* and (+Pc) is *P. psychrophila* inoculation. *represent *t*-value cannot be computed because the standard deviation of both group are 0.
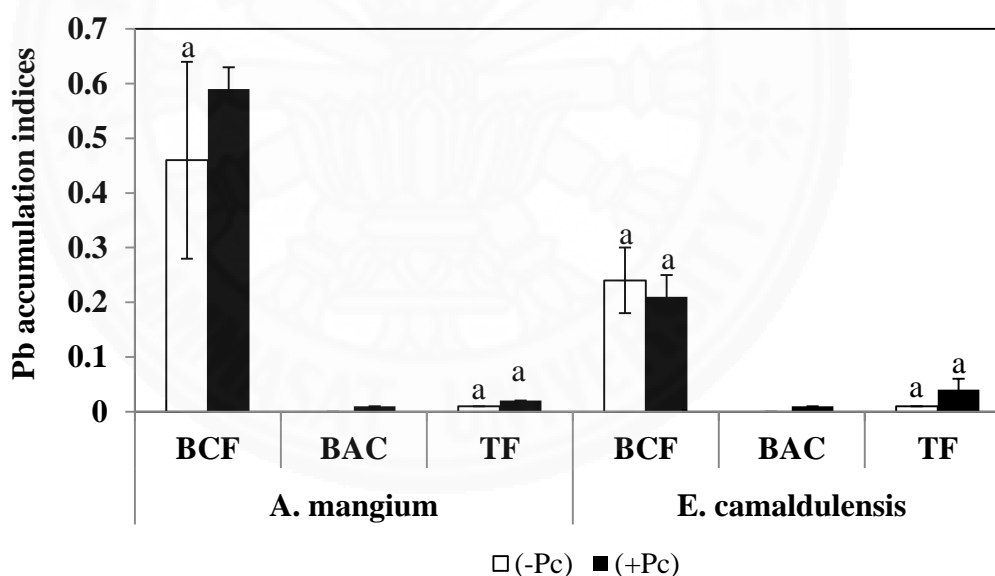
Figure 4.29 Effect of *P. psychrophila* inoculation on Pb accumulation indices. Each value is the mean of triplicates. Error bars represent standard deviation. The different letters above the bar graph denote a significant difference between inoculation and non-inoculation as determined by the *t*-test at $p \leq 0.05$. (-Pc) is un-inoculation with *P. psychrophila* and (+Pc) is *P. psychrophila* inoculation.

137

Chapter 5
Conclusions and Recommendations

5.1 Conclusions

The results of this study helped in assessing the potential of using fast-growing trees inoculated with Pb-tolerant endophytic bacteria for uptake from the artificially Pb-spiked soil. It also helps in identifying the trees and endophytic bacterium for Pb phytoremediation.

The investigations from screening of fast-growing trees by hydroponic experiment showed that all trees (*A. indica*, *A. mangium*, *E. camaldulensis* and *S. siamea*) had high Pb tolerance with TI value > 70. Only *A. mangium* and *E. camaldulensis* grown in 50 mg/L of Pb solution accumulated very high Pb concentration in roots (49004 and 40598 mg/kg, respectively). In addition, they could produce new white roots when grown in low Pb concentration. Moreover, they had TF values < 1, indicating that they were Pb excluders suitable for phytostabilization. Therefore, *A. mangium* and *E. camaldulensis* were the best candidates based on high Pb content in roots, and were chosen to be the new host of endophytic bacteria.

The screening of Pb-accumulating plants by field survey showed that *P. calomelanos* was the best host harboring Pb-tolerant endophytic bacteria based on high Pb content in the root (32633 mg/kg). In addition, a new Pb hyperaccumulator for phytoextraction, namely *B. pilosa* was also identified. It accumulated 1270 mg/kg Pb in shoot, and it showed a TF value of 1.79. Meanwhile, *T. latifolia*, *M. pudica*, and *Chrysopogon* sp. showed BCF values > 1, and BAC and TF < 1. These plants could be useful in phytostabilization.

Through the screening results of Pb-tolerant endophytic bacteria by characterization criteria, isolate Pc was selected. It showed high Pb mobilization in soil (2.23 mg/kg), and Pb solubilization in solution (18.2 mg/L). In addition, it could produce siderophore (1.63 of siderophore production index), solubilize inorganic phosphate (8.70 of phosphate solubilization index), high Pb tolerance (1875 of MIC value). This isolate was identified as *P. psychrophila*. Besides, it was not the local

138

strain of *A. mangium* and *E. camaldulensis* and it was not a pathogen itself, making it as a secure candidate to assist the trees for phytostabilization.

The results of inoculation of *P. psychrophila* into the roots of *A. mangium* and *E. camaldulensis* by pruned-root dip method showed that inoculated *P. psychrophila* could enter and colonize in the roots of the new hosts. The appeared colony morphology from recovery extraction and 16S rRNA gene identification successfully confirmed that *P. psychrophila* colonized inside the roots before immersing in Pb solution. Besides, the effect of inoculation on Pb accumulation in roots of plants grown in hydroponic culture, it was found that *P. psychrophila* seemed to be effective in promoting the Pb uptake of *A. mangium* by enhancing Pb accumulation in roots. However, *P. psychrophila* was not useful for Pb uptake of *E. camaldulensis* based on reduction of Pb content in root. The difference of Pb content in plants with and without *P. psychrophila* inoculation also implied that the inoculation technique was successful and the inoculated *P. psychrophila* could colonize inside the roots of new hosts.

The results of recovery extraction after 15 d of inoculation showed that *P. psychrophila* could enter and colonize inside the root of *A. mangium* and *E. camaldulensis*. This implies that *P. psychrophila* could also colonize inside the root tissues of new plant hosts after 60 d of inoculation grown in Pb contaminated soil of pot experiment.

The results of inoculation on Pb accumulation in pot experiment showed that there were no influences on plant growth and Pb phytotoxicity for both plants inoculated with *P. psychrophila*. *P. psychrophila* significantly increased Pb accumulation in shoots of *A. mangium* and *E. camaldulensis*. Besides, *P. psychrophila* increased Pb accumulation in roots of *A. mangium*, but slightly reduced that of *E. camaldulensis*. With translocation factor (TF) <1, *A. mangium* and *E. camaldulensis* were identified as excluders that are suitable for phytostabilization. Inoculation of *P. psychrophila* slightly increased bioconcentration factor (BCF) and TF in *A. mangium*. However, inoculation of *P. psychrophila* in *E. camaldulensis* showed lower BCF and TF compared to *A. mangium*. The overall findings of this study suggested that *P. psychrophila* was effective in promoting the phytostabilization potential of *A.*

mangium, which may be used for remediation of Pb contaminated site. However, it was not useful for phytostabilization by *E. camaldulensis*.

5.2 Recommendations

Based on the results of this study, following recommendations are suggested.

1. Plants used in this study should be uniform and grown from seed germination under control conditions to reduce variation.

2. The protocol of surface disinfection technique should be validated to obtain a diversity of endophytic bacteria by comparing the conditions used such as reagent and time. There is no protocol of this technique to remove all epiphytes without penetrating the interior tissues, it can kill internal endophytic bacteria.

3. In order to indicate true endophytic bacteria, the recovery extraction is not enough for judgement. Thus, the visualization of *P. psychrophila* with green fluorescence protein inside plant tissue should be carried out. This will not only help in confirming colonization of *P. psychrophila* inside the plant roots, but also will helped in localizing *P. psychrophila* in different plant tissues.

4. For characterization, other plant growth promoting bacteria such as nitrogen fixation, IAA production, ACC deaminase enzyme, antibiotics, etc. should be tested. Besides, these properties should be tested under Pb stress.

5. The exposure time of pot experiment should be longer than 2 months to increase the probability of obtaining the significant difference.

6. For Pb analysis, the digestion method should be verified.

7. *A. mangium* inoculated with *P. psychrophila* should be further planted in the real Pb contaminated site in a long-term (2 years) to verify the beneficial effects of this strain on Pb phytostabilization. This time is suitable to get high heating value according to Department of Alternative Energy Development and Efficiency, Thailand. Besides, *A. mangium* and *E. camaldulensis* did not found in the Song Tho Pb mine area, so they will be classified as the invasive plants of this area. To prevent the infestation of these plants, the management plan needs to be prepared. Starting from, the study area should be limited clearly as the experiment zone about 8 x 8 m^2.

This zone has 4 treatments including trees with and without inoculation. Each treatment has 4 replicates. Plant height, biomass and Pb concentration in shoots and in soil will be determined every 3 months. Importantly, if the new seedlings or sprouts can growth, they will be pulled the roots out to eliminate the potential for reintroduction. After finished 2 years (trees have high heating value as firewood), all plants must be get rid of from the area. The stem will be mechanical cut to use as firewood. The roots will be removed by digging the root out. Once removed, the roots should be left in the sun to dry out. Then, they should be bagged and taken to the hazardous landfill in order to protect reentering of Pb to the environment. Otherwise, the roots accumulated Pb should be done in the phytomining process to obtain the Pb.

8. Pb speciation in plant tissue and in soil should be further studied, because it is mainly the important factor for Pb mobilization.

References

Ahemad, M. (2015). Phosphate-solubilizing bacteria-assisted phytoremediation of metalliferous soils: a review. *Biotechology*, *5*, 111-121.

Aini Syuhaida, A. W., Sharifah Norkhadijah, S. I., Praveena, S. M., & Suriyani A. (2014). The comparison of phytoremediation abilities of water mimosa and water hyacinth. *Asian Research Publishing Network Journal of Science and Technology*, *4*(12), 722-731.

Alford, É. R. Pilon-Smits, E. A. H., & Paschke, M. W. (2010). Metallophytes-a view from the rhizosphere. *Plant Soil*, *337*, 33-50.

Ali, H., Khan, E., & Sajad, M. A. (2013). Phytoremediation of heavy metals-concepts and applications. *Chemosphere, 91*, 869-881.

Alkhatib, R., Bsoul, E., Blom, D. A., Ghoshroy, K., Creamer, R., & Ghoshroy, S. (2013). Microscopic analysis of lead accumulation in tobacco (*Nicotiana tabacum* var. Turkish) roots and leaves. *Journal of Microscopy and Ultrastructure*, 1, 57-62.

Alloway, B. J. (Ed.). (2013). *Heavy metals in soils*. Dordrecht: Springer.

Almeida, A. A. F., Valle, R. R., Mielke, M. S., & Gomes, F. P. (2007). Tolerance and prospection of phytoremediator woody species of Cd, Pb, Cu and Cr. *Brazilian Journal of Plant Physiology*, *19*(2), 83-98.

Amos-Tautua, B. M. W., Onigbinde, A. O., & Ere, D. (2014). Assessment of some heavy metals and physicochemical properties in surface soils of municipal open waste dumpsite in Yenagoa, Nigeria. *African Journal of Environmental Science and Technology*, *8*(1), 41-47.

Anjum, N. A., Hasanuzzaman, M., Hossain, M. A., Thangavel, P., Roychoudhury, A., Gill, S. S., et al. (2015). Jacks of metal/metalloid chelation trade in plants-an overview. *Frontiers in Plant Science*, *6*, Retrieved Febury 3, 2017, 1-17 from https://www.frontiersin.org/articles/10.3389/fpls.2015.00192/full

APHA, AWWA & WEF. (2012). *Standard Method for the Examination of Water and Wastewater*. New York: American Public Health Association.

Ashraf, M. A., Maah, M. J., & Yusoff, I. (2013). Evaluation of natural phytoremediation process occurring at ex-tin mining catchment.

Chiang Mai Journal of Science, *40*, 198-213.

Babu, A. G., Kim, J. D., & Oh, B. T. (2013). Enhancement of heavy metal phytoremediation by *Alnus firma* with endophytic *Bacillus thuringiensis* GDB-1. *Journal of Hazardous Material*, *250-251*, 477-483.

Babu, A. G., Shea, P. J., Sudhakar, D., Jung, I. B., & Oh, B. T. (2015). Potential use of *Pseudomonas koreensis* AGB-1 in association with *Miscanthus sinensis* to remediate heavy metal(loid)-contaminated mining site soil. *Journal of Environmental Management*, *151*, 160-166.

Badr, N., Fawzy, M., & Al-Qahtani, K. M. (2012). Phytoremediation: an ecological solution to heavy-metal-polluted soil and evaluation of plant removal ability. *World Applied Sciences Journal*, *16*(9), 1292-1301.

Baker, A. J. M. (1981). Accumulators and excluders-strategies in the response of plants to heavy metals. *Journal of Plant Nutrition*, *3*, 643-654.

Baker, A. J. M., & Brooks, R. R. (1989). Terrestrial higher plants which hyperaccumulate metallic elements-a review of their distribution, ecology and phytochemistry. *Biorecovery*, *1*, 8-126.

Batty, L. C. (2005). The potential importance of mine sites for biodiversity. *Mine Water and the Environment*, *24*, 101-103.

Bech, J., Roca, N., Boluda, R., Tume, P., Duran, P., Poma, W., & Sanchez, I. (2014). Hyperaccumulator of Pb in native plants growing on Peruvian mine tailings. Retrieved September 23, 2015, from http://meetingorganizer.copernicus.org/EGU2014/EGU2014-1376.pdf

Bhatnagar, S., & Kumari, R. (2013). Bioremediation: A sustainable tool for environmental management-A review. *Annual Review & Research in Biology*, *3*(4), 974-993.

Black, C. A. (Ed.). (1965). *Methods of soil analysis, Part 2: Chemical and Microbiological Properties*, Wisconsin: American Society Agronomy.

Bray, R. H., & Kurtz, L. T. (1945). Determination of total, organic and available forms of phosphorus in soils. *Soil Science*, *59*, 39-45.

Bressan, W., & Borges, M.T. (2004). Delivery methods for introducing endophytic bacteria into maize. *BioControl, 49*, 315-322.

Brooks, R. R., Chambers, M. F., Nicks, L. J., & Robinson, B. H. (1998). Phytomining

Trends in Plant Science, *3*, 359-362.

Brunner, I., Luster, J., Gunthardt-Goerg, M. S., & Frey, B. (2008). Heavy metal accumulation and phytostabilisation potential of tree fine roots in a contaminated soil. *Environmental pollution*, *152*(3), 559-568.

Bull, C. T., De Boer, S. H., Denny, T. P., Firrao, G., Fischer Le, S. M., Saddler, G. S., et al. (2012). Comprehensive list of names of plant pathogenic bacteria, 2008-2010. *Journal of Plant Pathology*, *94*(1), 21-27.

Butcher, D. J. (2009). Phytoremediation of lead in soil: Recent applications and future prospects. *Applied Spectroscopy Reviews*, *44*, 123-139.

Champomier-Verges, M. C., Stintzi, A., & Meyer, J. M. (1996). Acquisition of iron by the non-siderophore producing *Pseudomonas fragi*. *Microbiology, 142* (5), 1191-1199.

Chehregani, A., & Malayeri, B. E. (2007). Removal of heavy metals by native accumulator plants. *International Journal of Agriculture and Biology*, *9*, 462-465.

Chen, L., Luo, S., Xiao, X., Guo, H., Chen, J., Wan, Y., et al. (2010). Application of plant growth-promoting endophytes (PGPE) isolated from *Solanum nigrum* L. for phytoextraction of Cd-polluted soils. *Applied Soil Ecology, 46*(3), 383-389.

Chen, W. M., Tang, Y. Q., Mori, K., & Wu, X. L. (2012). Distribution of culturable endophytic bacteria in aquatic plants and their potential for bioremediation in polluted waters. *Aquatic Biology,* 15, 99-110.

Chen, L., Luo, S. L., Li, X. J., Wan, Y., Chen, J. L., Liu, C. B. (2014). Interaction of Cdhyperaccumulator *Solanum nigrum* L. and functional endophyte *Pseudomonas* sp. Lk9 on soil heavy metals uptake. *Soil Biology and Biochemistry*, *68*, 300-308.

Cheng, S. (2003). Effects of heavy metals on plants and resistance mechanisms. *Environmental Science and Pollution Research, 10*(4), 256-264.

Cheraghi, M., Lorestani, B., & Yousefi, N. (2011). Introduction of hyperaccumulator plants with phytoremediation potential of a lead- zinc mine in Iran. *International Journal of Environmental, Chemical, Ecological, Geological and Geophysical Engineering*, *5*(5), 163-168.

Cherian, S., Weyens, N., Lindberg, S., & Vangronsveld, J. (2012). Phytoremediation of trace element–contaminated environments and the potential of endophytic bacteria for improving this process. *Critical Reviews in Environmental Science and Technology*, *42*(21), 2215-2260.

Clemens, S., Palmgren, M. G., & Krämer, U. (2002). A long way ahead: understanding and engineering plant metal accumulation. *TRENDS in Plant Science*, *7*(7), 309-315.

Compant, S., Clément, C., & Sessitsch, A. (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. *Soil Biology and Biochemistry*, *42*, 669-678.

Doty, S. L. (2008). Enhancing phytoremediation through the use of transgenics and endophytes. *New Phytologist*, *179*, 318-33.

Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K. H., & Stackebrandt, E. (Eds.). (2006). *The prokaryotes*. New York: Springer-Verlag.

Edi Premono, M., Moawad, A. M., & Vlek, P. L. G. (1996). Effect of phosphate-solubilizing Pseudomonas putida on the growth of maize and its survival in the rhizosphere. *Indonesian journal of crop science*, *11*, 13-23.

Enamorado-Báez, S. M., Abril, J. M., & Gómez-Guzmán, J. M. (2013). Determination of 25 trace element concentrations in biological reference materials by ICP-MS following different microwave-assisted acid digestion methods based on scaling masses of digested samples. *ISRN Analytical Chemistry, 2013*, 1-14. Retrieved November 3, 2014, from https://www.hindawi.com/archive/2013/851713/

Fahr, M., Laplaze, L., Bendaou, N., Hocher, V., Mzibri, M. E., Bogusz, D., & Smouni, A. (2013). Effect of lead on root growth. *Frontiers in Plant Science*, 4, 1-7. Retrieved August 3, 2014, from https://www.frontiersin.org/articles/10.3389/fpls.2013.00175/full

Fässler, E., Evangelou, M. W., Robinson, B. H., & Schulin, R. (2010). Effects of indole-3-acetic acid (IAA) on sunflower growth and heavy metal uptake in combination with ethylene diamine disuccinic acid (EDDS). *Chemosphere*, 80, 901-907.

Favas, P. J. C., Pratas, J., Varun, M., D'Souza, R., & Paul, M. S. (2014). Phytoremediation of soils contaminated with metals and metalloids at mining areas: potential of native flora. *Environmental Risk Assessment of Soil Contamination INTECH, 17*, 485-517. Retrieved December 3, 2016, from https://www.intechopen.com/books/environmental-risk-assessment-of-soil-contamination

Francesconi, K., Visoottiviseth, P., Sridokchan, W., & Goessler, W. (2002). Arsenic species in an arsenic hyperaccumulating fern, *Pityrogramma calomelanos*: a potential phytoremediator of arsenic-contaminated soils. *Science of the Total Environment*, *284*, 27-35.

Furini, A. (Ed.). (2012). Plants and Heavy Metals, Netherlands: Springer.

Gao, Z., Han, J., Mu, C., Lin, J., Li, X., Lin, L., & Sun, S. (2014). Effects of saline and alkaline stresses on growth and physiological changes in oat (*Avena sativa* L.) seedlings. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, *42*(2), 357-362.

Gerhardt, K. E., Gerwing, P. D., & Greenberg, B. M. (2017). Opinion: Taking phytoremediation from proven technology to accepted practice, *Plant Science*, *256*, 170-185.

Ghosh, M., & Singh, S. P. (2005). A review on phytoremediation of heavy metals and utilization of it's by products. *Applied Ecology and Environmental Research*, *3*, 1-18.

Gill, M. (2014). Heavy metal stress in plants: a review. *International Journal of Advanced Research*, *2*(6), 1043-1055.

Glick, B. R. (2010). Using soil bacteria to facilitate phytoremediation. *Biotechnology Advances*, *28*, 367-374.

Gomes, M. A., Hauser-Davis, R. A., de Souza, A. N., & Vitória, A. P. (2016). Metal phytoremediation: General strategies, genetically modified plants and applications in metal nanoparticle contamination. *Ecotoxicology and Environmental Safety, 134*, 133-147.

González-Oreja, J. A., Rozas, M. A., Alkorta, I., & Garbisu, C. (2008). Dendroremediation of Heavy Metal Polluted Soils. *Reviews on Environmental Health*, *23*(3), 1-13.

Hallmann, J., Quadt-Hallmann, A., Mahaffee, W. F., & Kloepper, J. W. (1997). Bacterial endophytes in agricultural crops. *Canadian Journal of Microbiology*, 43, 895-914.

Handayanto, E., Muddarisna, N., & Krisnayanti, B. D. (2014). The potential of local trees for phytostabilization of heavy metals in gold cyanidation tailing contaminated soils of west Lombok, Indonesia. *American-Eurasian Journal of Sustainable Agriculture, 8*(7), 15-21.

Harris, J. M. (2015). Abscisic Acid: Hidden Architect of Root System Structure. *Plants, 4*, 548-572.

He, L. Y., Chen, Z. J., Ren, G. D., Zhang, Y. F., Qian, M., & Sheng, X. F. (2009). Increased cadmium and lead uptake of a cadmium hyperaccumulator tomato by cadmium-resistant bacteria. *Ecotoxicology and Environmental Safety*, *72*, 1343-1348.

He, H. D., Ye, Z. H., Yang, D. J., Yan, J. L., Xiao, L., Zhong, T., et al. (2013). Characterization of endophytic *Rahnella* sp. JN6 from *Polygonum pubescens* and its potential in promoting growth and Cd, Pb, Zn uptake by *Brassica napus*. *Chemosphere*, *90*, 1960-1965.

Henning, J. A., Weston, D. J., Pelletier, D. A., Timm, C. M., Jawdy, S. S., & Classen A.T. (2016). Root bacterial endophytes alter plant phenotype, but not physiology. *PeerJ, 4*, 1-20. Retrieved April 3, 2017, from https://peerj.com/articles/2606/

Ho, W. M., Ang, L. H., & Lee, D. K. (2008). Assessment of Pb uptake, translocation and immobilization in kenaf (*Hibiscus cannabinus* L.) for phytoremediation of sand tailings. *Journal of Environmental Sciences*, *20*, 1341-1347.

Hunt, A. J, Anderson, C. W. N., Bruce, N., García, A. M., Graedel, T. E., Hodson, M., et al. (2014). Phytoextraction as a tool for green chemistry. *Green Processing and Synthesis*, 3, 3-22.

Jackson, M. L. (1958). *Soil Chemical Analysis*. New Jersey: Prestige-Hall, Inc.

Jarosławiecka, A., & Piotrowska-Seget, Z. (2014). Lead resistance in micro-organisms. *Microbiology,* 160, 12-25.

Jebara, S. H., Saadani, O., Fatnassi, I. C., Chiboub, M., Abdelkrim, S., & Jebara, M. (2015). Inoculation of *Lens culinaris* with Pb-resistant bacteria shows

potential for phytostabilization. *Environmental Science and Pollution Research*, *22*, 2537-2545.

Jeger, M. J., & Spence, N. J. (Eds.). (2001) Biotic interactions in plant-pathogen associations. United Kingdom: Cabi publishing.

Jeong, S., Moon, H. S., Nama, K., Kim, J. Y., & Kim, T. S. (2012). Application of phosphate-solubilizing bacteria for enhancing bioavailability and phytoextraction of cadmium (Cd) from polluted soil. *Chemosphere, 88*, 204-210.

Jha, P. N., Gupta, G., Jha, P., & Mehrotra, R. (2013). Association of rhizospheric/endophytic bacteria with plants: A potential gateway to sustainable agriculture. *Greener Journal of Agricultural Sciences*, *3*(2), 73-84.

JÍdrzejczyk-KoryciÒska, M. (2006). Floristic diversity in calamine areas of the Silesia-Cracow Monocline. *Biodiversity: Research and Conservation*, *3-4*, 340-343.

Kabata-Pendias, A. (2011). Trace Elements in Soils and Plants. Florida: CRC Press.

Kangwankraiphaisan, T., & Suntornvongsagul, K. (2013). Cu accumulation in the rhizosphere of *Lindenbergia philippensis* (Cham.) Benth. growing in the contaminated sediment. *Journal of Applied Sciences*, *13*, 743-748.

Karami, A., & Shamsuddin, Z. H. (2010). Phytoremediation of heavy metals with several efficiency enhancer methods. *African Journal of Biotechnology*, *9*(25), 3689-3698.

Karimi, L. N., Khanahmadi, M., & Moradi, B. (2012). Accumulation and phytotoxicity of lead in *Cynara scolymus*. *Indian Journal of Science and Technology*, 5, 3634-3641.

Karpagam, T., & Nagalakshmi, P. K. (2014). Isolation and characterization of phosphate solubilizing microbes from agricultural soil. *International Journal of Current Microbiollogy and Applied Science*, *3*(3), 601-614.

Khan, Z., & Doty, S. (2011). Endophyte-assisted phytoremediation. *Current Topics in Plant Biology*, *12*, 97-195.

Khan, S., Afzal, M., Iqbal, S., & Khan, Q. M. (2013). Plant-bacteria partnerships for the remediation of hydrocarbon contaminated soils. *Chemosphere*, *90*, 1317-1332.

Khaokaew, S., & Landrot, G. (2015). A field-scale study of cadmium phytoremediation in a contaminated agricultural soil at Mae Sot District, Tak Province, Thailand: (1) Determination of Cd-hyperaccumulating plants. *Chemosphere*, *138*, 883-887.

Kim, I. S., Kang, K. H., Johnson-Green, P., & Lee, E. J. (2003). Investigation of heavy metal accumulation in *Polygonum thunbergii* for phytoextraction. *Environmental Pollution*, *126*, 235-243.

King, E. D., Ward, M. K., & Raney, D. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescin. *Journal of Laboratory and Clinical Medicine*, *44*, 301-307.

Kramer, U. (2010). Metal hyperaccumulation in plants. *Annual Review of Plant Biology*, *61*, 517-534.

Laghlimi, M., Baghdad, B., Hadi, H. E., & Bouabdli, A. (2015). Phytoremediation mechanisms of heavy metal contaminated soils: A review. *Open Journal of Ecology*, 5, 375-388.

Li, H. Y., Wei, D. Q., Shen, M., & Zhou, Z. P. (2012). Endophytes and their role in phytoremediation. *Fungal Diversity, 54*(1), 11-18.

Liang, X., He, C. Q., Ni, G., Tang, G. E, Chen, X. P., & Lei, Y. R. (2014). Growth and Cd accumulation of *Orychophragmus violaceus* as affected by inoculation of Cd-tolerant bacterial strains. *Pedosphere, 24*(3), 322-329.

Lindsay, W. L., & Norvell, W. A. (1978). Development of a DTPA test for zinc, iron, manganese, and copper. *Soil Science Society of America Journal*, *42*, 421-428.

Lisle, L., Gaudron, J., & Lefroy, R. (1993). *Laboratory Techniques for Plant and Soil Analysis*. Canberra: Australian Center for International Agricultural Research.

Liu, D., Li, S., Islam, E., Chen, J. R., Wu, J. S., Ye, Z. Q., et al. (2015). Lead accumulation and tolerance of Moso bamboo (*Phyllostachys pubescens*)

seedlings: applications of phytoremediation. *Journal of Zhejiang University SCIENCE B - Biomedicine and Biotechnology, 16*, 123-130.

Lodewyckx, C., Vangronsveld, J., Porteous, F., Moore, E. R. B., Taghavi, S., Mezgeay, M., & der Lelie, D. V. (2002). Endophytic bacteria and their potential applications. *Critical Reviews in Plant Sciences*, *21*, 583-606.

Luo, S. L., Chen, L., Chen, J. L., Xiao, X., Xu, T. Y., Wan, Y., et al. (2011). Analysis and characterization of cultivable heavy metal-resistant bacterial endophytes isolated from Cd-hyperaccumulator *Solanum nigrum* L. and their potential use for phytoremediation. *Chemosphere*, *85*, 1130-1138.

Luo, Z. B., He, J., Polle, A., & Rennenberg, H. (2016). Heavy metal accumulation and signal transduction in herbaceous and woody plants: Paving the way for enhancing phytoremediation efficiency. *Biotechnology Advances, 34*(6), 1131-1148.

Ma, Y., Rajkumar, M., Luo, Y. M., & Freitas, H. (2011a). Inoculation of endophytic bacteria on host and non-host plants effects on plant growth and Ni uptake. *Journal of Hazardous Materials*, *196*, 230-237.

Ma, Y., Rajkumar, M., Vicente, J. A. F., & Freitas, H. (2011b). Inoculation of Ni-resistant plant growth promoting bacterium *Psychrobacter sp*. strain SRS8 for the improvement of nickel phytoextraction by energy crops. *International Journal of Phytoremediation*, *13*(2), 126-139.

Ma, Y., Rajkumar, M., Rocha, I., Oliveira, R. S., & Freitas, H. (2015). Serpentine bacteria influence metal translocation and bioconcentration of *Brassica juncea* and *Ricinus communis* grown in multi-metal polluted soils. *Frontiers in Plant Science, Plant Biotechnology, 5*(757), 1-13. Retrieved January 3, 2017, from

https://www.frontiersin.org/articles/10.3389/fpls.2014.00757/full

Ma, Y., Rajkumar, M., Zhang, C., & Freitas, H. (2016). Beneficial role of bacterial endophytes in heavy metal phytoremediation. *Journal of Environmental Management*, *174*, 14-25.

Mackova, M., Dowling, D., & Macek, T. (Eds.). (2006). Phytoremediation Rhizoremediation, Dordrecht: Springer.

Madhaiyan, M., Poonguzhali, S., & Sa, T. (2007). Metal tolerating methylotrophic bacteria reduces nickel and cadmium toxicity and promotes plant growth of tomato (*Lycopersicon esculentum* L.) *Chemosphere*, *69*, 220-228.

Mahar, A., Wang, P., Ali, A., Awasthi, M. K., Lahori, A. H., Wang, Q., et al. (2016). Challenges and opportunities in the phytoremediation of heavy metals contaminated soils: A review. *Ecotoxicology and Environmental Safety, 126*, 111-121.

Mahmood, T. (2010). Phytoextraction of heavy metals-the process and scope for remediation of contaminated soils. *Soil and Environment*, *29*(2), 91-109.

Malá, J., Cvrčková, H., Máchová, P., Dostál, J., & Šíma, P. (2010). Heavy metal accumulation by willow clones in short-time hydroponics. *Journal of Forensic Sciences*, *56*, 28-34.

Małachowska-Jutsz, A., & Gnida, A. (2015). Mechanisms of stress avoidance and tolerance by plants used in phytoremediation of heavy metals. *Archives of Environmental Protection*, *41*(4), 104-114.

Malar, S., Manikandan, R., Favas, P. J. C., Sahi, S. V., & Venkatachalam, P. (2014). Effect of lead on phytotoxicity, growth, biochemical alterations and its role on genomic template stability in *Sesbania grandiflora*: A potential plant for phytoremediation. *Ecotoxicology and Environmental Safety*, *108*, 249-257.

Malik, R. N., Husain, S. Z., & Nazir, I. (2010). Heavy metal contamination and accumulation in soil and wild plant species from industrial area of Islamabad, Pakistan. *Pakistan Journal of Botany*, *42*, 291-301.

Marcos, F. C. C., Iório, R. P. F., Silveira, A. P. D., Ribeiro, R. V., Machado, E. C., & Lagôa, A.M.M.A. (2016). Endophytic bacteria affect sugarcane physiology without changing plant growth. *Bragantia*, *75*(1), 1-9. Retrieved May 3, 2017, from http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0006-8705 201 6000100001

Meeinkuirt, W., Pokethitiyook, P., Kruatrachue, M., Tanhan, P., & Chaiyarat, R., (2012). Phytostabilization of a Pb-contaminated mine tailing by various tree species in pot and field trial experiments. *International Journal of Phytoremediation, 14*, 925-938.

Meeinkuirt, W., Kruatrachue, M., Tanhan, P., Chaiyarat, R., & Pokethitiyook, P. (2013). Phytostabilization potential of Pb mine tailings by two grass species, *Thysanolaena maxima* and *Vetiveria zizanioides*. *Water Air Soil Pollution*, *224*, 1750-1757.

Memon, A. R., Aktoprakligil, D., Ozdemir, A., & Vertii, A. (2001). Heavy metal accumulation and detoxification mechanisms in plants. *Turkish Journal of Botany*, *25*, 111-121.

Mendez, M. O., & Maier, R. M. (2008). Phytostabilization of mine tailings in arid and semiarid environments - An emerging remediation technology. *Environmental Health Perspectives*, *116*(3), 278-283.

Mganga, N., Manoko, M. L. K., & Rulangaranga, Z. K. (2011). Classification of plants according to their heavy metal content around north mara gold mine, tanzania: implication for phytoremediation. *Tanzania Journal of Science*, *37*, 109-119.

Mohanty, M. (2016). Post-harvest management of phytoremediation technology. *Journal of Environmental and Analytical Toxicology, 6*(398), 1-8. Retrieved January 18, 2017, from https://www.omicsonline.org/open-access/postharvest-management-of-phytoremediation-technology-2161-0525-1000398.php?aid=80025

Nagajyoti, P. C., Lee, K. D., Sreekanth, T. V. M. (2010). Heavy metals, occurrence and toxicity for plants: A review. *Environmental Chemistry Letters*, *8*(3), 199-216.

Nalik, M. M., & Dubey, S. K. (2013). Lead resistant bacteria: Lead resistance mechanisms, their applications in lead bioremediation and biomonitoring. *Ecotoxicology and Environmental Safety, 98*, 1-7.

Nautiyal, C. S. (1999). An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiology Letters*, 170, 265-270.

Navarro-Torre, S., Mateos-Naranjo, E., Caviedes, M. A., Pajuelo, E., & Rodriguez-Llorente, I. D. (2016). Isolation of plant-growth promoting and metal resistant cultivable bacteria from *Arthrocnemum macrostachyum* in the

Odiel marshes with potential use in phytoremediation. *Marine Pollution Bulletin*, *110*, 133-142.

Ngamau, C. N., Matiru, V. N., Tani, A., & Muthuri, C. W. (2012). Isolation and identification of endophytic bacteria of bananas (*Musa spp.*) in Kenya and their potential as biofertilizers for sustainable banana production. *African Journal of Microbiology Research,* 6(34):6414-6422.

Ngorwe, E. N., Nyambaka, H., Murungi, J., & Ongera, T. (2014). Effects of physico-chemical properties on uptake of lead and cadmium in tobacco grown in medially polluted soils. *The International Journal of Science and Technoledge*, *2*(8), 146-153.

Nicolaus, B., Poli, A., Donato, P. D., Romano, I., Laezza, G., Gioiello, A., et al., (2016). Pb^{2+} effects on growth, lipids, and protein and DNA profiles of the Thermophilic Bacterium *Thermus Thermophilus*. *Microorganisms, 4*(45), 1-14. Retrieved December 8, 2016, from

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5192528/

Nirola, R., Megharaj, M., Palanisami, T., Aryal, R., Venkateswarlu, K., & Naidu, R., (2015). Evaluation of metal uptake factors of native trees colonizing an abandoned copper mine-a quest for phytostabilization. *Journal of Sustainable Mining*, *14*, 115-123.

Niu, Z. X., Sun, L. N., Sun, T. H., Li, Y. S., & Wong, H. (2007). Evaluation of phytoextracting cadmium and lead by sunflower, ricinus, alfalfa and mustard in hydroponic culture. *Journal of Environmental Sciences (China)*, *19*, 961-967.

Pandey, V. C., Bajpai, O., & Singh, N. (2016). Energy crops in sustainable phytoremediation. *Renewable & Sustainable Energy Reviews*, *54*, 58-73.

Park, J. H., Bolan, N., Megharaj, M., Naidu, R., & Chung, J. W. (2011). Bacterial-assisted immobilization of lead in soils: Implications for remediation. *Pedologist, 54*, 162-174.

Peverill, K. I., Sparrow, L. A., & Reuter, D. J. (1999). *Soil Analysis: An Interpretation Manual*. Victoria: ASPAC, CSIRO Publishing.

Pierzynski, G. M., Schnoor, J. L., Youngman, A., Licht, L., & Erickson, L. E. (2002). Poplar trees for phytostabilization of abandoned zinc-lead smelter. *Practice*

Periodical of Hazardous, Toxic, and Radioactive Waste Management, *6*,
177-183.

Pollard, A. J., Reeves, R. D., & Baker, A. J. M. (2014). Facultative
hyperaccumulation of heavy metals and metalloids. *Plant Science, 217-218*,
8-17.

Pothisat, S. (Ed.). (2000). Ore Lead - Zinc in Kanchanaburi province, Bangkok:
Department of Mineral Resources.

Pourrut, B., Shahid, M., Dumat, C., & Winterton, P. (2011). Lead uptake, toxicity,
and detoxification in plants. *Reviews of Environmental Contamination and
Toxicology*, *213*, 113-136.

Prasad, M. N. V., & Hagemeyer, J. (Eds.). (1999). *Heavy metal stress in plants*.
Berlin: Springer-Verlag Berlin Heidelberg.

Prasad, M. N. V., & Freitas, H. M. (2003). Metal hyperaccumulation in plants-
biodiversity prospecting for phytoremediation technology. *Electronic
Journal of Biotechnology, 6*(3), 285-321.

Prasad, M. N. V. (2004). Phytoremediation of metals in the environment for
sustainable development. *Proceedings of the Indian National Science
Academy - Part B: Biological Sciences*, *70*, 71-98.

Pulford, I. D., & Watson, C. (2003). Phytoremediation of heavy metal-contaminated
land by trees-a review. *Environment International*, *29*, 529-540.

Rajkumar, M., Ae, N., & Freitas, H. (2009). Endophytic bacteria and their potential to
enhance heavy metal phytoextraction. *Chemosphere*, *77*, 153-60.

Rajkumar, M., Ae, N., Prasad, M. N. V., & Freitas, H. (2010). Potential of
siderophore-producing bacteria for improving heavy metal phytoextraction.
Trends in Biotechnology, 28(3), 142-149.

Rajkumar, M., Sandhya, S., Prasad, M. N. V., & Freitas, H. (2012). Perspectives of
plant-associated microbes in heavy metal phytoremediation. *Biotechnology
Advances*, *30*, 1562-1574.

Rajkumar, M., Ma, Y., & Freitas, H. (2013). Improvement of Ni phytostabilization by
inoculation of Ni resistant *Bacillus megaterium* SR28C. *Journal of
Environmental Management*, *128*, 973-980.

Ramı́rez-Bahena, M. H., Cuesta, M. J., Tejedor, C., Igual, J. M., Ferna´ndez-Pascual, M., & Peix, A´. (2015). *Pseudomonas* endophytica sp. nov., isolated from stem tissue of *Solanum tuberosum* L. in Spain. *International Journal of Systematic and Evolutionary Microbiology*, *65*, 2110-2117.

Rascio, N., & Navari-Izzo, F. (2011). Heavy metal hyperaccumulating plants: How and why do they do it? And what makes them so interesting? *Plant Science, 180*(2), 169-181.

Ribeiro de Souza, S. C., López de Andrade, S. A., Anjos de Souza, L., & Schiavinato. M. A. (2012). Lead tolerance and phytoremediation potential of Brazilian leguminous tree species at the seedling stage. *Journal of Environmental Management*, *110*, 299-307.

Rieuwerts, J. S., Thornton, I. Farago, M. E., & Ashmore, M. R. (1998). Factors influencing metal bioavailability in soils: preliminary investigations for the development of a critical loads approach for metals. *Chemical Speciation & Bioavailability, 10*(2), 61-75.

Romeiro, S., Lagôa, A. M. M. A., Furlani, P. R, Abreu, C. A, Abreu, M. F., & Erismann, N. M. (2006). Lead uptake and tolerance of *Ricinus communis* L. of *Brazilian Journal of Plant Physiology*, *18*(4), 483-489.

Rosenblueth, M., & Martínez-Romero, E. (2006). Bacterial endophytes and their interactions with hosts. *Molecular Plant-Microbe Interactions, 19*(8), 827-837.

Rotkittikhun, P., Kruatrachue, M., Chaiyarat, R., Ngernsansaruay, C., Pokethitiyook, P., Paijitprapaporn, A., & Baker, A. J. (2006). Uptake and accumulation of lead by plants from the Bo Ngam lead mine area in Thailand. *Environmental Pollution*, *144*, 681-688.

Ryan, R. P., Germaine, K., Franks, A., Ryan, D. J., & Dowling, D. N. (2008). Bacterial endophytes: recent developments and applications. *FEMS Microbiology Letters*, *278*, 1-9. Retrieved August 8, 2014, from https://academic.oup.com/femsle/article/278/1/1/515835

Sahi, S. V., Bryant, N. L., Sharma, N. C., & Singh, S. R. (2002). Characterization of a lead hyperaccumulator shrub, *Sesbania drummondii*. *Environmental Science and Technology, 36*, 4676-4680.

Saifullah, Meers, E., Qadir, M., de Caritat, P., Tack, F. M. G., Du Laing, G., & Zia, M. H. (2009). EDTA-assisted Pb phytoextraction. *Chemosphere, 74*, 1279-1291.

Salazar, M. J., & Pignata, M. L. (2014). Lead accumulation in plants grown in polluted soils. Screening of native species for phytoremediation. *Journal of Geochemical Exploration, 137*, 29-36.

Sani, R.K., Rastogi, G., Moberly, J. G., Dohnalkova, A., Ginn, T. R., Spycher, N., et al. (2010). The toxicity of lead to *Desulfovibrio desulfuricans* G20 in the presence of goethite and quartz. *Journal of Basic Microbiology, 50*, 160-170.

Santoyo, G., Moreno-Hagelsieb, G., Orozco-Mosqueda, M. C., & Glick, B. R. (2016). Plant growth promoting bacterial endophytes. *Microbiological Research*, *183*, 92-99.

Sarma, H. (2011). Metal hyperaccumulation in plants: A review focusing on phytoremediation technology. J*ournal of Environmental Science and Technology, 4*, 118-138.

Sarwar, N., Imran, M., Shaheen, M. R., Ishaque, W., Kamran, M. A., & Matloob, A. (2017). Phytoremediation strategies for soils contaminated with heavy metals: Modifications and future perspectives. *Chemosphere*, *171*, 710-721.

Schaechter, A. (Ed.). (2003). *The desk encyclopedia of microbiology*. China: Elsevier.

Schwyn, B., & Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry*, *160*, 47-56.

Searle, L. J., Méric, G., Porcelli, I., Sheppard, S. K., & Lucchini, S. (2015). Variation in siderophore biosynthetic gene distribution and production across environmental and faecal populations of *Escherichia coli*. *PLoS ONE*, *10*(3), e0117906. Retrieved June 8, 2015, from http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0117906

Segura, A., Rodríguez-Conde, S., Ramos, C., & Ramos, J. L. (2009). Bacterial responses and interactions with plants during rhizoremediation. *Microbial Biotechnology, 2*(4), 452-464.

Selamat, S. N., Abdullah, S. R., & Idris, M. (2014). Phytoremediation of lead (Pb) and arsenic (As) by *Melastoma malabathricum* L. from contaminated soil

in separate exposure. *International Journal of Phytoremediation*, *16*, 694-703.

Sessitsch, A., Kuffner, M., Kidd, P., Vangronsveld, J., Wenzel, W. W., Fallmann, K., & Puschenreiter, M. (2013). The role of plant-associated bacteria in the mobilization and phytoextraction of trace elements in contaminated soils. *Soil Biology and Biochemistry*, *60*, 182-194.

Sgroy, V., Cassán, F., Masciarelli, O., Florencia Del Papa, M., Lagares, A., & Luna, V. (2009). Isolation and characterization of endophytic plant growth-promoting (PGPB) or stress homeostasis-regulating (PSHB) bacteria associated to the halophyte *Prosopis strombulifera*. *Applied Microbiology and Biotechnology, 85*(2), 371-381.

Sharma, P., & Dubey, R. S. (2005). Pb toxicity in plants. *Brazilian Journal of Plant Physiology*, *17*, 35-52.

Shaw, A. J. (Ed.). (1990). Heavy metal tolerance in plants: Evolutionary aspects. Florida: CRC Press Inc.

Sheng, X. F., Xia, J. J., Jiang, C. Y., He, L. Y., & Qian, M. (2008). Characterization of heavy metal-resistant endophytic bacteria from rape (*Brassica napus*) roots and their potential in promoting the growth and lead accumulation of rape. *Environmental Pollution*, *156*, 1164-1170.

Sheoran, V. A. S. Sheoran, A. S., & Poonia, P. (2013). Phytomining of gold: A review. *Journal of Geochemical Exploration*, *128*, 42-50.

Sherene, T. (2010). Mobility and transport of heavy metals in polluted soil environment. *Biological Forum-An International Journal*, *2*(2), 112-121.

Shi, X., Zhang, X., Chen, G., Chen, Y., Wang, L., & Shan, X. (2011). Seedling growth and metal accumulation of selected woody species in copper and lead/zinc mine tailings. *Journal of Environmental Sciences*, *23*(2), 266-274.

Shin, M. N. , Shim, J., You, Y., Myung, H., Bang, K. S., Cho, M., et al. (2012). Characterization of lead resistant endophytic *Bacillus sp*. MN3-4 and its potential for promoting lead accumulation in metal hyperaccumulator *Alnus firma*. *Journal of Hazardous Materials*, *199-200*, 314-320.

Shu, W. S., Ye, Z. H., Lan, C. Y., Zhang, Z. Q., & Wong, M. H. (2001). Acidification of lead/zinc mine tailings and its effect on heavy metal mobility. *Environment International*, *26*, 389-394.

Simmons, R. W. (2004). Review of the current contamination status of Potentially Toxic Elements (PTEs) in the Greater Mekong sub-region. Retrieved December 3, 2016, from http://publications.iwmi.org/pdf/H038811.pdf

Soongsombat, P., Kruatrachue, M., Chaiyarat, R., Pokethitiyook, P., & Ngernsansaruay, C. (2009). Lead tolerance and accumulation in *Pteris vittata* and *Pityrogramma calomelanos*, and their potential for phytoremediation of lead-contaminated soil. *International Journal of Phytoremediation*, *11*(4), 396-412.

Srivastava, S., & Paul A. K. (2016). Associated microflora of medicinal ferns: biotechnological potentials and possible applications. *International Journal of Bioassay*, *5*(3), 4927-4943.

Stępniewska, Z., & A. Kuźniar, A. (2013). Endophytic microorganisms-promising applications in bioremediation of greenhouse gases. *Applied Microbiology and Biotechnology, 97*, 9589-9596.

Stout, L., & Nu¨sslein, K. (2010). Biotechnological potential of aquatic plant-microbe interactions. *Current Opinion in Biotechnology, 21*, 339-345.

Su, C., Jiang L. Q., & Zhang, W. J. (2014). A review on heavy metal contamination in the soil worldwide: Situation, impact and remediation techniques. *Environmental Skeptics and Critics, 3*(2), 24-38.

Subhashini, V., Swamy, A. V. V. S., & Krishna, R. H. (2013). Pot experiment: To study the uptake of zinc by different plant species in artificially contaminated soil. *World Journal of Environmental Engineering, 1*(2), 27-33.

Sun, Y. B., Zhou, Q. X., Wang, L., Liu, W. T., & Liu, R. (2009). Characteristics of cadmium tolerance and bioaccumulation of *Bidens pilosa* L. seedlings. *Huan Jing Ke Xue, 30*(10), 3028-3035.

Sun, L. N., Zhang, Y. F., He, L.Y., Chen, Z. J., Wang, Q. Y., Qian, M., & Sheng, X. F. (2010). Genetic diversity and characterization of heavy metal-resistant-

endophytic bacteria from two copper-tolerant plant species on copper mine wasteland, *Bioresource Technol. 101*, 501-509.

Sura-de Jong, M., Reynolds, R. J. B., Richterova, K., Musilova, L., Staicu, L. C., Chocholata, I., et al. (2015). Selenium hyperaccumulators harbor a diverse endophytic bacterial community characterized by high selenium resistance and plant growth promoting properties. *Frontiers in Plant Science, 6*, 1-17. Retrieved August 8, 2016, from

https://www.frontiersin.org/articles/10.3389/fpls.2015.00113/full

Surapat, W., Pukahuta, C., Rattanachaikunsopon, P., Aimi, T., & Boonlue, S. (2013). Phosphate-solubilizing bacteria isolated from agricultural chili soil and their efficiency on the growth of chili (*Capsicum frutescens* L. cv. Hua Rua). *Chiang Mai Journal of Science*, *40*(1), 11-25.

Sylvain, B., Mikael, M. H., Florie, M., Emmanuel, J., Marilyne, S., Sylvain, B., & Domenico M. (2016). Phytostabilization of As, Sb and Pb by two willow species (*S. viminalis* and *S. purpurea*) on former mine technosols. *Catena, 136*, 44-52.

Tale, S., & Ingole, S. (2015). A review on role of physico-chemical properties in soil quality. *Chemical Science Review and Letters*, *4*(13), 57-66.

Tamura K., Stecher G., Peterson D., Filipski A., & Kumar S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution, 30*, 2725-2729.

Tangahu, B. V., Abdullah, S. R. S., Basri, H., Idris, M., Anuar, N., & Mukhlisin, M. (2011). A review on heavy metals (As, Pb, and Hg) uptake by plants through phytoremediation. *International Journal of Chemical Engineering, 2011*, 1-31. Retrieved May 18, 2014, from

https://www.hindawi.com/journals/ijce/2011/939161

Tanhan, P., Kruatrachue, M., Pokethitiyook, P., & Chaiyarat, R. (2007). Uptake and accumulation of cadmium, lead and zinc by Siam weed [*Chromolaena odorata* (L.) King & Robinson]. *Chemosphere, 68*, 323-329.

Tchounwou, P. B., Yedjou, C. G., Patlolla, A. K., & Sutton, D. J. (2012). Heavy metals toxicity and the environment. *Experientia Supplementum*, *101*(133–164), 1-30. Retrieved May 18, 2016, from

159

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4144270/

Thangavel, P., & Sridevi, G., (Eds.). (2015). *Environmental Sustainability*. India: Springer.

Thongsri, T., Visoottiviseth, P., & Kosiyachinda, P. (2010). Accumulation and distribution of heavy metals into Thai Jasmine rice in Mae Sod paddy field, Thailand. Retrieved December 18, 2016, from http://www.iaea.org/inis/collection/NCLCollectionStore/_Public/41/131/41 131186.pdf

Ullah, A., Heng, S., Munis, M. F. H., Fahad, S., & Yang, X. (2015). Phytoremediation of heavy metals assisted by plant growth promoting (PGP) bacteria: A review. *Environmental and Experimental Botany, 117*, 28-40.

United State Environmental Protection Agency. (2007). Microwave assisted acid digestion of sediments, sludges, soils and oils. (Method 3051A). Retrieved April 15, 2014, from https://www.epa.gov/sites/production/files/2015-12/documents/3051a.pdf

Vamerali, T., Bandiera, M., & Mosca, G. (2010). Field crops for phytoremediation of metal-contaminated land: A review. *Environmental Chemistry Letters, 8*, 1-17.

van der Ent, A., Baker, A. J. M., Reeves, R. D., Pollard, A. J., & Schat, H. (2013). Hyperaccumulators of metal and metalloid trace elements: Facts and fiction. *Plant Soil*, *362*, 319-334.

Verma V., & Gange A. (Eds.). (2014). Advances in endophytic research. New Delhi: Springer.

Walkley, A., & Black, I. A. (1947). Chronic acid titration method for determination of soil organic matter. *Soil Sci. Amer. Proc*. 63, 257.

Wang, S., Shi, X., Sun, H., Chen, Y., Pan, H., Yang, X., & Rafiq, T. (2014) Variations in metal tolerance and accumulation in three hydroponically cultivated varieties of *Salix integra* treated with lead. *PLoS ONE*, *9*(9), 1-11. Retrieved June 15, 2015, from http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0108568

Waranusantigul, P., Kruatrachue, M., Pokethitiyook, P., & Auesukaree, C. (2008). Evaluation of Pb phytoremediation potential in *Buddleja asiatica* and *B. paniculata*. *Water, Air, Soil Pollution, 193*, 79-90.

Weyens, N., Daniel van der Lelie, Taghavi, S., & Vangronsveld, J. (2009). Phytoremediation: plant-endophyte partnerships take the challenge. *Current Opinion in Biotechnology*, *20*, 248-254.

Whiting, S. N., Reeves, R. D., Richards, D., Johnson, M. S., Cooke, J. A., Malaisse, F., et al. (2004). Research priorities for conservation of metallophyte biodiversity and their potential for restoration and site remediation. *Restoration Ecology, 12*, 106-116.

Wu, C., Liao, B., Wang, S. L., Zhang, J., & Li, J. T. (2010). Pb and Zn accumulation in a Cd-hyperaccumulator (*Viola Baoshanensis*). *International Journal of Phytoremediation*, *12*(6), 574-585.

Wuana, R. A., & Okieimen, F. E. (2011). Heavy metals in contaminated soils: A review of sources, chemistry, risks and best available strategies for remediation. *ISRN Ecology*, 2011, 1-20. Retrieved December 15, 2016, from https://www.hindawi.com/archive/2011/402647/

Xu, C., Chen, X., Duan, D., Peng, C., Le, T., & Shi, J. (2015). Effect of heavy metal resistant bacteria on enhanced metal uptake and translocation of the Cu-tolerant plant, *Elsholtzia splendens*. *Environmental Science and Pollution Research, 22*, 5070-5081.

Yang, X. E., Long, X. X., Ni, W. Z., & Fu, C. X. (2002). *Sedum alfredii* H: A new Zn hyperaccumulating plant first found in China. *Chinese Science Bulletin, 47*, 1634-1637.

Yanqun, Z., Yuan, L., Schvartz, C., Langlade, L., & Fan, L. (2004). Accumulation of Pb, Cd, Cu and Zn in plants and hyperaccumulator choice in Lanping lead–zinc mine area, China. *Environment International*, *30*, 567-576.

Yanqun, Z., Yuana, L., Jianjun, C., Haiyan, C., Li, Q., & Schvartz, C. (2005). Hyperaccumulation of Pb, Zn and Cd in herbaceous grown on lead-zinc mining area in Yunnan, China. *Environment International*, *31*, 755-762.

Yao, Z., Li, J., Xie, H., & Yu, C. (2012). Review on remediation technologies of soil contaminated by heavy metals. *Procedia Environmental Sciences, 16*, 722-

729.

Yaowakhan, P., Kruatrachue, M., Pokethitiyook, P., & Soonthornsarathool, V. (2005). Removal of lead using some aquatic macrophytes. *Bulletin of Environmental Contamination and Toxicology*, *75*, 723-730.

Yoon, J., Cao, X., Zhou, Q., & Ma, L. Q. (2006). Accumulation of Pb, Cu, and Zn in native plants growing on a contaminated Florida site. *Science of the Total Environment*, *368*, 456-64.

Yumoto, I., Kusano, T., Shingyo, T., Nodasaka, Y., Matsuyama, H., & Okuyama, H. (2001). Assignment of *Pseudomonas sp.* strain E-3 to *Pseudomonas psychrophila* sp. nov., a new facultative psychrophilic bacterium. *Extremophiles, 5*, 343-349.

Zacchini, M., Pietrini, F., Mugnozza, G. S, Iori, V., Pietrosanti, L., & Massacci, A. (2009). Metal tolerance, accumulation and translocation in poplar and willow clones treated with cadmium in hydroponics. *Water Air Soil Pollution*, *197*, 23-34.

Zaidi, A., Wani, P., & Khan, M. (Eds.). (2012). *Toxicity of Heavy Metals to Legumes and Bioremediation*. Vienna: Springer.

Zárubová, P. Tlustoš, P., Vondráčková, S., Mrnka, L., Száková, J., & Pavlíková. D. (2012). Restoration of contaminated sites with the aid of fast growing trees. *European Chemical Bulletin*, *1*(9), 391-396.

Zhang, Y. F., He, L.Y., Chen, Z. J., Zhang, W. H., Wang, Q. Y., Qian, M. & Sheng, X. F. (2011). Characterization of lead-resistant and ACC deaminase-producing endophytic bacteria and their potential in promoting lead accumulation of rape. *Journal of Hazardous Materials, 186*(2-3), 1720-1725.

Zhenhua, X., Dongmei, G., Xiuli, S., & Ying, X. (2012). Review of endophyte and its use and function. *International Conference on Environmental Engineering and Technology Advances in Biomedical Engineering, 8*, 124-130.

Zhivotovsky, O. P, Kuzovkina, J. A., Schulthess, C. P., Morris, T., Pettinelli, D., & Ge, M. (2011). Hydroponic screening of willows (*Salix* L.) for lead tolerance and accumulation. *International Journal of Phytoremediation*, *13*, 75-94.

Appendices

Appendix A
Experimental plants

1. *Acacia mangium* **Willd.** (Synonym: *Abrus mangium*)

Classification	
Kingdom	Plantae
Subkingdom	Viridiplantae
Superdivision	Embryophyta
Division	Tracheophyta
Subdivision	Spermatophytina
Class	Magnoliopsida
Superorder	Rosanae
Order	Fabales
Family	Fabaceae
Genus	Acacia
Species	*Acacia mangium*

Common Names: Sabah salwood, Brown salwood, Black wattle, and Kra thin tepa. Trade name is brown salwood.

Characteristic: *A. mangium* is a fast-growing tree, dicotyledon, flowering seed plant, single-stemmed and irregular shape.

Biogeography: *A. mangium* is terrestrial (coastal forest, riverine, grassland, savannah, and scrubland) and mangrove forest plants. It prefers tropical climate zone. It is a native to Australia, Indonesia and Western Papua New Guinea. It prefers waterlogged, well-drained, saline, poor infertile, fertile loamy and acidic soils (pH 4.5 - 6.5).

Plant Morphology: The growth form is tree with medium tall (16-30 m). Foliar type is simple or unifoliate with entire margin and acute apex. Foliage composes of stipes (20-30 cm long), fronds oblong, acuminate apex, bi-pinnate and fern blade (long: 15-30 cm, wide: 10-15 cm). The colors of mature foliage are green, silver, and grey, and texture is smooth with raised veins. Trunk type is woody. Bark in mature trees is rough, hard, slightly fissured near the base, greyish-brown to dark brown, and in

164

young trees is smooth and greenish bark. Root type is underground with tap and fibrous root. *A. mangium* is bisexual flowers. Inflorescence composes of many tiny white-cream flowers in spikes. It is a polycarpic plant that flowering period is yearly towards each of rainy season. Fruit is classified as simple with brown in mature. Fruit type is dehiscent dry fruit. Seeds are black and shiny with many shapes. They are arranged longitudinally and attached to the pods by an orange to red folded funicle.

Plant propagation: *A. mangium* need full sun with moderate water and maintenance. Propagation methods are seed, leaf cutting, grafting, and tissue culture.

Application: *A. mangium* has phytoremediation potential for cleaning soil and water contaminated by Cr, Cu, Fe, Pb and Ni. It employs in soil conservation. Besides, it is used as shade tree, wind or firebreak. In Malaysia, it is a widely planted at roadside, while, it is recommended for wider use in urban forestry as ornamental tree in Thailand. Germinating seeds are vegetable food, while young shoots and leaves are browsed by cattle as fodder. Pulp is readily bleached to high brightness levels and is excellent for paper making. Wood with density (530-690 kg/m^3 at 15% moisturizer content) is used for construction, boat building, and attractive furniture and cabinets, door and window components. It has a calorific value of 4800-4900 kcal/kg for good quality charcoal. Sawdust provides a good quality substrate for the profitable production of shiitake mushrooms.

References:

Orwa, C., Mutua, A., Kindt, R., Jamnadass, R., & Anthony, S. (2009). *Acacia mangium*. Retrieved December 1, 2016, from http://worldagroforestry.org/treedb/AFTPDFS/Acacia_mangium.PDF

Nparks flora and fauna web. (2013). *Acacia mangium Willd*. Retrieved December 1, 2016, from https://florafaunaweb.nparks.gov.sg/special-pages/plant-detail.aspx?id=2695

The Integrated Taxonomic Information System report (on-line database). *Acacia mangium Willd*. Retrieved December 1, 2016, from https://www.itis.gov/servlet/SingleRpt/SingleRpt#null

2. *Azadirachta indica* A. Juss. (Synonyms: *Aglaia azadirachta*)

Classification	
Kingdom	Plantae
Subkingdom	Viridiplantae
Superdivision	Embryophyta
Division	Tracheophyta
Subdivision	Spermatophytina
Class	Magnoliopsida
Superorder	Rosanae
Order	Sapindales
Family	Meliaceae
Genus	Azadirachta
Species	*Azadirachta indica*

Common Names: Neem tree, Nim tree, Indian lilac, and Margosa tree

Characteristic: *A. indica* is a dicotyledon with flowering seed plants. It is a fast-growing tree with a single-stem. Its size is a small to medium (15-30 m tall) with a round, large crown (10-20 m diameter).

Biogeography: *A. indica* is a terrestrial growing almost anywhere in the lowland tropics. It is a native to India and the Indian subcontinent (Nepal, Pakistan, Bangladesh, and Sri Lanka). It grows in islands located in the southern part of Iran. It can grow under many soil types (dry, drought, well-drained, and fertile loamy soils). It is highly drought tolerant, but it quickly dies in waterlogged soils. It also grows on a neutral to alkaline soils with the best at pH 6.2-7.0.

Plant Morphology: Plant growth form is tree with small to medium sized. Mature bark is thick, deeply fissured and flaking with dark grey outer bark and reddish inner bark. There are scattered tubercles on the surface of the bark. Foliage is alternate, dark green leaves (20-40 cm long), and pinnately compound with 4-7 pairs of leaflets. They have short petioles (2-7 cm long) and 2 pairs of glands at the base of the leaf blade. Opposite or subopposite, glossy leaflets are lanceolate with serrate margin (7 cm long, 2.5 cm wide). Color of mature foliage is green with glossy and shiny texture. Leaves are clustered near the branch tips. Flowers are white that composes of 5-

166

petalled flowers with a light and honey-like fragrance. They are arranged in branched inflorescences known as panicles. Fruits are fleshy, pitted fruit is known as a drupe. Color of mature fruit is green-light green. Fruits are ellipsoidal with a thin skin and 1-2 seeds (1-2 cm long).

Plant propagation: *A. indica* prefers full sun with moderate water. Propagation methods are seed, stem cutting, sucker, and marcotting.

Application: *A. indica* is used for cleaning soil and water contaminated by Cr. It employs in soil conservation since it has drought resistance with a well-developed root system. It is suitable for shelter, and uses as a windbreak. Fruits are eaten by fresh or cooked. The young twigs and flowers are consumed as vegetables. Leaves and fruit are used as a fodder. Charcoal is excellent quality. Oil is burned in lamps throughout India. The wood (density: 720-930 kg/m^3 at 12% moisturizer content) is used to make wardrobes, bookcases and closets, and packing cases, and it is used as firewood for a long time.Stem is also widely used to make posts for construction or fencing. Gum or resin has potential as a food additive. The seed oil and its byproducts are used in soap, cosmetics, pharmaceuticals and other non-edible products paper glue, pesticides, and fertilizer. Neem extract can be used as natural pesticide. Crushed seed kernels are also used as a dry pesticide application. The neem cake uses as an organic manure and soil amendment to enhance the efficiency of nitrogen fertilizers.

References:

Orwa, C., Mutua, A., Kindt, R., Jamnadass, R., & Anthony, S. (2009). *Azadirachta indica*. Retrieved December 1, 2016, from
http://worldagroforestry.org/treedb/AFTPDFS/*Azadirachta_indica* .PDF

Nparks flora and fauna web. (2013). *Azadirachta indica A. Juss*. Retrieved December 1, 2016, from
https://florafaunaweb.nparks.gov.sg/Special-Pages/plant-detail.aspx?id=2738

The Integrated Taxonomic Information System report (on-line database). *Azadirachta indica A. Juss*. Retrieved December 1, 2016, from
https://www.itis.gov/servlet/SingleRpt/SingleRpt#null

3. *Eucalyptus camaldulensis* Dehnh

Classification	
Classification	
Kingdom	Plantae
Subkingdom	Viridiplantae
Superdivision	Embryophyta
Division	Tracheophyta
Subdivision	Spermatophytina
Class	Magnoliopsida
Superorder	Rosanae
Order	Myrtales
Family	Myrtaceae
Genus	Eucalyptus
Species	*Eucalyptus camaldulensis*

Common Names: River red gum and Red gum

Characteristic: *E. camaldulensis* is a dicotyledon with flowering seed plants. It is a fast-growing tree with irregular shape. It grows to 20-50 m tall, a trunk diameter of 1-2 m and large crown of 15 m in diameter.

Biogeography: *E. camaldulensis* is a terrestrial plant. Although it is found in a wide variety of biomes, but it is most common along riverine areas. It is a native to Australia. It also is a common forestry tree in seasonally dry parts of Southeast Asia. It grows under a wide range of climatic conditions (temperate to hot and humid to arid zones). The suitable soils are waterlogged soils or drains site. It grows best on deep, silt or loamy soils with a clay base and accessible water table. It tolerates to waterlogging periodic flooding, and acid soils.

Plant Morphology: *E. camaldulensis* grows to 20-50 m tall with a trunk diameter of 1-2 m. Bark is smooth, many colors (white, grey, yellow-green, grey-green or pinkish grey), shedding in strips or irregular flakes. Juvenile leaves are opposite at first, then alternate. Adult leaves are lanceolate (10-15 cm long), pendulous as with most eucaplypts. Leaves often curved or sickle shaped, tapering, and short pointed at base. Leaf texture is smooth with dull green-blue green color. Inflorescence axillary composes of 7-11 white or creamy flowered. Flower buds are white with shape in

168

ovoid-conical. Fruit is very small, dry, woody, and explosive capsule at the end of thin stalks with exerted valves containing minute seeds.

Plant propagation: *E. camaldulensis* prefers full sun with moderate water and maintenance. Propagation methods are seed and stem cutting.

Application: *E. camaldulensis* is valuable for bee populations making it is a major source of honey. Especially, It contains a compound (1,8-cineole leaf oil), making a potential sources of medicinal-grade eucalyptus oil. The oils are used as an inhalant with steam for relief of colds and influenza symptoms. Because of its refreshing odor and its efficiency in killing bacteria, the oil is also used as an antiseptic. This tree is a generally planted as a shade tree along roadside and ornamental tree. Moreover, it is used for pulp and paper production. It is also planted for hardboard, fibreboard and particleboard. The wood is hard and durable. The density of the wood is 900-980 kg/m^3 at 12% moisturizer content. Wood has been used for many purposes, including railway sleepers, flooring, fencing, plywood, veneer, firewood and charcoal. However, the firewood is suitable for industrial use in brick kilns. It is not preferred for domestic use due to smoky and burns too fast. It makes good-quality charcoal.

References:

Orwa, C., Mutua, A., Kindt, R., Jamnadass, R., & Anthony, S. (2009). *Eucalyptus camaldulensis*. Retrieved December 1, 2016, from
http://worldagroforestry.org/treedb/AFTPDFS/*Eucalyptus_camaldulensis*.PDF

Nparks flora and fauna web. (2013). *Eucalyptus camaldulensis Dehnh*. Retrieved December 1, 2016, from
https://florafaunaweb.nparks.gov.sg/Special-Pages/plant-detail.aspx?id=2885

The Integrated Taxonomic Information System report (on-line database). *Eucalyptus camaldulensis Dehnh*. Retrieved December 1, 2016, from
https://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=502465#null

4. *Senna siamea* (Lam.) H.S. Irwin & Barneby

Classification	
Kingdom	Plantae
Subkingdom	Viridiplantae
Superdivision	Embryophyta
Division	Tracheophyta
Subdivision	Spermatophytina
Class	Magnoliopsida
Superorder	Rosanae
Order	Fabales
Family	Fabaceae
Genus	Senna
Species	*Senna siamea*

Common Names: Kassod tree, Busuk-busuk, Petai belalang

Characteristic: *S. siamea* is a dicotyledon with flowering seed plants. Plant growth form is woody tree.

Biogeography: *S. siamea* is a terrestrial plant that is found in lowland forests, river banks, waste area and along roadsides. It grows in a range of climatic conditions. It is susceptible to cold and frost and does not do well at altitudes above 1300 m. It is a native to Brunei, Cambodia, China, India, Laos, Malaysia, Myanmar, Nepal, Philippines, Sri Lanka, Thailand, and Vietnam. This species is fast-growing and tolerant of infertile soils, drought and flooding. It grows well in deep well-drained fertile soils with pH 5.5-7.5. It also is intolerant of saline soils.

Plant Morphology: *S. siamea* is medium-sized evergreen tree growing up to 18 m tall with a straight trunk of up to 30 cm in diameter. Low-branching trunk has grey with smooth bark. The root system consists of a few thick roots, growing to considerable depth, and a dense mat of rootlets in the top 10-20 cm of soil, which may reach a distance of 7-15 m from the stem in 1 year. Foliage is alternate leaves that are pinnately compound, 23-33 cm long, with slender, green-reddish, and tinged axis composed of 6-12 pair of leaflets on short stalks of 3 mm. Leaflet is oblong (7 cm long, 12-20 mm wide) rounded at both ends, with tiny bristle tip. Color of mature

170

foliage is green. Flowers having yellow or golden color are arranged in inflorescences known as racemes. Inflorescences are located at branch tips or in leaf axils. Trees begin to flower at 2-3 years of age. Fruits are long, flattened pods with many ellipsoid seeds. Seeds are bean shaped, shiny, dark brown, 8 mm long, with distinct areole.

Plant propagation: *S. siamea* prefers full sun with moderate water. It can be propagated by seed having a maximum storage life of 3 years. Fresh seeds have a high germination rate. Stored seeds should be soaked in concentrated sulfuric acid or boiling water for 10-30 min. Stem or root cuttings does not work well.

Application: *S. siamea* is planted as a shade tree, windbreak, or ornamental tree. In Thailand, young fruits and leaves are eaten as a vegetable. Leaves are used as green manure. This plant is widely grown for fodder. The dark-color wood makes good fuel. The energy value of the wood is 22400 kJ/kg, and hard wood density is 600-1010 kg/m³ at 15% moisturizer content. The wood was formerly preferred for locomotive engines. Charcoal is also of excellent quality. The dark heart wood is used for joinery, cabinet making, inlaying, handles, sticks and other decorative uses. The wood has also been used for poles, posts, bridges, mine poles and beams. This tree effectively increases topsoil infiltration, reducing runoff and combating soil erosion. It uses extensively for rehabilitation of degraded land such as Al mine tailings.

References:

Orwa, C., Mutua, A., Kindt, R., Jamnadass, R., & Anthony, S. (2009). *Senna siamea*. Retrieved December 1, 2016, from http://worldagroforestry.org/treedb/AFTPDFS/ *Senna_siamea*.PDF

Nparks flora and fauna web. (2013). *Senna siamea (Lam.) H. S. Irwin & Barneby*. Retrieved December 1, 2016, from https://florafaunaweb.nparks.gov.sg/Special-Pages/plant-detail.aspx?id=3118

The Integrated Taxonomic Information System report (on-line database). *Senna siamea (Lam.) H.S. Irwin & Barneby*. Retrieved December 1, 2016, from https://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=505177#null

5. *Pityrogramma calomelanos* (L.) Link

Classification	
Kingdom	Plantae
Subkingdom	Viridiplantae
Superdivision	Embryophyta
Division	Tracheophyta
Subdivision	Polypodiophytina
Class	Polypodiopsida
Subclass	Polypodiidae
Order	Polypodiales
Family	Pteridaceae
Genus	Pityrogramma
Species	*Pityrogramma calomelanos*

Common Names: Silver fern, Silverback fern, and Dixie silverback fern

Characteristic: Plant growth form is shrub (creeper). Shape is shrubby.

Biogeography: *P. calomelanos* is a terrestrial plant preferred tropical, sub-tropical, and monsoonal climate zone. It is a native to Mexico, Caribbean, and South America. In Thailand, it distributes in northern (Mae Hong Son, Chiang Mai and Tak), in south-western (Kanchanaburi and Prachuap Khiri Khan), in south-eastern (Trat), and in southern (Nakhon Si Thammarat, Phangnga, Trang, Satun, Yala, and Narathiwat). Its ecology is on open mountain slopes in recently felled areas or along new roads at low or medium attitudes.

Plant Morphology: Growth form is short (30-45 cm tall). Erect rhizome covered with scales and upright fronds is shortly creeping up to 10 mm in diameter. Scales are bright brown, linear, narrow (3-6 mm long), thin, and margin entire. Fronds are tufted, arching, firmly herbaceous. Foliage composes of stipes (20-30 cm long), dark purple, polished smooth with a few scales at the base, glabrous upwards, covered with white powder in young stage, fronds oblong, acuminate apex, bi-pinnate and fern blade (15-30 cm long and 10-15 cm wide). The colors of mature foliage are green, silver, and grey. Foliage retention is evergreen. Stem type is Acaulescent having no apparent stem above ground. Root type is underground with fibrous root. Sporangia are placed

172

along the veins and freely throughout the lower surface of fronds. Lamina is oblong, with acuminate apex, and 2-3 pinnatifid. Rachis is black to chestnut brown, shiny, glabrous, grooved on upper surface; lateral pinnae gradually smaller upwards; lower ones stalked, linear-subtriangular, acuminate to long-tailed at apex; pinna-rachis slender, grooved; grooves decurrent to those on rachis. Pinnules is oblong to oblong-lanceolate, cuneate at base, acute to acuminate at apex, pinnatifid to varying degree, margins shallowly toothed and in rolled in dried material, dark green above and covered with a yellow powder below, glabrous on both surfaces. Lobes is oblanceolate to spathulate, acute and dentate at apical portion, herbaceous, light green, glabrous but coated with white waxy powder; veins free, pinnate in larger ones, to several times forked. Sori is about 3 mm long, situated along the veins in the outer half of the lamina between the costule and margin, often difficult to see because of the yellow powder; exindusiate. Sporangia are placed along veins throughout the lower surface without any protection.

Plant propagation: *P. calomelanos* prefers full sun or semi-shade of light with moderate water. It can propagate using spore.

Application: *P. calomelanos* quickly accumulates As. Foliage can be used as ornamental leaf.

References:

Encyclopedia of life. *Pityrogramma calomelanos dixie silverback fern*. Retrieved December 13, 2016, from http://www.eol.org/pages/597264/overview

Nparks flora and fauna web. (2013). *Pityrogramma calomelanos (L.) Link*. Retrieved December 13, 2016, from https://florafaunaweb.nparks.gov.sg/special-pages/plant-detail.aspx?id=1561

The Integrated Taxonomic Information System report (on-line database). *Pityrogramma calomelanos (L.) Link*. Retrieved December 13, 2016, from https://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=17661#null

Appendix B

Media preparation

1. Plant medium (25% Hoagland's nutrient solution)

Hoagland's nutrient solution (Hoagland and Arnold, 1950) was modified by changing the amount of 1M $NH_4H_2PO_4$ from 0.25 mL to 0.01 mL to protect the precipitation. Moreover, 0.5% iron citrate was change to use 0.02 mL of 5 mg/L of $FeCl_3 \cdot 6H_2O$. The composition and amount of each compound to make the 25% modified Hoagland's nutrient solution was as follows:

Chemical compounds	Formula	Stock concentration	25% Solution/L
Macronutrient stocks			
1. Potassium nitrate	KNO_3	1M	1.50 mL
2. Calcium nitrate tetrahydrate	$Ca(NO_3)_2 \cdot 4H_2O$	1M	1.00 mL
3. Ammonium dihydrogen phosphate	$NH_4H_2PO_4$	1M	0.01 mL
4. Magnesium sulfate heptahydrate	$MgSO_4 \cdot 7H_2O$	1M	0.50 mL
5. Micronutrient stock as follow:			0.25 mL
Boric acid	H_3BO_3	2.86 g/L	
Manganese(II) chloride tetrahydrate	$MnCl_2 \cdot 4H_2O$	1.81 g/L	
Zinc sulfate heptahydrate	$ZnSO_4 \cdot 7H_2O$	0.22 g/L	
Copper sulfate(II) pentahydrate	$CuSO_4 \cdot 5H_2O$	0.08 g/L	
Molybdenum(VI) acid monohydrate	$H_2MoO4 \cdot H_2O$	0.02 g/L	
Iron stock			
6. Iron(III) chloride hexahydrate	$FeCl_3 \cdot 6H_2O$	5 mg/L	0.02 mL

The stock solutions (1-6) were prepared to reach the final concentration. For the micronutrient stocks (5), the amount of all compounds was combined in a total volume of 1 L of deionized water. To make up the iron stock, 5 mg/L of $FeCl_3 \cdot 6H_2O$ was prepared by dissolved in 500 mL deionized water, mix thoroughly to dissolve all salt. The final pH of the solution was adjusted to 5.5 with 1N HCl or 1N

NaOH. The 25% modified Hoagland's nutrient solution was prepared by adding the specified amount of each stock solution. All stock solutions were kept in 4°C.

2. Endophytic bacterial culture media

There were many brands of LB (Luria Bertani) powder that were used in this study. Therefore, each medium was prepared according to the manufacture's guideline.

2.1 LB broth

LB powder was weighted as each brand, and dissolved in 1 L of miliQ water or deionized water. The condition of miliQ water used thoroughly in this thesis was 15Ω.cm@25°C. Then, medial solution was heated in the microwave to complete the desolations, and sterilized by autoclaving. The condition of sterilization by autoclave used thoroughly in this thesis was 121°C for 15 min. The LB broth was cooled for overnight to check the contamination before use.

2.2 LB agar (1.5% of Bactor agar)

LB powder was weighted as each brand. 15 g of Bactor agar powder (Bacto™) was added and dissolve in 1 L of miliQ water or deionized water. Then, medial solution was heated in the microwave to complete the desolations, and sterilized by autoclaving. The media was cooled to 50°C before pouring in plates. These agar plates were kept in 4°C before use.

2.3 King's B agar (Sigma-Aldrich)

Dissolve the King's B powder 33 g in 990 mL distilled water and add 10 mL glycerol. Sterilize by autoclaving at 121°C for 15 minutes. Then, the medial solution was heated in the microwave to complete the desolations, and sterilized by autoclaving. The media was cooled to 50°C before pouring in plates. These agar plates were kept in 4°C before use.

Appendix C
Test media and solution preparation

1. Pb stock solution

The very high Pb concentration (10000 mg/L) was prepared by dissolving analytical grade of lead acetate trihydrate [Pb(CH$_3$COO)$_2$·3H$_2$O] 18.3 g in deionized water and adjusting the volume to 1000 mL by a volumetric flask. This stock solution was acidified by adding a few drop of concentrated nitric acid (69% HNO$_3$) in order to reduce precipitation of the metal ion and kept in plastic bottle at 4°C until used. The concentration was expressed in term of lead ion (Pb^{2+}) in mg/L of solution.

2. Pb standard solution

Pb standard solutions used for calibration were freshly prepared by diluting a stock solution and adjusting the volume with deionized water in a 25 mL of volumetric flask. The suitable Pb concentration was difference in each experiment and also depended on the model of FAAS.

3. Pb media for screening the Pb tolerant PGPE

LB agar was prepared as described in Appendix B. Pb stock solution was added to reach the final Pb concentration 20 mg/L prior to autoclave. The media was cooled to 50°C before pouring in plates. These plates were kept in 4°C before use.

4. Pb media for testing the MIC value

LB agar was prepared as described in Appendix B. Pb stock solution was added to reach the final Pb concentration 50, 100, 200, 400, 600, 800, 1600, 1800, 1825, 1850, 1875, 1900, 2000, and 3200 mg/L prior to autoclave. The media was cooled to 50°C before pouring in plates. These plates were kept in 4°C before use.

5. National Botanical Research Institute's phosphate growth medium

This medium was prepared to screen the phosphate solubilizing PGPE according to Nautiya (1999). The composition and amount of each compound to make this medium was presented as follows:

Chemical compound	Formula	Amount (g/L)
Glucose	$C_6H_{12}O_6$	10.0
Tricalcium phosphate	$Ca_3(PO_4)_2$	5.00
Magnesium chloride hexahydrate	$MgCl_2.6H_2O$	5.00
Magnesium sulfate heptahydrate	$MgSO_4.7H_2O$	0.25
Potassium chloride	KCl	0.20
Ammonium sulfate	$(NH_4)_2SO_4$	0.10
Agar (Bacto^{TM})	-	15.0

All ingredients were orderly dissolved in miliQ water ($15\Omega.cm@25°C$) and adjusted volume to 1,000 mL in a volumetric flask. Media was sterilized by autoclaving for 121°C for 15 min, they were plated and kept for further study.

6. Chrome azurol S (CAS) agar

This medium was prepared to screen the siderophore production PGPE according to modify from Schwyn and Neilands (1987). The composition and amount of each compound to make this medium was presented as follows:

Chemical compound	Formula	Amount (g or mL)
Chrome Azurol S	$C_{23}H_{13}Cl_2Na_3O_9S$	0.065 g
Iron(III) chloride hexahydrate	$FeCl_3.6H_2O$	0.0027 g
Hydrochloric acid	HCl	10 mM 10 mL
Hexadecyltrimethylammonium bromide (HDTMA)	$CH_3(CH_2)_{15}N(Br)(CH_3)_3$	0.073 g
Glycerol	$HOCH_2CH(OH)CH_2OH$	10 mL
King Agar B	-	33 g

To make the chrome azurol S (CAS) agar, the separately 4 solutions such as Chrome Azurol S, $FeCl_3.6H_2O$, HDTMA (Hexadecyltrimethylammonium bromide) and King Agar B medium were prepared as follows:

Solution	Preparation
1. Chrome Azurol S solution	0.065 g of Chrome Azurol S was dissolved in 50 mL of milliQ water (15 MΩ.cm@25°C).
2. $FeCl_3.6H_2O$ solution	0.0027 g of $FeCl_3.6H_2O$ (Wako) was dissolved in 10 mL of 10mM HCl (Wako, conc 36%).
3. HDTMA solution	0.073 g of HDTMA (Sigma) was dissolved in 40 mLof milliQ water (15 MΩ.cm@25°C).
4. King Agar B	33 g of King Agar B (Fluka) was dissolved in 990 ml of milliQ water (15 MΩ.cm@25°C), and 10 mL glycerol (Wako) was added. This solution was mixed well, and pH was adjusted to 6.8±0.02. It was heated in a microwave to ensure that the agar was completely melted before autoclaving at 121°C for 15 min. The solution should be clear amber, no precipitation, and let it cool about 50°C.

Then, solution 1, 2, and 3 were used for preparing the blue dye by mixing solution 1 with 10 mL of solution 2. Then, 40 mL of solution 3 was gently added. Now, solution should be a dark blue color. This solution was sterilized by autoclaving at 121°C for 15 min, and let it cool about 50°C. Then, 900 mL of King Agar B medium was gently added. Now, solution should be blue green color. Finally, this medium was poured in the plates, and kept at 4°C until use.

Appendix D

Phylogenetic tree reconstruction

A phylogenetic tree is a representation of the evolution of a set of species, which the species are grouped based on similarity. The species correspond to leaves of the tree, and internal nodes correspond to hypothetical common ancestors of these species. The branch lengths are a measure of the evolutionary distance between the species (Jensen, 2008). There are two main categories of tree-building methods (discrete characters and distance method). The former is molecular sequences from individual taxa. This category composes of the maximum parsimony (MP) and maximum likelihood (ML) methods. The latter is the amount of dissimilarity between pairs of sequences, computed on the basis of sequence alignment. It assumes that all sequences involved are homologous and that tree branches are additive, meaning that the distance between two taxa equals the sum of all branch lengths connecting them. This category composes of Un-weighted Pair Group Method Using Arithmetic Average (UPGMA), Neighbor Joining (NJ), Fitch–Margoliash (FM) and Minimum Evolution (ME) method (Xiong, 2006).

Among them, ML method has proven itself superior to distance methods like UPGMA and NJ, and the MP by consistently yielding better trees. Besides, ML method is able to handle sequencing errors and ambiguities (Jensen, 2008). ML uses probabilistic models to choose a best tree that has the highest probability of reproducing the observed data. It finds a tree that most likely reflects the actual evolutionary process. ML is an exhaustive method that searches every possible tree topology and considers every position in an alignment. ML calculates the total likelihood of ancestral sequences evolving to internal nodes and eventually to existing sequences. It also incorporates parameters that account for rate variations across sites. ML has advantages (all the sequence data is used, and every the possible trees is searched). It also has disadvantages (choosing an unrealistic substitution model may lead to an incorrect tree, and impossible to use when the number of taxa increases to a modest size (Xiong, 2006). Therefore, ML is suitable used in this study.

To reconstruct the biology tree, the dataset was prepared. It composed of the partial 16S rRNA gene sequences of each isolates as unknown (Pc, Pd, Pe, Ai, Aj, and El), homologous candidate, tester and out group sequences. For 26 candidate sequences, they were obtained by blasting of 16S rRNA gene sequence from each isolate in nr/nt database with magablast. Sequences with 98-100% identity and non-redundancy were chosen. For 3 tester sequences, *Pseudomonas migulae*, *Bacillus cereus*, and *Serratia grimesii* were randomly duplicated. An out group sequence belonged to *Amoeba proteus* was used to generate the root. The program MEGA version 6 (Tamura et al., 2013) was used to implement the tree. Starting from, the homologous dataset (fasta file) was opened MEGA 6, all sequences were aligned by MUSCLE algorithm with default parameters. For ML method, an initial tree was first built. Its branch lengths were adjusted to maximize the likelihood of the data set so that tree topology was under the desired model of evolution. Then variants of the topology were created to search for topologies. Maximum-likelihood branch lengths were computed for these variant tree topologies and the greatest likelihood retained as the best choice so far. This search continues until no greater likelihoods were found. This method also generated the model feature that permitted selecting the most suitable substitution model. The reliability of the phylogeny was evaluated by bootstrap analysis with 1000 replicates. Finally, the rooted tree was viewed as cladogram, which is unscaled with the branches lengths, neatly, but has no phylogenetic meaning.

References

Jensen, B. R. (2008). *Tree reconstruction by maximum likelihood using prior knowledge*. Retrieved December 20, 2016, from
http://users-birc.au.dk/cstorm/students/RohdeJensen_Oct2008.pdf

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, *30*, 2725-2729

Xiong, J. (2006). *Essential bioinformatics*. Retrieved December 20, 2016, from
http://www.aun.edu.eg/molecular_biology/Procedure%20Bioinformatics22.23-4-2015/Xiong%20-%20Essential%20Bioinformatics%20send%20by%20Amira.pdf